

## Chapter 1 : Literature review

### 1.1 Introduction to Stem Cells

Over recent years, stem cells have captivated the scientific community with their potential to be used in regenerative medicine, disease modelling and other biological applications (Roelen & Lopes, 2008). Stem cells are defined as cells that are able to both self-renew, and to divide to create daughter cells that will differentiate into at least one other cell type (Hou & Singh, 2008; Lanza & Gearhart, 2006; Roelen, 2011; Weissman, Anderson, & Gage, 2001). Different types of stem cells exist, with their identity determined by origin and by differentiation potential (Roelen, 2011). Stem cells are most commonly found in early stage embryos and are able to be recovered from the germinal ridge of the blastocyst. However, stem cells are also found in far lower quantities in adult tissue, where they are involved in tissue maintenance and repair, and include cell types such as spermatogonial stem cells which give rise to sperm in the adult testis (Kuijk, Chuva de Sousa Lopes, Geijsen, Macklon, & Roelen, 2011; Lanza & Gearhart, 2006).

The idea of cell 'potency' or 'stemness', refers to the potential ability of stem cells to differentiate into different cell types. The classification of stem cells into these three groups is based on their differentiation potential. Unipotent stem cells are only able to differentiate into one cell type. Multipotent stem cells are able to differentiate into multiple cell types, usually of the same germ lineage. Pluripotent stem cells are able to differentiate into all cell types of all three germ lineages, mesoderm, endoderm and ectoderm (Roelen, 2011).

### 1.2 Pluripotent Stem Cells

Pluripotent stem cells have the ability to create the precursor cells for the formation of an entire organism. They have the potential to differentiate into any cell in the body when exposed to the correct conditions. The most common source of pluripotent cells are embryonic stem cells (ES cells) (Chen, L. & Liu, 2009). However, once the embryonic development stage is complete, embryonic stem cells become adult stem cells and no longer have an unlimited potential to develop into all cell types, and instead appear to be restricted to particular cell types (Rippon & Bishop, 2004).

As pluripotent stem cells have the potential to differentiate into all cells types in the body, they are of immense interest for regenerative medicine and other scientific endeavours, such as the study of

cell lines and research into developmental biology (Chen, L. & Liu, 2009). Pluripotent stem cells have the potential to be used in regenerative medicine by providing a new source of healthy cells and tissue to treat a wide variety of diseases and injuries. Currently, however the therapeutic use of stem cells is restricted, particularly when the use of embryonic stem cells are involved. There are obvious ethical issues associated with destroying an embryo to obtain pluripotent stem cells. Additionally, using pluripotent stem cells from sources other than the patient's own body creates the problem of rejection of the cells by interactions with the immune system (Svendsen, 2013).

Pluripotent cells from either embryonic or induced origins must meet stringent criteria, cells not meeting minimum criteria cannot be deemed to be fully pluripotent. As a minimum, pluripotent stem cells must be able to undergo unlimited proliferation (also *in vitro*) while maintaining a normal diploid karyotype (number and appearance of chromosomes); additionally they must express pluripotency markers including OCT4 and NANOG. Furthermore, pluripotent stem cells must be able to differentiate into cell types from all three germ layers (ectoderm, mesoderm and endoderm) *in vivo* as embryoid bodies and *in vitro* through the formation of teratomas in immune deficient mice. More stringent tests of pluripotency include the contribution of these cells to chimera organisms, where, after the injection of pluripotent cells into blastocyst stage embryos, the resulting organism has at least two genetically distinct cell populations and the injected cells must contribute to the germline. Ideally, pluripotent stem cells are able contribute to tetraploid complementation (Maherali & Hochedlinger, 2008). When a tetraploid stage embryo is combined with a pluripotent stem cell and then transplanted to a foster mother, the extra embryonic structures, including the placenta and umbilical cord, will be derived from the embryo, while the subsequent foetus is completely derived from the transplanted pluripotent stem cell (Roelen, 2011).

Although stem cells from different cellular environments have different physiological demands, and therefore different molecular profiles, there are certain gene expression markers that are shared by all stem cells. Through transcriptional profiling of stem cells, markers and genes expressed exclusively by stem cells have been identified. Stem cells are believed to express many downstream signalling components involved in the transduction of growth factors and signalling molecules. Signalling molecules involved in maintaining pluripotency in stem cells include the TGF, Notch, Wnt and Jak/Stat family members. These cells also express components involved in cell cycles and telomere maintenance, and have increased telomere activity. Pluripotent stem cells must also express transcription factors that are central to pluripotency, including Oct3/4, SOX2 and NANOG

(Lanza & Gearhart, 2006; Roelen, 2011). These factors will be discussed in more detail later in this review.

### 1.2.1 Embryonic stem cells

The most common form of pluripotent stem cells are those derived from early stage embryos and are known as embryonic stem cells (ES cells). The first murine ES cells were isolated and cultured in 1981 (Evans & Kaufman, 1981; Martin, 1981), while the first human ES cells were isolated and cultured in 1994, with the first human ES cell line established in 1998 (Thomson et al., 1998). Embryonic stem cells are formed through the cleavage of the zygote before formation of the blastocyst, they are harvested from the inner cell mass (ICM) of the blastocyst, in a process which results in the destruction of the embryo.

The differentiation potential of ES cells can be assessed *in vivo* through the ability of these cells to contribute to all cell lines including the germline, through the formation of chimeras. In instances where this process is unethical or not appropriate (i.e. the study of human ES cells), embryoid bodies may be formed *in vitro* or ES cells can be injected into immune compromised mice to form teratomas, a form of tumour with all three germ layers present. In addition, ES cells must be shown to express well known markers for pluripotency including *OCT4* and *NANOG* (these factors along with other will be discussed further) (Bishop, Buttery, & Polak, 2002; Biswas & Hutchins, 2007).

Embryonic stem cell lines have now been derived from numerous species including primates. However, attempts to derive ES cells from livestock species such as sheep and cattle have not been successful (Keefer, Pant, Blomberg, & Talbot, 2007; Renard, Maruotti, Jouneau, & Vignon, 2007; Telugu, Ezashi, & Roberts, 2010). Culture conditions for these cells has been predominantly derived from mouse and human protocols, are likely to be suboptimal as cultured ES-like cells vary widely in morphology, expression profiles and ability to differentiate (Cibelli et al., 1998; Mitalipova, Beyhan, & First, 2001; Wang, L. et al., 2005). Recently, an important step towards determining the culture conditions required for bovine ES cells has been made. Verma, Huang, Kallingappa, and Oback (2013) cultured bovine ES cells, isolated from the inner cell mass of bovine embryos, to create a screening platform for factors that encourage long-term culture and growth of bovine ES cells. This study found that the culture of bovine ES cells, supplemented with dual kinase inhibitors, were able to proliferate in culture for several weeks and differentiate to all three germ layers (Verma et al., 2013).

Due to the controversy surrounding the use of embryonic stem cells in human research, and the difficulties presented in isolating and culturing ES cells from livestock species, the use of stem cells from other origins, including adult tissue and induced pluripotent stem cells (iPS cells), offers an alternative source of stem cells.

### **1.2.2 Nuclear Reprogramming of Somatic Cells to Pluripotency**

Until recently the fate of cells was believed to be fixed, and once differentiation had occurred, it was assumed cell fate was not able to be reversed or altered. However, in 2006 it was demonstrated that it is possible to reprogram somatic, differentiated cells, to a state of pluripotency, through the introduction of transcription factors which are specific to embryonic stem cells. These artificially induced stem cells are known as induced pluripotent stem (iPS) cells, and are believed to be very similar to embryonic stem cells, with the exception of their origin (Takahashi & Yamanaka, 2006; Yu, Junying et al., 2007). This discovery has the potential to provide a different path for the use of stem cells in regenerative medicine. Additionally, iPS technology has the added benefit of being able to create patient specific pluripotent stem cells which avoid immune system rejection. The first clinical trials for iPS cell use in humans were approved in 2013, where retinal cells, derived from human iPS cells, were used to treat patients suffering from macular degeneration (Cyranski, 2013).

There are currently three successful methods which have been established to generate induced pluripotent stem (iPS) cells from somatic cells. These are; nuclear transfer, fusion of somatic cells with embryonic stem cells, and most recently the forced expression of defined factors. These three methods of producing pluripotent stem cells, somatic cell nuclear transfer (SCNT), somatic cell and embryonic cell fusion, and reprogramming by defined factors, are shown in Figure 1-1, and will be discussed in further detail.

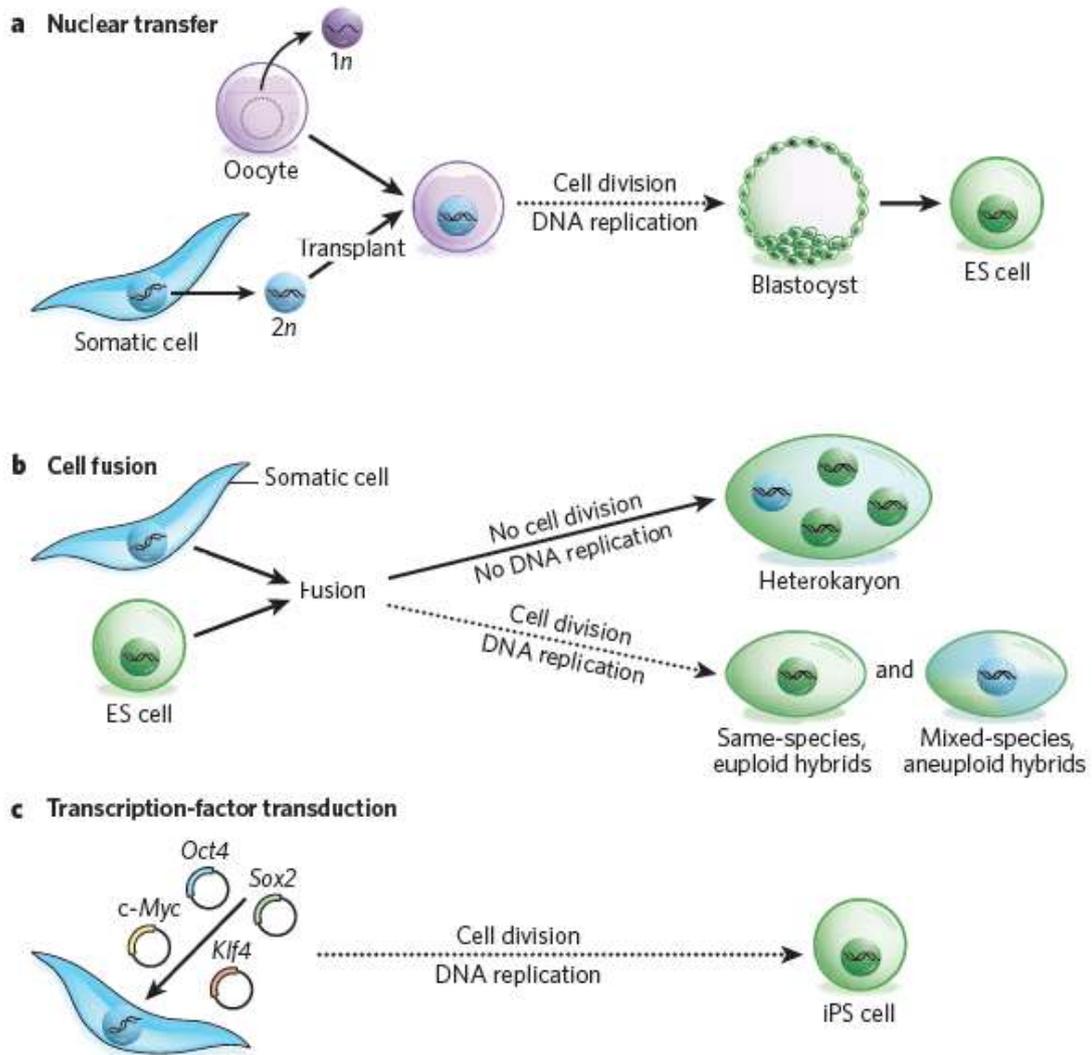


Figure 1-1. Three methods used for nuclear reprogramming of somatic cells to a pluripotent state, nuclear transfer (SCNT), fusion of somatic and embryonic stem cells, and reprogramming by defined factors (Yamanaka, Shinya & Blau, 2010).

### 1.2.2.1 Somatic Cell Nuclear Transfer (SCNT)

Somatic cell nuclear transfer (SCNT) is the basis for cloning mammalian cells, and involves the transfer of the nucleus of a differentiated somatic cell into an enucleated egg cell (Lee & Prather, 2013). After the nucleus is inserted into the egg cell it is reprogrammed to pluripotency and is stimulated to divide to form a blastocyst with identical DNA to the original organism (Jaenisch & Young, 2008). The stem cells produced by the blastocyst can then be harvested for use.

Alternatively, the blastocyst can be implanted into a pseudo-pregnant foster mother and left to develop into an entire organism as was the case in the first mammalian clone “Dolly” the sheep (Wilmut, Schnieke, McWhir, Kind, & Campbell, 2007). However, SCNT is also associated with a high proportion of embryonic, foetal and neonatal deaths, with the likelihood of a successful pregnancy by SCNT less than 5% (Lee & Prather, 2013; Palmieri, Loi, Ptak, & Salda, 2008; Wilmut, Beaujean, De, & Dinnyes..., 2002).

The use of SCNT for stem cell research offers an opportunity to create patient specific stem cells from a cloned embryo that are genetically matched to the patient in order to avoid rejection when used in disease therapies or clinical applications (Geoffrey & Natalie, 2013) . However due to ethical concerns and the availability of human oocytes, this method has not been used in human models (Yamanaka, S., 2009) . However, the efficiency of producing of pluripotent cell lines using SCNT is relatively low (Wilmut et al., 2002). Additionally, the demands placed on the oocyte cytoplasm during the process of reprogramming a somatic nucleus, is believed to be far greater than traditional fertilisation using sperm. As a result, the inappropriate expression of genes that exert harmful effects at different stages of development is often seen (Whitworth, K. M. & Prather, 2010). Recently, SCNT derived stem cells were produced from human adult somatic cells (Yamada et al., 2014). The group managed to derive a line of stem cells from a type 1 diabetic donor, that were able to be successfully cultured and differentiated into cells that secreted insulin. This study demonstrates the potential to produce patient specific stem cells derived from SCNT for use in therapeutic applications and disease modelling.

#### **1.2.2.2 Fusion of Somatic and Embryonic Stem Cells**

Epigenetic reprogramming of somatic nuclei to a pluripotent state has been achieved through the fusion of a somatic cell with an embryonic stem cell to form a single entity (Yamanaka, Shinya & Blau, 2010). Early cell-fusion studies were the first to indicate that the cell fate of differentiated somatic cells is not fixed, but is instead plastic (Blau, Chiu, & Webster, 1983; Blau et al., 1985 ). Later studies into cell fusion have demonstrated that differentiated somatic cells can be reverted back to a pluripotent state under certain conditions for both mouse (Tada, Takahama, Abe, Nakatsuji, & Tada, 2001) and human cells (Cowan, Atienza, Melton, & Eggan, 2005). Kimura *et al* (2004) reported that thymocyte-ES hybrid cells acquired ES like epigenetics, with the promoter regions of several genes including Oct3/4 undergoing histone acetylation and methylation. This result indicated that the

genome is at least partially reprogrammed by fusion of the cells. However it is unclear whether the genome of the somatic cell is ever completely reprogrammed following fusion with ES cells.

The main problem associated with the use of ES-fused cells in a clinical sense is that the ES cell derived chromosomes still persist in the fused cell. These chromosomes may cause rejection of cells upon transplant to the receiving organism. In order to avoid this, ES chromosomes must first be removed. This process would be technically challenging and the cells may not remain pluripotent after the removal of the ES chromosomes (Matsumura et al., 2007). Little is understood about the molecular processes of reprogramming through fusion with ES cells. The use of these cells for clinical purposes may not be of value due to the possibility of host rejection and the ethical issues associated with the use of embryonic stem cells.

### ***1.2.2.3 Reprogramming by Transcription Factors***

In 2006 researchers at the Institute for Frontier Medical Sciences at Kyoto University determined that it was possible to reprogram somatic cells to a pluripotent state using transcription factors unique to embryonic stem cells (Takahashi & Yamanaka, 2006). The group screened 24 transcription factors thought to be responsible for inducing pluripotency, and introduced combinations of these factors into somatic mouse embryonic fibroblasts and tail-tip fibroblasts using a retroviral vector. It was determined that while no single factor was effective in inducing pluripotency, the simultaneous expression of Oct3/4, SOX2, c-MYC and KLF4 (Yamanaka factors) was capable of reprogramming these somatic fibroblasts to an embryonic stem cell like state. These cells exhibited ES cell specific marker genes including NANOG and Oct3/4 and the transplantation of these cells into nude mice resulted in the formation of teratomas with all three germ cell layers, consistent with a pluripotent state. However, these initial iPS cells were not able to produce to chimeric mice and were therefore not fully reprogrammed (Takahashi & Yamanaka, 2006; Yildirim, 2012).

Yu et al (2007) demonstrated that it was possible to produce human iPS cells using a different combination of transcription factors to those described by Yamanaka. OCT4, SOX2, NANOG and LIN28 factors were used in reprogramming human somatic foetal fibroblasts, thus eliminating the need to use the oncogene c-MYC, which is known to cause tumours and cell death. The group cloned the genes for the four factors (Thompson factors) into a lentiviral vector. The resulting cells exhibited ES cell like characteristics had normal karyotype and expressed cell surface markers and genes that are commonly expressed by ES cells. The cells were able to differentiate into all three

germ indicated by the production of teratomas in nude mice. However, due to ethical issues involving the use of human embryos, chimeric tests could not be conducted.

Since the production of the first generation of mouse and human iPS cells, many groups have had increasing success in producing iPS cells more efficiently. This has led to the production of iPS cells from an increasing number of species, including other primates, dogs (Shimada et al., 2010; Whitworth, D., Ovchinnikov, & Wolvetang, 2012), pigs (West, F. et al., 2011; Wu et al., 2009), horses (Nagy et al., 2011), sheep (Li, Y., Cang, Lee, Zhang, & Liu, 2011; Sartori et al., 2012; Song et al., 2013), goats (Song et al., 2013) and cattle (Cao et al., 2012; Han et al., 2011; Sumer, Liu, & Malaver-Ortega..., 2011).

### 1.2.3 Reprogramming Factors

Transcription factors are proteins that bind to DNA to affect the transcription of specific genes. The first reprogramming experiments were initially performed using the retroviral transduction of 24 candidate genes that were believed to be involved in the establishment and maintenance of pluripotency (Takahashi & Yamanaka, 2006). It has now been established that there are six reprogramming factors known to function as transcription factors for the reprogramming of somatic cells to pluripotency, these are; OCT4 (O), SOX2 (S), KLF4 (K), c-MYC (M), LIN28 (L) and NANOG (N). When these transcription factors are introduced to a cell in combination, usually as OSKM (Yamanaka factors) or OSNL (Thompson factors), they induce the expression of genes normally expressed by pluripotent stem cells, rather than somatic cells. The downstream effect of the expression of these genes leads to a cascade of transcriptional activity, and the subsequent reprogramming of the somatic cell to a state of pluripotency, thereby producing induced pluripotent stem cells (Maherali & Hochedlinger, 2008).

**OCT4 (OCT3/4)** is encoded by the gene *POU5F1*, and is expressed at high levels in undifferentiated embryonic stem cells, compared to somatic cell types. OCT4 expression is essential for maintaining pluripotent stem cells (both embryonic and induced) in an undifferentiated pluripotent state. If OCT4 expression is decreased or switched off, it results in the spontaneous differentiation of the stem cells. However the expression of OCT4 alone is not sufficient in inducing pluripotency (Yamanaka, S., 2009). Importantly, to date, no iPS cells have been generated without the use of OCT4.

**SOX2** is involved in maintaining pluripotency in embryonic stem cells. It is known to work with OCT4 to co-regulate the expression of genes involved in the maintenance of pluripotency (including UTF1, Fgf4 and Fbx15). SOX2 is essential for the maintenance of pluripotency and it has been suggested that the primary role of SOX2 is to maintain OCT4 expression (Yamanaka, S., 2007).

**KLF4** (Kruppel-like factor 4) is a zinc finger protein that is highly expressed in undifferentiated embryonic stem cells and to a lesser extent in adult somatic cell types such as those found in the gut and testis. It functions to regulate cell proliferation and differentiation and it also has an impact on cell survival (Rowland, B. D. & Peeper, 2006). Additionally, studies have shown that KLF4 acts as both a tumour suppressor and a oncogene (Rowland & Peeper, 2005).

**c-MYC** is a known proto- oncogene, but also plays a role in the pluripotency of cells. The c-MYC gene codes for a transcription factor that regulates numerous genes involved in the control of cell proliferation, growth, differentiation and apoptosis. The abnormal expression of c-MYC is associated with tumour formation and cancer development. It has been shown that c-MYC is dispensable as a reprogramming factor (Yu, Junying et al., 2007), although some studies have shown that reprogramming is far less efficient without introduction of exogenous c-MYC (Nakagawa, M. et al., 2008).

**NANOG** is another transcription factor involved in inducing and maintaining pluripotency. It was used in the initial cocktail (OSNL), by Yu et al (2007), to create human iPS cells. It is believed to be involved in self-renewal of undifferentiated ES cells and maintaining pluripotency (Chambers et al., 2003). While NANOG is thought to be a dispensable transcription factor for iPS generation (Takahashi & Yamanaka, 2006), it has been suggested that its addition as a transcription factor is essential for the reprogramming of bovine somatic cells to pluripotency (Sumer, Liu, Malaver-Ortega, et al., 2011). NANOG expression is often used as a selection factor for identifying fully reprogrammed iPS colonies (Yu, Junying et al., 2007).

**LIN28** is a protein marker of undifferentiated embryonic stem cells and has been used as part of the Thompson factor reprogramming cocktail. LIN28 binds to and promotes the translation of IGF-2 (Insulin like growth factor 2). LIN28 is believed to be involved in the self-renewal of pluripotent stem cells (Bang & Carpenter, 2008).

### 1.2.4 Factors affecting iPS cell generation

Reprogramming somatic cells to pluripotency is a highly inefficient process. Most reports claim less than 0.1% of target cells undergo the reprogramming process, most cells are not reprogrammed, or instead undergo incomplete reprogramming (Stadtfield & Hochedlinger, 2010). Different factors have been shown to influence the efficiency of the reprogramming process, including the choice of target cell, combinations of reprogramming factors, method of factor delivery, and cell culture conditions.

#### **Choice of Target Cell**

The type of target cell chosen for reprogramming has been shown to have a significant effect on the efficiency of reprogramming. Since the initial experiments using murine embryonic fibroblasts for iPS cell generation (Takahashi & Yamanaka, 2006), numerous different cell types from different species have been reprogrammed to pluripotency, with different reprogramming efficiencies achieved. Adult and embryonic fibroblasts are a popular choice of cell due to the ease of collection from donors (Takahashi & Yamanaka, 2006; Yu, Junying et al., 2007). However keratinocytes (Aasen et al., 2008), neural progenitors (Eminli, Utikal, Arnold, Jaenisch, & Hochedlinger, 2008; Kim, D. et al., 2009), adipocytes, blood cells (Loh et al., 2009) and mesenchymal stem cells (Niibe et al., 2011; Yan et al., 2010) have all been used for reprogramming. It is now believed that some cell types are more efficiently reprogrammed than others, in some cases this is thought to be due to the high endogenous expression of pluripotency genes in particular cell types. Kim, D. et al. (2009) and Kim, J. et al. (2009) showed that neural progenitor cells which express high levels of Myc and KLF4 are able to be reprogrammed using only OCT4 and SOX2.

The differentiation status of the cells is also of particular importance for the efficiency of reprogramming. Different cell types, including adult stem cells, and different sub-populations of fibroblasts, have been shown to be more amenable to reprogramming than others (Aasen et al., 2008; Byrne, Nguyen, & Reijo Pera, 2009; Eminli et al., 2009; Niibe et al., 2011). Generally, younger cells show more efficient reprogramming than the same adult cell type (Okada & Yoneda, 2011). It is thought that younger cells are less differentiated adult cells and are therefore easier to reprogram to a pluripotent state. It is possible that older cells, having undergone numerous passages, may have increased levels of DNA damage compared to younger cells and this may impede reprogramming (Marión et al., 2009). In addition there is evidence that suggests that fully differentiated cells retain epigenetic memory, and as such are more difficult to reprogram to pluripotency. There is a tendency of these reprogrammed cells to differentiate into cells of the same lineage as the somatic cell type,

rather than all three cell lineages (Bar-Nur, Russ, Efrat, & Benvenisty, 2011; Kim, K. et al., 2010; Polo et al., 2010).

In some studies of bovine iPS generation, embryonic fibroblasts have been found to have higher reprogramming efficiency than foetal and adult fibroblasts (Huang, B. et al., 2011). However, adult bovine fibroblasts have been reprogrammed in previous studies (Huang, B. et al., 2011; Sumer, Liu, Malaver-Ortega, et al., 2011).

The purpose for reprogramming cells to pluripotency, generally dictates the target cell type chosen. For large scale iPS generation and for the production of patient specific stem cells, easily accessible cell types are preferred. It is for this reason that fibroblast cell types are so often used.

### **Combination of Reprogramming Factors**

Initial reprogramming experiments used a reprogramming cocktail of OCT4 (POU5F1), SOX2, KLF4 and c-MYC to reprogram murine fibroblast cells (Takahashi & Yamanaka, 2006). Reprogramming has also been achieved using a slightly different combination of four reprogramming factors, OCT4, SOX2, LIN28 and NANOG (Yu, Junying et al., 2007). It is therefore believed that OCT4 and SOX2 are essential for reprogramming while KLF4, c-MYC, NANOG and LIN28 are supportive factors. Different combinations of these six factors have been used to reprogram numerous cell types from many different species (Deng et al., 2012; Liu, Haisong et al., 2008; Park, I.-H. et al., 2008; Park, K.-M., Cha, Ahn, & Woo, 2013; Takahashi & Yamanaka, 2006).

The expression of NANOG by cells is essential for pluripotency and is used as a marker of fully reprogrammed iPS cells (Yu, Junying et al., 2007). As such, the addition of NANOG to the OSKM factor cocktail has been shown to greatly improve reprogramming in some cell types. The time taken for iPS colonies to form following transfection of mouse B cells by a reprogramming cocktail including NANOG was reported to approximately half when compared to the time taken with OSKM alone (Hanna et al., 2009). However, there have also been claims that NANOG does not improve efficiency of reprogramming in some cell types (Schwarz, Bar-Nur, Silva, & Hochedlinger, 2014) and it is not an essential factor for inducing pluripotency (Takahashi & Yamanaka, 2006). Recently it was suggested that NANOG was essential for the production of fully reprogrammed bovine iPS cells (Sumer, Liu, Malaver-Ortega, et al., 2011). This claim is yet to be validated, with other reports of successful bovine iPS cell generation without the addition of exogenous NANOG (Cao et al., 2012).

Reprogramming using a combination of all six reprogramming factors has also been shown to improve reprogramming efficiency 10 fold compared to the OSKM factor combination (Liao et al., 2008). Although NANOG and LIN28 are not believed to be essential for reprogramming, they are thought to enhance the reprogramming (Onder et al., 2012; Theunissen, Thorold et al., 2011).

The use of oncogenes c-MYC and KLF4 in the reprogramming cocktail has inherent problems. The formation of tumours in mice transplanted with differentiated iPS cells derived by the introduction of these genes has been associated with their re-activation in the host.

As such the elimination of these two factors from the reprogramming cocktail is desirable. Cell types that express high endogenous levels of c-MYC and KLF4 have been reprogrammed without the need for delivery of exogenous genes (Kim, J. et al., 2008; Nakagawa, M. et al., 2008; Wernig, Meissner, Cassady, & Jaenisch, 2008). However the efficiency of producing iPS cells without the addition of KLF4 and c-MYC is far lower than if using the four factor combination of OSKM (Nakagawa, M. et al., 2008; Wernig, Meissner, et al., 2008).

Additionally, a number of small molecules that can replace the function of traditional reprogramming factors have been identified. These include RepSox which is able to replace SOX2 by inducing endogenous NANOG expression (Ichida et al., 2009), additionally, TGF- $\beta$  inhibitor, 616452, has also been shown to replace SOX2 (Li, Yanqin et al., 2011). KLF4 can be replaced by oestrogen-related receptor beta (ESRRB) (Feng, B., Ng, Heng, & Ng, 2009). In addition l-Myc, n-Myc and GLIS1 have also been found to replace c-MYC and have a lower chance of tumour formation (Blelloch, Venere, Yen, & Ramalho-Santos, 2007; Maekawa et al., 2011).

### **Cell culture conditions**

Generally, cell culture conditions for the derivation of iPS cells from different species, are based on defined culture conditions for embryonic stem (ES) cells of the same species. Culture conditions for human and murine ES cells are well defined and the conditions for iPS generation have been extrapolated from ES culture (Takahashi & Yamanaka, 2006; Thomson et al., 1998). In contrast, conditions for the culture of embryonic stem cells from other species including livestock, have not been determined and as such the culture requirements of iPS cell derived from these species have not been defined (Gong, Roach, Jiang, Yang, & Tian, 2010; Jin et al., 2012). Therefore, as a result, culture conditions adapted from human or murine iPS cell culture, have been used and modified to produce iPS cells in different species. Hence, current culture conditions for different species may be sub-optimal, and this may explain the difficulties faced in producing iPS cells in species such as the bovine.

Culture conditions may be altered and optimised to improve reprogramming efficiency. The use of serum or serum replacement, media choice, growth factors and the use of feeders or a feeder free matrix, as well as levels of oxygen and CO<sub>2</sub>, can all effect iPS cell generation.

Additionally small molecule supplementation may be used to target molecular pathways involved in reprogramming to improve reprogramming efficiency. These will be discussed in further detail at a later stage.

The use of serum in iPS culture has been shown to enhance the generation of iPS cells in some cases (Cheng, Dutra, Takesono, Garrett-Beal, & Schwartzberg, 2004; Horii, Nagao, Tokunaga, & Imai, 2003; Ruth et al., 2010). However the components of serum are not well defined and may vary between different batches. Additionally it is suspected that some components of serum may induce differentiation of pluripotent cells (Horii et al., 2003). Serum replacement is able to improve the growth of mouse ES cells and can enhance mouse iPS cell generation (Blelloch et al., 2007). However, serum replacement cannot be used to produce reprogrammed cells for all cell types (Mannello & Tonti, 2007).

The use of feeder layers or feeder free culture also plays a role in the derivation of iPS cells. iPS cells are generally derived in co-culture with a layer of inactivated fibroblast feeders. However, feeder free conditions where iPS cultures are grown on a matrix have been successful in deriving iPS cells in mice (Stadtfield, Nagaya, Utikal, Weir, & Hochedlinger, 2008; Sugii, Kida, Berggren, & Evans, 2011), and in humans (Nakagawa, Masato et al., 2014; Sugii et al., 2010; Warren, Luigi, Ni, Wang, & Guo, 2012), but has yet to be established in other species. Although murine embryonic fibroblasts (MEFs) are most commonly used as feeder layers for iPS cell derivation in numerous species, it has been suggested that the use of species specific feeders may be more appropriate for the culture of iPS cells (Nagy et al., 2011).

The levels of oxygen and carbon dioxide have also been shown to influence the culture of ES and iPS cells, as pluripotent cells prefer a hypoxic environment (Fischer & Bavister, 1993; Lim et al., 2011; Yuichiro, Keita, Akihiro, & Tomomasa, 2004). Hypoxic conditions have been found to maintain self-renewal and prevent differentiation of pluripotent cells in culture (Forristal, Wright, Hanley, Oreffo, & Houghton, 2010; Lim et al., 2011), and have also improved reprogramming efficiency of human and murine iPS cells (Bae et al., 2012; Shimada, Hashimoto, Nakada, Shigeno, & Nakamura, 2012; Yoshida, Y., Takahashi, Okita, Ichisaka, & Yamanaka, 2009).

### 1.2.5 Methods for Factor Delivery

Initial experiments used retroviral vectors to introduce transcription factors to target cells. However, this method involves the integration of not only the transcription factors to random areas of the host cell's genome, but also the integration of the viral DNA (Lowry et al., 2008; Takahashi & Yamanaka, 2006; Yu, Junying et al., 2007). As a result, other non-integrating methods of transcription factor delivery have since been implemented so that iPS cells that can be used in a clinical setting. Common methods of transcription delivery will be discussed in further detail.

#### 1.2.5.1 Viral Reprogramming

##### **Retroviruses**

As previously discussed, the first iPS cells were produced using retroviral vectors containing the transcription factors (Takahashi & Yamanaka, 2006). Retroviral viral vectors work by inserting the transcription factors into the host cell's DNA. This results in the expression of these factors, and a subsequent cascade of transcription, that leads to reprogramming the cells to a pluripotent state (Maherali & Hochedlinger, 2008). Retroviral vectors generally result in relatively high reprogramming efficiencies of between 0.01-1% (Stadtfield & Hochedlinger, 2010). Complete silencing of the transgene following reprogramming to pluripotency is an essential determinant of full reprogramming of iPS cells. Retrovirally derived iPS cells have numerous transgene integration sites and frequent incomplete silencing of even one transgene compromises the development of the iPS cell (Brambrink et al., 2008; Stadtfield & Hochedlinger, 2010). The use of retroviral vectors to produce iPS cells has been criticized, as it not only involves the permanent integration of the transcription factors, but also the viral DNA into random locations of the host cells genome. Integration may lead to insertional mutations, disrupt endogenous gene activity and can result in the reactivation of pluripotency genes after differentiation has occurred and thus leads to tumour formation (Li, M. & Izpisua Belmonte, 2012; Miura et al., 2009; Okita, Ichisaka, & Yamanaka, 2007) .

##### **Lentiviruses**

Lentiviruses are another viral vector system used for the delivery of transgenes to host cells, with the first lentiviral vectors used for human iPS cell generation (Yu, Junying et al., 2007). Lentiviral vectors have a higher cloning capacity and infection efficiency than retroviruses, due to their ability

to infect both dividing and non-dividing cells. Furthermore, the efficiency of iPS derivation using lentiviruses is comparable to that of retroviral reprogramming, with efficiencies of between 0.1-1% achieved both constitutive and inducible vector systems (Stadtfield & Hochedlinger, 2010). However, lentiviral transgenes are less effectively silenced in the resulting iPS cells (Yao et al., 2004; Yu, Junying et al., 2007). As a result, inducible lentiviral systems, where the transgenes can be turned on and off using a promoter, are preferred. Inducible lentiviruses require an inducer such as doxycycline to turn on the transcription of the transgene, enabling reprogramming of cells to occur in the presence of the inducer. Once cells are fully reprogrammed and endogenous expression of reprogramming factors is activated, the inducer may be removed from culture, thereby silencing the transgenes and resulting in a competent iPS cell (Hockemeyer et al., 2008; Markoulaki et al., 2009; Sommer et al., 2009; Yildirim, 2012).

In addition, the use of polycistronic constructs containing all necessary transcription factors for reprogramming in one vector, has led to the more efficient production of lentiviral cells. Although the use of a single polycistronic construct limits the number of transgene integration sites in the host genome and thereby reduces the risk of disruption of endogenous gene activity, there is still a risk of tumour formation after implantation (Okita et al., 2007).

### **Non-Integrating Viral Vectors**

In an attempt to limit viral integration into the host genome during reprogramming, adenoviruses have been used as an alternative vector to retroviruses. There is no evidence to suggest that this virus undergoes plasmid integration with the host genome, thereby eliminating the problem posed by the use of retroviruses (Okita, Nakagawa, Hyenjong, Ichisaka, & Yamanaka, 2008). Fully reprogrammed mouse and human iPS cells have been derived using adenoviral vectors (Fusaki, Hiroshi, Akiyo, Koichi, & Mamoru, 2009; Okita et al., 2007). However, the reprogramming efficiencies of adenoviral vectors is far lower than integrative methods, approximately 0.001% compared to 0.1-1% (Stadtfield & Hochedlinger, 2010). Additionally a high proportion of iPS cells with chromosomal abnormalities have been reported when derived using adenoviral methods (Stadtfield et al., 2008). As a result, screening of iPS clones is therefore essential to ensure they are void of viral DNA.

In 2009 another non-integrating viral vector was used to create iPS cells from human fibroblasts (Fusaki et al., 2009). The Sendai virus vector is an RNA virus that does not integrate into the genome. These viruses replicate in the form of single stranded RNA in infected cells. A high reprogramming efficiency of up to 1% was reported with the use of Sendai viral vectors (Fusaki et al., 2009). Additionally, the cells derived from this method exhibited decreased transgene activity with each division and the virus was easily removed by antibody mediated negative selection for surface

markers on the virus. The iPS cells created in this study were able to be differentiated into cells of all three germ layers both in embryoid bodies and in teratomas. However, no other more stringent tests for pluripotency were conducted to assess these cells (Fusaki et al., 2009).

#### **1.2.5.2 Non-Viral Reprogramming**

To minimise the risk of insertional mutations, and thereby allow iPS cells to be used in a clinical setting, non-integrating methods of delivering reprogramming factors have become popular. These approaches include episomal delivery and direct delivery of RNA and proteins. Unfortunately, most non-integrating methods are less efficient than retroviral or lentiviral mediated reprogramming (González, Boué, & Izpisúa Belmonte, 2011).

#### **Excisable Vectors**

The *piggyBac* transposon system has also been used to introduce reprogramming factors to cells where they are integrated into the host cells genome, however, after reprogramming the transgenes can be completely excised (Chen, Y.-T. et al., 2010; Woltjen et al., 2009). The *piggyBac* system generally involves the addition of both a donor plasmid containing the genes of interest, along with a helper plasmid that contains transposase. The transposase recognises the inverted terminal repeat sequence of the transposon and integrates the gene of interest into TTA chromosomal sites. Once reprogramming occurs the transgenes are removed through the transfection of another helper plasmid that contains transposase to remove the transposon from the genome. Reprogramming efficiencies of approximately 0.1% are expected from the transposon system (Stadtfeld & Hochedlinger, 2010). While this method results in lower reprogramming efficiency than viral methods, it is capable of delivering large gene constructs and polycistronic plasmids with a lesser effect on reprogramming efficiency than viral vectors (Chen, Y.-T. et al., 2010; Kaji et al., 2009; Patel & Yang, 2010). The laborious screening processes to confirm absence of transgene integration following transposon removal and the potential genomic toxicity caused by transposase activity has limited the use of this method of reprogramming. However, transposons have been used to generate iPS cells from numerous species including humans (Chen, Y.-T. et al., 2010; Lacoste, Berenshteyn, & Brivanlou, 2009), mouse (Kaji et al., 2009), horse (Nagy et al., 2011) and pig (Kues, Wilfried et al., 2013; Kues, W. A, Nowak-Imialek, M, Haridoss, S, & Niemann, H, 2010; Woltjen et al., 2009) .

### **Episomal Vectors - Plasmid and Minicircles**

It has become popular to use episomal vectors as an alternative to integrating or non-integrating viral vectors. Episomal vectors may be replicating or non-replicating, and have the advantage of being relatively easy to use, and their use eliminates the need for time-consuming viral production. Many studies have had success with this method, using plasmids that contain either single transcription factors, or polycistronic versions with all needed transcription factors present on the same plasmid (Chou, B.-K. et al., 2011; González et al., 2011; Kaji et al., 2009; Okita et al., 2011; Si-Tayeb et al., 2010; Yu, J. et al., 2009).

Yu, Hu et al (2009) reported the first use of a non-integrating plasmid vector derived from the Epstein-Barr virus to introduce the transcription factors OCT4, SOX2, NANOG and Lin 28 into human somatic cells. The single transfection of the episomal vectors successfully produced fully reprogrammed iPS cells. These vectors are removed naturally from the cell through division. The first iPS cells derived using this method were found to have similar morphology to ES cells, had normal karyotypes, expressed ES cell specific markers and genes, and were able to differentiate into cells of all three germ layers.

Kaji *et al* (2009) also demonstrated that it is possible to reprogram cells with a single cassette of reprogramming factors, in a plasmid that was linked to 2A peptides. This study found that the exogenous reprogramming factors can be completely removed from the cell via *Cre*-transfection. However, following *Cre* extraction, part of the vector backbone remained, causing the potential problem of insertional mutations in the resulting iPS cells, thus making them unsuitable for clinical use.

Although success has been shown for generating iPS cells using plasmid transfection, low reprogramming efficiencies are common (between 0.001-0.01%) most likely due to the large size of the plasmid vectors (Stadtfeld & Hochedlinger, 2010; Yu, Junying, Chau, Vodyanik, Jiang, & Jiang, 2011; Yu, J. et al., 2009). In an attempt to circumvent this problem, the minicircle was created. In 2010, a minicircle vector was used to reprogram adult adipose stem cells without the need for a viral vector (Jia et al., 2010). Minicircle vectors are derived from supercoiled DNA molecules that have been processed to remove the bacterial backbone, thereby producing a vector that lacks any bacterial DNA. Transfection with minicircle vectors results in higher transfection efficiencies and longer ectopic expression, than episomal plasmids, and as such minicircle vectors are an attractive option for introducing reprogramming factors to cells. The group used the four Thompson factors

(OCT4, SOX2, LIN28 and NANOG) along with a GFP reporter gene, to reprogram adult human adipose stem cells. Cells produced were similar to human embryonic stem cells in morphology, were able to differentiate into embryoid bodies that expressed markers from all three germ lineages, and were able to form teratomas in nude mice. Southern blot analysis of these colonies showed no integration of the vector into the host genome. However this type of reprogramming only resulted in an reprogramming efficiency of approximately 0.005% which is relatively low compared to viral methods (between 0.1-1%) (~0.01% Okita et al., 2007; Stadtfeld & Hochedlinger, 2010; Wernig, Meissner, et al., 2008). Conveniently, the minicircle vector used in these experiments is commercially available for purchase, making it an easy and convenient method for producing human iPS cells, and may be used to produce iPS cells from other species (Bioscience, 2013)

### **Reprogramming with mRNA**

Although newer methods of generating iPS cells have been directed at using non-viral vectors for reprogramming, and hence reducing the risk of integration and genetic mutation, they have still used DNA based constructs. As a result there is still a risk of integration into the host genome and cell lines need to be thoroughly examined for integrations before their potential use. The use of RNA as a method of reprogramming, offers an alternative to eliminate this problem.

The use of RNA to generate iPS cells has a varying success rate. Yakubov, Rechavi et al. (2010) and Plews et al. (2010) were able to create iPS cell like colonies using modified RNA molecules with polyA tails. The success of reprogramming of these cells is still in question with few stringent tests applied to assay the derived colonies. In Addition, some iPS colonies exhibited arrested or slow growth.

Warren, L. et al. (2010) developed a system that claims to efficiently generate human iPS cells using the direct delivery of synthetic mRNA molecules by a cationic vehicle. The efficiency rate of generation of these cells was reported to be far higher than even viral systems, with up to 2% of cells reprogrammed in a relatively short period of time (17 days). This system involved the development of *in vitro* transcribed RNAs for reprogramming factors (OSKM and LIN28). These mRNA molecules were modified, through incorporation of ribonucleoside base substitution of 5-methylcytidine for cytidine and pseudouridine, in order to overcome endogenous antiviral cell defences and thereby increase efficiency of transcription. This system is appealing due to the commercial availability of the modified mRNA molecules, along with specialised media needed for reprogramming success. However, this method is time consuming and laborious with transfection of mRNA molecules needed daily for two weeks. However, in 2012, the same group developed a method to accelerate

the mRNA reprogramming process whilst eliminating the need for a feeder layer (Warren, Luigi et al., 2012).

### **Protein Mediated Transfection**

Another alternative to using DNA mediated reprogramming is to deliver the reprogramming factors directly to the cells as proteins. There has been success with this approach by the use of purified recombinant proteins (Kim, D. et al., 2009; Zhou, H. et al., 2009). Proteins for reprogramming factors can be bound to a cell penetrating peptide (CPP), containing a high proportion of basic amino acids (Arg, Lys) and as such are able to be transported through the cell membrane (Kim, D. et al., 2009). Alternatively, proteins with a poly-arginine tail have been used to successfully generate mouse iPS cells (Zhou, H. et al., 2009). Unfortunately, both of these methods of protein delivery are highly inefficient in producing iPS cells compared to viral transfection (approximately 0.0001% compared to 0.1-1% respectively) (Stadtfield et al., 2008). Additionally, recombinant proteins are difficult to purify in the large amounts required for reprogramming, and this makes them difficult to produce and use in a standard laboratory (González et al., 2011).

### **Chemical Reprogramming- Small Molecules**

The use of small molecules for improving reprogramming efficiency has been well documented, as has the use of small molecules in chemical reprogramming. Small molecules are naturally occurring, or synthetic, chemicals that act on molecular pathways associated with reprogramming. As such, small molecules have the potential to replace traditional transcription factors through the activation or repression of pathways normally affected by the addition of transcription factors. The use of small molecules is particularly appealing due to their ability to replace oncogenes c-MYC and KLF4 thereby limiting the risk of tumorigenesis (Efe & Ding, 2011; O'Doherty, Greiser, & Wang, 2013). The use of small molecules for reprogramming has the potential to replace all traditional reprogramming factors, thereby alleviating the concerns associated with genetic integration of the factors and their downstream effects. However, this is yet to be demonstrated successfully. (O'Doherty et al., 2013). Signalling pathways that may be targeted by small molecules are discussed below.

#### **1.2.6 Signalling Pathways in Reprogramming**

Although early experiments used a defined set of transcription factors to reprogram somatic cells to a pluripotent state, little was actually known about the molecular pathways and cellular mechanisms involved in the reprogramming process. The identification of signalling pathways including LIF-JAK-

STAT, BMP-SMAD, WNT- $\beta$ -catenin, TGF $\beta$  and MEK/ERK has provided insight into the mechanisms of cellular reprogramming.

### **LIF-JAK-STAT**

The JAK-STAT pathway has been shown to be important in mouse embryonic stem (ES) cells for self-renewal, and is activated by leukaemia inhibitory factor (LIF). The heteromeric LIF complex consists of gp130 and the LIF receptor, Janus Kinase (JAK) is a tyrosine kinase which constitutively binds to the intercellular domain of the LIF receptor, initiating phosphorylation of the gp130 region, which, in turn recruits signal transducers and transcription activators STAT1 and STAT3. STAT proteins are then phosphorylated by JAK mediated phosphorylation where they form homodimers, and act as transcription factors in the nucleus (Auernhammer & Melmed, 2000; Okita & Yamanaka, 2006; Sumer, Liu, & Verma, 2010). STAT3 has been found to be important for the maintenance of pluripotency of embryonic stem cells (Boeuf, Hauss, Graeve, Baran, & Kedinger, 1997; Niwa, Burdon, Chambers, & Smith, 1998) and works by targeting genes involved in pluripotency, including c-MYC (Cartwright et al., 2005). However, LIF alone is not sufficient to result in ES cell proliferation and pluripotency maintenance, instead factors including those found in serum are required (Ying, Q., Nichols, Chambers, & Smith, 2003). Unlike its role in pluripotency of mouse ES cells, LIF does not promote self-renewal or maintain pluripotency in human or primate ES cells (Humphrey et al., 2004; Sumi, Fujimoto, Nakatsuji, & Suemori, 2004).

### **BMP-SMAD Pathway**

The BMP-SMAD pathway is activated by SMAD4 binding to its plasma membrane receptor which facilitates the phosphorylation of SMAD proteins which, in turn, translocate to the nucleus to act as transcription factors. It is thought that BMP4 and LIF work together to maintain pluripotency in mouse ES cells, with self-renewal achieved by a balance of LIF and BMP4 (Ying, Q. et al., 2003).

### **TGF $\beta$ -Activin**

The TGF $\beta$  (transforming growth factor  $\beta$ )-Activin pathway is activated by BMP ligands binding to type II tyrosine kinase receptors. This in turn promotes the phosphorylation of SMAD 2/3 which then initiates a cascade of regulatory genes including NANOG. These regulatory genes are involved in self-renewal in mouse ES cells (Heldin, Miyazono, & ten Dijke, 1997; Ichida et al., 2009).

Inhibition of TGF- $\beta$  also facilitates the mesenchymal to epithelial transition (MET) pathway. It is essential that cells undergo an epithelial to mesenchymal transition (EMT) for the correct differentiation of cells to occur during early embryonic development (Chaffer et al., 2007). The

transition of mesenchymal to epithelial lineage (MET) has been shown to result in the reprogramming of somatic fibroblasts to iPS cells (Li, R. et al., 2010).

### **Wnt- $\beta$ -catenin**

$\beta$ -catenin is a cytoplasmic protein that functions as a cell adhesion molecule and also as an intracellular signalling molecule in the Wnt signalling pathway. The absence of Wnt activation results in the phosphorylation of  $\beta$ -catenin which is then degraded keeping the cytoplasmic levels of  $\beta$ -catenin low. Wnt binds to its receptors of the Frizzled family of cell surface receptors (Sato, Meijer, Skaltsounis, Greengard, & Brivanlou, 2004). The mechanisms that follow the binding of Wnt are poorly understood, but are believed to result in the activation of the Dishevelled family of proteins. This activation results in the halt in  $\beta$ -catenin degradation, allowing it to accumulate in the cell.  $\beta$ -catenin is able to regulate and maintain pluripotency through its association with transcription factors lymphoid enhancer factor (LEF) and T-cell factor (TCF) (Sumer et al., 2010).

### **PI3 Kinase**

Phosphatidylinositol 3 (PI3) kinases are lipid kinases involved in the phosphorylation of inositol phospholipids. Activation of class 1A PI3 kinases occurs by binding of numerous receptors including FGF and insulin to tyrosine receptors. This induces cell proliferation and suppresses apoptosis (Okita & Yamanaka, 2006; Sumer et al., 2010). Additionally, the inhibition of this pathway in mouse and human ES cells induces differentiation, even in the presence of LIF (Paling, Wheadon, Bone, & Welham, 2004).

### **ERK/MAPK**

The ERK/MAPK (extracellular signal-regulated kinase/mitogen activated protein kinase) pathway is activated by the binding of growth factors, including EGF and FGF, to cell surface receptors. This results in the activation of MAPK by phosphorylation which regulates transcription factors such as c-MYC (Binétruy, Heasley, Bost, Caron, & Aouadi, 2007). Conversely, MEK/ERK signalling pathways promote differentiation and suppress self-renewal in mouse ES cells (Okita & Yamanaka, 2006). Inhibition of this pathway enhances reprogramming by promoting growth of reprogrammed iPS cells (Shi, Yan et al., 2008).

### **Chromatin State**

Epigenetic mechanisms, including modifications to DNA chromatin packaging, play a key role in the reprogramming of somatic cells to pluripotency, through their effects on gene transcription. (Gan, Yoshida, McDonald, & Owens, 2007; Zuccotti, Piccinelli, Giorgi Rossi, Garagna, & Redi, 1995). DNA methylation is one of the ways cells regulate transcription of genes. Un-methylated genes undergo transcription, while methylation of genes halts their transcription (Altun, Loring, & Laurent, 2010). During embryonic development, DNA methylation declines, allowing for the transcription of genes associated with lineage differentiation. Histone deacetylation is regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs) through the addition or removal of histones respectively. Embryonic stem cells have a transcriptionally permissive state which is associated with histone deacetylation and DNA methylation (Azuara et al., 2006). Inhibition of deacetylation of histones can facilitate the return of the genome to a transcriptionally permissive state (Zhao, J. et al., 2010). Treatment with histone deacetylase inhibitors including valproic acid, 5'-azacytidine and butyrate, has been shown to significantly improve reprogramming efficiency of iPS cells (Huangfu et al., 2008; Liang, Taranova, Xia, & Zhang, 2010; Ware et al., 2009).

### **1.2.7 Enhancing Pluripotency by Pathway Targeting**

The discovery of small molecules and growth factors that target these reprogramming and signalling pathways, has allowed these pathways to be targeted to enhance reprogramming.

The MEK/ERK pathway is believed to activate lineage committed differentiation of iPS cells (Barbosa, Fernandes, Dias, Diogo, & Cabral, 2012; Kunath et al., 2007; Li, J. et al., 2007; Zhang, X., Peterson, Liu, McMahon, & Ohba, 2013). It is also thought to regulate the activity of c-MYC along with other transcription factors including NANOG (Wu, B., Li, W., Wang, L., Liu, Z.-h., & Zhao, X.-y., 2013). Inhibition of the MEK/ERK pathway enhances cellular reprogramming and self-renewal. Small molecules that inhibit the MEK/ERK pathway include PD0325901, PD98059 and PD184352 and the ERK inhibitor PD184352 enhances (Li, W. et al., 2009; Ying, Q.-L. et al., 2008). The effects of these molecules are greatest when supplemented in the later stages of reprogramming, to promote growth of iPS cells (Shi, Yan et al., 2008).

Inhibition of TGF- $\beta$  with small molecules such as SB431542 and AM-83-01 has been shown to result in increased reprogramming efficiency (Ichida et al., 2009; Li, W. et al., 2009; Maherali & Hochedlinger, 2009). Additionally the supplementation of AM-83-01 has been shown to result in cellular reprogramming with transduction of OCT4 only (Yuan, Xu. et al., 2011). Wnt pathway

activation by small molecules including 6-bromoindirubin-3'-oxime (BIO) (Sato et al., 2004), and CHIR99021 (Davidson et al., 2012) has also been shown to enhance iPS reprogramming in mice and humans.

Other small molecules act by affecting the epigenetics of somatic cells to enhance plasticity and improve reprogramming. The regulation of expression and suppression of genes is primarily determined by the structure of chromatin, in particular, the acetylation or deacetylation of lysine in the tails of the histone cores. Acetylation and deacetylation is controlled by histone deacetylases (HDACs) and histone acetyltransferases (HATs) respectively (Marks, Richon, Miller, & Kelly, 2004; Monneret, 2005).

HDAC inhibitor butyrate has been shown to decrease the number of partially reprogrammed iPS cells and increase the number of fully reprogrammed iPS (Liang et al., 2010). Valproic acid (VPA), also an HDAC inhibitor, is generally considered to be the most potent HDAC inhibitor compared to similar small molecules including trichostatin A (TSA). Studies have shown the supplementation of VPA in iPS cultures is capable of improving reprogramming efficiency by more than 100 fold, (Kim, Y., Ahn, K., Kim, M., & Shim, H., 2011). 5'-azacytidine, a DNA methyltransferase inhibitor, have been shown to increase efficiency of reprogramming of 10-fold (Huangfu et al., 2008; Kim, Y. et al., 2011).

Additionally the inhibition or activation of multiple pathways involved in the reprogramming process can have synergistic effects in enhancing reprogramming. The activation of the Wnt pathway and inhibition of the ERK/MAPK pathway by small molecule combination CHIR99021 (Wnt) and PD0325901 (ERK/MAPK) known as 2i media, has been used to successfully derive iPS cells from numerous species. These species include those that have previously proven difficult to produce fully reprogrammed iPS cells from, such as rat (Buehr et al., 2008), pig (Esteban et al., 2009) and bovine (Huang, B. et al., 2011). The combination of MEK inhibitor PD0325901 and TGF- $\beta$  inhibitor SB431542 has been shown to considerably improve the generation of human iPS cells. Reprogramming efficiencies were improved more than 100 fold, resulting in efficiencies of over 1% in iPS cultures supplemented with this combination of small molecules (Lin et al., 2009). The combination of PD032901, SB431542 and HDAC inhibitor sodium butyrate (NaB) facilitates more efficient reprogramming of human iPS cells, through increasing reprogramming kinetics and significantly decreasing the numbers of partially reprogrammed colonies (Zhang, Zhonghui et al., 2011).

Figure 1-2 shows signalling pathways involved in cellular reprogramming and the small molecule inhibitors and chromatin modifiers that influence these pathways to enhance reprogramming (Sumer et al., 2010).

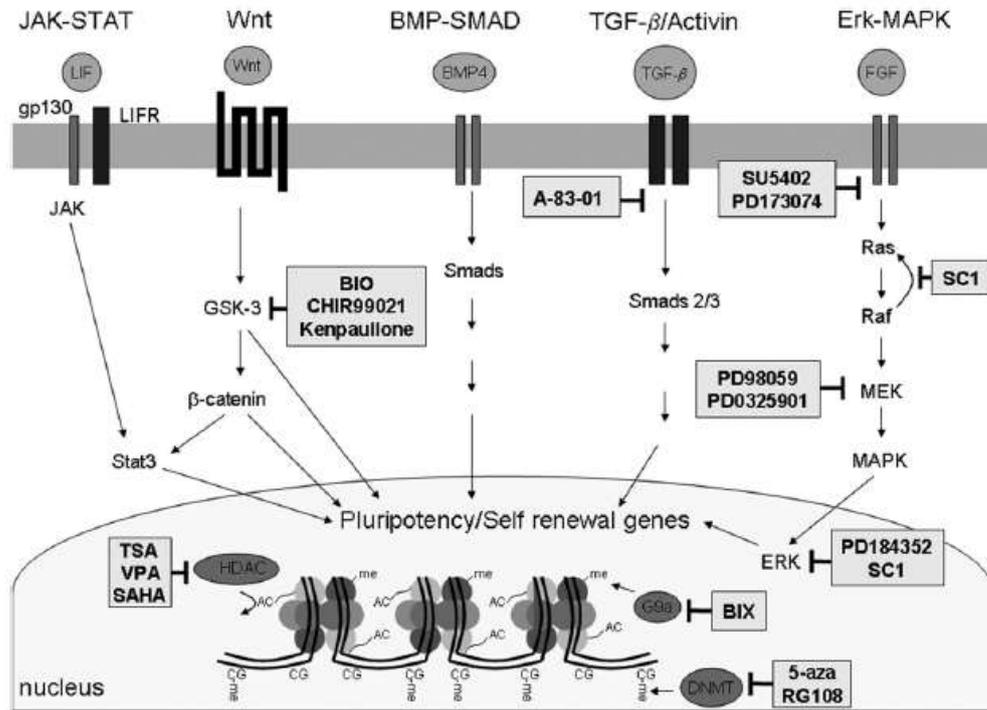


Figure 1-2. Signalling pathways and the influence of small molecules and growth factors on promoting pathways involved in reprogramming (Sumer et al., 2010).

### 1.2.8 Evaluation of iPS Cells

Since the first generation of iPS cells, there has been much debate as to how similar iPS cells are to embryonic stem cells, and whether they are in fact truly pluripotent. Several criteria have been proposed to determine whether iPS cells are in fact fully reprogrammed. iPS cells must express unique pluripotent features, including ES cell like morphology, and molecular and function attributes associated with pluripotency, such as the ability to differentiate into cell types from all three germ lineages (Yamanaka, S., 2009). At a minimum, cells must display all morphological attributes of pluripotent stem cells, including unlimited self-renewal, express key pluripotency genes, show transgene silencing, and have proof of pluripotent differentiation at the highest feasible stringency (Jaenisch & Young, 2008).

#### 1.2.8.1 Morphology

Cells must appear similar in morphology to ES cells from the same species; that is they must produce colonies with tight morphology, well-defined borders and prominent nucleoli. It is also essential that these cells demonstrate unlimited self-renewal (Maherali & Hochedlinger, 2008).

### **1.2.8.2 Molecular Attributes**

Fully reprogrammed iPS cells should display gene expression profiles indistinguishable from that of ES cells. iPS cells must have protein expression levels of the key pluripotency factors (OCT4, SOX2, NANOG) similar to ES cells, as well as ES cell specific surface antigen expression (e.g. SSEA and Tra). It has been determined that both OCT4 and NANOG are involved in the maintenance of pluripotency and self-renewal of cells. As a result, selection of pluripotent cells expressing high levels of OCT4 or NANOG, has led to an increase in quality of pluripotent cells. Selection of high quality iPS cells has been shown to result in generation of chimeric mice where the iPS cell genome is able to contribute to the germline (Maherali & Hochedlinger, 2008; Okita et al., 2007; Wernig, Meissner, et al., 2008). Genuine iPS cells must not express any of the exogenous factors delivered to the cell during reprogramming. After a certain period of time, the cells own endogenous factors should be expressed instead to maintain pluripotency. This can be assessed by DNA de-methylation of the promoters of pluripotency genes (Maherali & Hochedlinger, 2008).

### **1.2.8.3 Genome Integrity**

Genomic integrity is essential for producing high-quality iPS cells. This is especially important for iPS cells that are potentially to be used in a clinical setting, in order to avoid alterations that may result in diseases such as cancer.

Stem cells that are grown in *in vitro* culture systems are exposed to high levels of selection pressure. This can result in random genomic alterations of varying levels (Ben-David, Uri . & Benvenisty, 2012; Martins-Taylor & Xu, 2012). It is believed that different culture conditions including the use of feeder layers and serum type, can affect the occurrence of mutations. However, no culture technique is able to eliminate the occurrence of genomic alterations completely (Ben-David, U. & Benvenisty, 2011). The increased risk of tumorigenesis due to genomic integration in iPS cells hinders their potential use in clinical applications. Additionally, the use of iPS cells in general research and disease modelling may also be affected by genomic mutations, as these cells may not display normal development or functionality. Additionally the risk of insertional mutations in iPS cells derived from retroviral and lentiviral vectors, obviously poses a threat for the use of iPS cells in clinical practice. Although the use of non-integrating and non-viral vectors have become more popular ways of deriving iPS cells, it is still important that iPS cells be assessed for insertional mutations, if the iPS

cells were derived using DNA delivery (Amabile & Meissner, 2009). It is also important to consistently monitor iPS cells after passages, as it has been shown that continuous passaging of iPS cells leads to more frequent chromosomal abnormalities (Aasen et al., 2008). Therefore, an important measure of iPS cell quality is a normal karyotype.

Differences in genome profiles of iPS cell and ES cell lines have been well documented. It has been hypothesised, that the micro-environment of *in vitro* culture systems can have a large impact on the production and viability of iPS cells (Newman & Cooper, 2010). Recently it has become common practice to conduct whole transcriptome profiling of iPS cell lines, especially in the field of human iPS generation. This method allows comparisons to be made between different iPS cells lines and between iPS and ES cell lines, as small as point mutations. This allows for improved characterization of different cell populations (Amabile & Meissner, 2009). The assessment of similarities between iPS and ES cell lines will become more essential in the future, for the safe use of iPS cells in clinical applications (Gore et al., 2011; Newman & Cooper, 2010). However, this approach is costly, and it is limited, in that it can only be used to compare cell types from human and murine species at this point in time (Ben-David, Uri . & Benvenisty, 2012)

#### **1.2.8.4 Epigenome Modifications**

The reprogramming of somatic cells to a state of pluripotency is due to epigenetic modifications, not genetic transformation. As such, the regulation of chromatin structure and epigenetic modifications play an essential role in the evaluation of iPS cells. Naturally, the epigenetics of somatic cells and pluripotent cells differ dramatically. However, iPS cells should be epigenetically similar to embryonic stem cells (ES cells), and DNA de-methylation should be seen at the promoters of genes associated with pluripotency (Amabile & Meissner, 2009; Maherali & Hochedlinger, 2008). The use of whole-genome methylation sequencing has allowed for the comparison of methylation profiles between ES cell lines and iPS cell lines, to determine how similar these pluripotent stem cells actually are. Studies have claimed to produce human iPS cell lines that were almost identical to human ES cell lines in regard to epigenetic modifications (Guenther et al., 2010). Conversely, numerous studies have identified significant differences in the epigenome of iPS cell lines and pluripotent potential of these cell lines when compared to ES cells (Akiko et al., 2009; Jie et al., 2009; Kim, K. et al., 2010; Lister et al., 2011). Additionally, it is also believed that iPS cells possess somatic cell memory, where they have not undergone complete de-methylation. Instead, some iPS cells still exhibit methylation of some genes from the original somatic cell type (Kim, K. et al., 2010; Vaskova, Stekleneva,

Medvedev, & Zakian, 2013). Somatic cell memory may present problems for the use of iPS cells as the differentiation of these cells may be preferential toward the original cell lineage (Bar-Nur et al., 2011). Additionally, there is a potential for cells to differentiate back to their original cell type following directed differentiation (Hochedlinger & Plath, 2009). This may result in the formation of tumours when cells are used in clinical applications. The assessment of the epigenome of iPS cells will therefore become increasingly important to ensure safe use of iPS cells in clinical applications.

#### **1.2.8.5 Functional Attributes**

Functionally, iPS cells should behave as, and share functional attributes of, embryonic stem cells. In a test of pluripotent ability, iPS cells must be able to demonstrate the ability to differentiate into all three lineages of the embryonic germ layers (endoderm, mesoderm, and ectoderm). This measure of pluripotency may be tested in numerous ways. In order of increasing stringency these include; the expression of alkaline phosphatase and pluripotency markers, *in vitro* differentiation into embryoid bodies; *in vivo* differentiation into teratomas in immune-compromised mice; chimera contribution (not ethically possible for human cells); germline transmission and finally, tetraploid complementation (Jaenisch & Young, 2008; Kang, Lan. & Gao, 2012; Yamanaka, S., 2009).

Alkaline phosphatase activity and the expression of pluripotency markers including *POU5F1* (OCT4), *SOX2* and *NANOG*, are less stringent test for pluripotency, however, it is essential that pluripotent stem cells exhibit these attributes (Feng, C., Jia, & Zhao, 2013; Kang, Lan. & Gao, 2012). A slightly more stringent test of pluripotency is the ability to differentiate iPS cells into embryoid bodies that express markers from all three germ lineages. Embryoid bodies are aggregates of pluripotent stem cells that are able to differentiate into cell types from all three germ lineages (Itskovitz-Eldor et al., 2000). Although it is not feasible to differentiate iPS cells into all different cell types *in vitro*, the ability to differentiate cells into cells representative of all three germ lineages provides evidence of pluripotency. Formation of embryoid bodies is routinely carried out as test for pluripotent potential of putative iPS cells, though it is not considered to be thorough enough to predict full reprogramming of cells (Kang, Lan. & Gao, 2012) .

The formation of teratomas is considered to be the minimum requirement for evaluating pluripotency of human iPS cells. In this assay, iPS cells are injected under the skin of immunodeficient mice where they should develop into teratomas that contain cell types from all three germ layers (Kang, Lan. & Gao, 2012). However, the formation of teratomas does not

necessarily guarantee complete reprogramming of cells, with reports that cells that are capable of forming teratomas are not always able to produce chimeric offspring (Yamanaka, S., 2009).

The production of chimeras and subsequent germline transmission with iPS cells is a more rigorous test of pluripotency. This assay involves the injection of iPS cells into a host blastocyst. This results in the production of offspring with cells from both the host blastocysts genotype and from the iPS cell (Kang, Lan. & Gao, 2012). However, chimera formation is not appropriate for assaying human iPS cells due to the formation of a human embryo (Okita et al., 2008; Wernig, Lengner, et al., 2008).

Tetraploid complementation assay first involves the production of a tetraploid embryo by the fusion of two early stage embryos (two cell stage). The tetraploid embryo develops normally and is able to implant in the uterus. Tetraploid cells are able to produce the extra-embryonic tissue, but do not develop into a foetus. The iPS cell to be assayed is then fused with the tetraploid embryo and should result in an embryo that develops normally, with the foetus derived exclusively from the iPS cell, and the extra embryonic tissue derived from the tetraploid embryo (Feng, C. et al., 2013; Kang, Lan. & Gao, 2012). Tetraploid complementation that results in viable offspring deems the iPS cells used as being equivalent to embryonic stem cells (Kang, Lan. & Gao, 2012; Kang, Lan, Wang, Zhang, Kou, & Gao, 2009; Roelen, 2011; Yamanaka, S., 2009). This was shown first in 2009 when through the process of tetraploid complementation mouse iPS cells were used to produce full term offspring. This suggested that these mouse iPS cells were fully pluripotent (Kang, Lan et al., 2009).

### **1.2.9 Induced Pluripotent Stem Cells from Livestock Species**

Since the first reprogramming experiments were conducted on murine and then human cells, iPS cell have been generated from numerous species, with differing levels of success. The potential for iPS cells generated from livestock species to be used in transgenic breeding, and for large animal disease models, has long been recognised. Many attempts to isolate and culture embryonic stem cells from livestock species, including bovine and ovine species, have proven difficult (Lu, Mumaw, West, & Stice, 2012). Additionally attempts to generate livestock iPS cells have also had limited success (Huang, B. et al., 2011; Lu et al., 2012; Zhao, Yuncheng et al., 2011).

Livestock iPS cells have arguably been most successfully derived from porcine species. A number of groups have reported the successful generation of porcine iPS cells (Esteban et al., 2009; Ezashi, Matsuyama, Telugu, & Roberts, 2011; Wu et al., 2009). However, to date, there have been no

reports of complete transgene silencing in porcine iPS cells, indicating these cells do not meet the stringent rules for assessing fully reprogrammed iPS. This has resulted in limitations for the use of these cells in clinical applications.

To date, there have been no authentic embryonic stem cell lines established in sheep, therefore the production of iPS cells offers a possible substitute (Lu et al., 2012). iPS cells have been derived from sheep, because of an interest in these cells for transgenic breeding and genetic modification. Ovine iPS cells were first produced using lentiviral transduction of a combination of six transcription factors (OCT4 (POU5F1), SOX2, KLF4, c-MYC, NANOG and LIN28) (Bao et al., 2011). However, although these putative iPS cells had ES cell like morphology and expressed traits of pluripotent cells, they were not able to be expanded in culture. Further experiments have shown that putative ovine iPS cells can be produced using drug-inducible lentiviral transduction of four factors, OCT4, SOX2, KLF4 and c-MYC. However, these ovine iPS cells did not show transgene silencing even in the absence of doxycycline activation (Li, Yang. et al., 2011; Liu, J. et al., 2012). The first reports of livestock iPS cells that undergo transgene silencing were made in ovine iPS cells, with Liu, J. et al. (2012) reporting transgene silencing was possible following numerous passages in long term culture.

The production of bovine iPS cells has been especially difficult. From the few reports to date, claiming to have produced bovine iPS cells, no reproducible protocol has been developed.

The first report claiming to have produced bovine iPS cells used lentiviral transduction of four factors, OCT4, SOX2, KLF4 and c-MYC, in bovine adult dermal fibroblasts. Interestingly this report claimed that NANOG was essential for the derivation of bovine iPS cells. However this claim has yet to be substantiated as other reports claim to have produced bovine iPS cells in the absence of exogenous NANOG (Cao et al., 2012). Although these cells showed characteristics of pluripotent cells and were able to be expanded in culture, transgene silencing in reprogrammed cells was not achieved. Non-viral bovine iPS cell generation has also been reported. This study used a polycistronic vector to deliver bovine transcripts for OCT4, SOX2, KLF4 and c-MYC to bovine fibroblasts (Huang, B. et al., 2011). These cells displayed ES cell like morphology and expressed pluripotent markers and proteins, but were unable to be expanded in culture.

Cao et al. (2012) have also reported the production of bovine iPS cells using lentiviral transduction. The iPS cells derived in this experiment showed typical ES cell like morphology, and expressed markers and proteins of pluripotency as well as being able to differentiate into cell types for all three germ layers both *in vivo* and *in vitro*. Additionally, it was claimed that these cells were able to be differentiated into female germ cells, giving hope that these cells may be used in transgenic breeding

and the production of genetically modified animals (Cao et al., 2012). There is speculation as to the extent to which bovine iPS cells have been reprogrammed, as characteristics necessary for characterisation as fully reprogrammed iPS cells, such as transgene silencing have yet to be demonstrated (Cao et al., 2012; Huang, B. et al., 2011; Sumer, Liu, Malaver-Ortega, et al., 2011).

iPS cell have also been generated from equine fibroblasts using non-viral *piggyBac* transposon delivery of factors OCT4, SOX2, KLF4 and c-MYC and supplementation with a cocktail of small molecules (Nagy et al., 2011). These cells displayed pluripotent stem cell characteristics including the differentiation into cell types of all three germ layers. However, they did not undergo transgene silencing. Further studies have shown the derivation of equine fibroblasts is possible without c-MYC. These cells also showed pluripotent characteristics but also failed to undergo transgene silencing (Khodadad et al., 2012).

Despite numerous attempts to produce livestock iPS cells, there has been little success in the production of a reproducible protocol to produce these cells. Culture conditions needed for the generation of livestock iPS cells from different species have yet to be defined, and it is currently unclear as to why there is such a variation in the success of producing iPS cells between different groups. The lack of transgene silencing, even in cells that are otherwise fully reprogrammed, has resulted in limitation of the use of these cells in clinical applications such as transgenic breeding. It is unclear as to why livestock cells appear to be more difficult to reprogram than mouse or human cells. It is hoped that improvements to iPS cell reprogramming technologies, and understanding of the molecular mechanisms involved in cellular reprogramming may enhance future efforts of reprogramming livestock cells.

### **1.3 Stem Cells from Adult Tissue**

Adult stem cells are thought to be undifferentiated cells found among the differentiated cell types, of tissues and organs, in a space that is called the 'stem cell niche'. These cells are generally clonal cells (single cells) that are able to self-renew and divide to produce daughter cells that differentiate into one or more specific cell types, for tissue renewal and repair. The differentiation potential of stem cells derived from adult tissue depends on the stem cell type. For example, hematopoietic stem cells are able to differentiate into all blood cell type's i.e. red, white, plasma and platelet blood cells, making them multipotent stem cells. Similarly, mesenchymal stem cells are also multipotent, and are able to generate bone, cartilage, fat and other blood supporting cells. In contrast

spermatogonial stem cells found in the testis are only able to differentiate in to the progenitors for sperm cells (Roelen, 2011). Stem cells have been found in almost every tissue of the body including the brain, heart, muscle, adipose tissue, skin, teeth and testes. However, adult stem cells are found in very small populations in each tissue, and culture of these cells *in vitro* is often difficult. One promising source of adult stem cells is adipose tissue with large numbers of adipose derived stem cells able to be derived from fat samples. The application of adult stem cells in research and for clinical purposes is a popular option when compared to embryonic stem cells (Bunnell, Flaat, Gagliardi, Patel, & Ripoll, 2008).

Spermatogonial stem cells (SSCs) are of particular interest to the field of reproduction. The use of these cells for improving reproductive technologies in livestock offers huge possibilities including the use of these cells in transgenic and artificial breeding applications (Lu et al., 2012). However, far less is known about the germline stem cells of livestock species, in particular bovine species, than in humans or mice. As a result it is important to study these cells in an attempt to better understand their regulation and growth and to be able to manipulate these cells in culture for future reproductive technologies (Aponte, Pedro et al., 2008).

### **1.3.1 Spermatogonial Stem Cells**

Spermatogonial stem cells (SSCs) are adult stem cells located in the testis and provide the basis of male sexual reproduction through the continual production of spermatozoa throughout the lifetime of the male (Aponte, Pedro, van Bragt, de Rooij, & van Pelt, 2005). In non-primate mammals SSCs are clonal cells located on the basal membrane of the seminiferous tubules. They make up a very small proportion of the total cell population of the male testis, and like all stem cells are characterized by their ability to both undergo self-renewal and differentiation into daughter cells. The processes of self-renewal and differentiation result in the production of billions of spermatozoa daily after sexual maturity is reached (Aponte, Pedro et al., 2008; Aponte, Pedro et al., 2005).

Spermatogonial stem cells (SSCs) arise in the testis from the differentiation of the primordial germ cells (PGCs) set aside in the early stages of embryo development. PGCs migrate via the hind gut to the genital ridges, during which time they proliferate and then colonise the genital ridges (Tam & Snow, 1981). Once these cells have colonized the genital ridges they become surrounded by tubules formed by precursor Sertoli cells. The cells are now known as gonocytes, and become arrested in the cell cycle until sexual maturity is reached. During puberty the gonocytes undergo proliferation and create the SSC population located on the basement membrane. Spermatogenesis is described using

the  $A_s$  model outlined in Figure 1-3. SSCs located on the basement membrane are referred to as type A-single Spermatogonia ( $A_s$ ). As spermatogonia are able to self-renew by division, to produce two new  $A_s$  spermatogonia, or are capable of division and differentiation into paired spermatogonia ( $A_{pr}$ ). Spermatogonia  $A_{pr}$  cells are then further able to divide to form chains of A-aligned ( $A_{al}$ ) spermatogonia, which then undergo differentiation to form type  $A_1$  spermatogonia. For most mammals, this process is then followed by six cellular divisions to form  $A_2$ ,  $A_3$ ,  $A_4$ , Intermediate ( $In$ ) and type B spermatogonia. Type B spermatogonia undergo division to produce spermatocytes, which undergo further division to produce haploid spermatids. Following differentiation, spermatozoa are released into the lumen of the seminiferous tubule. Type  $A_s$ ,  $A_{pr}$  and  $A_{al}$  spermatogonia are known as undifferentiated spermatogonia, while  $A_1$ ,  $A_2$ ,  $A_3$ ,  $A_4$ ,  $In$  and B spermatogonia are considered to be differentiated (Aponte, Pedro et al., 2005; De Rooij & Russell, 2000; Yoshida, S., 2010).

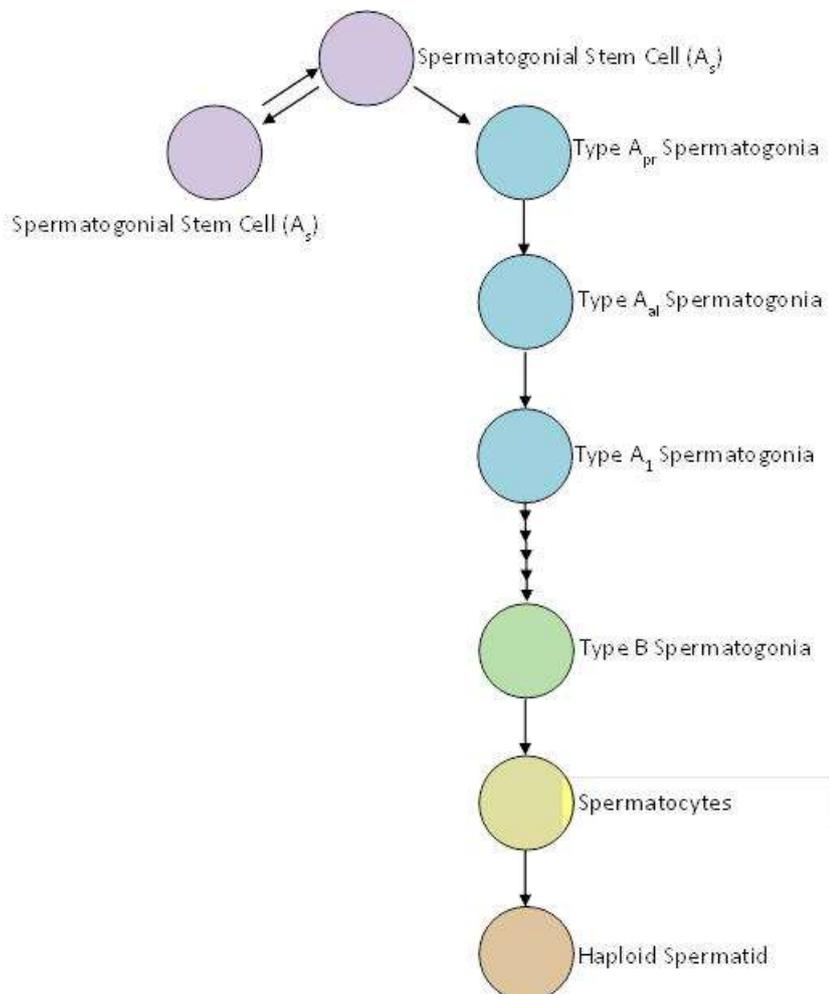
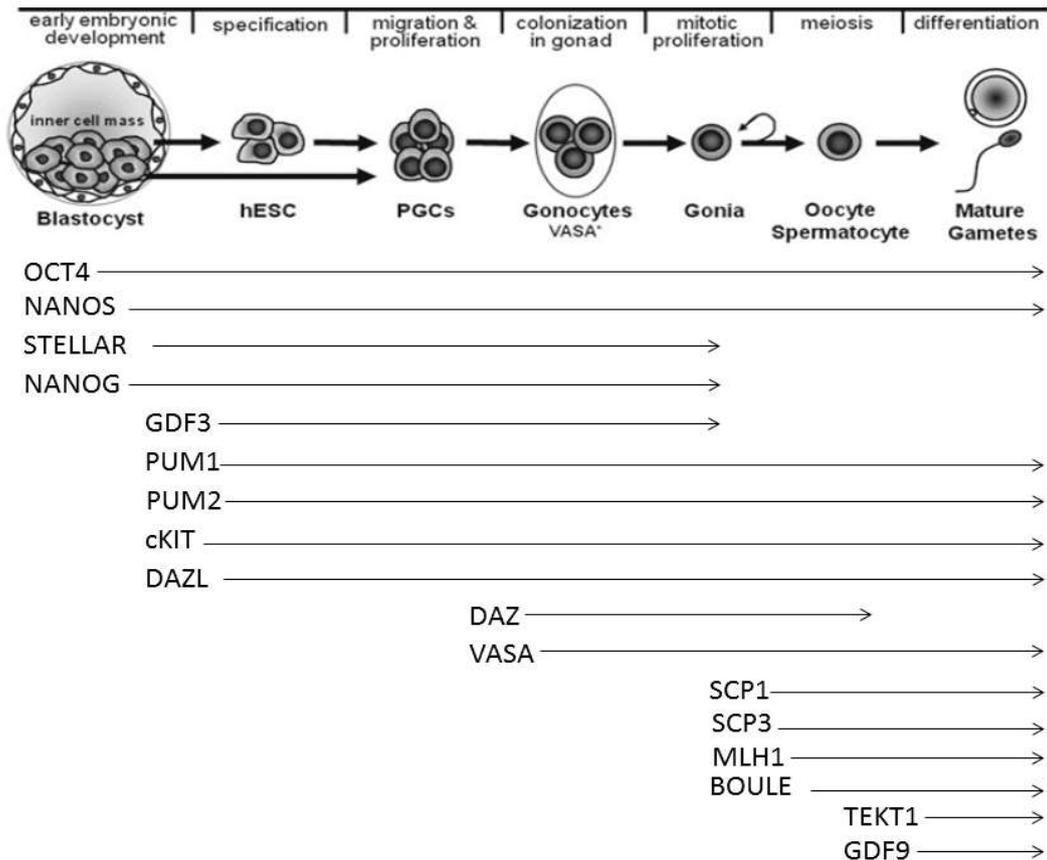


Figure 1-3: Basic schematic of  $A_s$  model of spermatogenesis.

The study of spermatogonial stem cells offers many applications including preservation of species, overcoming fertility problems, and treating diseases of the testis, such as cancer. There is also significant interest in the study of SSCs in livestock species as a method of improving genetics in livestock. However, little is actually known about SSCs and spermatogenesis in livestock species, especially in bovine species (Brinster, R. & Zimmermann, J., 1994).

### **1.3.2 Characterisation and Molecular Markers of Male Germ Cells**

Currently, the process of SSC self-renewal, spermatogenesis and molecular characteristics of undifferentiated spermatogonia, is not completely understood. This is especially true for the bovine. To be able to more closely study livestock SSCs, or to potentially use SSCs in clinical practice, it is essential that we are able to isolate a pure population of these cells and be able to characterise and maintain SSCs in culture. To achieve this, it is essential to first understand the molecular profiles and mechanisms of these unique cells (Aponte, Pedro et al., 2008; Hou & Singh, 2008). As germline cells differentiate toward the production of gametes, they exhibit different molecular profiles that allow for their identification and potential to separate cell types based on surface markers. The molecular profiles exhibited by human stem cells and germline cells throughout development are outlined in Figure 1-4. However, less is known about the expression of molecular profiles during livestock gamete development.



**Figure 1-4: Molecular markers expressed by human stem cells and germ cells at different stages of differentiation, established by *in vitro* and *in vivo* studies. The gene name is indicated on the left of the arrow, with the temporal expression of the gene indicated by the length of the arrow (Figure adapted from (Roode et al., 2012; Schuh-Huerta & Pera, 2011)).**

Additionally, no markers that uniquely identify SSCs in a population of undifferentiated spermatogonia have been found. Instead, markers are generally specific for subsets of spermatogonia, for differentiated or undifferentiated spermatogonia, or for spermatogonia in general (Oatley, J. M. & Brinster, 2006). These markers are useful for identifying and enriching certain spermatogonia subsets in a testis cell population, however they are unable to be used to identify a pure population of SSCs (Aponte, P. & de Rooij, 2008; Aponte, Pedro et al., 2005; Herrid, Davey, & Hill, 2007). Several molecular markers of undifferentiated spermatogonia have been identified for various species, with the majority of work having been carried out on murine SSCs (Aponte, Pedro et al., 2005; Hamra et al., 2005; Oatley, J. M. & Brinster, 2006).

### 1.3.2.1 Known Bovine Spermatogonia Markers

Few markers of bovine spermatogonia have been identified, however some markers identified in humans and rodents are believed to be conserved in the bovine. DDX4 (previously known as VASA)

has been shown to be expressed by a subset of undifferentiated spermatogonia in the bovine adult and foetal testis (Bartholomew & Parks, 2007; Fujihara, Kim, Minami, Yamada, & Imai, 2011). ZBTB16 (previously known as Plzf) has also been shown to be expressed by a population of early stage spermatogonia including spermatogonial stem cells (Borjigin, Davey, Hutton, & Herrid, 2010; Reding, Stepnoski, Cloninger, & Oatley, 2010). Recently, THY1 was also reported to be a marker for bovine undifferentiated spermatogonia (Reding et al., 2010). Additionally, KIT has been shown to identify a more differentiated population of bovine spermatogonia ( $A_{1-4}$ ), as it also does in the rodent testis (Izadyar, 2002; Izadyar, Den, Stout, & Stout..., 2003; van, de, & van, 1999).

In addition to markers that are conserved across species, novel markers for sub-sets of bovine spermatogonia have also been identified. *Dolichos biflorus* agglutinin (DBA) has been identified as a ligand that identifies bovine Type A spermatogonia through its specific affinity to  $\alpha$ -D -N-acetyl-galactosamine (Herrid et al., 2007; Wrobel, K., 2000). Additionally UCHL1 (previously known as PGP 9.5) was identified as a novel marker of undifferentiated spermatogonia in porcine testis (Luo, Megee, Rathi, & Dobrinski, 2006) and has also been shown to identify a sub-set of undifferentiated bovine spermatogonia (Goel et al., 2010; Herrid et al., 2007; Sandeep, Ranjeet Singh, Sanjay Kumar, Niranjana, & Hiroshi, 2010). However neither DBA or UCHL1 appear to be specific for identification of bovine SSCs (Wrobel, K.-H., Bickel, & Kujat, 1996). Again, although these markers are able to identify different subsets of spermatogonia, including undifferentiated spermatogonia which included SSCs, none are able to uniquely identify SSCs.

### 1.3.3 Spermatogonial Stem Cell Transplant

Since the first successful spermatogonial stem cell transplant (germ cell transplantation) was achieved, in mice in 1994 (Brinster, R. & Zimmermann, J., 1994), the practice has become an important technology that offers many opportunities for the study of spermatogenesis. The technology has allowed for improved assaying of the competence of spermatogonial stem cells as only true SSCs are able to colonise the host testis to produce sperm. Germ cell transplantation also has the potential to be used as a reproductive technology in livestock and other species (Dobrinski, Ina, 2005; Honaramooz, A., Behboodi, Blash, Megee, & Dobrinski, 2003; Schlatt, 2002).

Essentially, germ cell transplantation involves the castration and cell isolation of a fertile donor. These cells are then transplanted, through the rete testis, into the seminiferous tubules of an infertile recipient (either naturally infertile or induced). The transplanted cells, containing SSCs, will

migrate from the lumen of the tubules to the basement membrane where they occupy the residual niche spaces made by the recipients depleted SSCs. The transplanted cells are then capable of proliferation and spermatogenesis, resulting in the production of sperm with the genetic code of the donor animal (Honaramooz, Ali & Yang, 2011).

This technology offers many potential uses in the livestock industry where traditional breeding technologies such as embryo transfer and artificial insemination are not feasible due to the extensive nature of management systems. Germ cell transplantation can instead be used to transfer donor SSCs of higher genetic potential, or genetically altered cells, to recipient's animals to improve the genetics of herds. As an example, in the northern Australia, extensive cattle systems are dominated by the *Bos Indicus* breeds. These breeds, while suited to the tropical climate, are believed to produce beef of lesser quality than *Bos Taurus* breeds. Efforts to improve the genetics of these herds to improve beef quality have had limited success due to the extensive nature of the production systems, and the climate limiting the utilization of the more productive *Bos Taurus* breeds. The use of spermatogonial stem cell transplant may prove to be an important technology to deliver superior genetics to these systems through the transplantation of *Bos Taurus* SSCs into *Bos Indicus* bulls. Recipient *Bos Indicus* bulls would in turn produce *Bos Taurus* semen and therefore create cross breed calves that are both capable of surviving the tropical temperatures and also producing beef of improved quality.

Furthermore, the genetic modification of germline stem cells *in vitro*, which are then transplanted into a recipient by germ cell transplantation to generate transgenic sperm, offers an alternative method for efficiently producing transgenic livestock. Recently, germ cell transplantation has been used to create transgenic pigs through viral and non-viral transformation of transplanted testis cells (Zeng, Wenxian et al., 2013; Zeng, W. et al., 2012). These studies introduced transgenes livestock germline stem cells using viral and non-viral vectors to produce transgenic gametes following germ cell transplantation. Zeng, Wenxian et al. (2013) showed that both adenoviral and lentiviral transduction of transgenes into germline stem cells resulted in transgene transmission in pigs. While non-viral transgene delivery by nucleofection was also shown to result in transgene transmission to sperm when goat germline stem cells were genetically modified and transplanted into recipients (Zeng, W. et al., 2012) . Figure 1-5 shows a schematic representing the process of germ cell transplantation in cattle where the technology can be used for both generation of transgenic livestock by long-term culture and modification of germline stem cells, and the use of this technology in artificial breeding technologies (Hill & Dobrinski, 2006).

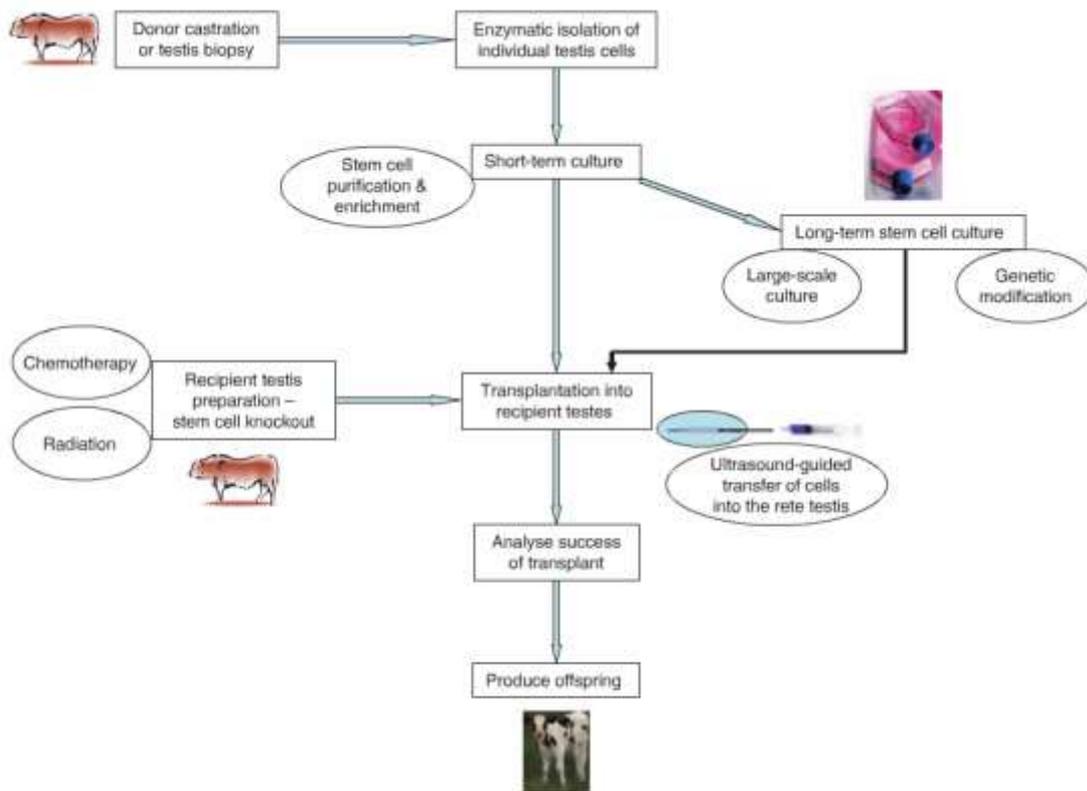


Figure 1-5. Schematic representation of the process of germ cell transplantation in cattle (Hill & Dobrinski, 2006).

Currently germ cell transplantation is an inefficient process, with a number of factors inhibiting the success of this technology in livestock species (Honaramooz, Ali, Megee, & Dobrinski, 2002). The first is the preparation of the recipient testis, the recipients endogenous SSCs must first be depleted in order to provide a space on the basal membrane for the colonisation of the donor SSCs (Brinster, R. & Zimmermann, J., 1994; Herrid, Vignarajan, Davey, Dobrinski, & Hill, 2006). The use of radiation to kill the recipients own SSCs has been used in numerous species including mice (Creemers et al., 2002), sheep (Herrid, Davey, Hutton, Colditz, & Hill, 2009) and pigs (Honaramooz, Ali et al., 2002). Chemotherapeutic agents such as busulfan have also been used for ablation of the recipients SSCs (Brinster et al., 2003; Honaramooz, Ali et al., 2005). Due to the size of bovine recipients, chemical methods of depletion may be more appropriate. However, with either treatment, a small population of the recipients own SSCs remain and these cells compete with the donor SSCs to re-populate the stem cell niche in the recipient's testis. As a result, the recipient produces spermatozoa from both its own SSCs and the donors SSCs creating a mixed population (Brinster et al., 2003).

Associated with the success of germ cell transplantation, is the need to transfer a relatively pure population of SSCs to the recipient (Shinohara & Brinster, 2000). Furthermore, the number of donor

cells needed to re-populate the recipient testis is quite high, while the number of SSCs present in the adult is relatively low, making up only 0.03% of all testis cells and 1.25% of all spermatogonia (Aponte, Pedro et al., 2005; Tegelenbosch & de Rooij, 1993). To obtain a large and pure population of bovine spermatogonial stem cells, effective enrichment and culture protocols for bovine spermatogonia are required (Hill & Dobrinski, 2006). However, culture conditions that allow for the reproducible, long term culture and propagation of bovine spermatogonia, have yet to be defined. Although progress has been made in the culture of SSCs, problems of low survival rates and differentiation in culture still exist (Aponte, Pedro et al., 2008; Izadyar, 2002). The efficient isolation and enrichment of bovine spermatogonia is difficult due to limited knowledge of their in situ properties. However, protocols for enrichment of bovine spermatogonia have been developed, but with varying results (Aponte, P. & de rooij, 2008; Herrid et al., 2009; Izadyar, Spierenberg, Creemers, den Ouden, & de Rooij, 2002). Finally, the developmental stage of the donor testis tissue is likely of effect on the transplantation success, as results using xenografting of bovine testis tissue in mice have shown best spermatogenic progression in grafts from pre-pubertal bulls (Huang, S., Sartini, & Parks, 2008). Methods of enrichment of undifferentiated spermatogonia will be discussed further.

#### **1.3.4 Enrichment of Spermatogonial Stem Cells**

As described previously, it has been shown that the efficiency of germ cell transplantation improves with the transplantation of a more enriched population of SSCs (Bugeaw et al., 2005; Shinohara, Avarbock, & Brinster, 1999; Shinohara & Brinster, 2000). Studies have demonstrated that mice showed a 10 fold increase in donor SSC colonisation when donor cells were enriched 10 fold prior to transplantation (Shinohara & Brinster, 2000). However, it is currently difficult to obtain a relatively pure population of bovine spermatogonia, and as a result the efficiency of colonisation of donor SSCs is also low. One study of bovine germ cell transplantation reported the number of donor spermatogonia colonising the recipients testis following transplantation was as low as 0.6-3.2% (Herrid et al., 2006). For successful application of testis cell transfer in the bovine the ability to identify and isolate a pure population of spermatogonial stem cells is required.

There are a number of methods currently used for enrichment of bovine spermatogonia (Herrid et al., 2009). Enrichment methods that identify and positively select cells based on cell surface markers include flow cytometry and Fluorescent Activated Cell Sorting (FACS), and Magnetic Activated Cell Sorting (MACS). Flow cytometry and FACS enrichment work by labelling cells of interest with a fluorescent antibody that binds to the specific cell surface protein (Herzenberg et al., 2002). Lasers

and electrical currents are used to identify labelled cells, or in the case of FACS are able to sort labelled cells. MACS also allows cells to be separated according to surface antigens, by using magnetic particles coated in the target antibody (Schmitz et al., 1994). A number of markers have been used to enrich murine SSC populations by MACS and/or flow cytometry methods. Kubota, Avarbock, and Brinster (2003) showed a 30 fold enrichment of mouse SSCs through selection of THY1 positive cells by MACS enrichment. While a 25 fold enrichment was observed by Shinohara et al. (1999) also selecting for THY1 positive cells in mouse testis. Success has also been achieved in selection populations for GFRA1 positive cells in both rodent and primate testes (Gassei, Ehmcke, Dhir, & Schlatt, 2010). Shinohara et al. (1999) used anti-  $\alpha 6\beta 1$  integrins to positively select for, and enrich, spermatogonial stem cells of mice. Positively selected cells were then transplanted into the testis of donor mice and resulted in 3.8-5 fold increase in colonisation area compared to non-selected/enriched cell transplants. Buageaw et al. (2005) also demonstrated that the GDNF family receptor  $\alpha 1$  (GFRA1) can be used as an antibody to select for spermatogonial stem cells of mice using MACS, with an enrichment of up to 4 fold observed, compared to the unsorted cell population. Additionally, DBA and THY1 have been used to produce SSC enriched populations of bovine testis cells by positive selection using MACS or FACS sorting (Herrid et al., 2007; Reding et al., 2010). In experiments conducted by Herrid et al. (2009), testes cells were stained with FITC-conjugated DBA to sort spermatogonial stem cells by FACS. These experiments resulted in a 4 fold increase in DBA positive cells. MACS has also been used to sort DBA-FITC labelled cells for MACS separation of bovine testicular cells, with reports of 4 to 6 fold enrichment (Herrid et al., 2009).

Alternative to molecular marker selection, physical properties of different testis cell populations may also be used to enrich SSC enriched populations. The use of techniques such as differential plating and Percoll discontinuous density separation gradients, are also established methods of enriching populations of bovine spermatogonia (Herrid et al., 2009; Izadyar, 2002). Differential plating utilises the tendency of Sertoli and somatic testis cells to adhere to cell culture plastic, resulting in an enriched spermatogonia population in the non-adherent culture fraction (Izadyar et al., 2002). Differential plating of bovine testis cells in un-coated flasks overnight, has been shown to enrich spermatogonia populations by 2-4 fold (25% to 45%) (Izadyar et al., 2002) (Herrid et al., 2009). Enrichment by differential plating can also be enhanced by coating cell culture flasks with various ligands (Dym et al., 1995; Herrid et al., 2006; Izadyar et al., 2002). Sertoli cells also adhere specifically to the lectin *Datura stramonium* agglutinin (DSA), therefore differential plating with DSA can enhance enrichment (Herrid et al., 2009). In mice, spermatogonia have been shown to bind to laminin by interactions with cell surface marker integrin  $\alpha 6\beta 1$  (Shinohara et al., 1999). At this stage

there has been no conclusive evidence for the expression of integrin  $\alpha 6\beta 1$  in bovine testis, however, differential plating by laminin coating has been reported to result in enrichment of 3.3 fold of DBA positive cells (Herrid et al., 2009).

Percoll discontinuous density gradient centrifugation allows the separation of cells or cellular particles based on their density. Percoll consists of colloidal silica beads coated in polyvinylpyrrolidone (PVP) and is believed to have no toxicity effects on cells (Hernandez-Lopez, Umland, Mondragon-Ceballos, & Nayudu, 2005). Percoll is prepared in layers of different densities; a mixed population of cells is layered on top of the prepared gradient and then centrifuged to separate different cell populations based on density. Percoll density gradients have been successfully used to separate different populations of testis cells in mixed populations from various species including bovine (Chang, Lee-Chang, Panneerdoss, MacLean, & Rao, 2011; Herrid et al., 2009; Izadyar et al., 2002; Schumacher, Schäfer, Holstein, & Hilz, 1978). After centrifugation, a relatively pure population of bovine spermatogonia (generally between 3-4 fold enrichment) may be found in the interface of gradients at densities from 1.0542 and 1.061  $\text{gmL}^{-1}$  and at 1.0513 and 1.0542  $\text{gmL}^{-1}$  (Herrid et al., 2009).

Similar to Percoll discontinuous gradient centrifugation, Velocity sedimentation separation using the STA-PUT method allows a heterogeneous population of testis cells to be separated through a linear BSA (bovine serum albumin) gradient. This method separates cells based on size, and allows for the collection of live enriched cell fractions, including un-differentiated spermatogonia, for further analysis or applications. The STA-PUT method allows for a high yield of enriched cell fractions, though it does not result in greatly enriched fractions of cell types. Additionally, enrichment by this method is relatively simple and only requires the specialised glassware allowing it to be conducted in most laboratories (Bryant, Meyer-Ficca, Dang, Berger, & Meyer, 2013; Hofmann, M.-C. C., Braydich-Stolle, & Dym, 2005; Luo et al., 2006).

The ability to enrich populations of spermatogonial stem cells is necessary not only for the study of SSCs, but also for the application of these cells in technologies such as testis cell transfer. Although the enrichment of spermatogonia from species such as mouse can result in a relatively pure population, morphological differences between species have prevented overly successful enrichment of bovine spermatogonia. The identification of markers for bovine spermatogonial stem cells or undifferentiated spermatogonia may assist in the enrichment of this population of cells. Furthermore the identification of cell surface markers is of particular interest to allow for the direct use of these markers in enrichment of SSCs including their use in MACS and FACS isolation.

## 1.4 Germ Cells from Pluripotent Stem cells

In 2003, Toyooka, Tsunekawa, Akasu, and Noce (2003) demonstrated that the differentiation of embryoid bodies (EBs) from mouse ES cells resulted in a small number of cells that expressed markers of male primordial germ cells (PGCs). It was possible to increase the efficiency of formation of these cells by co-culture with trophoblast cells or cell lines that expressed BMP4. These cells were able to contribute to spermatogenesis when transplanted into donor testis. The differentiation of primordial germ cells from pluripotent stem cells has since been achieved by numerous groups. Payer et al. (2006) demonstrated that EBs from mouse ES cells underwent spontaneous differentiation to express *Stella*. Additionally Wei et al. (2008) showed that the induction of *Stella* positive PGCs was enhanced by BMP4 and Wnt3a. Nicholas et al. (2009) showed that *Dazl* positive cells, isolated from EBs from mouse ES cells, expressed markers of spermatogonia including *VASA*. It has also been reported that human germline cells have been derived *in vitro* from ES cell lines (Eguizabal et al., 2011; Park, T. et al., 2009). Spermatogonia that differentiate to the spermatid stage have been produced from pluripotent stem cells *in vitro*. While Sertoli and Leydig cells have also been produced by differentiation of EBs (Geijsen et al., 2004). It has also been shown that the retinoic acid induces the production of PGCs and is able to induce the expression of markers for male PGCs, spermatogonia and spermatids (Chen, W. et al., 2012; Kerkis et al., 2007; Silva, C. et al., 2009). Additionally, supplementation of EBs with testosterone, or a combination of testosterone and retinoic acid, has also been shown to increase the expression of testis cell markers (Silva, C. et al., 2009). The production of a line of spermatogonial stem cells from pluripotent stem cells has been attempted through the differentiation of mouse ES cells, by treatment with retinoic acid. This treatment resulted in the production SSC like colonies. Further treatment of SSC like colonies with retinoic acid was reported to produce haploid cells that produced sperm like cells. However, the putative sperm cells produced abnormal pups that died within five months, possibly due to abnormalities in genetic imprinting (Nayernia et al., 2006). To date there has been no success in the production of a genuine line of spermatogonial stem cells derived from embryoid bodies of pluripotent stem cells (Imamura, Hikabe, Lin, & Okano, 2014).

The use of iPS cells to produce germline cells has enormous potential. Like ES cells, iPS cells are able to differentiate into all three germ layers, and have been shown to contribute to the germline in chimeras, suggesting that they are capable of differentiating into germline stem cells, at least *in vivo* (Hamanaka et al., 2011; Okita et al., 2007). iPS cells have been used in attempts to derive germ cells

by several groups. Kim, D. et al. (2009) were able to derive EBs from neural stem cells that could be differentiated into putative germ cells which expressed markers of early PGCs. Another group showed the selection and culture of *VASA* positive PGCs, derived from EBs from murine hepatocytes, resulted in oocytes like cells. The supplementation of *VASA* positive cells with BMP4, epidermal growth factor (EGF), glial cell-derived neurotrophic factor (GDNF) and stem cell factor (SCF), produced clumps of *VASA* positive cells that expressed markers for post-migratory PGCs and meiotic cells. It was suggested that the conditions of this experiment may have been optimal for the differentiation of early spermatogonia (Imamura et al., 2010).

Additionally, in 2011 the *in vitro* generation of murine primordial germ cell-like cells that had the ability to undergo spermatogenesis was achieved (Hayashi, Ohta, Kurimoto, Aramaki, & Saitou, 2011). This study explored the use of a progressive process which looked at the conditions under which ES and iPS cells were induced into pregastrulating epiblast-like cells, which in turn were used to produce primordial germ cell-like cells. Small molecules and cytokines were used to direct differentiation of ES and iPS cells toward epiblast like cells which resulted in an efficient culture system for the derivation of murine primordial germ cells *in vitro*.

It is clearly possible to produce germ cells, and putative stem cell like cells, by differentiation of pluripotent stem cells *in vitro*, at least in murine and humans. However, additional research is required to establish cells that more closely resemble true germline stem cells (Imamura et al., 2014). The potential to produce germline cells from livestock pluripotent stem cells, including iPS cells, offers huge potential in the field of transgenic breeding and genetic modification. These artificially produced germ cells also have the potential to be used in reproductive technologies including germ cell transfer, eliminating the need to castrate the donor animal.

There have been no reports claiming to have produced male germline cells from bovine pluripotent stem cells. However, Cao et al. (2012) reported that bovine iPS cells were capable of producing EBs that were able to differentiate female germ cells, by supplementation with retinoic acid and follicular fluid. These cells were shown to express markers of late stage germ cells including *VASA*, *Dazl*, *Gdf9*, *Nobox* and *Zp2*. This data suggests that it may be possible to produce male germline cells from bovine iPS cells, by the optimisation of culture conditions, and that these cells could be used for reproductive technologies, including germ cell transplantation in the future.

## 1.5 Conclusions

The use of bovine spermatogonial stem cells (SSCs) in reproductive technologies and transgenic breeding offers enormous potential for the livestock industries. However, the use of SSCs in technologies such as testis cell transfer are limited, due a lack of knowledge about bovine SSCs, with few markers currently available for bovine spermatogonia, and none for SSCs exclusively. This, along with inter-species variation, has resulted in limited enrichment and characterisation of these cells. Maintaining these cells and expansion in culture, has also been limited. Methods to improve enrichment of these cells may allow for the enhanced study of these cells and on understanding of how to potentially expand and maintain these cells. The identification of new markers of bovine SSCs may be achieved by proteomic analysis or enhanced enrichment procedures, allowing for the more accurate study of these cells. The ability to better characterise these cells may allow for the derivation of SSCs from pluripotent stem cells including iPS cells.

Induced pluripotent stem (iPS) cells provide an alternative source for pluripotent stem cells from livestock species where embryonic stem cell lines have to date failed to be established. There is a potential for bovine iPS cells to be used in numerous applications including the generation of germline cells for use in breeding applications, such as germ cell transplantation and transgenic breeding. However, the production of bovine iPS cells has proven difficult, the few reports claiming to have produced bovine iPS cells, and no protocol for the reproducible derivation of bovine iPS cells established. The production of bovine iPS cells has been limited by difficulties expanding cells in culture, inefficient derivation of colonies, and no transgene silencing of reprogrammed cells. The integration of transgenes into the host genome has resulted in limitations in the use of iPS cells derived by viral/ integrative methods. As such, non-viral/ non-integrative methods of iPS cell derivation have been established to produce iPS cells that are safe for clinical applications. However, the production of non-viral iPS cells is generally far less efficient than lentiviral or retroviral methods. Investigations into pathways involved in reprogramming have led to the identification of small molecules and growth factors that are able to enhance reprogramming efficiency by targeting these pathways. Small molecules have been shown to enhance reprogramming by increasing reprogramming efficiency, increasing the speed of reprogramming kinetics, and by converting partially reprogramming cells to fully reprogrammed iPS cells. However, the efficiency of iPS cell derivation still remains low. Continued research into the pathways involved in bovine iPS cell generation may assist in the improved derivation of these cells. It is unclear as to why the derivation of bovine iPS cells is less successful than other species. Numerous barriers that affect

reprogramming must be overcome to produce fully reprogrammed bovine iPS cells. This may be achieved by altering culture conditions, supplementation with small molecules and choice of cell type. Further study is required to establish a reproducible protocol for the derivation of bovine iPS cells so that they may be utilised in the future.

## **1.6 Aims**

The general aim of this thesis was to produce bovine induced pluripotent stem (iPS) cells that may be differentiated toward the germline, for potential use in reproductive technologies such as germ cell transplantation, and transgenic breeding applications.

Methods to improve enrichment of bovine SSCs are of interest in this thesis in order to improve the success of bovine germ cell transplantation. Additionally, the ability to better identify and characterise bovine testis cells, specifically SSCs, by the identification of new markers is important. New markers that uniquely identify SSCs or different sub-sets of spermatogonia, in the bovine testis would also contribute to knowledge of characterisation of the cells and may provide better methods for enrichment. This thesis aims to characterise putative bovine spermatogonia markers, identified from proteomic analysis of enriched populations of bovine testis (Colgrave et al., 2013). These markers may be of use in identifying bovine germline cells produced by differentiation of bovine iPS cells.

Within the scope of this thesis, one aim was to compare different methods of iPS cell generation for their relative effectiveness in the production of bovine iPS cells. This thesis aimed to determine if the combination of reprogramming factors introduced to different fibroblast cells types (adult and embryonic) resulted in a difference in reprogramming efficiency. Additionally, this thesis aimed to produce non-viral bovine iPS cells that were free of transgene integration, and could therefore potentially be used in clinical applications including reproductive technologies. In order to further improve the derivation of bovine iPS cells the effects on reprogramming of different combinations of small molecules was assessed.

Additionally, the use of commercially available technologies including non-viral vectors, for the production of bovine iPS cells offers potential for producing these cells in a reproducible manner.

## Chapter 1: Literature Review

Reprogramming with a commercially available non-viral minicircle vector, designed for human iPS cell generation, was trialled for the generation of bovine iPS cells.

Furthermore, the differentiation of iPS cells toward the germline offers numerous applications for in reproductive technologies including artificial breeding and the production of transgenic livestock. The directed differentiation of embryoid bodies toward the germline was also attempted as part of this thesis through rudimentary methods. EBs derived from putative non-viral bovine iPS cells that were produced as part of this thesis were exposed of the meiosis inducing agent, retinoic acid, to encourage the production of bovine germ cells *in vitro*.

## **Chapter 2 : Enrichment of Bovine Spermatogonia and Spermatogonial Stem Cells**

### **2.1 Introduction**

Spermatogonia, specifically spermatogonial stem cells (SSCs), are the progenitors for sperm production in mammals. These multipotent stem cells have the ability to self-renew and to generate a series of differentiating germ cells that lead to the production of sperm. However, little is known about the processes of spermatogenesis or the detailed characteristics of spermatogonia and SSCs, especially in livestock species (Herrid et al., 2009). The study of livestock SSCs can provide insights into the process of spermatogenesis in livestock may identify specific markers for different subsets of bovine spermatogonia and could improve the potential use of these cells in new transgenic and artificial breeding technologies. To better understand cellular development and function of spermatogonial stem cells, and to better characterise these cells, the isolation of a large, relatively pure population of spermatogonial stem cells is essential (Aponte, Pedro et al., 2008).

In 1994, the technique of germ cell transplantation was developed in mice models, where donor germ cells were transplanted into a recipient resulting in sperm of the donor genome produced (Brinster, R. & Zimmermann, J., 1994; Jiang & Short, 1995). This technique involves the isolation of single cells from donor testis and the transplantation of the cell suspension into the seminiferous tubules, rete testis or large efferent duct of the testis of a male recipient. A small percentage of donor SSCs are then able to colonise the stem cell niche on the basement membrane where they are able to proliferate and differentiate to produce sperm of the donor's genotype. The ability to colonise recipient testis and produce viable sperm is the hallmark of a male germline stem cell, hence germ cell transplantation is used as defining proof of a germline stem cell identification (Schlatt, 2002).

Germ cell transplantation has also generated immense interest in the field of livestock reproduction. Successful germ cell transplantation has been reported in numerous livestock species including pigs (Honaramooz, Ali et al., 2002), goats (Honaramooz, A. et al., 2003), cattle (Herrid et al., 2006; Izadyar et al., 2003) and sheep (Oatley, J. et al., 2005). Germ cell transplantation has been suggested as a

potential alternative to artificial insemination and is also a possible route to produce transgenic animals, potentially more efficiently than traditional methods (Herrid et al., 2009; Honaramooz, Ali & Yang, 2011). SSCs could be genetically modified and transplanted into a recipient to produce potentially thousands of sperm that contain the genetic modifications, thereby generating transgenic offspring in an efficient manner. This method of producing transgenic offspring is of particular interest in livestock where the generation interval is quite long compared to laboratory based animals (in cattle, nine months gestation and a further minimum of eight months from birth to puberty, depending on the breed) (Aponte, P. & de rooij, 2008). However, the enrichment of a relatively pure population of SSCs is necessary to be able to manipulate these cells *in vitro* (Dobrinski, I., Avarbock, & Brinster, 2000; Kim, K. J. et al., 2013). The inability to isolate and culture livestock SSCs currently limits the potential use of these cells in transgenic breeding applications. Germ cell transplantation also has the potential to be used to enhance the dissemination of genetics of high quality animals, possibly more efficiently than current artificial insemination techniques. This application may be of value in the extensive grazing systems of northern Australia, where *Bos Indicus* bulls could be transplanted with *Bos Taurus* testis cells to produce cross bred calves with higher genetic value (Dobrinski, I., 2008).

Spermatogonial stem cells are found in relatively low quantity in adult testis with the total spermatogonia population in bovine (including differentiated spermatogonia) estimated to be approximately 10% (Herrid et al., 2007), additionally, SSCs are estimated to make up only 0.03% of all germ cells and 1.25% of all spermatogonia (Aponte, Pedro et al., 2005; Tegelenbosch & de Rooij, 1993) . Furthermore the efficiency of colonisation of donor SSCs is also low, with one study of bovine germ cell transplantation reporting the number of donor spermatogonia following transplantation between 0.6-3.2% (Herrid et al., 2006). It has been shown that the efficiency of germ cell transplantation improves with the transplantation of a more enriched population of SSCs (Bugeaw et al., 2005; Shinohara et al., 1999; Shinohara & Brinster, 2000). Moreover, the contamination of enriched SSC populations with other testis cells has adverse effects on the survival and maintenance of SSCs, thereby decreasing efficiency of transplantation (Kubota, Avarbock, & Brinster, 2004; Nagano, Ryu, Brinster, Avarbock, & Brinster, 2003). Additionally, large scale culture of SSCs is currently not feasible and as such SSCs cannot be expanded in culture to obtain a large number of these cells. The use of enrichment methods to obtain a pure population of SSCs will allow the improved study of these calls and may provide insights into the culture of these cells (Aponte, Pedro et al., 2008). The successful application of germ cell transplantation and study of spermatogonial

stem cells will rely on the ability to identify and isolate a pure population of spermatogonial stem cells for transplantation.

There are various methods used to enrich populations of spermatogonia, each with varying degrees of success. SSCs from mixed populations of testis cells can be enriched through positive or negative selection of physical or functional characteristics of different cell types in the testis. Enrichment methods take advantage of cell surface markers, cell density and the ability of cell types to adhere to certain surfaces or chemicals (Herrid et al., 2009). Although protocols have been developed to achieve highly enriched populations of spermatogonia in the mouse (Bellvé et al., 1977; Mito, Shinya, & Takashi, 2004; Shinohara & Brinster, 2000; Takashi, Kyle, Mary, & Ralph, 2000), morphological differences between species have prevented equally successful enrichment of bovine spermatogonia (Herrid et al., 2007; Herrid et al., 2009). Numerous enrichment methods have been trialled for the isolation of bovine spermatogonia, with differing levels of success.

Cell surface markers staining for various subsets of spermatogonia have been used for positive enrichment of spermatogonia populations in various species. In cattle a *Dolichos biflorus* agglutinin (DBA) is a cell surface ligand that has specific affinity to  $\alpha$ -D -N-acetyl-galactosamine, a cell surface marker expressed by spermatogonia type A, a type of undifferentiated spermatogonia which includes SSCs (Herrid et al., 2007; Wrobel, K., 2000). This marker has been used to identify the relatively undifferentiated subset of spermatogonia by fluorescent staining, and has been used to enrich populations using magnetic activated cell sorting (MACS) and florescent activated cell sorting (FACS) (Herrid et al., 2009). For successful testis cell transfer in large animal models a large number of cells are required to be isolated without compromising cell viability. The use of methods such as MACS and FACS yield only comparatively limited numbers of enriched cells in comparison to numbers required for germ cell transplantation. Methods such as differential plating, velocity sedimentation method (STA-PUT), or discontinuous density gradient centrifugation through Percoll may be more appropriate for yielding enriched cell populations large enough to be used for transfer (Bryant et al., 2013; Dirami, Ravindranath, Pursel, & Dym, 1999; Herrid et al., 2009; Izadyar et al., 2003; Kokkinaki et al., 2009; Luo et al., 2006).

Differential plating has been used to enrich spermatogonia populations by both positive and negative selection in numerous species including the bovine (Dym et al., 1995; Herrid et al., 2009; Herrid et al., 2006; Izadyar et al., 2002; Izadyar et al., 2011). Differential plating utilises the

adherence of either spermatogonia or somatic testis cells including Sertoli cells, to a specific ligand or to cell culture plastic. Sertoli cells and other somatic testis cells have a tendency to adhere to cell culture plastic *in vitro* (Galdieri, Ziparo, Palombi, Russo, & Stefanini, 1981), and hence plating a mixed population of testis cells on un-coated cell culture plates enriches spermatogonia in the non-adherent fraction of the culture (Herrid et al., 2009; Izadyar et al., 2002). Additionally Sertoli cells adhere specifically to ligands including the lectin *Datura stramonium* agglutinin (DSA) and gelatine, coating cell culture plastic with these compounds can therefore be used to further enhance the enrichment of spermatogonia in the non-adherent cell fraction (Herrid et al., 2009). In mice spermatogonia are characterised by cell surface markers including the integrin  $\alpha 6\beta 1$  which binds to laminin (Shinohara et al., 1999). As a result laminin coating of cell culture plates has been used to enrich spermatogonia in the adherent culture fraction (Shinohara et al., 1999). At this stage there has been no conclusive evidence for the expression of integrin  $\alpha 6\beta 1$  in bovine testis; however, differential plating by coating with laminin, a integrin ligand, has shown enrichment of bovine spermatogonia (Herrid et al., 2009). However, a comparison of enrichment methods for bovine spermatogonia found that there was no difference in enrichment seen between differential plating on un-coated cell culture plates and those coated with different substances to improve enrichment (Goel et al., 2010; Herrid et al., 2009).

Percoll density gradient centrifugation has also been used to enrich populations of spermatogonia in a mixed population of testis cells, and is based on separation of cells according to their buoyancy in a discontinuous gradient of Percoll. Percoll gradients have been used to enrich different populations of testis cells in various livestock species including sheep (Rodriguez-Sosa, Dobson, & Hahnel, 2006), pigs (Dirami, Poulter, & Cooke, 1991) and cattle (Aponte, P. & de rooij, 2008; Herrid et al., 2009; Rafeeqi & Kaul, 2013). Populations of spermatogonia are found in interface of Percoll gradients, with bovine spermatogonial cells reported to be isolated in the interface between 35-43% Percoll gradients ( $1.0654-1.0542 \text{ g ml}^{-1}$ ) (Rafeeqi & Kaul, 2013), while other reports have claimed to isolated the most enriched fraction at the interface of 20-35% Percoll gradients ( $1.0413-1.0542 \text{ g ml}^{-1}$ ) (Aponte, P. & de rooij, 2008; Herrid et al., 2009; Izadyar et al., 2002).

The highest reported percentage of spermatogonia obtained after enrichment was 76% following differential plating with BSA coated plates (control plates) (Herrid et al., 2009). However, other studies using uncoated or BSA coated plates for enrichment have not reported enrichment values as high, and instead report that enrichment is enhanced by differential plating followed by separation

of the differentially plated enriched fraction on a Percoll gradient (Goel et al., 2010; Izadyar et al., 2002). Table 2-1 summarises the findings from previous reports of the enrichment of bovine spermatogonia using different methods.

**Table 2-1. Previous reports of fold increase and final spermatogonia percentage of bovine spermatogonia in enriched populations using different enrichment methods.**

Author/ Date	Marker for enrichment	Enrichment Method	% Positive cells in enriched population	Fold Increase Spermatogonia
<b>Herrid et al. (2009)</b>	<b>DBA</b>	Differential Plating		
		Laminin (Adherent)	52.9 %	3.3
		DSA (Non-Adherent)	52.5 %	2.9
		BSA (2hr incubation, Non-Adherent)	76.4 %	4.7
		Percoll (Interface 1.0413-1.0542 g ml <sup>-1</sup> )	50 %	2
		MACS	21.1 %	4.6
		FACS	16 %	4.1
<b>Izadyar et al. (2002)</b>		Differential Plating		
		Un-Coated Plates	45% ± 3.1	Not Specified
		Differential Plating and Percoll Gradient (30%, 1.0542 g ml <sup>-1</sup> )	73% ± 2.9	Not Specified
<b>(Aponte, P. &amp; de rooij, 2008)</b>		Differential Plating	48.27 %	Not Specified
		Differential Plating and Percoll Gradient (30%-1.0542 g ml <sup>-1</sup> )	71.86 %	Not Specified
<b>Goel et al. (2010)</b>	<b>UHL-1 For Gonocytes</b>	Differential Plating		
		Gelatine	36.3 % ± 2.4	2
		BSA	38.6 % ± 2.1	
		Un-Coated Plates	38.1 % ± 2.2	
		Differential Plating and Percoll Gradient	55.3 % ± 3.4	3

Regardless of enrichment methods, it seems to be difficult to isolate highly purified spermatogonia populations. Cell to cell interactions mediated through adherence molecules that can fuse different cells from the testis population together could be a reason for this (Prozialeck & Edwards, 2007; Salaheldeen, Kurio, Howida, & Iida, 2012). It has been observed that the incubation of testicular cells overnight for differential plating allows more Sertoli cells to bind to germ cells making enrichment of a pure population of spermatogonia difficult (Luo et al., 2006). Tight junction proteins are also observed in the testis between adjacent Sertoli cells to form the blood-testis barrier and to establish

the stem cell niche (Mital, Hinton, & Dufour, 2011). Recently it was found that Claudin-8, a tight junction protein, is expressed by bovine spermatogonia and a subset of Sertoli cells, and is believed to be responsible, at least in part, for the observation of adherence of spermatogonia to Sertoli cells in culture (McMillan et al., 2013; Mruk & Cheng, 2010). It is thought that Sertoli cells expressing Claudin-8 adhere to spermatogonia and are co-selected with the associated spermatogonia during enrichment procedure, thereby preventing the ability to acquire a highly enriched population of bovine spermatogonia (McMillan et al., 2013).

It has been documented that the C terminus of the enterotoxin produced by bacteria *Clostridium perfringens* (C-CPE), is able to bind to members of the Claudin family, including Claudin-8 in some species, and can disrupt tight junctions that are formed by these proteins (Sonoda et al., 1999; Yuan, Xiaoqin et al., 2009). C-CPE has been used in certain cancer treatments to facilitate drug delivery by breaking tight junction bounds between different members of the Claudin family in found to form tight junction barriers in various tumours (Cocco et al., 2010; Kondoh, Masuo, Takahashi, Azusa, Fujii, Makiko, Yagi, Kiyohito, & Watanabe, Yoshiteru 2006; Yuan, Xiaoqin et al., 2009). Additionally, studies have shown that the exposure of tight junction barriers, formed by Claudin's, to of the binding site of C-CPE (consisting of amino acids 290-319) (Kondoh, Masuo et al., 2006) is sufficient to enable binding and facilitate tight junction interruption. Additionally, this smaller fragment, referred to as C-CPE<sub>290-319</sub>, retains full binding affinity for Claudin-3 and 4 and has no cytotoxic effects (Cocco et al., 2010; Yuan, Xiaoqin et al., 2009). It may therefore be possible to break interactions formed by Claudin-8 between Sertoli cells and spermatogonia using the C-CPE peptide (Kimura, J. et al., 2010) to obtain a more enriched population of spermatogonia.

In summary, improved enrichment methods of bovine spermatogonial stem cells are necessary to be better able to identify, characterise and use these cells in artificial breeding and transgenic technologies (Herrid et al., 2009). At this stage no one method of enrichment has been found to be most successful in isolating a pure population of bovine spermatogonia. Additionally, enrichment may be hampered by the adherence of Sertoli cells to spermatogonia in suspension, resulting in the contamination of spermatogonia enriched cell populations with adherent Sertoli cells (Luo et al., 2006).

This chapter aims to compare enrichment methods for deriving a population of undifferentiated bovine spermatogonia identified through DBA binding activity. It is hypothesised that the use of differential plating followed by further separation of the enriched fraction of testis cells by Percoll discontinuous density gradient will result in enhanced enrichment compared to using these methods individually.

Additionally, the ability of the C-CPE<sub>290-319</sub> to enhance enrichment of bovine spermatogonia using MACS enrichment was assessed. It is hypothesised that the treatment of initial testis cell isolations with the C-CPE<sub>290-319</sub> peptide will enhance enrichment by interfering with bonds between Sertoli cells and spermatogonia, possibly formed by the presence of the adhesion molecule Claudin-8.

## **2.2 Materials and Methods**

### **2.2.1 General**

All cell culture materials were purchased from Life Technologies (USA) unless otherwise stated. MACS reagents were purchased from Miltenyi Biotec (Germany). Chemicals and reagents were purchased from Sigma-Aldridge Chemical Industries (USA). All animal experiments were conducted with approval from under the F D McMaster Laboratory Animal Ethics Committee, CSIRO Animal Food and Health Sciences (Approval number 10/14).

### **2.2.2 Testis Collection and Enzymatic Isolation of Testis Cells**

Pubescent Angus bull calves (approximately 6 months old) were castrated under general anaesthesia. Testes were collected and stored on ice until dissection. Enzymatic digestion of testis tissue and testis cell isolation was conducted using the method described by Herrid et al. (2006). Briefly, the tunica albuginea and epididymis were removed from testes and testes were washed in Dulbecco's phosphate buffered saline (DPBS). Approximately 20g of tissue was dissected from each testes, and placed in DMEM/F-12 (1:1) containing 100 U/ml penicillin and 100 mg/ml streptomycin (in the following referred to as DMEM/F-12). The tissue was finely chopped using sterile scissors and

placed into a sterile tea strainer where it was ground into a paste using the plunger of a syringe. The resulting paste was then transferred into DMEM/F-12, the suspension was allowed to settle, the supernatant was removed and the sedimented material was washed with DMEM/F-12 three times. The tissue material was resuspended in DMEM/F-12 supplemented with 1mg/ml collagenase (Sigma-Aldridge, USA) and incubated at 37°C in a shaking water bath for approximately 15 minutes, or until individual tubules could be observed using a microscope. The suspension was allowed to settle and was then washed five times with DPBS. Cells were then resuspended in DPBS containing 2.5 mg/ml trypsin, and incubated at 37°C for up to 10 minutes or until a single cell suspension was obtained. An equal volume of DMEM/F-12 with 5% foetal bovine serum (FBS) was added to the suspension to inactivate the trypsin. The cell suspension was then filtered through a cell strainer with two layers of nylon mesh (80 and 55 µm) then centrifuged at 1000× g for 5 minutes. The cell pellet was resuspended in DMEM/F-12 + 5% FBS. Cell viability was assessed by trypan blue staining. Isolated cells were used for enrichment protocols.

### **2.2.3 Differential Plating Enrichment**

$7 \times 10^6$  freshly isolated testis cells were plated in T125 culture flasks with 10ml DMEM/F-12 + 10% FBS and incubated overnight at 37°C 5% CO<sub>2</sub> in a humid environment. After this time flasks were lightly tapped to dissociate loosely attached cells, and the supernatant collected. The flask was rinsed three times with DPBS to dislodge other non-adherent cells. All supernatant fractions were collected and centrifuged at 1500x g for 5 minutes. Cell pellets were resuspended in 5ml DMEM/F-12 +10% FBS, cell viability was assessed using trypan blue staining and cells were counted. Cells smears were made from the non-adherent cell fractions, the remaining cells were analysed for DBA binding activity and GATA4 staining by flow cytometry analysis. Alternatively, remaining cells were used in Percoll discontinuous density gradient centrifugation for further enrichment.

### **2.2.4 Discontinuous Percoll Gradient Enrichment**

Percoll discontinuous density gradients were prepared as follows; a solution of 10x DMEM/F-12 was made by dissolving a packet of 10x DMEM/F-12 in 100 ml deionised water with 2.43 g sodium bicarbonate and 0.1 g BSA (pH 7.4). A 90% Percoll solution (Sigma-Aldrich Chemical Industries, USA)

was prepared using 10x DMEM/F-12 (Life Technologies, USA) solution and six different density gradients were prepared according to Table 2-2.

Table 2-2. Preparation of Percoll gradient Fractions with 10x DMEM: F12.

Fraction	Fraction Density (g ml <sup>-1</sup> )	Percentage Of Percoll	Refractive Index	Percoll Stock (mL)	DMEM/F-12 (mL)	Volume (mL)
I	1.096	90	9.4	5.0	0.0	4.0
II	1.070	68	6.8	6.8	2.2	8.0
III	1.065	43	6.2	4.3	4.7	8.0
IV	1.055	35	5.2	3.5	5.5	8.0
V	1.047	20	4.5	2.0	7.0	8.0
VI	1.034	0	3.2	0.0	9.0	8.0

Using a 10ml syringe with a 10cm piece of polyethylene tubing, 4ml of freshly prepared Percoll gradient, starting at lowest density, were carefully layered from the bottom into a 50ml centrifuge tube.  $7 \times 10^6$  testis cells ( $5-10 \times 10^6$  cells/ml in DMEM: F12, 1% BSA) from the initial isolate or the enriched fraction of differently plated cells were carefully loaded onto the Percoll gradient as illustrated in Figure 2-1.

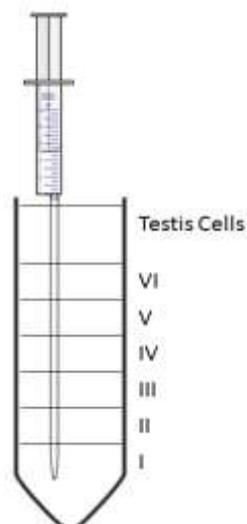


Figure 2-1. Diagram illustrating the preparation of Percoll discontinuous density gradient used in enrichment experiments for bovine testis cells.

The Percoll gradients containing testis cells were centrifuged at 2400 rpm for 30 minutes at 4°C. After spinning two visible cell bands located on the interface of gradients were collected using a syringe with a 13 gauge needle. The collected cell fractions were diluted in 40 ml DMEM/F-12 + 1% BSA, and centrifuged for a further 5 minutes at 1500 rpm at 4°C. The resulting cell pellet was resuspended in DMEM: F12 with 10% FBS. Cell smears were prepared and remaining cells were analysed by antibody staining and flow cytometry analysis.

### **2.2.5 Immunocytochemistry**

Cell smears were fixed in MDF (Modified Davidson's Fixative: 14% ethyl alcohol, 37.5% formalin, 6.25% glacial acetic acid) for 2 minutes and rinsed with TBS-T (TBS 0.05 M + 0.05% Tween 20). Primary antibodies for DBA-HRT (Vector Laboratories, USA; 1:20), GATA4 (Vector Laboratories, USA; 1:300), were diluted in TBS containing 0.5% bovine serum albumin (BSA). Smears were incubated with primary antibodies for 30 minutes at room temperature. Primary antibodies were replaced with buffer for negative controls. Smears were incubated with secondary mouse or rabbit IgG for 30 minutes (Dako, Denmark), slides were rinsed and incubated for 2-5 minutes with diaminobenzidine (DAB) (Dako, Denmark), until sufficient cell staining had occurred. Smears were counterstained with Haematoxylin for 30 - 60 seconds or until sufficient counterstaining was achieved. Smears were washed in water and dehydrated through graded ethanol and xylene washes. Smears were examined using a compound microscope (Zeiss, Germany) equipped with an AxioImager and AxioVision software (Carl Zeiss, Oberkochen, Germany).

### **2.2.6 Flow Cytometry Analysis**

$1 \times 10^7$  cells from the initial isolate and each fraction of differential plating and Percoll gradients were resuspended in 100  $\mu$ l MACS buffer (DPBS containing 0.5% BSA +2 mM EDTA). Cell suspensions were stained with 10  $\mu$ l DBA-FitC (Vector Laboratories, USA) and incubated on ice for 10 minutes. 1ml MACS buffer was added to stained cells and suspensions were centrifuged for 2 minutes at 400x g at room temperature. Supernatant was removed and the cell pellet was fixed and permeabilised using BD cytofix/cytoperm kit (BD Biosciences, USA) with 200  $\mu$ l Fix & Perm solution (Life Technologies, USA). Cell suspensions were centrifuged for 2 minutes at 1500x g supernatant was removed and the pellet was washed again in Fix & Perm solution. Cell pellets were resuspended in 50  $\mu$ l primary

antibody, GATA4 (Santa Cruz, USA 1:100), and incubated on ice for 30 minutes. Cell suspensions were washed twice with 200 µl staining buffer (Life Technologies, USA). Cell pellets were resuspended in Dylight-649 conjugated anti-mouse IgG (Abcam, USA, 1:500) secondary antibody and incubated on ice for 30 minutes. Suspensions were washed three times with MACS buffer, after which they were resuspended in 200 µl MACS buffer and analysed using BD LSR II flow cytometer (BD Biosciences, USA), Data was analysed using FCS Express software (De Novo Software, USA).

### 2.2.7 MACS Enrichment of Undifferentiated Spermatogonia in the Presence of C-CPE

The C-CPE<sub>290-319</sub> peptide described by Yuan, Xiaoqin et al. (2009), and a control peptide, C- CPE<sub>ALA</sub>, with every third amino acid substituted for Alanine (A), were synthesised by Peptide 2.0 Incorporated. Table 2-3 shows sequences and characteristics of peptides used in Chapter 2. Peptides were dissolved in 70% ethanol and stored at -20°C until use. 20 µg of either the C-CPE<sub>290-319</sub> or control peptide were added to 1x10<sup>7</sup> freshly isolated testis cells suspended in 3 ml of sterile DPBS. Cells with peptide treatment and cells from the initial isolate (no-peptide treatment) were passed through a 40 µM steriflip filter unit (Merck Millipore, Germany) to separate cells and allow access to Claudin receptors. Cells were incubated at 4°C overnight in T75 flasks in 10ml DMEM/F-12 + 10% FBS.

**Table 2-3: Amino acid sequence and characteristics of C-CPE<sub>290-319</sub> and control peptides.**

	<b>Amino Acid Sequence</b>	<b>Chemical Formula</b>	<b>Molecular Weight</b>	<b>Isoelectric Point</b>
C-CPE <sub>290-319</sub>	SLDAGQYVLVMKANSSYSYGNYPYSILFQKF	C <sub>157</sub> H <sub>232</sub> N <sub>36</sub> O <sub>46</sub> S <sub>1</sub>	3391.82	8.97
C- CPE <sub>ALA</sub>	ALDAGQAVLAMKANSAYSANYAYSALFAKF	C <sub>143</sub> H <sub>215</sub> N <sub>35</sub> O <sub>42</sub> S <sub>1</sub>	3128.53	9.03

Following incubation cells were washed with MACS buffer (DPBS containing 0.5% BSA +2 mM EDTA), centrifuged 300x g for 5 minutes and re-suspended in MACS buffer (100 µl). DBA-FITC beads (10 µl, Vector Laboratories, USA) were added to each cell suspension and incubated on ice for 15 minutes. Cells were washed twice with 1 ml MACS buffer and re-suspended in 90 µl MACS buffer per 10<sup>6</sup> cells.

Anti-FITC MicroBeads (20  $\mu$ l, Miltenyi Biotec, USA) were added per  $10^6$  cells and suspensions were incubated on ice for a further 15 minutes. Cells were washed in MACS buffer (1 ml) and resuspended in MACS buffer (500  $\mu$ l).

Suspensions were prepared by filtration through a MACS Separator (Miltenyi Biotec, USA) and rinsed with MACS buffer (3 ml). The cell suspensions were then loaded onto MACS LS columns fitted to the MACS platform (Miltenyi Biotec, USA) and allowed to pass through the column, the column was then rinsed three times with 3ml MACS buffer. The DBA negative cells (depleted of spermatogonia) were collected as the MACS negative fraction. The LS column was removed from the MACS platform, and DBA labelled cells (spermatogonia) were removed from column by adding 5ml MACS buffer and, using the plunger supplied, cells were collected as the MACS positive fraction. Cells from MACS positive and negative fractions were resuspended at  $1 \times 10^6$ /ml in MACS buffer supplemented with 1  $\mu$ l propidium iodine (3 $\mu$ M Sigma-Aldrich Chemical Industries, USA) immediately before analysis with flow cytometry. Cell death (PI positive) and DBA-FITC binding activity was assessed using BD LSR II flow cytometer (BD Biosciences, USA). Flow cytometry compensation was performed using three colour beads (BD Biosciences, USA) and PeakFlow claret flow cytometer reference beads (Life Technologies, USA). Data was analysed using FCS Express software (De Novo Software, USA).

### **2.2.8 Data Analysis**

Differences in positive cell counts for treatments were analysed using ordinary one-way ANOVAs using GraphPad Prism<sup>®</sup> software (GraphPad Software, USA)  $P < 0.05$  was considered to be significant. All graphs were created using GraphPad Prism with error bars presented as means  $\pm$  SEM.

## 2.3 Results

### 2.3.1 Enrichment of Bovine Spermatogonia by Percoll Discontinuous Density Gradient Centrifugation Further Enhances Enrichment of Differentially Plated Cells

Testis cell isolations from four bull calves were differentially plated in un-coated cell culture dishes overnight, after which the non-adherent fractions of the cultures, or initial isolate cultures were centrifuged in a Percoll discontinuous density gradient prepared according to Table 2-2.

Cell smears were made from the non-adhered cell population of the initial testis cell isolate following differential plating overnight, and from cell populations present at the interface between Percoll gradients after Percoll discontinuous gradient centrifugation. Enrichment for undifferentiated spermatogonia was confirmed by DBA binding activity, while Sertoli cells were stained with GATA4. Photographs of cell smears showing controls and specific staining of DBA-HRT and GATA4 are shown in Figure 2-2.

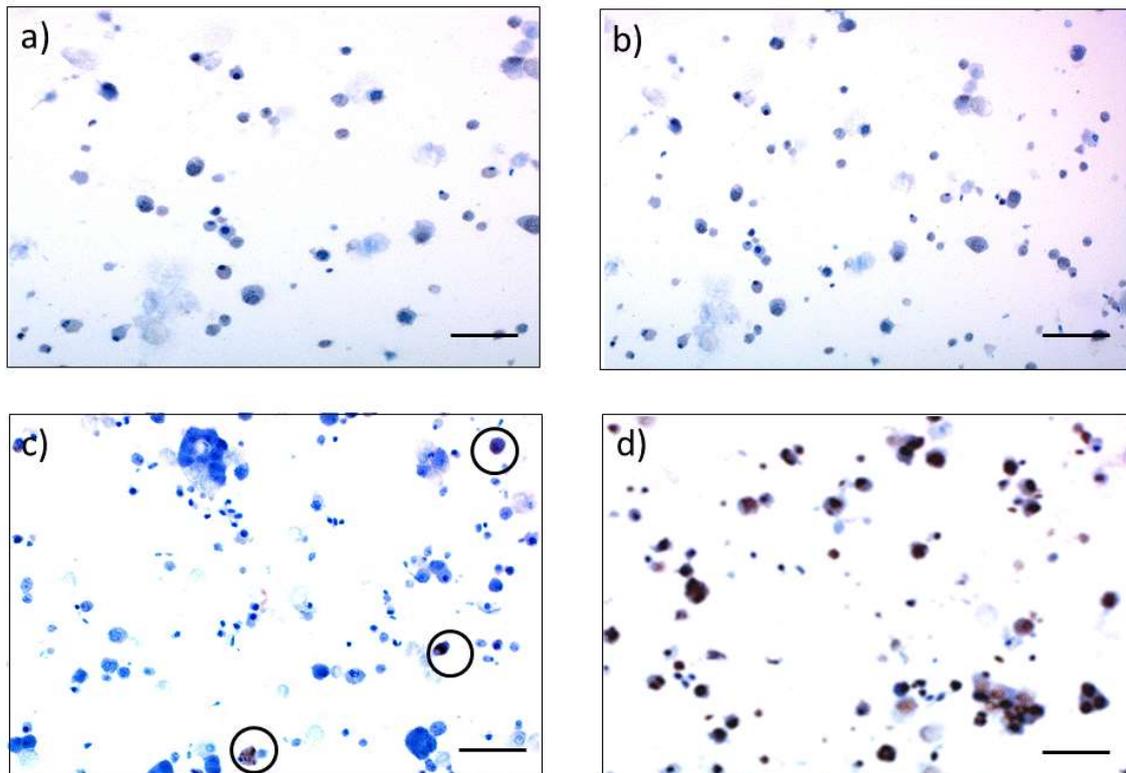


Figure 2-2. Examples of cell smears from testis cell isolates. a, b) No primary antibody, smears stained with secondary antibodies for mouse and rabbit IgG respectively (controls). c) Smear showing specific staining for DBA-HRT, brown cells circled cells exhibit DBA binding activity (un-differentiated spermatogonia). d) Smear showing specific staining for GATA4, brown cells positively express GATA4 (Sertoli cells). Scale bars represent 50µm.

Average cell counts from stained smears from the four testis isolates (200 cells counted for each smear) indicated an enrichment of approximately 2 fold of DBA positive cells in the non-adherent fraction of differentially plated cultures (Table 2-4). After Percoll discontinuous gradient centrifugation an enrichment of DBA positive cells of over 10 fold was observed in the cell population located in the interface between Percoll fractions 35-43% ( $1.065\text{-}1.055\text{ g ml}^{-1}$ ). Additionally, approximately half the number of GATA4 positive cells were observed in this population of cells compared to the initial isolate (Table 2-4). Another population of cells were observed in the interface between Percoll fractions 20-35% ( $1.047\text{-}1.055\text{ g ml}^{-1}$ ). Approximately 91% of these cells stained positively for Sertoli cell marker GATA4, with 5 fold more DBA positive cells found in the initial isolate than in this fraction of Percoll (Table 2-4).

To further analyse the enrichment of undifferentiated spermatogonia in the different cell populations, live cells from the initial isolate, non-adherent cells from the differential plated culture and both fractions of Percoll isolated cells were stained with DBA and GATA4 and analysed using flow cytometry.

Cell counts obtained by flow cytometry for stained cells in each population varied from the cell smear counts of the same isolations, and varied most widely for GATA4 staining. However similar trends were observed, as with results from cell smears, the largest enrichment for DBA positive cells was observed in the interface between 35-43% Percoll fractions with a 4 fold increase in DBA positive cells compared to the initial isolate. An enrichment of 1.7 fold was observed in the non-adherent fraction of differentially plated cells. While the cell population isolated in the interface between 20-35% Percoll fractions showed an almost 3 fold decrease in the number of DBA positive cells compared to the initial isolate and instead had relatively high percentage of GATA4 positive cells (Table 2-4). The percent of PI positive cells did not differ within treatments (DBA or GATA4 stained cells) for the initial isolate and enriched populations, however, the number of PI positive cells in samples stained with GATA4 was higher than those stained with DBA ( $16.3\% \pm 4.1$  compared to  $9.4\% \pm 3.2$ ).

Table 2-4. Cell counts for DBA positive (+) and GATA4 positive (+) cells in initial isolate, differentially plated non-adherent fraction, Percoll gradients interface between 43-35% and 35-20%. Different characters beside treatments indicate a significant difference in the percentage of cells observed between treatments in the same column (n=4, P<0.05).

	Flow Cytometry				Smear Counts			
	DBA+ (%)	Fold change from ii	GATA4 + (%)	Fold change from ii	DBA+ (%)	Fold change from ii	GATA4 + (%)	Fold change from ii
<b>Initial Isolate (ii)</b>	6.3 ± 1.2 <sup>c,d</sup>	NA	24.1 ± 6.6 <sup>a</sup>	NA	3.7 ± 0.6 <sup>d</sup>	NA	96.4 ± 1.9 <sup>a</sup>	NA
<b>Differentially Plated</b>	10.6 ± 2.8 <sup>c</sup>	1.6	3.0 ± 1.1 <sup>b</sup>	0.1	7.8 ± 1.3 <sup>c</sup>	2.1	86.3 ± 3.0 <sup>a</sup>	0.9
<b>ii Fraction 3-4 (43-35%)</b>	18.1 ± 2.0 <sup>b</sup>	2.9	6.76 ± 2.0 <sup>b</sup>	0.8	27.1 ± 2.6 <sup>b</sup>	7.3	52.0 ± 9.2 <sup>c</sup>	0.5
<b>ii Fraction 4-5 (35-20%)</b>	4.9 ± 1.2 <sup>c,d</sup>	0.8	28.8 ± 4.6 <sup>a</sup>	1.1	0.8 ± 0.9 <sup>d</sup>	0.2	60.3 ± 11.5 <sup>b</sup>	0.6
<b>DP Fraction 3-4 (43-35%)</b>	26.1 ± 5.8 <sup>a</sup>	4.1	5.1 ± 1.1 <sup>b</sup>	0.2	36.8 ± 2.6 <sup>a</sup>	9.9	48.7 ± 6.3 <sup>b</sup>	0.5
<b>DP Fraction 4-5 (35-20%)</b>	2.2 ± 1.1 <sup>d</sup>	0.34	23.3 ± 5.1 <sup>a</sup>	1.0	0.7 ± 0.8 <sup>d</sup>	0.2	91.1 ± 3.8 <sup>a</sup>	0.9

### 2.3.2 Treatment of Bovine Testis Cell Populations with C-CPE<sub>290-319</sub> Does Not Result in Enhanced Enrichment of Spermatogonia by MACS Isolation

Initial testis cell isolations were incubated with either C-CPE<sub>290-319</sub> peptide or control peptide C-CPE<sub>ALA</sub> prior to enrichment procedures to determine if enrichment was enhanced in the presence of C-CPE during MACS isolation. Samples of testis cells incubated with C-CPE<sub>290-319</sub> or C-CPE<sub>ALA</sub>, along with untreated testis cells, were enriched for DBA-biotin binding activity using MACS. MACS bound (DBA positive) and unbound (DBA negative) fractions of cells were analysed for cell viability through propidium iodine staining and the quantity of DBA positive cells in each fraction was determined using flow cytometry analysis

Flow cytometry analysis of MACS bound and unbound fractions of each treatment showed an enrichment of DBA positive cells in the MACS bound fractions of each treatment compared to the initial isolate (No-peptide 5 fold, C-CPE<sub>290-319</sub> 4.1 fold, C-CPE<sub>ALA</sub> 3.1 fold). There was no statistically significant difference observed in enrichment of DBA positive cells in MACS bound fraction of cells treated with the C-CPE<sub>290-319</sub> peptide compared to those treated with C-CPE<sub>ALA</sub> peptide or no peptide treatment ( $P>0.05$ ) (Figure 2-3). Additionally, there was no difference in the number of DBA positive cells observed in between the initial isolate and MACS unbound fractions ( $P>0.05$ ).

The number of propidium iodine (PI) positive cells also increased dramatically in all MACS bound treatments (No-peptide 6.5 fold, C-CPE<sub>290-319</sub> 7.3 fold, C-CPE<sub>ALA</sub> 6.6 fold), indicating a large proportion of dead cells in MACS bound fractions. There were no statistically significant differences in the percentages of dead cells observed between MACS bound cells treated with either C-CPE<sub>290-319</sub> or C-CPE<sub>ALA</sub> peptide or no peptide treatment ( $P>0.05$ ). C-CPE<sub>290-319</sub> MACS unbound cells showed a higher percentage of PI positive cells than the initial isolate and no-peptide MACS unbound treatments ( $P<0.05$ ), although there was no statistically significant difference in the percentage of PI stained cells between C-CPE<sub>290-319</sub> or C-CPE<sub>ALA</sub> (Figure 2-3).

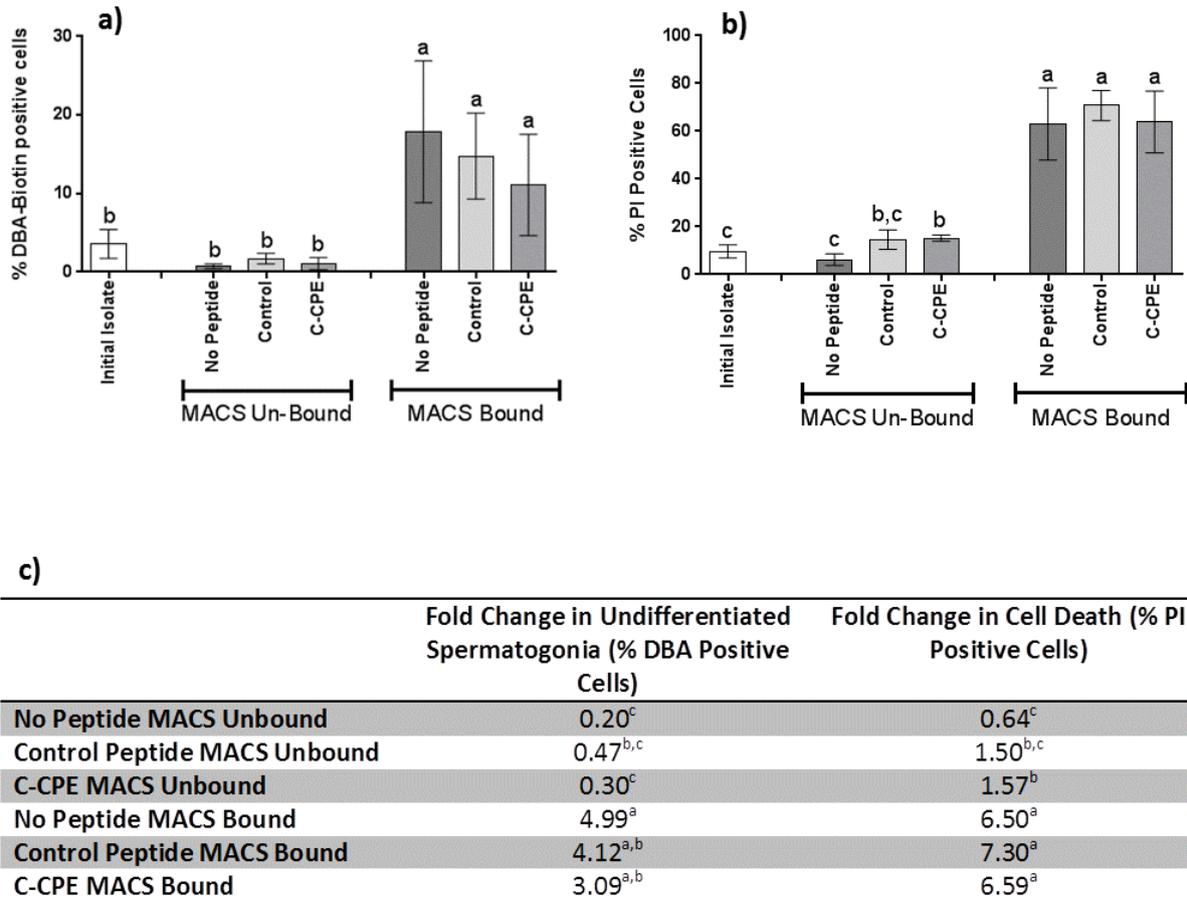


Figure 2-3. a) Percent (%) of DBA positive cells observed in initial isolate, and MACS bound and unbound fractions of testis cells treated with C-CPE (C-CPE<sub>290-319</sub>) control peptide (C-CPE<sub>ALA</sub>) or no peptide treatment. b) Percent (%) of propidium iodine (PI) positive cells observed in initial isolate, and MACS bound and unbound fractions of testis cells treated with C-CPE (C-CPE<sub>290-319</sub>) control peptide (C-CPE<sub>ALA</sub>) or no peptide treatment. Different characters above treatment means indicate a significant difference in DBA or PI positive cells between treatments (n=4, P<0.05). Error bars represent ± SEM. c) Table showing fold change in undifferentiated spermatogonia as determined by flow cytometry analysis (% DBA positive cells) and cell death (% PI positive cells) compared to initial isolate cells with no peptide treatment or treatment with control or C-CPE<sub>290-319</sub> peptides following MACS separation.

## 2.4 Discussion

The ability to enrich pure populations of bovine spermatogonial stem cells will aid in advancing reproductive technologies including testis cell transplantation (Herrid et al., 2009). Previous studies have used numerous methods to enrich populations of bovine spermatogonia, but no method has proven successful in obtaining a pure population of spermatogonia or more importantly spermatogonial stem cells. Previous studies comparing the efficiency of enrichment methods for bovine spermatogonia have reported conflicting results as to which method of enrichment produces the most enriched population of bovine spermatogonia. Some studies have shown that the most effective enrichment of bovine testis cells by differential plating is achieved without ligand coating

(Goel et al., 2010; Herrid et al., 2009). Herrid et al. (2009), reported greatest enrichment of undifferentiated bovine spermatogonia was achieved by this method of differential plating, obtaining an enriched population of 76.4% DBA+ cells. Differential plating of bovine testis cells, has been successfully in other studies, though lower enrichment results were achieved than the Herrid et al. (2009) study (Goel et al., 2010; Izadyar et al., 2002). Results presented in this chapter indicate a far lower proportion of DBA positive cells were obtained by differential plating than previous reports. An enrichment of between 7.8-10.6% DBA positive cells was achieved in the experiments outlined in this chapter, equating to an enrichment increase of 1.6-2.1 fold, also lower than that reported by Herrid et al. (2009) (4.7 fold). However, differential plating still resulted in consistent enrichment of DBA positive cells.

Enrichment of bovine spermatogonia using density gradients has been described in various studies, with the enriched population of spermatogonia isolated in interface of Percoll fractions with a density of between 1.0542-1.0564 g ml<sup>-1</sup> (30-45% Percoll) (Aponte, P. & de rooij, 2008; Goel et al., 2010; Herrid et al., 2009; Izadyar et al., 2002; van Pelt et al., 1996). These studies have all reported enrichment of bovine spermatogonia by Percoll density gradient ranging from approximately 50-73% spermatogonia in the isolated population (Aponte, P. & de rooij, 2008; Goel et al., 2010; Herrid et al., 2009; Izadyar et al., 2002). Additionally, studies by Izadyar et al. (2002), Aponte, P. and de rooij (2008) and Goel et al. (2010) found that enrichment by Percoll gradient centrifugation resulted in the highest enrichment of other treatments trialled. Results from this study confirm the enrichment of bovine spermatogonia in the Percoll fractions with a density of 1.055-1.065 g ml<sup>-1</sup> (35-43%), and corresponded to similar densities reported previously, differing due to fewer gradient steps. Lower enrichment percentages were observed in the results from this study compared to previous studies using Percoll for enrichment, with the highest proportion of DBA positive cells observed approximately 37%.

Results of the experiments outlined in this chapter suggest that Percoll gradient enrichment is more efficient at obtaining a population of testis cells high in spermatogonia than differential plating with un-coated plates. Additionally, the enrichment of DBA positive cells increased with differential plating followed by Percoll discontinuous gradient centrifugation with an enrichment of 4.1-9.9 fold observed in cultures that were differentially plated followed by centrifugation on a Percoll gradient, compared to cultures that were subjected to Percoll gradient without prior differential plating (2.9-7.3 fold increase).

Flow cytometry analysis and stained cell smears were used to analyse the proportion of DBA and GATA4 positive cells in each population. The result of these analyses showed differences in the percentages of positively stained cells; especially in the case of GATA4 where flow cytometry analysis indicated a significantly lower proportion of GATA4 positive cells were present in populations than analysis of cell smears. It has been suggested that some cell types are under-represented in flow cytometry analysis compared to traditional methods such as staining cell smears. Smock, Perkins, and Bahler (2007) report that FACS analysis of plasma cells from bone marrow resulted in consistently lower numbers of plasma cell percentages than plasma cell percentages determined morphologically by stained cell smears. These differences were believed to be in part due to the sample quality and also due to cellular processing involved in the process of flow cytometry. Reports that differences in percentage of cells observed in FACS analysis compared to cell smears up to  $69\% \pm 28\%$ , with only 10% of specimens having a similar cell count for both FACS and cell smears have been made (Smock et al., 2007). Studies using flow cytometry for the analysis of Sertoli cells have found that if morphological changes occur in the Sertoli cells or in surrounding tubules, Sertoli cells cannot be detected by flow cytometry (Kostakopoulos et al., 2002). Furthermore, GATA4 stained cells analysed for cell viability with PI staining indicated a higher proportion of cell death than samples stained with DBA. It is possible that the preparation of Sertoli cells by staining with GATA4 prior to flow cytometry analysis may have resulted in cell damage that did not allow cells to be recognised by flow cytometry. Previous studies where spermatogonia were analysed using flow cytometry did not report the analysis of Sertoli cells by GATA4 staining; it is therefore difficult to determine whether the under-representation of GATA4 positive cells is commonly observed in the analysis of bovine testis populations. Numbers of spermatogonia observed by flow cytometry and cell smear analysis were more closely correlated, possibly because spermatogonia are less affected by the processing required prior to flow cytometry analysis. Hence, it is possible that flow cytometry analysis is more suitable for the analysis of DBA positive cells than for GATA4 positive cells in bovine testis populations.

All enrichment methods tested resulted in a decrease in GATA4 positive cells in the spermatogonia enriched fractions of culture, indicating the decreased presence of Sertoli cells in these populations. The increased expression of DBA binding activity, along with the decreased expression of GATA4 in enriched populations indicate that both differential plating and Percoll discontinuous density gradient centrifugation produced enriched populations of bovine spermatogonia.

The interactions between Sertoli cells and spermatogonia by adhesion molecules may result in the inhibition of enrichment by the contamination of adhered Sertoli cells in enriched spermatogonia populations (Luo et al., 2006). The experiment outlined in this chapter tested whether it was possible to improve enrichment of spermatogonia by treatment of initial testis cell isolations with the C-CPE<sub>290-319</sub> peptide to interfere with bonds formed between Sertoli cells and spermatogonia by Claudin-8 interaction. Results indicate that treatment of testis cell isolations with C-CPE<sub>290-319</sub> did not result in an increase of DBA positive cells in the MACS enriched fraction of cells compared to treatment with the control peptide or no peptide treatment. There was no difference in the percentage of DBA positive cells in the MACS bound fractions of any treatments.

To date, most C-CPE/ Claudin binding has been determined in mouse and human models and not in livestock species. There are 24 members of the Claudin family, and so far CPE has been shown to interact with Claudins-3-4-5-6-7-8-9 and 14 to differing degrees (Mitchell & Koval, 2010). Furthermore, difference in the ability of CPE to bind to members of the Claudin family also differs between species, with CPE only able to recognise murine Claudin-8 and not the human protein. These differences in binding ability are believed to be due to the convergence of the amino acid sequence of Claudins' between species (Mitchell & Koval, 2010). The binding motif of Claudins' that is recognised by CPE is found in the extracellular loop domains of the protein and must conform to the amino acid sequence NP(L/V)(L/V)(P/A) to facilitate binding to CPE (Mitchell & Koval, 2010). The proline (P) amino acid is believed to be most important in binding of C-CPE as it stabilising the loop of the protein necessary for binding (Winkler et al., 2009). The human sequence of Claudin-8 shows significant convergence from the murine sequence and is therefore unrecognised by CPE.

Conversely, the bovine amino acid sequence of binding motif has more similarity to the murine sequence of Claudin-8 and contains the proline amino acid needed to stabilise the loop in the binding site of the protein. However, evidence supporting the binding of bovine Claudin-8 to CPE has not yet been reported. It is therefore possible that the C-CPE<sub>290-319</sub> peptide was not able to bind to bovine Claudin-8. Additionally, a positive control was not available for this experiment. It has been well documented in previous studies that the C-CPE<sub>290-319</sub> peptide binds effectively to Claudins 3 and 4 from humans and mice (Cocco et al., 2010; Kondoh, M., Takahashi, A., Fujii, M., Yagi, K., & Watanabe, Y., 2006), however no studies have been conducted on bovine models. Therefore a positive control using the C-CPE<sub>290-319</sub> peptide on mouse or human cells was deemed uninformative

as it would not prove the ability of this peptide to bind to bovine Claudins. This experiment had been designed to be a preliminary test for whether it was possible to disrupt the tight junction bonds in the bovine testis. Further investigations into the mechanistic aspects of the C-CPE peptides ability to bind to bovine Claudin-8 were not pursued in the scope of this thesis. Replication of the original experiment C-CPE<sub>290-319</sub> peptide would have addressed the question whether the peptide was correctly synthesized and folded. In the scope of this thesis this investigation was not further pursued.

Peptide labelling with compounds such as Biotin may be used in future experiments in order to determine if the C-CPE peptide was able to bind to bovine Claudin-8. Additionally, it is possible that the assumed Claudin-8 mediated bonds formed between Sertoli cells and spermatogonia were not broken to a sufficient degree to allow C-CPE access the binding sites of Claudin-8. Testis cell isolations were filtered through cell strainers while in contact with the C-CPE<sub>290-319</sub> peptide in an attempt to break bonds between Sertoli cells and spermatogonia and allow C-CPE access to the binding site. However this filtration process may not have been sufficient to effectively break these bonds.

Furthermore, the role of Claudin-8 on the cell surface of bovine spermatogonia and a subset of Sertoli cells is not sufficiently understood, and might not be responsible for connections between Sertoli cells and spermatogonia. The interaction of Sertoli cells and spermatogonia may be a result of several different adhesion molecules that are involved in the adherence of spermatogonia to Sertoli cells in the testis (Ziparo, Geremia, Russo, & Stefanini, 1980). These molecules include N-cadherin's and galactosyltransferase which bind Sertoli cells and spermatogonia via carbohydrate residues (Newton, Blaschuk, & Millette, 1993; Scully, Shaper, & Shur, 1987). It is therefore reasonable to assume that even if C-CPE<sub>290-319</sub> was able to bind to bovine Claudin-8 and break the bonds formed between Sertoli cells and spermatogonia, there may be other cell surface adhesion molecules that prevented the separation of these cells thereby inhibiting enrichment.

Of interest was the high proportion of propidium iodine positive cells that were observed in the MACS positive fractions (DBA-Biotin binding activity) of samples, indicating a high amount of cell death in these fractions. This is in contrast to results of the first experiment outlined in this chapter, where DBA positive cells did not show an increased incidence of PI positive cells compared to the initial isolate, while GATA4 stained cells did. GATA4 expression was not analysed in this experiment, with the focus instead on the enrichment of DBA positive cells. The incidence of increased cell death

observed in the C-CPE experiment may have been as a result of the necessary re-suspension of the peptides in 70% ethanol. Ethanol exposure has been shown to damage cells in culture (Castilla, González, Fouad, Fraga, & Muntané, 2004), however, apoptosis and necrosis of cells were observed at far higher levels than cells were exposed to in this study (10 mmol/l) (Castilla et al., 2004). Alternatively the processing of cells may have resulted in apoptosis of cells; the treatment of samples with an apoptosis inhibitor may improve the rate of cell survival in these fractions. Importantly, MACS unbound fractions of cells treated with the C-CPE<sub>290-319</sub> peptide also had a higher incidence of cell death than other MACS unbound fractions. Hence, it is possible that the C-CPE<sub>290-319</sub> peptide may cause cell death by an unknown mechanism even though it has been deemed to have no cytolytic effects in other cell types (Yuan, Xiaoqin et al., 2009). It is also possible that the C-CPE<sub>290-319</sub> peptide may bind to 'death receptors' on the cell surface of PI positive cells. Mammalian cells undergo apoptosis through the activation of procaspase, this activation can be triggered by proteins such as Fasligand, produced by killer lymphocytes, binding to death receptors on the cell surface, resulting in an intracellular cascade of apoptotic signalling resulting in subsequent cell death (Alberts, Johnson, & Lewis, 2002). Further investigation into the cause of cell death in this experiment could include identification of apoptotic cells using commercially available protocols, while comparison of Fasligand and C-CPE could also be made to determine if binding of C-CPE to cell surface death receptors is possible.

The results showed that addition of C-CPE peptide did not improve the MACS enrichment of DBA positive cells. In the context of this thesis, further understanding of the mechanisms of C-CPE effects on testis cells was not a priority, and therefore no more experiments were conducted involving C-CPE.

## 2.5 Conclusion

The enrichment of bovine spermatogonia to produce a pure population of cells will aid in the enhanced development of reproductive technologies for the bovine species including testis cell transfer. This chapter has described use of differential plating and Percoll discontinuous density gradient centrifugation on the enrichment of DBA positive cells (undifferentiated bovine spermatogonia). It was found that a combination of differential plating on un-coated cell culture dishes followed by further separation of the enriched fraction of cells using Percoll discontinuous density gradients was the most successful enrichment method, resulting in an increase in

enrichment of DBA positive cells of between 4-10 fold. In an attempt to further improve the enrichment of DBA positive cells the C-CPE<sub>290-319</sub> peptide was used to treat testis cell samples prior to MACS isolation. It was hypothesised that C-CPE<sub>290-319</sub> peptide would interfere with bonds between Sertoli cells and spermatogonia possibly caused by the existence of Caludin-8 a member of a family of tight junction proteins. However, there was no evidence observed that treatment with this peptide resulted in enhanced enrichment of DBA positive cells when using MACS enrichment.

As the enrichment of bovine spermatogonia and specifically spermatogonial stem cells still results in the contamination of enriched populations with Sertoli cells and other somatic testis cells, the identification of new makers, in particular cell surface markers that can uniquely identify undifferentiated bovine spermatogonia are required. These markers may be used to better understand the physiology of bovine spermatogonial stem cells and may be able to be used directly in enrichment methods.

## **Chapter 3 : Characterisation of Putative Spermatogonial Markers**

### **3.1 Introduction**

Spermatogonial stem cells (SSCs) make up a small subset of the undifferentiated, type A spermatogonia. These adult stem cells form the foundation for spermatogenesis, and sustain male fertility throughout adult life (Aponte, Pedro et al., 2005; Oatley, J. M. & Brinster, 2006). SSCs reside on the basal membrane of the seminiferous tubules of the testis, and are closely associated with Sertoli cells, which act as support cells providing nutrients for all stages of spermatogonia (Aponte, P. & de Rooij, 2008; Aponte, Pedro et al., 2005; Oatley, J. M. & Brinster, 2006; Phillips, Gassei, & Orwig, 2010). SSCs are of immense interest in mammalian reproduction, as they are the only cell type capable of restoring fertility (Aponte, Pedro et al., 2005). Additionally, SSCs are the only cell type that have the ability to colonise testis tissue when transplanted into a recipient, in a process known as germ cell transplantation (Brinster, R. & Zimmermann, J., 1994). This technology has potential applications in the livestock industry to more efficiently produce transgenic livestock and to improve breeding strategies (Herrid & McFarlane, 2013; Herrid et al., 2006; Hill & Dobrinski, 2006).

The development of germ cell transplantation in the bovine species has made the identification and isolation of a pure population of undifferentiated spermatogonia essential. The success of germ cell transplantation relies on the ability to isolate a large, relatively pure population of spermatogonial stem cells. However, SSCs are present in extremely low numbers in the adult testis, making up only approximately 0.03% of all germ cells and 1.25% of the total spermatogonia population in mice (Tegelenbosch & de Rooij, 1993). Although methods to assess, isolate, and characterise spermatogonia are well established in mice, there are no markers that identify SSCs exclusively (Aponte, Pedro et al., 2005). Furthermore, the characterisation of livestock germ cells is still rudimentary, with few bovine spermatogonial markers available, and none that uniquely identify SSCs. Therefore the detailed characterisation of bovine SSCs is still required (Herrid et al., 2007). The lack of cell surface markers for spermatogonia, and specifically spermatogonial stem cells, limits the ability to identify and purify these cells, thereby limiting their study and use. Identification of markers for bovine spermatogonia and SSCs, is of particular interest to enhance germ cell enrichment and culture, for the potential use of live cells in germ cell transplantation (Herrid et al., 2007).

A number of markers have been identified that characterise different subsets of the spermatogonia population. Different markers are able to identify the total spermatogonia population, undifferentiated spermatogonia types, or more differentiated cell types. Although the majority of these markers have been identified in murine models, studies have shown that most are conserved, at least in part, between species (Aponte, Pedro et al., 2005; Oatley, J. M. & Brinster, 2006). However, as no markers that uniquely identify SSCs have been discovered for any species, the identification of SSCs is limited. Additionally, spermatogonial stem cells are not easily identified, as they are not obviously morphologically different to other types of spermatogonia type A, or more differentiated spermatogonia, especially in cell suspension (Aponte, Pedro et al., 2005). Given the importance of purifying live populations of these cells, cell surface markers that identify SSCs specifically would be particularly beneficial (Colgrave et al., 2013; Izadyar et al., 2003). However, proteins located in the plasma membrane are generally low in abundance, further hindering the identification of such proteins as markers for spermatogonia (Colgrave et al., 2013).

In bovine testis UCHL1 (previously known as PGP9.5 - Protein Gene Product 9.5) (Fujihara et al., 2011; Wrobel, K., Bickel, Kujat, & Schimmel, 1995), and DBA (*Dolichos biflorus* agglutinin) binding activity (Ertl & Wrobel, 1992; Herrid et al., 2007; Izadyar et al., 2002; Wrobel, K., 2000) are expressed in gonocytes in the neonatal testis, and early stage spermatogonia in the adult testis (Fujihara et al., 2011). ZBTB16 (previously known as PLZF) has been shown to identify a small subset of undifferentiated bovine type A spermatogonia (Costoya et al., 2004; Reding et al., 2010), and is believed to be most representative of the SSC population (Kubota et al., 2003). DDX4 (previously known as VASA) has been shown to be expressed in gonocytes in the neonatal testis, and by more differentiated sub-sets of bovine spermatogonia in the adult testis (Bartholomew & Parks, 2007; Fujihara et al., 2011). Similarly, stem cell markers OCT4 and NANOG, have been shown to be expressed by bovine gonocytes, but are also expressed in more differentiated spermatocytes in the adult testis, with rare associations with undifferentiated spermatogonia sub-sets (Fujihara et al., 2011). Alternatively Sertoli cells can be identified by their expression of GATA4 (Imai et al., 2004) and Vimentin (Steger, Schimmel, & Wrobel, 1994; Wrobel, K. H., Bickel, & Kujat, 1995).

The identification of putative spermatogonia markers that may be unique to SSCs is difficult. Proteomics provides a feasible method to profile the presence and quantity, of protein expression in testis cell populations. Proteomics analysis have previously been used to study SSC populations in

rats (Guillaume et al., 2001), mice (Dihazi et al., 2011; Dihazi et al., 2009; Guo, X. et al., 2001) and humans (Harkness et al., 2008). This technology has provided critical information in understanding the complex biological processes of spermatogenesis (Chocu, Calvel, Rolland, & Pineau, 2012). Recently, proteomic analysis of bovine SSCs was conducted to identify potential candidates for protein markers of bovine spermatogonia and SSCs (Colgrave et al., 2013). This study analysed membrane enriched fractions of differentially plated bovine testis cell populations. The presence and abundance of proteins, in both the non-adherent fraction (spermatogonia enriched) and adherent fraction (Sertoli cell enriched) were evaluated by protein score and spectral counting. A short-list of potential candidates for bovine spermatogonia markers was compiled according to the likely expression by spermatogonia. Potential marker genes which showed expression patterns similar to early spermatogonia were evaluated for differential mRNA expression in bovine spermatogonia, by quantitative real time PCR (qRT-PCR) and immunohistochemistry. The analysis of six candidate markers from this short list, IQGAP1, TNL1, FSCN1, DDX6, TKTL1 and NAP1L4, are the focus of this chapter.

IQGAP1 is a scaffold protein expressed in numerous tissues and is involved in regulating various cellular processes including organisation of the actin cytoskeleton, transcription processes and is also involved in cellular adhesion, including the adhesion of Sertoli cells to germ cells by interactions with multiple proteins including; actins (Erickson, Cerione, & Hart, 1997), Cdc42 (Erickson & Cerione, 2001),  $\beta$ -catenin (Kuroda et al., 1998), E-cadherin (Kuroda et al., 1998) and N-cadherin (Lui, Mruk, & Cheng, 2005).

Fascin1 (FSCN1) is an actin binding protein, that forms tightly packed actin bundles by filamentous cross linking (Hashimoto, Kim, & Adams, 2011). Actin bundles are important for cellular structure and function, and are believed to be required for spermatogenesis and normal sperm morphology and function (Kolthur-Seetharam, Martianov, & Davidson, 2008). A study by Tubb et al. (2002) found that FSCN3 is a paralog of FSCN1, and is restricted to the mammalian testis where it is expressed by late stage spermatozoa and the sperm head. However, the functional role of FSCN3 is unknown (Hashimoto et al., 2011; Tubb et al., 2002).

Talin1 (TLN1) is an adaptor protein present in the cell membrane that connects cell adhesion molecules from the integrin family to cytoskeletal actin. TLN1 is specifically involved in cell adhesion and cell spreading. In *Drosophila* TLN1 is expressed in somatic gonadal precursor cells (SGPs), these

cells are present in the gonads but are not germ cells. It is thought that Talin1 plays an important role in establishing the stem cell niche and maintenance of cell position, during morphogenesis in the gonads of *Drosophila* (Tanentzapf, Devenport, Godt, & Brown, 2007). Another member of the Talin family, TLN2, has been found to be abundant in the mammalian testis, where it is expressed in elongating spermatids of the mature testis. However little is known about the function of this protein (Emmanuel et al., 2009).

DDX6, also known as rck/p54, is a member of the DEAD box protein family and functions as an RNA helicase. DDX6 is similar to DDX4 (VASA), which is also a marker for gonocytes, and more differentiated populations of germ cells (Fujihara et al., 2011). DDX6 may be involved in regulating the translation of mRNAs that are essential for spermatogenesis. The DDX6 protein has been previously shown to be expressed in murine testis tissue (Akao & Nakagawa, 2006; Matsumoto, Kwon, Kim, & Akao, 2005), specifically in the germinal epithelial cells lining the testis tubules, forming particles in spermatogonia and primary spermatocytes (Matsumoto et al., 2005).

NAP1L4 is a nucleosome assembly protein, that acts as a histone chaperone, by delivering histones from the cytoplasm to the chromatin assembly machinery (Li, S. et al., 2012). NAP1L4 has been reported to be localised mainly in the cytoplasm and in the nucleus of a small population of cells where it is believed to mediating nucleosome formation (Okuwaki, Kato, & Nagata, 2010). Additionally, NAP1L4 has been found to be expressed in human trophoblastic stem cells (Frost, Udayashankar, Moore, & Moore, 2010), Hu, R., Lee, Johnson, and Feinberg (1996) report that NAP1L4 is expressed at levels three fold higher in the testis, than in other tissue. Conversely, in porcine tissue, NAP1L4 is believed to be universally expressed, though at higher levels in the neonatal placenta, than in other tissues (Okuwaki et al., 2010). As a result it has been suggested that NAP1L4 may be involved in the reproductive performance and placental function of animals (Okuwaki et al., 2010).

TKTL1 is a transketolase, involved in linking the pentose phosphate pathway to the glycolytic pathway. TKTL1 has previously been reported to be highly expressed in human spermatogonia (von Kopylow et al., 2010; Wang, P., McCarrey, Yang, & Page, 2001). Additionally, it is characterised as a cancer/testis gene (CTG), meaning it is normally expressed in testis cells and is also activated in a range of cancer types (Hofmann, O. et al., 2008). The role of TKTL1 as a CTG, suggests its expression is necessary for rapidly dividing cells (Glazer et al., 2009; Xu, X., Zur Hausen, Coy, & Löchelt, 2009).

To characterise the expression of the putative markers, TALIN1, IQGAP1, FASCIN1, DDX6, TKTL1 and NAP1L4, in the bovine testis, the expression profiles of these markers in spermatogonia enriched and Sertoli cell enriched fractions of testis isolations were analysed using qRT-PCR. The expression of these markers in the spermatogonia and Sertoli cell enriched testis cell populations was compared to expression of established bovine spermatogonial markers DDX4 (VASA), UCHL1 (PGP9.5) and ZBTB16 (PLZF), and Sertoli markers, Vimentin and GATA4.

Putative bovine spermatogonial markers were identified by their association expression in the spermatogonia enriched fraction of testis cells. To further characterise protein expression of these markers by cells of the bovine testis, immunohistochemical staining for antibodies of these markers was conducted on sections of bovine testis from pre-pubertal, pubertal and post-pubertal *Bos Taurus* bulls.

Finally, cells expressing the potential markers in bovine testis cell isolates from different age groups were quantified by the immunocytochemical staining of the putative markers in testis cell smears from pre-pubertal, pubertal bulls.

## 3.2 Materials and Methods

### 3.2.1 General

All animal experiments were conducted with approval from under the F.D McMaster Laboratory Animal Ethics Committee, CSIRO Animal Food and Health Sciences (Approval number 10/14). All reagents for cell culture were purchased from Life Technologies (USA) unless otherwise stated. MACS reagents were purchased from Miltenyi Biotec (Germany). Chemicals and other reagents were purchased from Sigma-Aldridge Chemical Industries (USA). Primers for candidate genes were designed using Primer3 software, and ordered through Biosearch Technologies, Inc. Table 3-1 describes the primers used in Chapter 3.

### 3.2.2 Collection of samples and Enzymatic Isolation of Testis Cells

Four Angus bull calves, aged between 4-8 months, were castrated under general anaesthesia. A further four *Bos taurus* bull calves, less than 1 year old, were slaughtered at a local abattoir, and testes were removed after slaughter of animals. Testes were stored on ice until dissection.

For immunohistochemical analysis of testis tissue, sections of testis obtained by needle biopsy, or following castration, were collected from three Angus and Angus-cross animals from each age group, pre-pubertal (testis weights 16,17 and 19g), pubertal (testis weights 33, 38.8 and 65.8 g) and post-pubertal adults (testis weights 194, and two in excess of 300g). Sections were prepared using standard methods and embedded in paraffin for staining.

For collection of testis cells for smears, testes were collected from four *Bos Taurus* animals from pre-pubertal (23.2, 25.3, 22.1 and 20.5 g) and pubertal (44.7, 57.7, 56.4 and 77.8g) age groups, by castration under anaesthesia. Testes were stored on ice until dissection. Following testis cell isolation smears were made and were frozen at -80°C prior to immunocytochemical staining.

Enzymatic digestion of testis tissue, and testis cell isolation, was conducted using the method described by Herrid et al. (2006), outlined in Chapter 2 (Page 52). Briefly, the tunica albuginea and epididymis were removed from testes. 20g of tissue was dissected from each testes, followed by a two-step enzymatic digestion of tissue with collagenase (1mg/ml Sigma-Aldridge, USA), followed by treatment with trypsin (2.5 mg/ml Sigma-Aldridge, USA), to obtain a single cell suspension. The cell suspension was then filtered through a cell strainer with two layers of nylon mesh (80 and 55 µm). Cells were washed and resuspended in DMEM/F-12 + 5% FBS. Cell viability was assessed by trypan blue staining. Isolated cells were used for differential plating, in the case of those used for qRT-PCR analysis, or were used to make cell smears.

### 3.2.3 Differential Plating for Enrichment

Initial isolate cells were resuspended in 10 ml of DMEM/F-12 with 5% FBS and 1% Anti-Anti (10,000 U/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies,

USA) (Life Technologies) and transferred into a T75 flask. Cells were incubated overnight at 37°C 5% CO<sub>2</sub>. Following incubation, flasks were tapped lightly to dissociate loosely attached cells, and the supernatant was collected. The attached cell fraction was removed enzymatically by incubation with TrypLE™ Express (5 ml, Life Technologies, USA) for 5 minutes. Both cell fractions were centrifuged at 1000 xg for 5 minutes and the cell pellet was frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Cells obtained from abattoir samples were prepared in the same way described above, but stored in RNA later (Life Technologies, USA) for transport to the F D McMaster Laboratory, Armidale, NSW, for RNA extraction. Cells were resuspended for RNA extraction following the manufacturer's instructions.

### 3.2.4 RNA Extraction and cDNA Production

RNA extraction from frozen cell samples, or cell samples stored in RNAlater, was conducted using an RNeasy Midi Kit (Qiagen, USA), with an on-column DNase step, according to manufacturer's instructions. The resulting RNA from each sample was quantified spectrophotometrically using a NanoDrop spectrophotometer (Thermo Scientific, USA).

In preparation for qRT-PCR, RNA was converted to cDNA using SuperScript™ III First-Strand Synthesis SuperMix, as per manufacturer's instructions (Life Technologies, USA). The resulting cDNA was stored at -20°C until analysis by qRT-PCR.

### 3.2.5 qRT PCR

Quantitative RT-PCR (qRT-PCR) reactions were carried out in an iQ5 real time thermal cycler (Bio-Rad, USA). Reactions were prepared with 1x IQ SYBR Green Supermix (Bio-Rad, USA), 0.5 µM each of forward and reverse primer and cDNA transcribed from 10 ng RNA, with reactions performed in triplicate. Reaction efficiencies for each primer were determined using a serial dilution of cDNA (100 ng, 20 ng, 4 ng, 0.8 ng and 0.16 ng), from testis initial isolate cells the reactions were carried out in triplicate. The reference gene *RPS26* was previously identified as the best reference gene using the sheep GeNorm kit (PrimerDesign, UK) and was used in all qRT-PCR reactions.

### **3.2.6 Immunohistochemistry of Testis Sections**

Paraffin sections (5 µm) were deparaffinised and rehydrated through xylene and graded alcohol dilutions to water. Antigen retrieval was conducted by heating slides in 10 mM Tris base, 1 mM EDTA, 0.05% Tween 20 in an 800 W microwave on 30% power for 15 minutes. Slides were allowed to cool and were rinsed in tap water.

For immunofluorescence, protein blocking was performed by incubating sections in tris-buffered saline 0.05% Tween 20 (TBS-T) for 30 minutes. Cocktails of primary antibodies containing DBA-biotin (10 µg/mL, Vector Laboratories, USA) and another primary antibody, GATA-4 (1 µg/mL, Santa Cruz Biotechnology, USA), Vimentin (1/100, Zymed, USA), and DDX6 (2 µg/mL, Santa Cruz Biotechnology, USA), NAP1L4 (0.2 µg/mL, Abcam, UK) or TKTL1 (0.5 µg/mL, Santa Cruz Biotechnology, USA), were incubated for 45 minutes at room temperature, with antibodies diluted in TBS-T, 0.5% BSA. Parallel sections were used as controls and were incubated with a combination of rabbit and mouse immunoglobulin (2 µg/mL, Sigma-Aldrich Chemical Industries, USA). Slides were washed in TBS-T and then incubated with the secondary antibodies streptavidin-alexafluor 350 (10 µg/mL, Life Technologies, USA), goat anti-mouse Alex 488 (1:250, Life Technologies, USA), chicken anti-rabbit Alex 594 (1:1000, Life Technologies, USA) for 30 minutes. Slides were washed in TBS, cover slipped in Prolong Gold (Life Technologies, USA), and kept in the dark until photographed.

For the brightfield images, staining was visualised using the Expose Mouse and Rabbit Specific HRP/DAB detection kit (Abcam, UK) according to the manufacturer's instructions. Basically, sections were incubated with hydrogen peroxide block (supplied) for 10 minutes, and then washed twice in TBS. To block nonspecific background, staining sections were incubated with protein block (supplied), for 10 minutes at room temperature, then washed once with TBS. Sections were incubated with primary antibodies at the following concentrations for 30 to 60 minutes; DDX6 (2 µg/mL, Santa Cruz Biotechnology, USA), NAP1L4 (0.2 µg/mL, Abcam, UK), TKTL1 (0.5 µg/mL, Santa Cruz Biotechnology, USA), followed by four washes with TBS. Primary antibodies were replaced with TBS buffer for the negative controls. Sections were incubated with Biotinylated Goat Anti-Polyvalent (supplied) for 10 minutes at room temperature, followed by four washes in TBS. Sections were incubated with streptavidin peroxidase (supplied), for 10 minutes at room temperature and washed four times in TBS. 30 µl (1 drop) of DAB Chromogen (supplied) in 1.5 ml of DAB Substrate (supplied) was applied to tissue and incubated for 5 minutes, followed by four washes in TBS. Sections were

counterstained with haematoxylin, and dehydrated through alcohol and xylene then mounted with DPX (Merck, Darmstadt, Germany).

Photography of fluorescent and brightfield images was performed using a compound microscope with an AxioImager and AxioVision software (Carl Zeiss, Oberkochen, Germany).

### 3.2.7 Immunocytochemistry of Cell Smears

Frozen cell smears were equilibrated to room temperature prior to immunocytochemical staining. Brightfield staining was conducted using the Expose Mouse and Rabbit Specific HRP/DAB detection kit (Abcam) according to the manufactures instructions described above. Primary antibodies were used at the following concentrations DDX6 (2 µg/mL, Santa Cruz Biotechnology, USA), NAP1L4 (0.2 µg/mL, Abcam, UK), TKTL1 (0.5 µg/mL, Santa Cruz Biotechnology, USA), DDX4 (1:2000, Abcam, Cambridge, UK), DBA-HRT (1:20, Vector Laboratories, USA), GATA4 (1:300, Vector Laboratories, USA). Primary antibodies were replaced with TBS buffer for negative controls. Smears were counterstained with haematoxylin, and dehydrated through alcohol and xylene then mounted with DPX (Merck, Darmstadt, Germany).

Images were photographed using a compound microscope an AxioImager and AxioVision software (Carl Zeiss, Germany). Cell counts were performed manually from at least two fields of view with at least 200 cells counted per smear.

### 3.2.8 Data Analysis

Quantitative RT-PCR  $C_t$  results were converted into gene expression data as fold change relative to the reference gene *RPS26*, using Microsoft Excel add-in Genex (Bio-Rad, USA).

Statistical analysis of gene expression data from qRT-PCR was conducted using GraphPad Prism<sup>®</sup> software (GraphPad Software, USA) with unpaired t-tests used to determine significant difference expression in means of adherent and non-adherent fractions of testis populations.  $P < 0.05$  was considered to be significant, graphed results are presented as means  $\pm$  SEM.

Chapter 3: Characterisation of Putative Spermatogonia Markers

Table 3-1: Primers, expected amplicon size and efficiencies used in Chapter 3. 'Bv' indicates primer is specific for expression of bovine cDNA.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Amplicon Size (bp)	Efficiency (%)	Melt Peak (°C)
<b>Known Testis Cell Markers</b>					
Bv <i>ZBTB16 (Plzf)</i>	ATCCTCTCCACCGCAACGAG	AAGTCCTCTGCCTTGGCTTG	116	110.8	85
Bv <i>Vimentin</i>	CATGTCCACCAGGTCCGTG	GGGTGGATGTGGTCACGTAG	107	93.7	89.5
<i>DDX4 (VASA)</i>	TACGCCCAATTCGATGAAC	TTGCCACTTTTCTTTGTCAAG	225	89.4	83
<i>GATA4</i>	ACCAGCAGCAGTGAGGAGAT	TGGTGA CTGGCTGACAGAAG	190	86.5	89
<i>UCHL1 (PGP9.5)</i>	CCCCTGAAGACAGAGCAAAG	CCGACATTGGCCTTCTG	86	87.9	84.5
<b>Candidate Markers</b>					
<i>DDX6</i>	TGCCATTCTCTTGCTTTGTG	CTCACTCCTTTTGCCTGGAG	148	100.7	82
<i>NAP1L4</i>	CGGAGTTCTGGTTCACCATT	AGTCATCGGGTTCAAAGTGG	165	93.8	83.5
<i>TKTL1</i>	TGAGCGCTTCATCGAGTGTT	AGCGAAGGTGCAAGCAAAAAG	194	102.5	87.5
<i>IQGAP1</i>	AACTGCCCTATGACGTGACC	TCAGCACTTTGGCAATGAAG	171	99.1	86
<i>FASCIN1</i>	TGCCTTTCAA ACTGGAAACC	GGCCTCT TTGGGGAAATAAG	188	90.9	90
Bv <i>TALIN1</i>	AGAGGGTTCCTTCGTGGATT	CTTCATTTTCAGCAGCCACA	194	96	87.5

### 3.3 Results

#### 3.3.1 Differential plating results in enrichment of bovine spermatogonia.

To obtain an enriched population of bovine spermatogonia, testis samples from eight peri-pubertal bulls were differentially plated. To confirm the enrichment of spermatogonia in the non-adherent population of differentially plated cells, quantitative real time PCR (qRT-PCR) was carried out for the expression of known spermatogonia markers *DDX4* (previously known as *VASA*), *UCHL1* (previously known as *PGP9.5*) and *ZBTB16* (previously known as *PLZF*), and known Sertoli cell markers *GATA4* and *Vimentin*. Seven of the eight testis samples analysed, showed increased expression of known spermatogonia markers, *DDX4*, *UCHL1* and *ZBTB16*, in the non-adherent fraction of the culture, consistent with the pattern of expression expected for enrichment of spermatogonia (Figure 3-1). The testis cell isolate from one animal consistently did not show expression patterns expected in the enriched populations of differentially plated testis cells, and so was omitted from analysis (Data not shown).

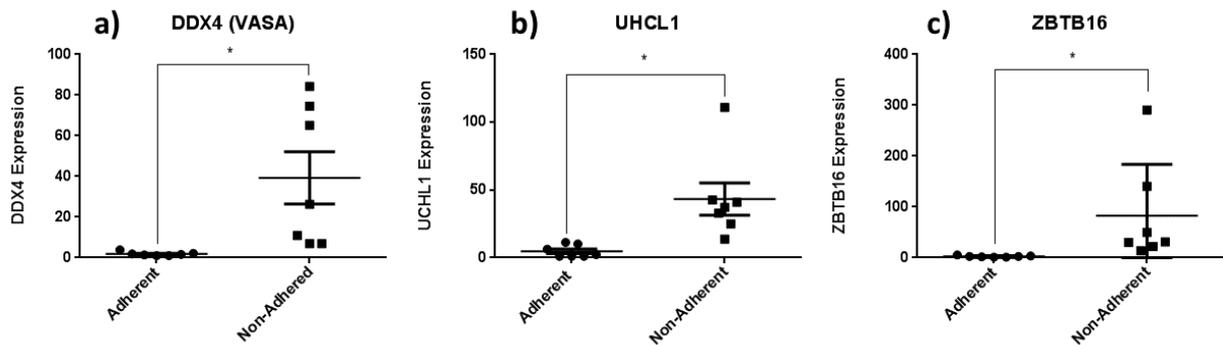


Figure 3-1. Expression of known spermatogonia markers a) *DDX4* (*VASA*), b) *UCHL1* (*PGP9.5*) and c) *ZBTB16* (*Plzf*), in adherent and non-adherent populations of differentially plated bovine testis cells relative to housekeeper gene *RPS26*. \* indicates a significant difference in the mean expression of adherent and non-adherent populations of testis cells. (P<0.05 n=7) Error bars represent  $\pm$  SEM.

Known Sertoli cell markers, *GATA4* and *Vimentin*, were expressed at lower levels in the non-adherent fraction compared to the adherent fraction of differentially plated bovine testis samples ( $P < 0.05$ ) (Figure 3-2).

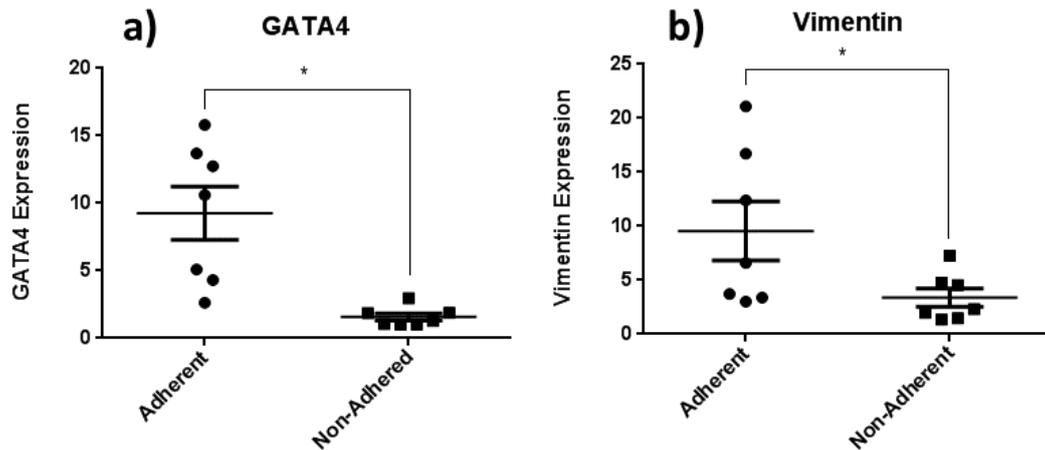


Figure 3-2. Expression of known Sertoli cell markers a) *GATA4* and b) *Vimentin*, in adherent and non-adherent populations of differentially plated bovine testis cells, relative to housekeeper gene *RPS26*. \* indicates a significant difference in the mean expression of adherent and non-adherent populations of testis cells ( $P < 0.05$   $n=7$ ). Error bars represent  $\pm$  SEM.

### 3.3.2 Putative bovine spermatogonia markers *DDX6*, *NAP1L4* and *TKTL1* are expressed in spermatogonia enriched fraction of bovine testis isolations.

Differentially plated testis samples from seven individual peri-pubertal bulls, that were successfully enriched for spermatogonia in the non-adherent fraction of differentially plated cells were used for initial analysis of expression of putative spermatogonia markers. The expression patterns of six genes identified in a previous study of proteomics profiling (Colgrave et al., 2013), *FSCN1*, *IQGAP1*, *TLN1*, *DDX6*, *NAP1L4* and *TKTL1*, in adherent and non-adherent testis cells fractions were analysed by qRT-PCR.

Of the six genes analysed three genes, *FSCN1*, *IQGAP1* and *TLN1* showed no difference in gene expression between the adherent (spermatogonia enriched) and non-adherent cell (Sertoli cell enriched) populations ( $P > 0.05$ ) (Figure 3-3a, b, c). Interestingly, one animal consistently showed increased gene expression of *FSCN1*, *IQGAP1*, *TLN1* in the non-adherent cell population compared to the other animals, however the difference in the expression of group means was not significant

( $P > 0.05$ ) (Figure 3-3a,b,c). *DDX6*, *NAP1L4* and *TKTL1* showed increased gene expression in non-adherent cell populations, of approximately 7 fold, 5 fold and 37 fold respectively ( $P < 0.05$ ) (Figure 3-3d, e, f).

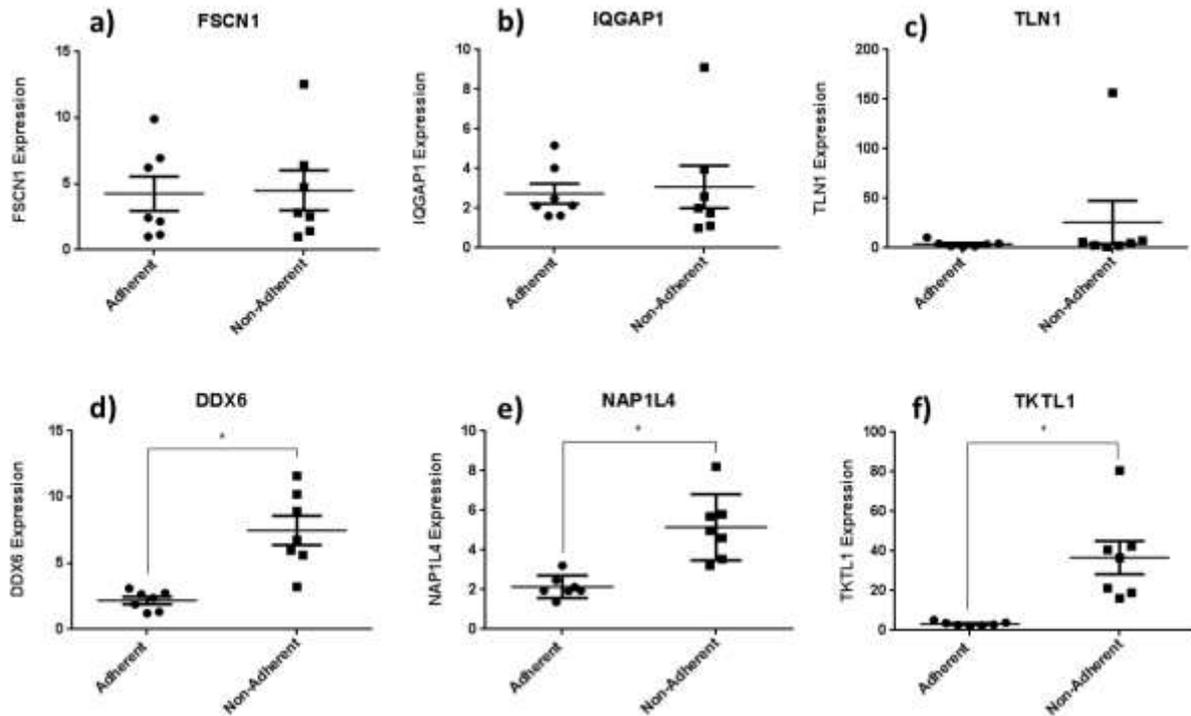


Figure 3-3. Expression of potential markers for bovine spermatogonia, a) *FSCN1*, b) *IQGAP1*, c) *TNL1*, d) *DDX6*, e) *NAP1L4*, and f) *TKTL1*, in adherent and non-adherent populations of differentially plated bovine testis cells, relative to housekeeper gene *RPS26*. \* indicates a significant difference in the mean expression of adherent and non-adherent populations of testis cells. ( $P < 0.05$  n=7). Error bars represent  $\pm$  SEM.

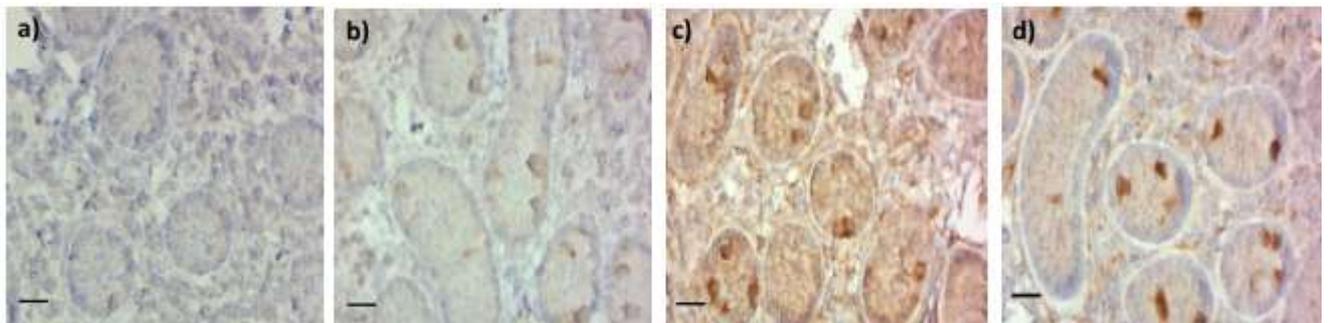
### 3.3.3 *DDX6*, *NAP1L4* and *TKTL1* are Expressed by Different Subsets of Spermatogonia in Pre-Pubertal, Pubertal and Post-Pubertal Bovine Testis

Analysis of qRT-PCR results indicated that the genes *DDX6*, *NAP1L4* and *TKTL1*, showed increased expression in the non-adherent, spermatogonia enriched population of differentially plated testis cells. This indicated that these genes may be putative markers for bovine spermatogonia. The protein expression of these markers in the testis of three different age groups of bulls, pre-pubertal, pubertal and post-pubertal was assessed by immunofluorescence co-staining of testis sections. Sections were stained for known spermatogonia markers, DBA and ZBTB16 and known Sertoli cell marker Vimentin, in conjunction with potential testis cell markers *DDX6*, *NAP1L4* and *TKTL1*.

Figure 3-4 shows the brightfield expression of putative markers DDX6, NAP1L4 and TKTL1 in pre-pubertal tissue. In pre-pubertal tissue, cells staining positively for NAP1L4, DDX6 and TKTL1 were located toward the centre of the tubule, with some cells migrating toward the basement membrane as indicated by bright field staining (Figure 3-4). Additionally, these markers were all expressed in the cytoplasm or nucleus of positively stained cells (Figure 3-4).

Figure 3-5 shows fluorescence control sections of bovine testis from each age group that were not stained with primary antibodies. A low level of background was observed and was obviously different from the specific staining observed from primary antibodies.

Figure 3-6 shows pre-pubertal testis tissue co-stained with putative markers and known spermatogonia marker DBA-biotin and sertoli cell marker Vimentin. Immunofluorescence staining of pre-pubertal tissue showed strong overlap of the putative markers DDX6, NAP1L4 and TKTL1 with early stage spermatogonia markers DBA-biotin (Figure 3-6). NAP1L4 was also weakly expressed by some cells co-staining with Vimentin, DDX6 and TKTL1 did not show co-staining with Vimentin (Figure 3-6) Figure 3-7 shows the expression of putative markers with spermatogonia markers ZBTB16 and DBA-biotin. Co-staining of ZBTB16 and putative markers was observed (Figure 3-7). Additionally, some cells co-stained for ZBTB16 and DBA-biotin.



**Figure 3-4. Brightfield images of bovine pre-pubertal testis sections stained for antibodies against putative spermatogonia markers a) No primary antibody (control). b) DDX6 staining of large cells located in the centre of the tubule. c) NAP1L4 staining of large cells located in the centre of the tubule, migrating toward the basement membrane. d) TKTL1 staining of large cells located in the centre of the tubule, migrating toward the basement membrane. Scale bars represent 50  $\mu$ m.**

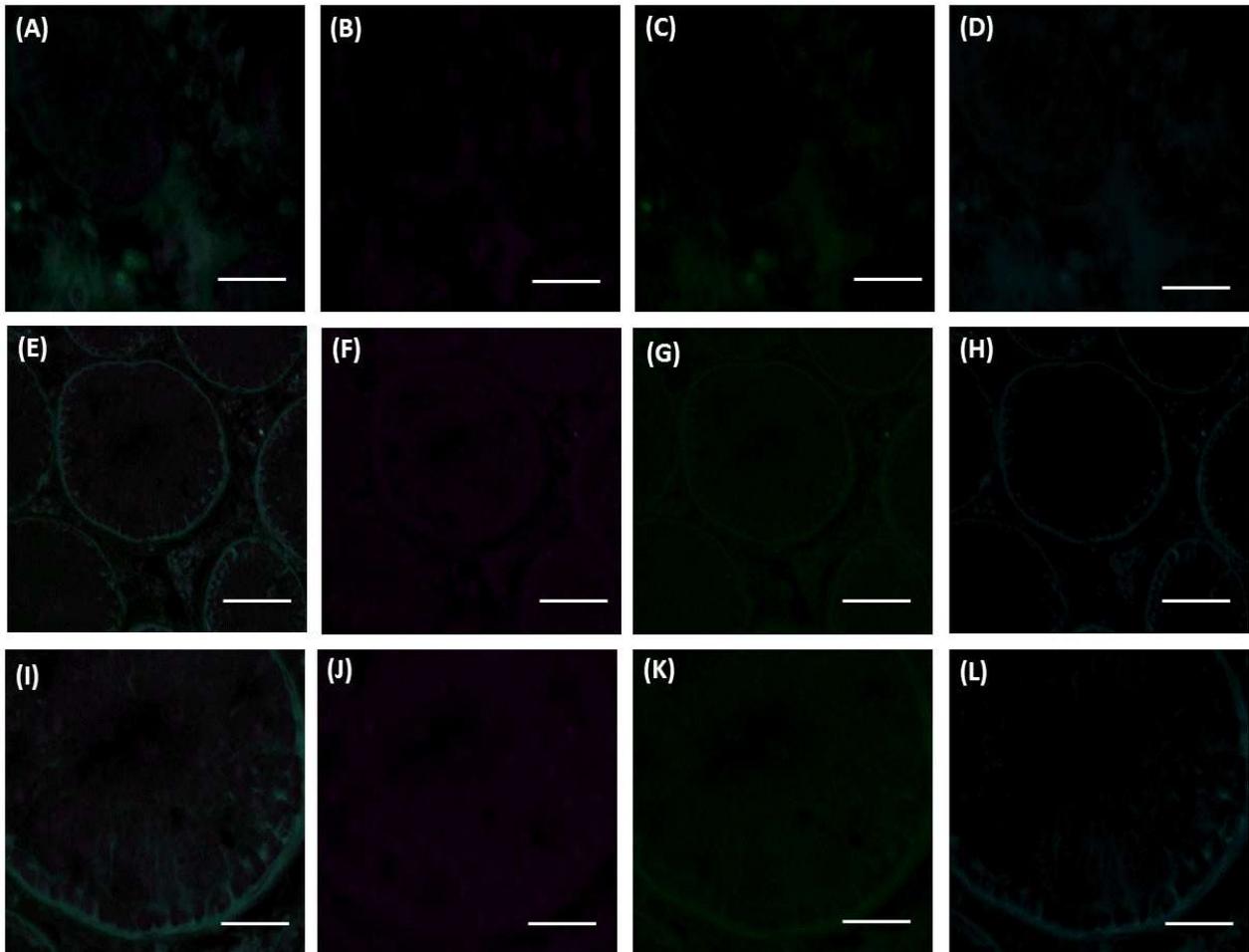


Figure 3-5. Immunohistochemistry control sections for background staining of (a-d) pre-pubertal, (e-h) pubertal, and (i-l) post-pubertal bovine testis. No primary antibody applied, secondary mouse and rabbit antibodies applied. Scale bars represent 50  $\mu$ m.

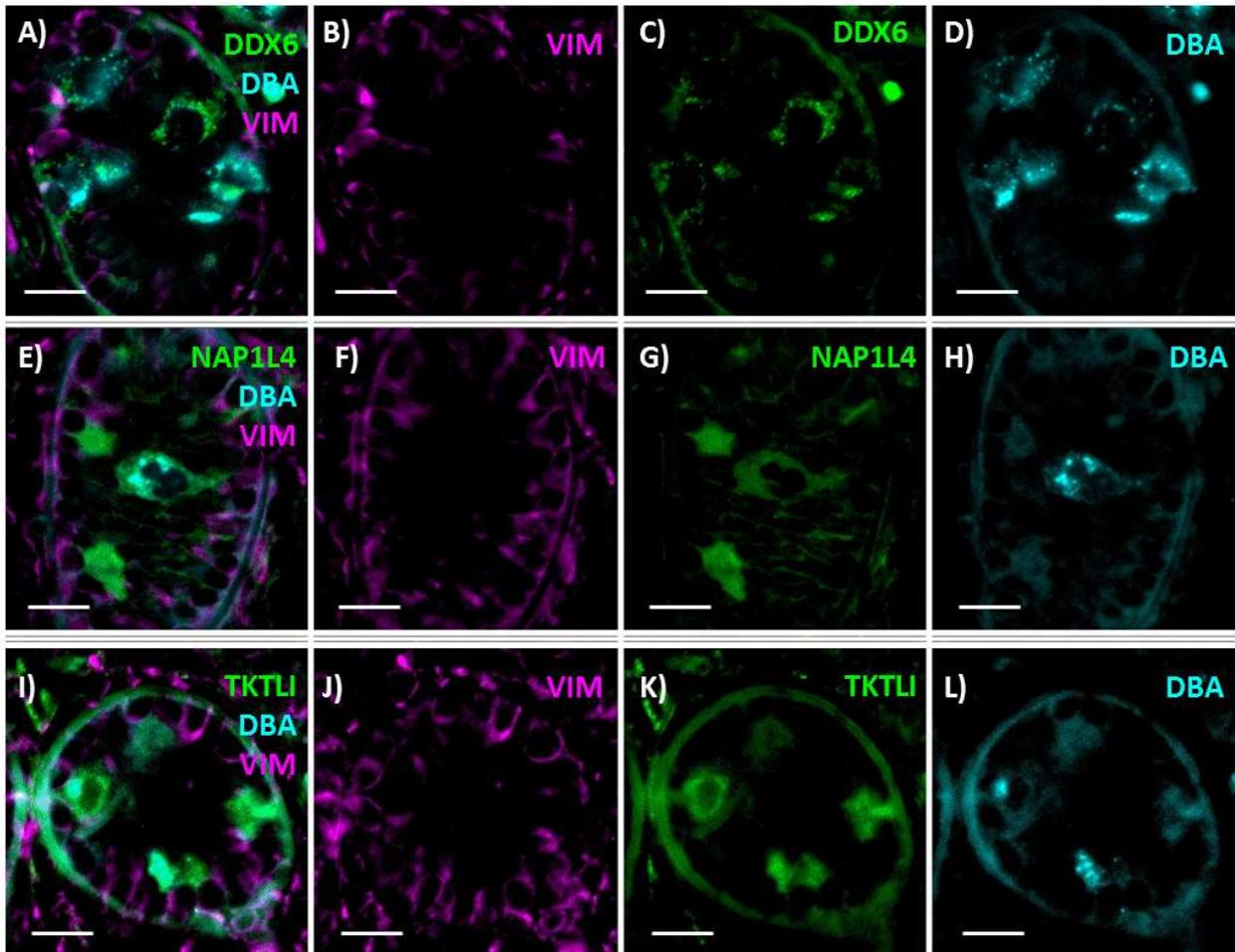


Figure 3-6. Expression of DBA-Biotin, Vimentin and putative markers DDX6, NAP1L4 and TKTL1 in bovine pre-pubertal testis sections. (A, E, I) Merge images of tissue stained with Vimentin, candidate marker (NAP1L4, DDX6 or TKTL1) and DBA-Biotin. (C) Tissue stained with DDX6, (G) Tissue stained with NAP1L4, and (K) tissue stained with TKTL1. (B, F, J) Tissue stained with Vimentin. (D, H, L) Tissue stained with DBA-Biotin. (C, D) Co-staining of DDX6 and DBA-Biotin. (G, H) Co-staining of NAP1L4 with DBA-Biotin. (K, L) Co-staining of TKTL1 with DBA-biotin. Scale bars represent 50  $\mu$ m.

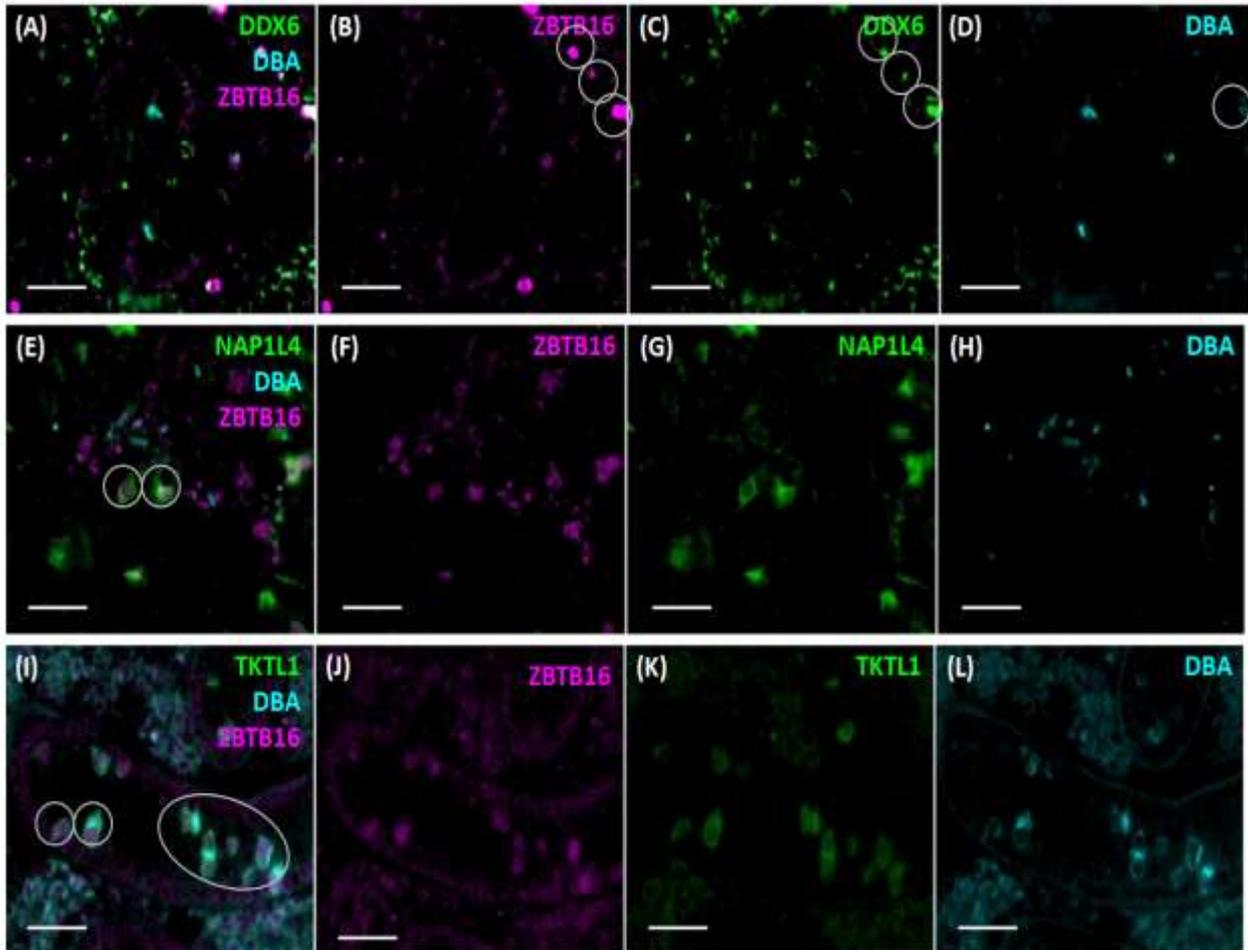
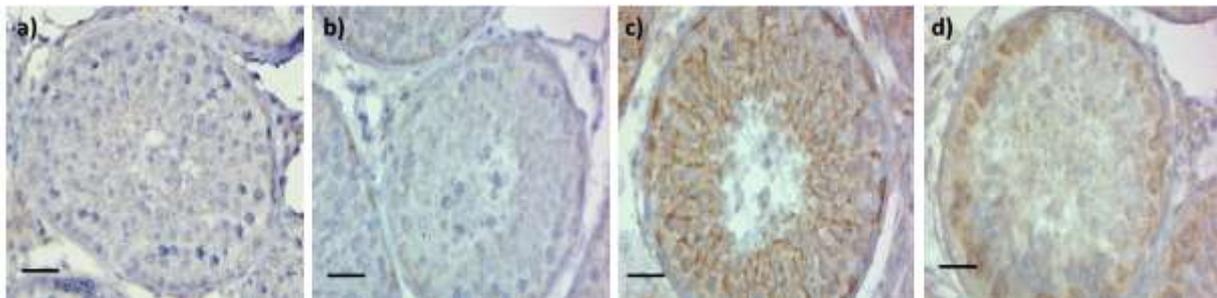


Figure 3-7. Expression of DBA, ZBTB16 and putative markers in bovine pre-pubertal testis sections. (A, E, I) Merge images of tissue stained with ZBTB16, candidate marker (NAP1L4, DDX6 or TKTL1), and DBA-Biotin. (C) Tissue stained with DDX6, (G) NAP1L4, and (K) tissue stained with TKTL1. (B, F, J) Tissue stained with ZBTB16. (D, H, L) Tissue stained with DBA-Biotin. (A, B, C) Co-staining of DDX6 and ZBTB16 shown by circled cells. (E) Co-staining of NAP1L4 with ZBTB16 shown by circled cells. (I) Co-staining of TKTL1 with DBA-biotin shown by circled cells. Scale bars represent 200  $\mu$ m.

Figure 3-8 shows brightfield expression of putative testis cell markers in bovine pubertal testis tissue. Brightfield staining for DDX6 positive cells, identified a small number of cells located close to the basement membrane of the tubules (Figure 3-8a). Staining for NAP1L4 positive cells identified a number of cells located on the basement membrane of the tubule, in addition to staining of extracellular space toward the centre of the tubule (Figure 3-8b). Brightfield images of TKTL1 staining identified positive cells that were one cell layer removed from the basement membrane (Figure 3-8c).

Figure 3-9 shows immunofluorescence staining of pubertal testis tissue with DBA-biotin, Vimentin and putative candidate markers, while Figure 3-10 shows staining of pubertal tissue with DBA-biotin, ZBTB16 and putative candidates. Immunofluorescence staining showed cells with strongest signal for both NAP1L4 and DDX6, co-stained with DBA-biotin (Figure 3-9) and ZBTB16 (Figure 3-10). Additionally, some cells only exhibited weaker staining for NAP1L4 or DDX6 (Figure 3-10). Cells expressing TKTL1 were not in contact with the basement membrane of the tubule, but were one cell layer removed (Figure 3-9 and Figure 3-10). TKTL1 positive cells did not co-stain with DBA-biotin or Vimentin, however some TKTL1 positive cells did co-stain with ZBTB16 (Figure 3-10i).



**Figure 3-8. Brightfield images of bovine pubertal testis sections stained for antibodies against putative spermatogonia markers. a) No primary antibody (control) .b) DDX6 weak staining of cells on the basement membrane. c) NAP1L4 staining of cells located on the basement membrane and expression pattern toward the centre of the lumen. d) TKTL1 staining of cells one cell layer removed from the basement membrane. Scale bars represent 50  $\mu$ m.**

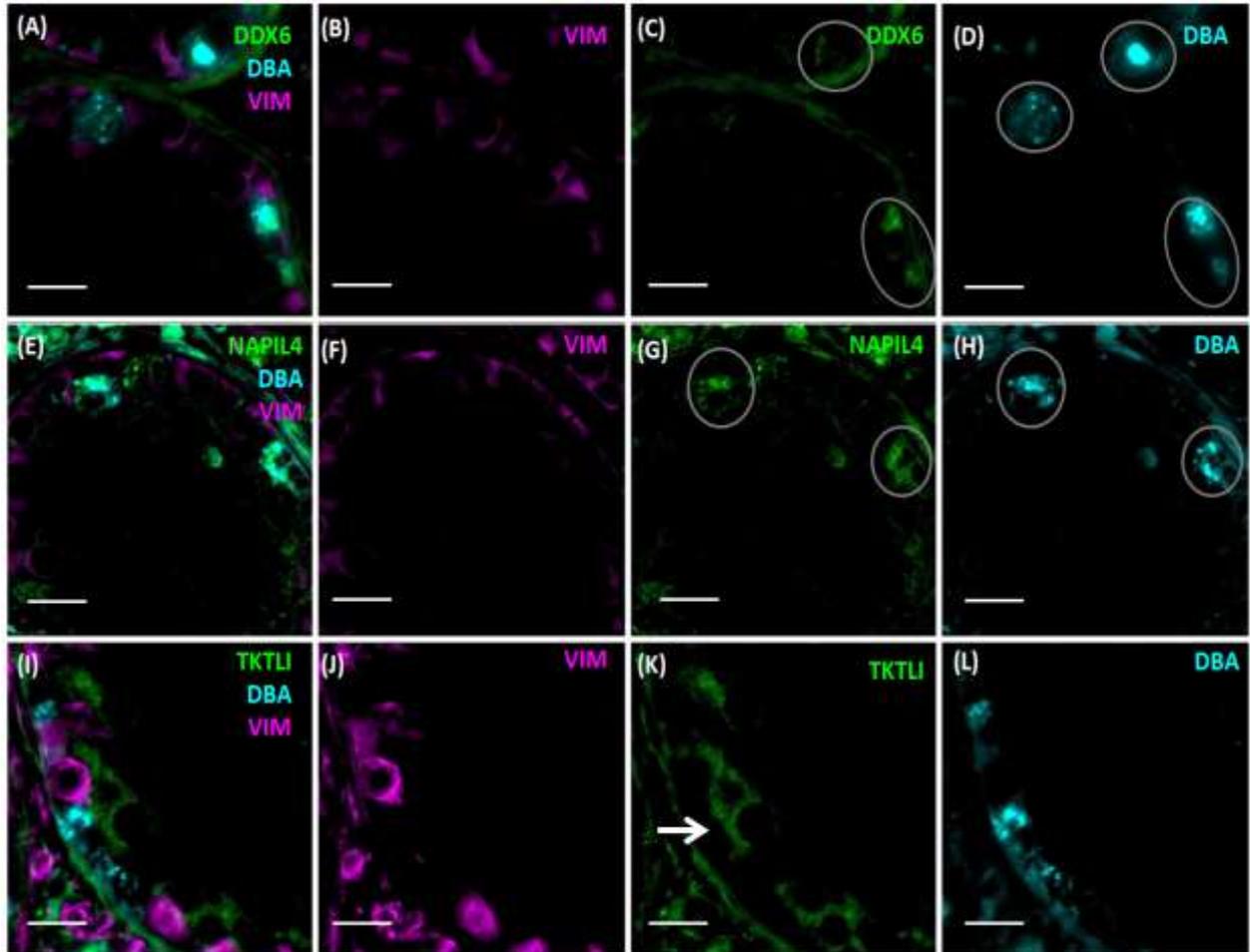


Figure 3-9. DBA-Biotin, Vimentin and putative markers DDX6, NAP1L4 and TKTL1 in bovine pubertal testis sections. (A, E, I) Merge images of tissue stained with Vimentin, candidate marker (NAP1L4, DDX6 or TKTL1), DBA-Biotin. (C) Tissue stained with DDX6, (G) Tissue stained with NAP1L4, (K) tissue stained with TKTL1, arrow shows cells located above basement membrane. (B, F, J) Tissue stained with Vimentin. (D, H, L) Tissue stained with DBA-Biotin. (C, D) Co-staining of DDX6 and DBA-Biotin shown by circled cells. (G, H) Co-staining of NAP1L4 with DBA-Biotin shown by circled cells. Scale bars represent 50  $\mu$ m.

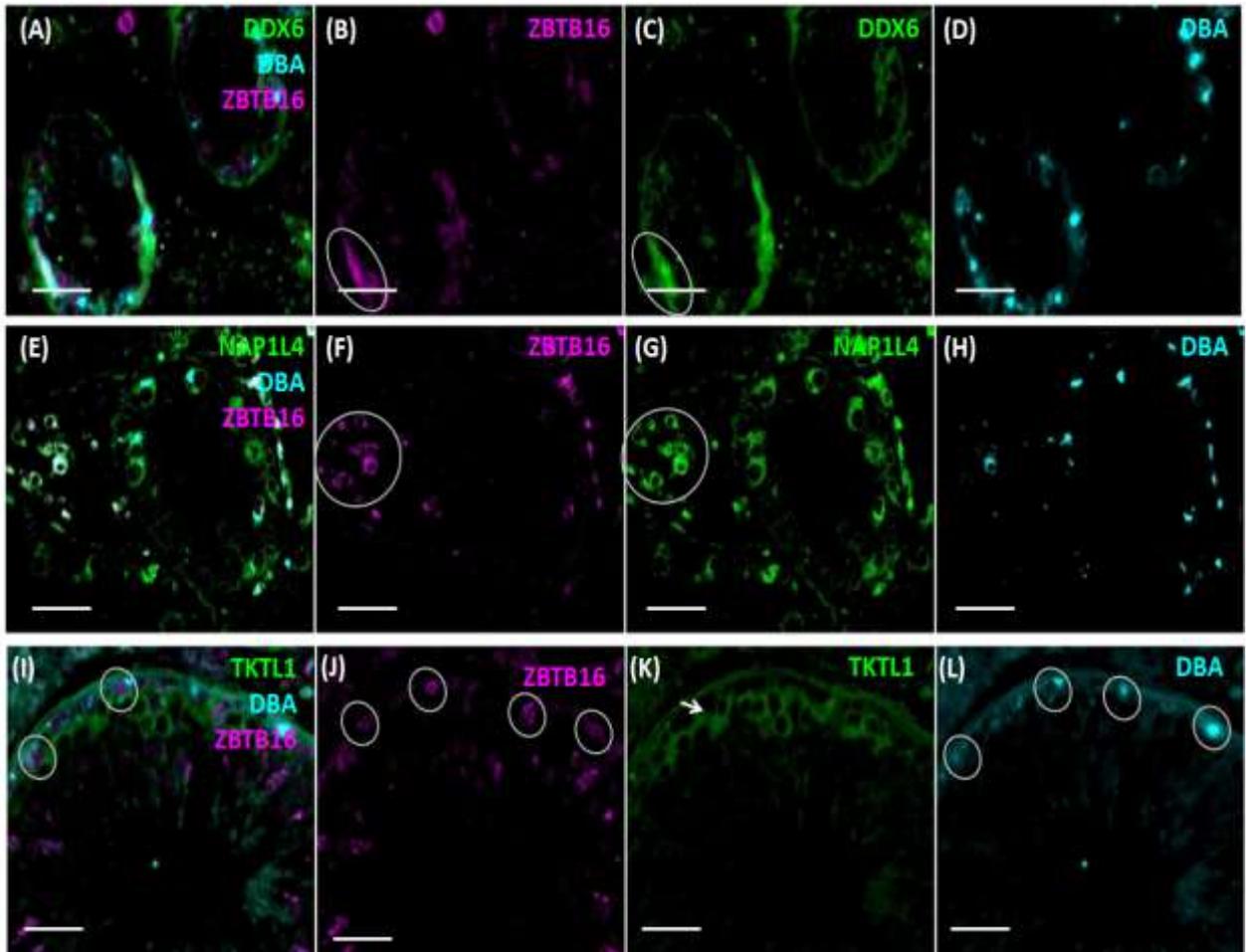
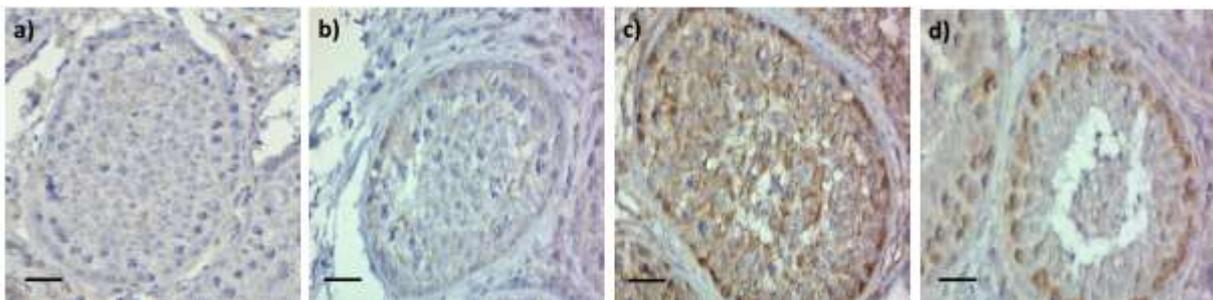


Figure 3-10. Expression of DBA, ZBTB16 and putative markers in bovine pubertal testis sections. (A, E, I) Merge images of tissue stained with ZBTB16, candidate marker (NAP1L4, DDX6 or TKTL1), and DBA-Biotin. (C) Tissue stained with DDX6, (G) NAP1L4, and (K) tissue stained with TKTL1. (B, F, J) Tissue stained with ZBTB16. (D, H, L) Tissue stained with DBA-Biotin. (A, B, C) Co-staining of DDX6 and ZBTB16 shown by circled cells. (F, G) Co-staining of NAP1L4 with ZBTB16 shown by circled cells. (I, J, L) Co-staining of ZBTB16 and DBA shown by circled cells. (K) TKTL1 cells appear to be one cell layer removed from the basement membrane as indicated by the arrow. Scale bars represent 200  $\mu\text{m}$ .

Figure 3-11 shows brightfield expression of putative testis cell markers in bovine post- pubertal testis tissue. Brightfield staining for DDX6 positive cells, identified a small number of cells located close to the basement membrane of the tubules (Figure 3-11a). Staining for NAP1L4 positive cells identified a number of cells located on the basement membrane of the tubule, but also showed staining of extracellular space toward the centre of the tubule (Figure 3-11b). Brightfield images of TKTL1 staining in post-pubertal testis identified positive cells that were located one cell layer above the basement membrane (Figure 3-11c). These patterns of staining were similar to those observed in pubertal testis tissue (Figure 3-8).

Figure 3-12 shows immunofluorescence staining of post-pubertal testis tissue with DBA-biotin, Vimentin and putative candidates, while Figure 3-13 shows staining of post-pubertal tissue with DBA-biotin, ZBTB16 and putative markers. In immunofluorescence staining of post-pubertal adult testis tissue, expression patterns of DDX6, NAP1L4 and TKTL1 were the same as shown in brightfield stains. Cells expressing NAP1L4 and DDX6 were also located on the basement membrane, as was seen in pre-pubertal and pubertal tissue. Additionally, NAP1L4 also exhibited a staining pattern in the centre of the lumen (Figure 3-12g and Figure 3-13g). While cells expressing TKTL1 were located one cell layer above the basement membrane (Figure 3-12 and Figure 3-13). Immunofluorescence staining identified some cells that expressed DDX6 and NAP1L4, co-stained with DBA-biotin (Figure 3-12) and/or ZBTB16 (Figure 3-13). While TKTL1 positive cells did not co-stain with DBA-biotin, ZBTB16, or Vimentin (Figure 3-12 and Figure 3-13).



**Figure 3-11. Brightfield images of post-pubertal testis sections stained for antibodies against putative spermatogonia markers. a) No primary antibody (control). b) DDX6 staining of cells located on the basement membrane. c) NAP1L4 staining of cells located on the basement membrane and expression pattern toward the centre of the lumen. d) TKTL1 staining of cells one cell layer removed from the basement membrane. Scale bars represent 50  $\mu$ m.**

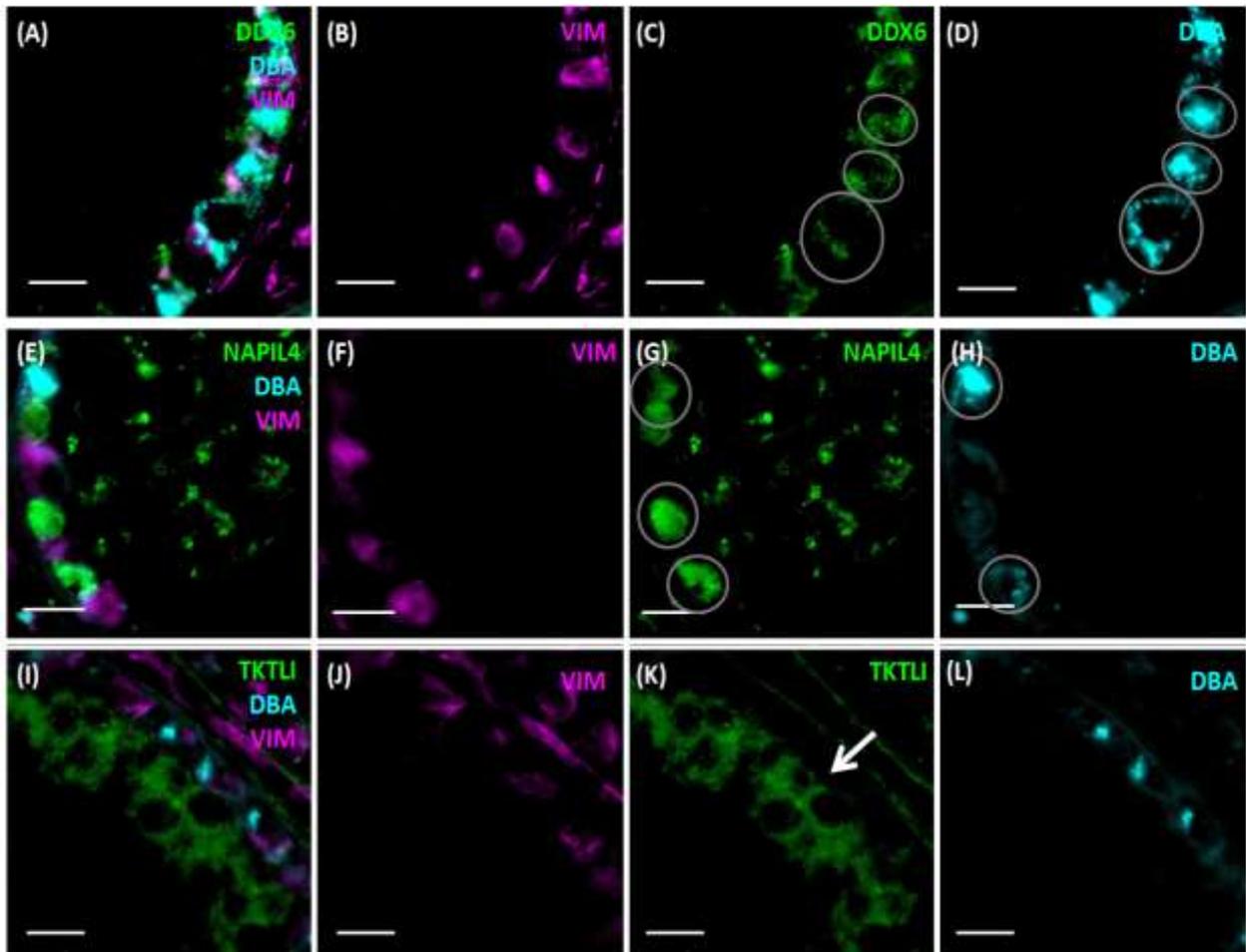


Figure 3-12. Expression of DBA-Biotin, Vimentin and putative markers DDX6, NAP1L4 and TKTL1 in bovine post-pubertal testis sections. (A, E, I) Merge images of tissue stained with Vimentin, candidate marker (DDX6, NAP1L4 or TKTL1), DBA-Biotin. (C) Tissue stained with DDX6, (G) Tissue stained with NAP1L4, (K) tissue stained with TKTL1, arrow shows cells located above basement membrane. (B, F, J) Tissue stained with Vimentin. (D, H, L) Tissue stained with DBA-Biotin. (C, D) Co-staining of DDX6 and DBA-Biotin, shown by circled cells. (G, H) Co-staining of NAP1L4 with DBA-Biotin, shown by circled cells. Scale bars represent 50  $\mu$ m.

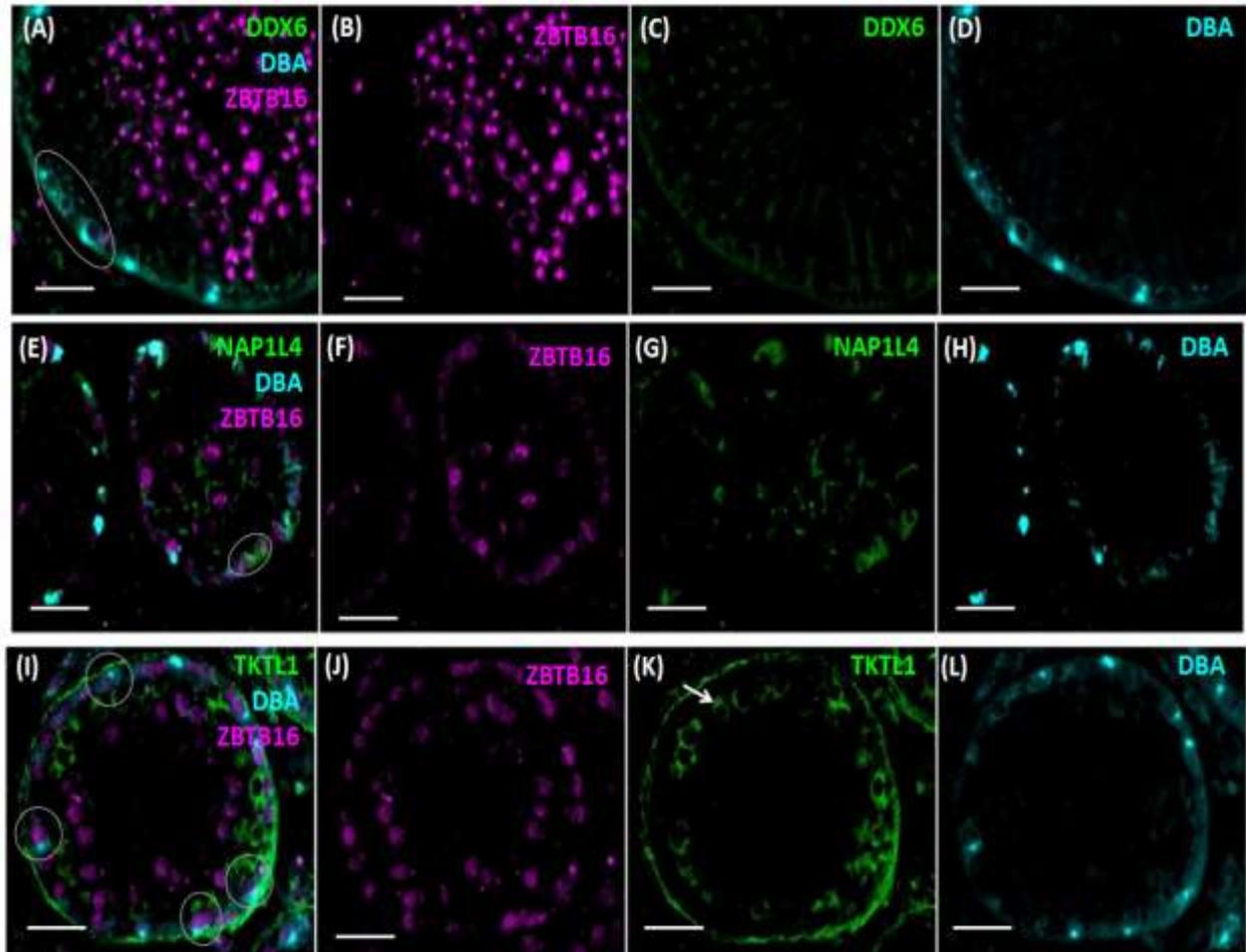


Figure 3-13. Expression of DDX6, ZBTB16 and putative spermatogonia markers bovine adult testis tissue. (A, E, I) Merge images of tissue stained with ZBTB16, candidate marker (NAP1L4, DDX6 or TKTL1) and DBA-Biotin. (C) Tissue stained with DDX6, (G) Tissue stained with NAP1L4, (K) tissue stained with TKTL1, arrow shows cells located above basement membrane. (B, F, J) Tissue stained with ZBTB16. (D, H, L) Tissue stained with DBA-Biotin. (A) Co-staining of DDX6 and DBA-Biotin, no co-staining with ZBTB16 was observed (E) Co-staining of NAP1L4 with DBA-Biotin and ZBTB16, shown by circled cells. (I) Co-staining of ZBTB16 and DBA shown by circled cells. (K) TKTL1 positive cells do not co-stain with DBA-biotin or ZBTB16 and appear to be one cell layer removed from the basement membrane as shown by the arrow. Scale bars represent 200  $\mu$ m.

### 3.3.3 Quantification of Expression of DDX6, NAP1L4 and TKTL1 in Pre-Pubertal, Pubertal and Post-Pubertal Bovine Testis

To further quantify the expression of putative spermatogonia markers, smears from four individual *Bos Taurus* bulls from pre-pubertal and pubertal age groups, were stained with antibodies for putative markers DDX6, NAP1L4, TKTL1, known spermatogonia markers DDX4 and DBA-HRT, and Sertoli cell marker GATA4. Figure 3-14 shows control smears for mouse and rabbit IgG, no non-specific staining was observed. Examples of stained smears from pre-pubertal and pubertal animals stained with antibodies for putative and known markers and are shown in Figure 3-15 and Figure 3-16 respectively.

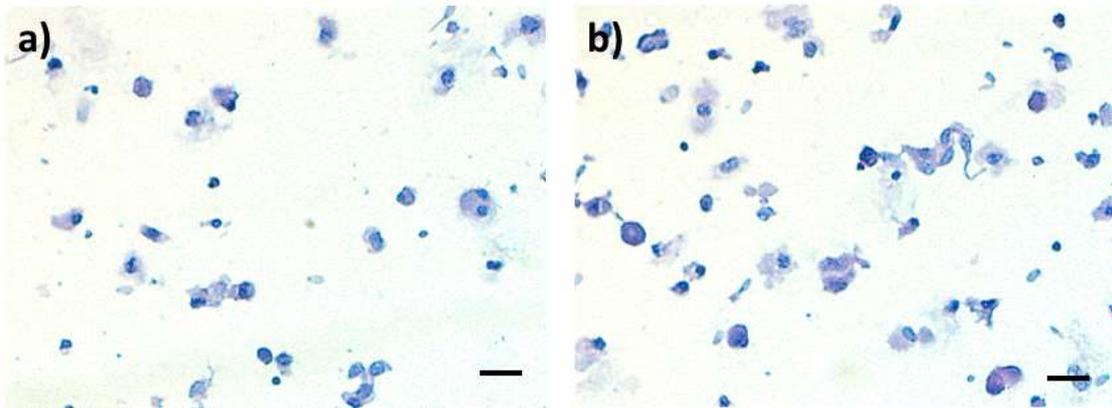


Figure 3-14. IgG controls of bovine testis cell smears from a) Pre-Pubertal and b) Pubertal animals, stained with mouse and rabbit IgG. No cells are stained positive, indicating no non-specific staining occurred. Scale bars represent 50  $\mu\text{m}$ .

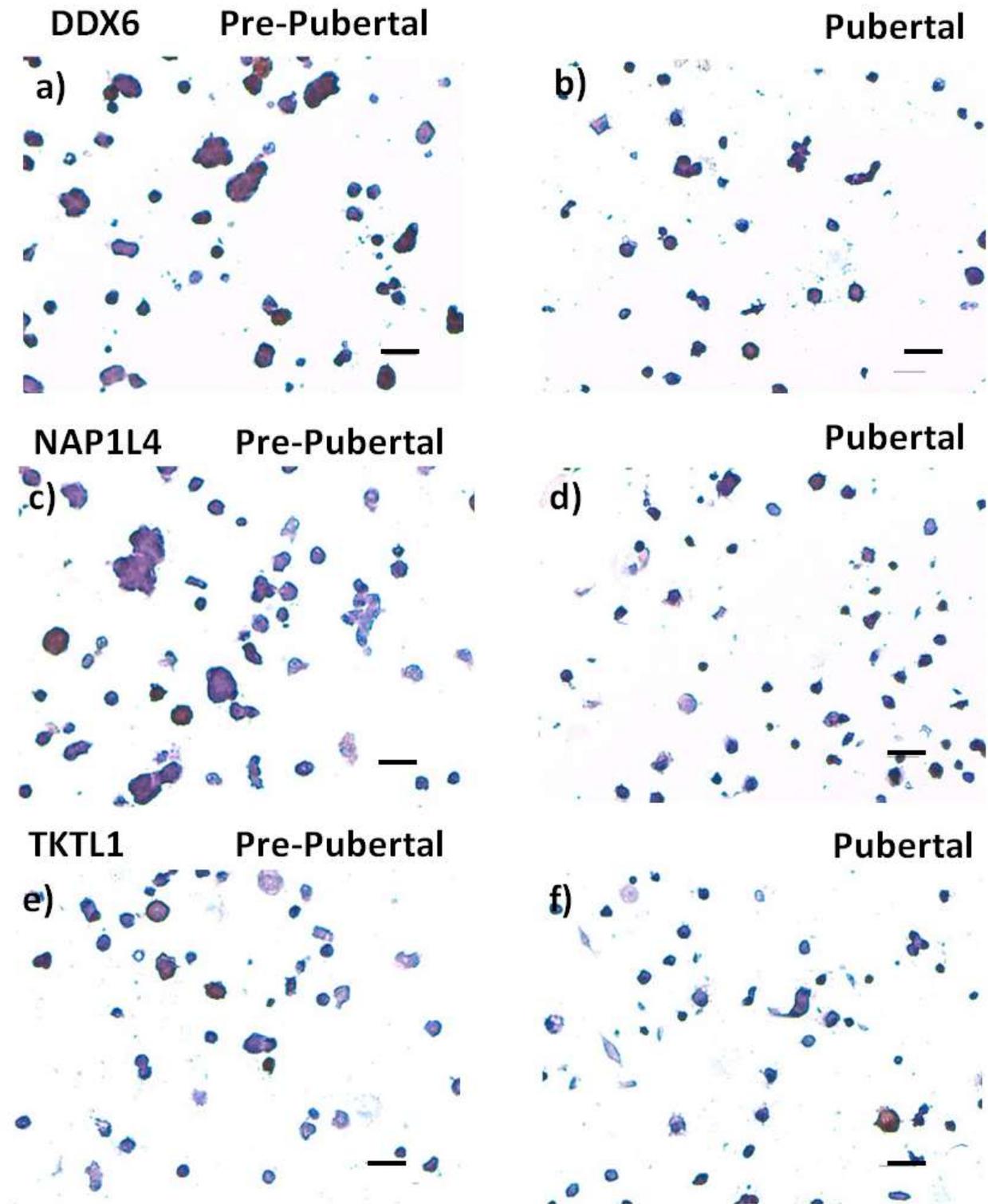


Figure 3-15. Samples of smear photographs of Pre-Pubertal, Pubertal and Post-Pubertal bovine testis stained with a) DDX6, b) NAP1L4 and c) TKTL1. Cells that are stained brown indicate cells expressing the marker of interest. Scale bars represent 50  $\mu$ m.

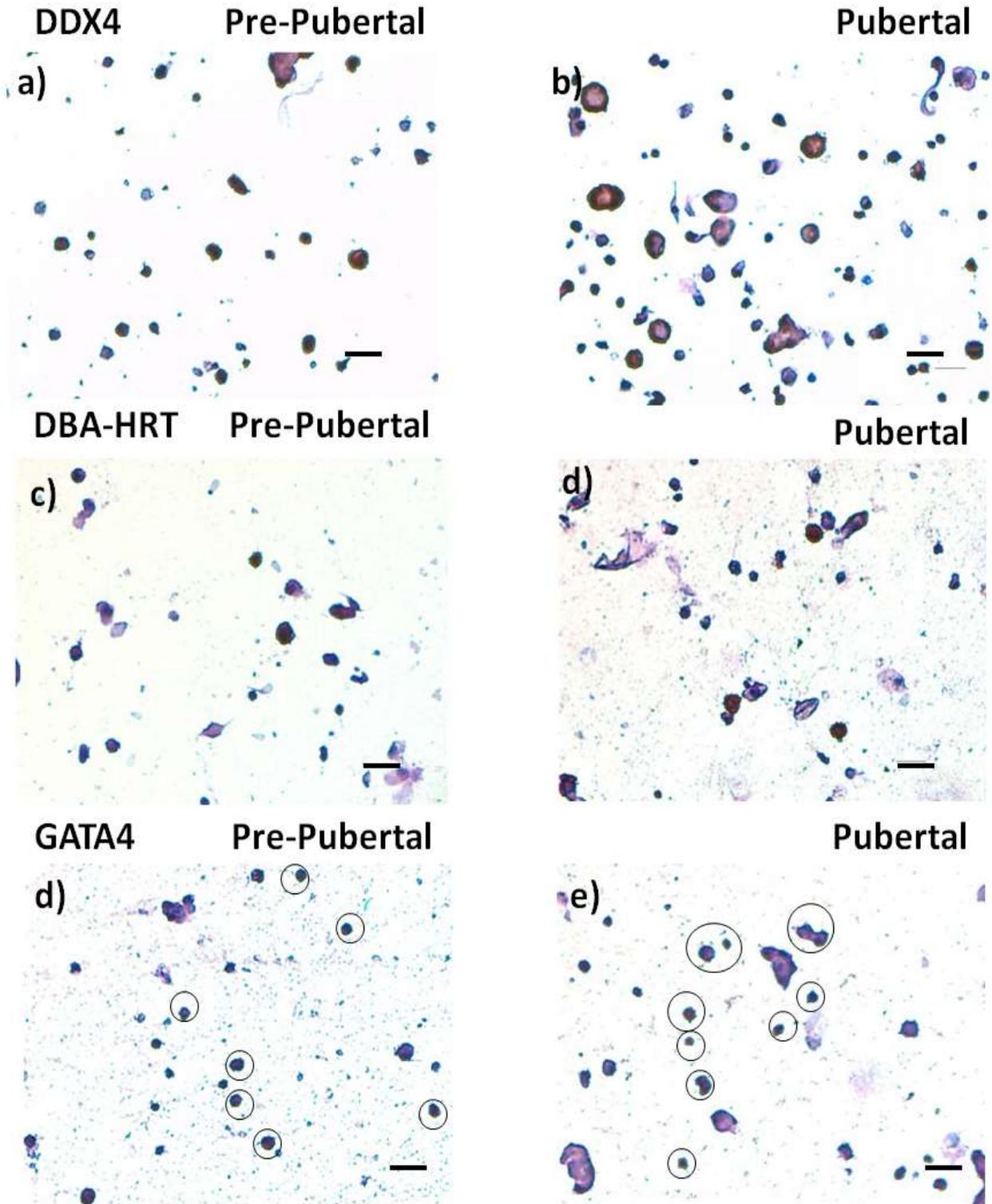
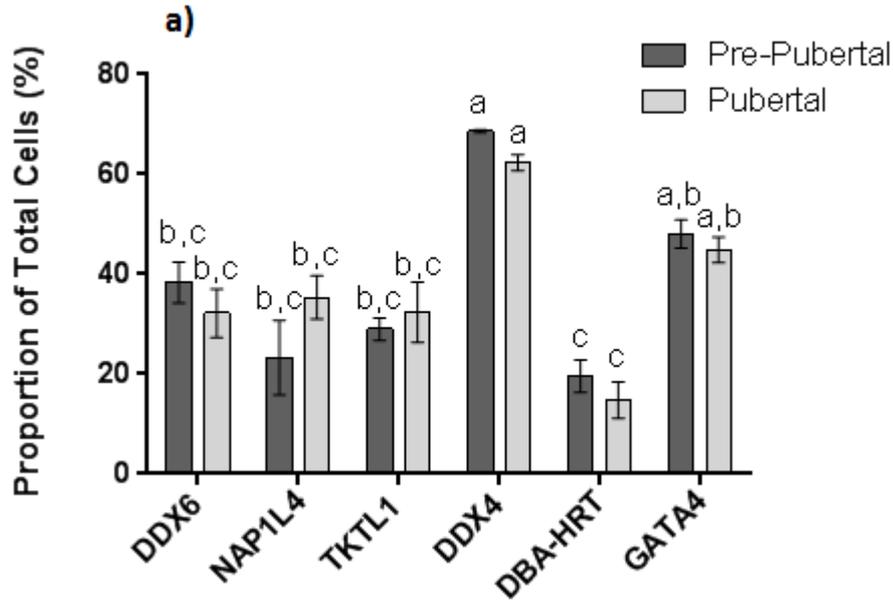


Figure 3-16. Samples of smear photographs of Pre-Pubertal, Pubertal and Post-Pubertal bovine testis stained with a) DDX4, b) DBA-HRT and c) GATA4. Cells that are stained brown indicate cells expressing the marker of interest. d, e) Staining for GATA4 positive cells was weak, circled cells indicate cells staining positively for GATA4. Scale bars represent 50  $\mu\text{m}$ .

Positively stained cells, in pre-pubertal and pubertal testis tissue, for each of the putative and known markers, were counted as a proportion of the total cell population. The proportions of cells expressing each marker were compared (Figure 3-17). There was no difference in the number of cells expressing putative DDX6, NAP1L4 or TKTL1 in either age group, with the proportion of positively stained cells ranging from 24.5-46.1% for DDX6, 15.9-46.3% for NAP1L4 and 19.5-23.1% for TKTL1 (Figure 3-17b)( $P>0.05$ ). However, there were a significantly higher proportion of cells that stained for DDX4 than any other known or putative markers ( $P<0.05$ ).

Additionally there was no difference in the proportion of cells expressing DDX4 in pre-pubertal or pubertal age groups, with an average of 64.5% of cells expressing DDX4 in pre-pubertal and pubertal testis (Figure 3-17b) ( $P>0.05$ ). Cells that expressed GATA4 were the second largest population in both age groups. However, the population of GATA4 positive cells was not significantly smaller than those staining for DDX4, and not significantly larger than those expressing putative markers, with 45% positive cells in pre-pubertal and pubertal testis smears (Figure 3-17) ( $P>0.05$ ).

Cells expressing DBA-HRT were the smallest population, with an average of 17% in pre-pubertal and pubertal testis. However, this population was not significantly smaller than putative markers DDX6, NAP1L4 or TKTL1 but was significantly smaller than those cell populations expressing GATA4 or DDX4 ( $P<0.05$ ) (Figure 3-17).



b)

		% Positively Stained Cells					
ID		DDX6	NAP1L4	TKTL1	DDX4	DBA-HRT	GATA4
<b>Pre-Pubertal</b>	1612	40.30	45.70	29.60	64.30	14.70	41.50
	1613	46.10	26.20	24.80	65.10	13.40	43.40
	1614	42.40	30.80	35.10	69.00	26.80	50.90
	1615	34.30	15.90	26.50	68.30	23.40	45.30
	<b>Mean</b>		40.78	29.65	29.00	66.68	19.58
<b>SEM</b>		2.47	6.19	2.26	1.16	3.28	2.03
<b>Pubertal</b>	1618	25.70	27.50	19.50	63.70	24.40	44.50
	1619	33.10	46.30	42.20	59.30	15.00	52.20
	1620	45.50	29.40	24.80	60.30	7.00	42.00
	1621	24.50	38.20	43.10	66.20	12.70	40.60
	<b>Mean</b>		32.20	35.35	32.40	62.38	14.78
<b>SEM</b>		4.82	4.33	6.02	1.59	3.62	2.59
<b>Total Mean</b>		<b>36.49</b>	<b>32.50</b>	<b>30.70</b>	<b>64.53</b>	<b>17.18</b>	<b>45.05</b>
<b>SEM</b>		2.99	3.66	3.05	1.22	2.44	1.52

Figure 3-17. a) Proportion of positively stained cells for pre-pubertal and pubertal testis cell smears stained with putative markers DDX6, NAP1L4 and TKTL1, known spermatogonia markers DDX4 (VASA), and DBA-HRT, and Sertoli cell marker GATA4. Different characters above treatment means indicates a significant difference in the percentage of cells expressing markers ( $P < 0.05$ ,  $n=4$ , Errors bars represent  $\pm$  SEM). b) Table shows percent positively stained cells of individual animals in pre-pubertal and pubertal age group testis cell smears stained with putative and known testis cell makers. Group means and SEM are given for each marker for pre-pubertal and pubertal age groups, the mean and SEM for total cells expressing markers in pre-pubertal and pubertal samples is also given.

### **3.4 Discussion**

The ability to specifically identify and isolate spermatogonial stem cells (SSCs) is essential for the success of technologies including germ cell transplantation. The identification of molecular markers, in particular cell surface markers unique to SSCs, would allow for the identification and enrichment of this small population of stem cells. Improved identification and enrichment of these cells, will potentially improve their use in transgenic breeding and reproductive applications, in addition to enhancing our general understanding of stem cell biology.

A number of markers for various subsets of spermatogonia, including those that identify the spermatogonia as a whole, and those that identify undifferentiated and differentiated spermatogonia types, have been established and characterised in previous studies. However, markers for different subsets of spermatogonia are not necessarily conserved between species, and few have been found to identify germ cells in the bovine. DBA binding activity, and UCHL1, have both been shown to identify gonocytes in the neo-natal bovine testis, and are restricted to undifferentiated spermatogonia in the pubertal and adult bovine testis (Fujihara et al., 2011; Herrid et al., 2007; Herrid et al., 2009; Izadyar et al., 2002). DDX4, identifies gonocytes in the neo-natal testis, however, in the pubertal and adult testis, it is not expressed by early stage spermatogonia, and is instead restricted to more differentiated populations of germ cells, including spermatocytes and round spermatids (Fujihara et al., 2011). ZBTB16 identifies gonocytes and undifferentiated spermatogonia in numerous species, including the bovine (Costoya et al., 2004; Herrid et al., 2007; Oatley, Jon, de Avila, Reeves, & McLean, 2004). However, markers that uniquely distinguish spermatogonial stem cells from other subsets of undifferentiated spermatogonia have not been identified in any species.

Methods for identifying new markers for spermatogonial stem cells include the use of proteomics profiling. Recently, global proteomics profiling of the membrane compartment of spermatogonia enriched bovine testicular cell populations was conducted. The resulting analysis identified a total of 1 387 proteins present in the membrane compartment of testis cells, of which 64 proteins, were found to be more highly expressed in the spermatogonia enriched fraction of differentially plated cells. Of these proteins, only 16 were expressed exclusively in the spermatogonia enriched fraction of testis populations. These preliminary results indicated, that these 16 proteins may be expressed

by un-differentiated bovine spermatogonia (Colgrave et al., 2013). There were very few genuine cell surface proteins identified as part of the original proteomics analysis, however, membrane proteins were identified and these proteins may be expressed on the cell surface. The majority of the proteins shortlisted from the original proteomics analysis were identified as membrane associated proteins which in humans samples were shown to be associated with the presence of SSCs.

This analysis identified a number of candidate markers that may be associated with bovine spermatogonia and spermatogonia stem cells. This chapter aimed to characterise the expression of six of the 16 candidate markers, *DDX6*, *NAP1L4*, *TKTL1*, *TLN1*, *IQGAP* and *FSCN1*, identified by the proteomics profiling (Colgrave et al., 2013), as being associated with enriched populations of bovine spermatogonia.

To confirm that enrichment of spermatogonia had been successful, testis isolations from eight peri-pubertal bulls that had differentially plated were analysed for gene expression of known spermatogonia and Sertoli cell markers. Results for qRT-PCR analysis presented in this chapter indicated that differentially plated testis isolations, from seven of the eight animals, showed increased expression of early stage spermatogonia markers *DBA*, *DDX4*, *UCHL1* and *ZBTB16* in the non-adherent, spermatogonia enriched fraction, compared to the adherent, sertoli enriched fractions. Additionally, the adherent fraction of the differentially plated cell culture showed higher expression of Sertoli cell markers *GATA4* and *Vimentin* (Imai et al., 2004; Zhang, Zhen, Hill, Holland, Kurihara, & Loveland, 2008), while the non-adherent fraction, expressed lower levels of these markers. These results indicated that spermatogonia enrichment was successful in the non-adherent fraction of differentially plated cells, and that there was a depletion of Sertoli cells in this fraction. One animal did not show the expected enrichment patterns in the adherent and non-adherent differentially plates testis cell populations, and so was eliminated from the analysis.

The successful enrichment by differential plating allowed for the analysis of gene expression of the candidate markers in the enriched populations of spermatogonia (non-adherent) or Sertoli enriched (adherent) fractions. Putative markers for spermatogonia and SSCs would be expected to show correlation with the known spermatogonia markers and exhibit increased expression in the spermatogonia enriched cell population, while lower expression would be expected in the Sertoli cell enriched population.

Initial characterisation of the six potential markers, DDX6, NAP1L4, TKTL1, TLN1, IQGAP and FSCN1, indicated that the expression of TLN1, IQGAP and FSCN1, did not correlate with high expression in spermatogonia enriched populations. There was no difference in gene expression of these three markers, in either the spermatogonia or Sertoli cell enriched testis populations, indicating that they were not associated with bovine spermatogonia alone. However, one animal consistently showed higher expression of TLN1, IQGAP1 and FSCN1, in the spermatogonia enriched fraction of the testis population. The reason for the different expression pattern displayed by this animal is unclear, as expression analysis of known markers for spermatogonia and Sertoli cells by this animal did not show an unusual profile. Additionally, these results did not suggest that the cell fractions obtained by differential plating of the testis cells from this animal resulted in a more or less enriched population of spermatogonia, compared to isolations from the other animals analysed. Indicating that the high expression of TLN1, IQGAP1 and FSCN1, in the spermatogonia enriched fraction of this animal was not due to a difference in enrichment, but instead may be due to unknown biological causes.

IQGAP1, was not shown to be expressed by Sertoli cells or germ cells alone, but was instead expressed by both Sertoli cells and spermatogonia enriched populations of testis cells. The IQGAP1 protein, has previously been found to localise on the on the cell surface of Sertoli and germ cells (Xia, Mruk, Lee, & Cheng, 2005). IQGAP is believed to be involved in the adherence of Sertoli and germ cells during spermatogenesis by assisting immigration of differentiating spermatogonia and spermatocytes to the centre of the tubule lumen. IQGAP1, along with Cdc42, and  $\beta$ -catenin are believed to be essential for Sertoli-germ cell interactions, but not Sertoli-Sertoli cell interactions (Lui et al., 2005). For this reason it is unsurprising that IQGAP1 was not expressed more highly in the spermatogonia enriched fractions of testis cells described in this chapter, as it is most likely expressed by both Sertoli cells and germ cells for the purposes of cell to cell adhesion.

The initial characterisation of expression of FSCN1 by bovine testis by qRT-PCR, showed no difference in the expression of FSCN1 in the Sertoli cell enriched or the spermatogonia enriched populations of bovine testis cells. It is possible that FSCN1 is also expressed by Sertoli cells due to its association with formation of actin-bundles for cell structure and function (Kolthur-Seetharam et al., 2008). Interestingly, a study has shown that FSCN3, a paralog of FSCN1, is expressed by late stage spermatozoa and the sperm head Tubb et al. (2002). It may be possible that analysis of FSCN3

would be interesting in the identification of late stage spermatogonia and sperm in the bovine. However, it is unlikely that this family of proteins would be expressed exclusively in SSCs.

Initial analysis of TLN1 gene expression, did not indicate increased expression in spermatogonia enriched testis cell populations, compared to the Sertoli cell enriched fraction of cells. TLN1 is an adaptor protein present in the cell membrane, and connects cell adhesion molecules from the integrin family to cytoskeletal actin. Integrin  $\alpha$ -6 is present in bovine testis cells, but it has not been confirmed if this protein is associated with early stage spermatogonia as it is in other species (de Barros et al., 2012). It is thought that Talin1 plays an important role in establishing the stem cell niche and maintenance of cell position (Tanentzapf et al., 2007). It is possible that Talins' act as ligands for Integrins' present in the bovine testis, but are not necessarily associated with spermatogonia alone due to their activity in establishing the stem cell niche.

In contrast to IQGAP1, FSCN1 and TNL1, initial characterisation of the expression of *DDX6*, *NAP1L4*, and *TKTL1*, did correlate with high expression in spermatogonia enriched cell fractions. These results suggested their expression may be limited to bovine spermatogonia, rather than Sertoli cells or other somatic testis cell types. The method of qRT-PCR gene expression analysis described in this chapter allows an initial screening platform for testing candidate genes for expression in bovine spermatogonia. However, this method is based on mRNA levels of the candidate genes and may not represent the protein expression of these populations accurately. The candidate genes *DDX6*, *NAP1L4* and *TKTL1* that showed increased expression correlated with enriched population of spermatogonia, were further analysed for protein expression using immunohistochemical analysis. This allowed for the further characterisation of these putative markers to determine if they were, in fact, markers specific for bovine spermatogonia or SSCs.

Additionally, the initial proteomics profiling analysis and qRT-PCR analysis conducted in this experiment used peri-pubertal testis tissue. It was of interest to characterise these markers in different age groups, pre-pubertal, pubertal and post-pubertal, to assess their robustness as markers for bovine spermatogonia during different stages of development. In order to characterise the expression of these markers in different age groups of bovine testis, immunohistochemistry was carried out for the protein expression of markers *DDX6*, *TKTL1* and *NAP1L4*, in conjunction with known markers *ZBTB16* (*PLZF*) and *DBA-Biotin* (spermatogonia markers), *GATA4* and *Vimentin* (Sertoli cells markers).

In the pre-pubertal testis, immunohistochemistry indicated that all three putative markers co-stained with known markers for spermatogonia, DBA-Biotin and Plzf, and did not co-stain with Sertoli cell markers GATA4 or Vimentin, indicating expression by germline cells. Cells expressing DDX6, NAP1L4 and TKTL1, were located toward the tubule centre. This indicates that in the pre-pubertal stage, expression of these markers may be restricted to gonocytes, which have not yet migrated to the basement membrane, where spermatogonial stem cells are located. Therefore, DDX6, TKTL1 and NAP1L4 are likely to identify gonocytes in pre-pubertal bovine testis.

Results from immunohistochemistry analysis in this study, suggest that DDX6 appears to be limited to the subset of early stage spermatogonia, indicated by co-expression of DDX6 with DBA and ZBTB16. In a small population of differentiating germ cells also appear to express DDX6, as indicated by the presence of cells with DDX6 in the absence of co-expression of DBA or ZBTB16. DDX6 expression in murine testis has been shown in spermatogonia and primary spermatocytes (Matsumoto et al., 2005), thus DDX6 may be expressed by a similar sub-set of cells in the bovine testis.

In this study, the immunohistochemistry analysis of NAP1L4 expression, in different stages of bovine testis development, indicated that the protein is expressed in early stage spermatogonia in pre-pubertal, pubertal and post-pubertal adult testis. However, NAP1L4 is also expressed, more weakly, in other testis cell types, and may be expressed by differentiating germ cells, as shown by its staining pattern towards the centre of the tubule. Histone modification is a necessary process in cell differentiation and spermatogenesis, and it can be hypothesised that NAP1L4, could play a role in epigenetic mechanisms during spermatogenesis by its role as a histone chaperone.

The expression of the TKTL1 protein in pre-pubertal testis indicates its association with earlier stage, un-differentiated spermatogonia or gonocytes. Conversely, the location of TKTL1 positive cells, one cell layer removed from the basement membrane, in conjunction with their occasional co-staining with ZBTB16, indicates that TKTL1 is expressed in the pubertal and post-pubertal testis in differentiating spermatogonia. Co-staining with DDX4 (VASA) would be needed to confirm that TKTL1 expression is developmentally dependent, and that expression of TKTL1, progresses from

early stage spermatogonia before puberty to differentiating spermatocytes from the onset of puberty.

To further characterise the population of cells expressing the markers DDX6, NAP1L4 and TKTL1, cell smears from pre-pubertal and pubertal animals were stained with antibodies against putative spermatogonial markers, known spermatogonia markers DDX4 (VASA) and DBA-HRT, and Sertoli cell marker GATA4. Unfortunately, adult testis samples could not be obtained at the time of this experiment and hence, were not included in this analysis. The characterisation of these putative markers in the adult testis would be of interest if this line of investigation were to be continued in future experiments.

Analysis of cell smears indicated that there was no difference in the proportion of cells staining positively for the putative markers DDX6, NAP1L4 and TKTL1 in either pre-pubertal or pubertal testis. Additionally there was no difference in the proportion of cells that stained for the different putative markers. However, the location and previous characterisation of these markers in this chapter, suggest that these markers do not necessarily stain the same sub-set of spermatogonia, and instead are expressed by similar sized populations of testis cells in the bovine. In comparison to the proportion of cells identified by known spermatogonia makers DBA- HRT and DDX4, these putative markers appear to represent cell populations that are neither as exclusive as those stained for DBA-HRT, or as variable as those stained for DDX4. This suggests that these markers identify different sub-sets of germ cells, that are more differentiated than the type-A spermatogonia that express DBA-HRT, and less differentiated than the later stage spermatogonia population that express DDX4.

As expected from protein structure prediction information, none of the putative spermatogonia markers, DDX6, TKTL1 or NAP1L4 displayed a staining pattern consistent with cell surface proteins, and instead showed expression in the cytoplasm, possible membrane associated, or nucleus of positively stained cells. Hence, these putative markers are not suitable for direct enrichment processes. However, they may be valuable in identifying different subsets of spermatogonia in the bovine testis at different stages of development.

### 3.5 Conclusion

The identification of new makers for bovine spermatogonia and spermatogonial stem cells (SSCs) may assist in enrichment of these populations of cells, and will improve our characterisation and knowledge of bovine testis cells.

The aim of this chapter was to characterise potential candidate markers for bovine spermatogonia that were identified by proteomics analysis of bovine testis cell populations (Colgrave et al., 2013). Initial screening by gene expression analysis indicated that three of the six makers analysed, *DDX6*, *NAP1L4* and *TKTL1*, were more highly expressed in the spermatogonia enriched fraction of testis cell populations. Conversely, there was no difference in expression of other candidate markers *IQGAP1*, *FCSN1* or *TNL1* in enriched or depleted populations of bovine spermatogonia.

Further analysis of the staining patterns of the putative markers *DDX6*, *NAP1L4* and *TKTL1*, indicated that in the pre-pubertal testis, these markers were associated with gonocytes. In the pubertal and post-pubertal testis, *DDX6* and *NAP1L4* were expressed by cells located on the basement membrane, and co-stained with known early stage spermatogonia markers DBA-biotin and ZBTB16. This indicated that *DDX6* and *NAP1L4* are also expressed by early stage bovine spermatogonia. However, a sub-set of *DDX6* positively stained cells, did not co-stain with DBA or ZBTB16, indicating that *DDX6* may also be expressed by a small population of more differentiated spermatogonia in addition to early stage spermatogonia. In addition to expression in early stage spermatogonia, *NAP1L4* also showed expression patterns toward the lumen of the tubule, this expression is most likely associated with differentiating germ cells. Conversely, although *TKTL1* is associated with gonocytes, it is not associated with undifferentiated spermatogonia, and is instead expressed by a restricted population of differentiating spermatogonia, located one cell layer removed from the basement membrane.

Quantification of the proportion of cells staining for putative markers *DDX6*, *NAP1L4* and *TKTL1*, further indicated that the population of cells expressing these putative markers were different than populations expressing known markers. It is likely that cells expressing *DDX6*, *NAP1L4* and *TKTL1* are more differentiated than those that exhibit DBA- binding activity, and less differentiated, or represent a smaller proportion, than the germ cell populations that express *VASA*.

It is clear that these three novel spermatogonial markers, DDX6, NAP1L4 and TKTL1, show specificity for different subsets of spermatogonia. These markers will be useful for further elucidating developmental processes in the bovine testis.

## Chapter 4 : Comparison of reprogramming factor combinations in producing Bovine iPS cells

### 4.1 Introduction

The production of induced pluripotent stem cells (iPS cells) is possible through the addition of a combination of reprogramming or transcription factors (Takahashi & Yamanaka, 2006). Many of the transcription factors used in the reprogramming process are normally expressed in the early stages of development, specifically, at high levels in embryonic stem cells (Takahashi & Yamanaka, 2006; Yu, Junying et al., 2007). The six reprogramming factors most commonly used to induce pluripotency are OCT4 (*POU5F1*), SOX2, KLF4, c-MYC, NANOG and LIN28. OCT4, SOX2 and NANOG are described as being core pluripotency factors and are involved in the maintenance of pluripotency (Chambers & Tomlinson, 2009; Esteban et al., 2009; Sumer, Liu, Malaver-Ortega, et al., 2011). However, no single reprogramming factor has been identified as being the sole reprogramming factor responsible for the induction or maintenance of pluripotency. The role of these six reprogramming factors in reprogramming and maintaining pluripotency is described in further detail below.

#### Reprogramming Factors

In 1990, Octamer-binding transcription factor 4 (OCT4 or OCT3/4), coded for by the gene *POU5F1*, was identified as being a novel protein of the OCT family. OCT4 is expressed at high levels in embryonic carcinoma cells, embryonic stem cells, early stage embryos and germ cells (Schöler, Hatzopoulos, Balling, Suzuki, & Gruss, 1989; Yamanaka, S., 2007). OCT4 is essential for the maintenance of pluripotency and the development of the inner cell mass *in vivo*. Precise levels of OCT4 determine the differentiation of embryonic stem cells and the development of primitive endoderm and mesoderm and play an important role in the differentiation of neural and cardiac cells (Nichols et al., 1998; Shi, G. & Jin, 2010). It is also thought that OCT4 directs cell fate away from tumorigenesis and toward embryonic stem (ES) cell like fate through activation and suppression of various genes and non-coding RNAs that are necessary for pluripotency (Shi, G. & Jin, 2010). The expression levels of OCT4 play an important role in cell fate. Within a narrow range of OCT4 expression, cells are able to maintain pluripotency and remain undifferentiated. Conversely, increased OCT4 expression by less than two fold, can result in the differentiation of cells toward the endoderm and mesoderm lineages, while the loss of OCT4 expression also causes loss of

pluripotency and differentiation (Niwa, Miyazaki, & Smith, 2000). Intriguingly, iPS cells have been produced by transfection of the OCT4 transcription alone. Studies have shown that somatic cells that express high endogenous levels of other reprogramming factors SOX2 and c-MYC, such as neural progenitors, can be reprogrammed to pluripotency with the addition of exogenous OCT4 alone (Kim, J. et al., 2008; Yamanaka, S., 2009). At this time it is believed OCT4 is essential for the reprogramming of cells to pluripotency and cannot be replaced by other reprogramming factors or growth factors (Shi, G. & Jin, 2010; Yuan, Xu. et al., 2011).

SOX2 (Sex-determining region Y-box 2) was identified in embryonic carcinoma cells in 1995 (Stevanovic, Zuffardi, Collignon, Lovell-Badge, & Goodfellow, 1994). It is important in maintaining pluripotency and is expressed in pluripotent, multipotent and omnipotent stem cells such as neural stem cells and early stage embryos. SOX2 is able to form a heterodimer with OCT4, additionally, gene binding sites for SOX2 closely overlap with those of both OCT4 and NANOG, this assists in the regulation of target genes including UTF1, FGF4 and FBX15 (Boyer et al., 2005; Yuan, H., Corbi, Basilico, & Dailey, 1995). The interaction between these factors highlights their importance in maintaining pluripotency. SOX2 functions through activation of genes necessary for inducing pluripotency. While low expression levels of the SOX2 protein are required for maintenance of pluripotent cells, it is thought reprogramming requires comparatively higher levels of SOX2 to be successful (Tiemann et al., 2011; Yamanaka, S., 2007). Additionally, it has been suggested that the primary role of SOX2 is to maintain *POU5F1* (OCT4) expression, and that the forced expression of *POU5F1* to produce OCT4 can compensate for the loss of SOX2 expression in ES cells (Nakagawa, M. et al., 2008).

KLF4 (Krüppel-like factor 4), is a zinc finger transcription factor that is highly expressed in differentiated, post-meiotic epithelial cells of the skin and gastrointestinal tract (Segre, Bauer, & Fuchs, 1999). KLF4 also is found in large quantities in undifferentiated cells in the growth arrest phase, and is undetected in cells that proliferate exponentially (Zhao, W. et al., 2004). KLF4 is able to both repress and activate transcription, depending on the target gene and interactions with other transcription factors. It is also believed to function both as a tumour suppressor and an oncogene, and in culture results in the inhibition of DNA synthesis and cell cycle progression (Zhao, W. et al., 2004). Rowland *et al* (2005) conducted studies to better understand the molecular mechanisms of KLF4, and found that the ectopic expression of KLF4 suppressed cell proliferation. Conversely the removal of just one target gene of KLF4, p21 (*Waf1* cyclin-dependent kinase inhibitor), was shown to

promote cell proliferation by down regulation of p53 (Rowland, B. et al., 2005; Yoon, Chen, & Yang, 2003). It can therefore be inferred that p21 is responsible for the outcome of KLF4 on cellular mechanisms. KLF4 is also believed to cooperate with OCT4 and SOX2 to activate the Lefty1 core promoter in murine ES cells (Nakatake et al., 2006). Although initially identified as an essential factor for the generation of iPS cells (Takahashi & Yamanaka, 2006). Yu, Junying et al. (2007) reported that KLF4 was unnecessary for generating human iPS cells, and can be replaced by other factors including NANOG and LIN28 (Yu, Junying et al., 2007).

c-MYC is a transcription factor that influences numerous cellular functions including cell-cycle regulation, proliferation, growth, differentiation and metabolism. It is believed that c-MYC plays a role in pluripotency by enhancing cell cycle mechanisms for self-renewal, modifying epigenetic patterns to promote dedifferentiation, and can be used for selection of rare cell populations with certain traits permitting pluripotency and self-renewal (Knoepfler, 2008). c-MYC was one of the first proto-oncogenes found in human cancers, and is believed to cause tumours in mice generated by iPS cells transfected with the c-MYC transcription factor, making the use of c-MYC problematic in the generation of iPS cells for use in clinical purposes (Miller, Thomas, Islam, Muench, & Sedoris, 2012). Studies have found that c-MYC is not essential for inducing pluripotency (Yu, Junying et al., 2007), yet cells generated without c-MYC were reported to have a decreased reprogramming efficiency of 500-fold compared to the same cells generated with exogenous c-MYC. This suggests that while c-MYC may not be necessary for inducing pluripotency, it does play an important role in increasing efficiency of iPS cell generation (Nakagawa, M. et al., 2008).

NANOG is another transcription factor first used to generate iPS cells as a part of the four factor cocktail originally described by Yu et al (2007) (OCT4, Sox2, LIN28 and NANOG). NANOG is involved in maintaining pluripotency and self-renewal of undifferentiated embryonic stem (ES) cells. The expression of NANOG in ES cells is thought to be the key factor for maintaining pluripotency (Chambers et al., 2003; Mitsui et al., 2003). Additionally, NANOG functions in co-operation with other transcription factors including OCT4 (*POU5F1*) and SOX2. Studies have shown that the overexpression of NANOG in ES cells results in self-renewal even in the absence of leukaemia inhibitory factor (LIF) (Cavaleri & Schöler, 2003). In contrast, the absence of NANOG causes stem cells to differentiate (Cavaleri & Schöler, 2003). Interestingly the addition of NANOG is not essential for iPS cell generation in most cell types from human and murine tissue, and is dispensable for murine iPS cell reprogramming under optimised conditions (Schwarz et al., 2014). However, the

expression of endogenous NANOG is seen as a hallmark of fully reprogrammed iPS cells and colonies (Takahashi & Yamanaka, 2006), and when used for selection of reprogrammed colonies results in far higher selection efficiency than other genes such as FBX15 (Wernig et al., 2007). Recent studies suggest that NANOG is a necessary component of the reprogramming cocktail for producing bovine iPS cells (Sumer, Liu, Malaver-Ortega, et al., 2011).

LIN28 is a protein marker of undifferentiated embryonic stem cells, and is used as part of the Thompson factor reprogramming cocktail (OSNL) (Yu, Junying et al., 2007). LIN28 binds to and promotes the translation of IGF-2 (Insulin like growth factor 2). In addition LIN28 also interacts with, and regulates microRNA Let-7, which plays an important role in suppressing tumours and also decreases “stemness” of stem cells by promoting differentiation (Poleskaya et al., 2007; Viswanathan & Daley, 2010). Let-7 is also believed to inhibit c-MYC, thereby inhibiting the maintenance of pluripotency, and therefore through the regulation of Let-7 by LIN28, the pluripotency of stem cells is believed to be preserved (Bang & Carpenter, 2008).

### **Reprogramming Factor Cocktails**

Reprogramming of somatic cells to pluripotency was initially performed in murine fibroblasts through the transduction of 24 candidate genes that were known to be implicated in pluripotency using a retroviral vector. The four transcription factors that were found to be sufficient to reprogram cells were identified as OCT4, SOX2, c-MYC and KLF4 (OSKM Yamanaka Factors) (Takahashi & Yamanaka, 2006). The following year reprogramming of human somatic cells was achieved using a slightly different combination of four transcription factors; OCT4 (*POU5F1*), SOX2, LIN28 and NANOG (OSNL Thompson factors) (Yu, Junying et al., 2007). This combination of factors led to the suggestion that OCT4 and SOX2 are essential for reprogramming while KLF4, c-MYC, NANOG and LIN28 are supportive factors.

During the years that have followed, a multitude of different cell types, from different species have been reprogrammed using various combinations of these six transcription factors. The OSKM factor combination has been reported to successfully reprogram many cells types from different species including mouse, human, rhesus monkey, pig and buffalo, among others (Deng et al., 2012; Liu, Haisong et al., 2008; Park, I.-H. et al., 2008; Park, K.-M. et al., 2013; Takahashi & Yamanaka, 2006).

The addition of NANOG to the OSKM factor cocktail has also been shown to improve reprogramming efficiency to a large extent in some cell types. The supplementation of NANOG to the OSKM factor cocktail to reprogram mouse B cells is reported to decrease the time taken for colonies to form (approximately halved) compared to the time taken with OSKM alone (Hanna et al., 2009). Additionally, reprogramming cocktails including all six reprogramming factors (OSKMNL) have also been used to reprogram cells to pluripotency, with higher efficiencies reported (approximately 10 fold higher) than the use of four factor combinations (Liao et al., 2008). It has been suggested that although NANOG and LIN28 are not essential for reprogramming, they play an important role in enhancing the reprogramming process (Onder et al., 2012).

Studies in more recent years have demonstrated that cell types with high endogenous expression of certain reprogramming factors, may be reprogrammed to pluripotency using fewer than four reprogramming factors. Mouse and human fibroblasts or neural stem cells that naturally express high endogenous levels of c-MYC and KLF4, have been reprogrammed without the addition of KLF4 and c-MYC exogenous factors, and in some cases with OCT4 (*POU5F1*) alone (Kim, J. et al., 2008; Nakagawa, M. et al., 2008; Wernig, Meissner, et al., 2008). This is of particular interest because it eliminates the need to introduce the exogenous oncogene c-MYC, and thereby decreases the inherent risk of tumour formation in iPS derived cells used for clinical purposes. However, the efficiency of producing iPS cells without the addition of KLF4 and c-MYC has been shown to be far lower than when using the four factor combination of OSKM (Nakagawa, M. et al., 2008; Wernig, Meissner, et al., 2008).

### **Inducible Vectors**

Inducible vector systems, where transgenes can be turned on and off using a promoter, are popular choices for reprogramming. Inducible vectors require the introduction of inducer, such as doxycycline, to turn on the transcription of the transgene. This method of controlling transgene transcription enables the initial reprogramming of cells by transgene expression, and enables transgene expression to be switched off when reprogramming is complete. This method is favourable as the complete silencing of transgenes in fully reprogrammed iPS cells is necessary for them to be deemed safe for use in clinical applications (Hockemeyer et al., 2008; Markoulaki et al., 2009; Sommer et al., 2009; Yildirim, 2012).

### **Polycistronic Vectors**

The introduction of individual factors or polycistronic vectors has been implemented for the production of iPS cells in various species. Both have merits and complications associated with their use in reprogramming. The original method of gene delivery for iPS generation used individual viral vectors to deliver each transcription factor (Takahashi & Yamanaka, 2006; Yu, Junying et al., 2007). As the stoichiometry of reprogramming factor expression levels are thought to be important in reprogramming, the use of single factors that can be added in different concentrations is sometimes used to optimise reprogramming (Papapetrou et al., 2009; Tiemann et al., 2011). However, the use of multiple vectors in a viral reprogramming system can result in high numbers of genomic integration sites, increasing the risk that the transgene will interrupt endogenous gene function (Lai et al., 2011).

Polycistronic vectors simplify the reprogramming process as they express multiple genes located on the same plasmid. This makes introducing all reprogramming factors to the target cells by infection or transfection far more efficient than introducing numerous single factors into the same cell (Carey et al., 2009; Gonzalez et al., 2009). Many polycistronic vectors also have separate promoters and self-cleaving peptides for the individual genes to enhance the efficiency of expression of the genes expressed by the vector.

### **Livestock iPS cells**

The production of iPS cells from livestock has been more challenging than the production of mouse and human iPS cells. There have been numerous studies that have successfully generated iPS like cells from livestock that exhibit most characteristics of fully reprogrammed iPS cells, with the exception of transgene silencing (Esteban et al., 2009; Ezashi et al., 2009; Sumer, Liu, Malaver-Ortega, et al., 2011). However, the successful production of fully reprogrammed bovine iPS cells has been especially difficult, with very few reports to date able to produce bovine iPS cells with a protocol that is reproducible. Furthermore the unanswered question, to which extent have bovine iPS cells been reprogrammed remains. Characteristics that are necessary for fully reprogrammed iPS cells such as the ability to passage cells indefinitely (Cao et al., 2012; Huang, B. et al., 2011) and transgene silencing have yet to be demonstrated in bovine iPS cells (Huang, B. et al., 2011; Sumer, Liu, Malaver-Ortega, et al., 2011). It is unclear why reprogramming methods that successfully produce fully reprogrammed iPS cells in other species are not as successful in bovine cells.

Although most reprogramming factors are highly conserved between mammalian species, they can differ slightly. Transcription factors are proteins that work by binding to the promoter sequence of genes to activate or repress transcription of endogenous genes (Cooper, 2000). Differences in the genomic sequence of transcription factors between species can result in altered protein structure of the transcription factor, thereby changing the binding affinity of the transcription factor to promoters or repressor in the host cell. For this reason homotrophic reprogramming factors are generally used in the production of iPS cells from mice and human somatic cells, i.e. human reprogramming factors are used to reprogram human cells. However, access to livestock reprogramming vectors is limited. Instead, numerous studies have demonstrated the use of human or mouse transcription factors for reprogramming somatic cells from other species including livestock species. This suggests that both human and mouse transcription factors are conserved enough between these species to result in reprogramming (Bao et al., 2011; Cao et al., 2012; Li, Yang, et al., 2011; Liu, J. et al., 2012; Sartori et al., 2012; Sumer, Liu, Malaver-Ortega, et al., 2011).

Of most interest to this study is the relatively recent claim that NANOG is essential for reprogramming bovine somatic cells to a pluripotent state. The first report of successful bovine iPS cell generation claimed that NANOG, in addition to OCT4, SOX2, KLF4 and c-MYC constructs, was essential to generate fully reprogrammed bovine iPS cells (Sumer, Liu, Malaver-Ortega, et al., 2011). This claim has yet to be substantiated, with other reports having been made for the successful derivation of bovine iPS cell derivation without the use of NANOG (Cao et al., 2012; Han et al., 2011). Interestingly, given the suggested importance of NANOG, very few studies have used the OSNL factor combination to produce iPS cells from livestock species (Table 4-1).

Chapter 4: Comparison of Reprogramming Factor Combinations in Producing Bovine iPS Cells

Table 4-1. Reported production of iPS cells from different livestock species.

Species	Reference	Cell Type	Reprogramming Factors	Vector Type
<b>Bovine</b>	(Sumer, Liu, Malaver-Ortega, et al., 2011)	Bovine adult fibroblasts	Individual human O,S,K,M,N	p-MX plasmids in retrovirus
<b>Bovine</b>	(Han et al., 2011)	Bovine foetal fibroblasts	Polycistronic Bovine OSKMNL Bovine OSKM Human OSKM	p-MX polycistronic plasmids in Retrovirus
<b>Bovine</b>	(Huang, B. et al., 2011)	Embryonic, Foetal and Adult bovine Fibroblasts	Polycistronic bovine OSKM and KSOM non-viral plasmids	Non-viral poly-promoter plasmid
<b>Bovine</b>	(Cao et al., 2012)	Bovine Foetal Fibroblasts	Individual human O,S,K,M	Fusion Proteins in Lentivirus
<b>Bovine</b>	(Deng et al., 2012)	Buffalo Foetal Fibroblast	Individual buffalo O,S,K,M,N,L and O,S,K,M	pMX-Plasmid in Retrovirus
<b>Ovine</b>	(Li, Yang. et al., 2011)	Sheep foetal fibroblasts	Murine OSKM	Polycistronic Tet-inducible Lentivirus
<b>Ovine</b>	(Bao et al., 2011)	Ovine fibroblasts	Polycistronic human OSMK, and OSMKNL plasmids	Doxycycline Tet-inducible lentivirus Constitutive expression lentivirus
<b>Ovine</b>	(Sartori et al., 2012)	Sheep Embryonic fibroblasts	Individual human O,S,K,M	p-MX plasmids in retrovirus
<b>Ovine</b>	(Liu, J. et al., 2012)	Sheep fibroblasts	Individual human O,S,K,M	pMX plasmids in Retrovirus
<b>Goat</b>	(Song et al., 2013)	Foetal goat ear fibroblasts	Individual human O,S,K,M	Lentivirus
<b>Porcine</b>	(Ezashi et al., 2009)	Porcine foetal fibroblasts	Individual O,S,K,M	Lentivirus
<b>Porcine</b>	(Ruan et al., 2011)	Porcine embryonic fibroblasts	Individual O,S,K,M	pMX plasmids in VSV-G envelope-coated pantropic retrovirus
<b>Equine</b>	(Nagy et al., 2011)	Foetal horse fibroblasts	Polycistronic MKOS plasmid	Electroporation of Piggy-BAC transposon plasmids

The roles of the six different reprogramming factors, OCT4 (*POU5F1*), SOX2, KLF4, c-MYC, NANOG and LIN28, in establishing and maintaining pluripotency is complex. As such, different combinations of reprogramming factors may more efficiently reprogram different cell types and ages to pluripotency than others. With the recent claims that NANOG is required for reprogramming bovine cells to pluripotency, it was of interest to compare how different reprogramming cocktails may affect reprogramming of bovine somatic cells to pluripotency.

The Tet-OSKM construct used in this chapter for cellular reprogramming, was previously used for lentiviral reprogramming of putative bovine iPS cultures at the F D McMaster Laboratory, Armidale, NSW (McMillan, 2012). To enable a comparison of previous work into the derivation of bovine iPS cells from this laboratory, Tet-OSKM was also used in reprogramming experiments outlined in this thesis.

Furthermore, it was previously determined that reprogramming of bovine iPS cells could be enhanced through the addition of a small molecule combination, of HDAC inhibitor sodium butyrate (NaB), MEK inhibitor PD0325901 (PD) and TGF- $\beta$  inhibitor SB431542 (SB). This combination was found to significantly accelerate the kinetics of reprogramming, resulting in lentivirally derived colonies observed in 14 days as opposed to over 20 days (McMillan, 2012). As such, these small molecules were added to the iPS media used in this chapter in accordance to supplementation described by McMillan (2012).

Currently an optimal protocol for the derivation of bovine iPS cells has not been described. Of the reports claiming to have produced bovine iPS no protocol has been reported to be successfully reproduced. Reports of the derivation of bovine iPS cells to date have only used the OSKM combination of reprogramming factors, with or without the additional transfection of NANOG. In light of the claim that NANOG is needed for producing fully reprogrammed bovine iPS cells, it is of interest to determine if the use of reprogramming factors may lead to the more efficient derivation of these cells. Additionally as cell type, and the age of cells is thought to effect reprogramming, (Okada & Yoneda, 2011), the difference in reprogrammability of bovine dermal fibroblasts (BDFs) and bovine embryonic fibroblasts (BEFs) was also of interest.

It was hypothesised that reprogramming cocktails containing NANOG would result in increased reprogramming efficiency of bovine somatic fibroblast to a pluripotent state. To test this hypothesis, bovine fibroblasts were lentivirally transduced with one of three polycistronic reprogramming constructs carrying three different combinations of the six transcription factors, Tet-OSKM, OSNL and OSKMNL.

## **4.2 Materials and Methods**

### **4.2.1 General**

All animal experiments were conducted with approval under the F D McMaster Laboratory Animal Ethics Committee, CSIRO Animal Food and Health Sciences (Approval number 11/20). All cell culture reagents were purchased from Life Technologies (USA), chemicals and reagents were purchased from Sigma Chemical Industries (USA) where indicated, small molecules were purchased from Stemgent (USA) unless otherwise stated. All plasmids were acquired from plasmid repository database Addgene. Table 4-2 describes plasmids used in Chapter 4. Primers used in Chapter 4 were designed using Primer3 software to be specific for bovine mRNA, and ordered through Biosearch Technologies, Inc. Primers used in Chapter 4 are described in Table 4-3. This experiment was repeated three times with two technical replicates for each treatment per experiment.

### **4.2.2 Derivation of Bovine Fibroblasts**

#### ***4.2.2.1 Bovine Dermal Fibroblasts (BDFs)***

Skin samples from male Angus calves were taken during castration performed under general anaesthesia. Primary cell cultures were prepared using standard methods. In brief, the epidermis of skin samples were cut into small pieces and incubated in 1% Trypsin at 37°C in a water bath for one hour under slow agitation. The suspension was filtered through a 30 µm cell filter and cells were collected by centrifugation at 400 xg for 5 minutes. Cells were resuspended in fibroblast growth media (DMEM, 10% Foetal Bovine Serum (FBS) (Life Technologies, USA), 2 mM GlutaMAX™, 100 µM non-essential amino acids (NNEA) (Life Technologies, USA) with 1% anti-anti (10,000 U/mL of

penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, USA), and cultured at 37°C, 5% CO<sub>2</sub>, in a humid atmosphere. Media was changed every second day and cells were passaged approximately every 3-4 days.

#### **4.2.2.2 Bovine Embryonic Fibroblasts (BEFs)**

A male *Bos Taurus* foetus was sourced from a local abattoir from slaughtered cattle. The foetus measured approximately 18cm crown to rump, giving an estimated age of 60-70 days gestation. Primary skin cultures were prepared as described above in 4.2.2.1.

#### **4.2.3 Plasmid Preparation**

Plasmids were grown and isolated as per the manufactures instructions for the Qiagen High Speed Plasmid Maxi Kit (Qiagen, Netherlands). Basically, bacterial culture spikes obtained from gene repository database, Addgene, were plated on ampicillin treated LB agar plates and grown overnight at 37°C. The following morning a starter culture was prepared, with inoculation of a single colony from the culture plates in 10 ml LB nutrient broth supplemented with 100 µg/ml ampicillin (Life Technologies, USA) and incubation for 8 hours at 37°C with shaking at 300 rpm. Following the 8 hour incubation, the starter culture was diluted 1 in 500 with LB broth supplemented with 100 µg/ml ampicillin and grown overnight at 37°C with shaking at 300 rpm. The following morning cells were harvested by centrifugation at 6000 xg for 15 minutes at 4°C. The cell pellet was then resuspended, lysed and neutralised using buffers supplied by the manufacturer. QIAfilter cartridges were used to bind and elute DNA by gravity flow. The resulting DNA was then precipitated and washed using ethanol and resuspended in buffer (provided). Plasmid DNA concentrations were measured spectrophotometrically using a NanoDrop spectrophotometer (Thermo Scientific, USA). Purified plasmid DNA was stored at -20°C until use.

#### 4.2.4 Lentiviral Production

HEK293 cells were grown in fibroblast growth media in T25 flasks to 60-80% confluence on the day of lentiviral production. Transfection of the target plasmids was conducted as per the manufacturer's instructions for the LTX Lipofectamine™ PLUS reagent kit (Life Technologies, USA). Essentially, fibroblast growth media was replaced with fibroblast media without antibiotics (DMEM, 10% FBS, 1% non-essential amino acids (NNEA), 1% GlutaMAX™). Reagents were brought to room temperature, target plasmid (1 µg Tet-OSKM, M2RtTA, OSNL, OSKMNL or eGFP), pMD2.g (1 µg) and psPAX2 (2 µg) were diluted in OPTIMEM (500 µl), PLUS reagent (4 µl) was added to each reaction and incubated at room temperature for 5 minutes. Lipofectamine™ LTX (8 µl) was added to each reaction and further incubated at room temperature for 30 minutes. The mixture was then added drop-wise directly onto the HEK293 cells which were then incubated at 37°C 5% CO<sub>2</sub> for 12-16 hours, after which the media was replaced with lentiviral collection media (DMEM, 10%, 2 mM GlutaMAX™, 100 µM NNEA (Life Technologies, USA)). Cells were incubated for a following 16-24 hours after which Lenti-X™ GoStix™ (Clontech Laboratories, 2014) were used, according to the manufacturer's instructions, to confirm the presence of lentivirus in all viral preparations. Basically, 20 µl of each lentiviral supernatant was applied to the sample well of a GoStix™ cassette. Three drops of Chase Buffer (supplied) was added to the sample well and allowed to run through the cassette until bands developed. After 10 minutes if lentivirus is present in the applied supernatant a control and test band appear on the cassette to confirm lentiviral titre. If lentivirus was present the media containing infective lentivirus was collected, filtered through a 45 µm cell filter and the supernatant was then snap frozen in liquid nitrogen and stored at -80°C until use.

#### 4.2.5 Lentiviral Infection of Fibroblasts

5x10<sup>4</sup> BEFs or BDFs were plated in each well of a 6 well dish in fibroblast growth media (DMEM, 10% FBS, 1% PSA, 1% non-essential amino acids (NNEA), 1% GlutaMAX™) and incubated at 37°C, 5% CO<sub>2</sub> for 12-24 hours. Fibroblast media was then replaced with lentiviral supernatant diluted 1:2 with fibroblast media (i.e. 500µl OSNL, OSKMNL or eGFP in 500µl Fibroblast media; or, 250 µl Tet-OSKM, 250 µl M2rtTA, 500 µl fibroblast media). 8 µg/ml of Polybrene (hexadimethrine bromide; Sigma-Aldrich Chemical Industries, USA) was added to each well and the cells were incubated for 24 hours. Infection was repeated the following day as described above, and cells were incubated for a following 24 hours. Following incubation the media was replaced by iPS media (MEM-Alpha, 20%

FBS, 0.5% PSA, 1% NEAA, 1% GlutaMAX™, 0.1 mM β-mercaptoethanol, 10 ng/ml bFGF, 4 ng/ml hLIF, and small molecules 0.5 mM sodium butyrate (Sigma-Aldrich Chemical Industries, USA), 0.5 μM PD0325901 (Stemgent, USA) and 2 μM SB431542 (Stemgent, USA)) and grown for a further 24 hours. Presence of green fluorescence protein in cells infected with the eGFP plasmid was observed using fluorescent microscopy to confirm lentiviral infection had been successful. Cells were then enzymatically passaged using TrypLE™ Express into T75 flasks and grown for 3-5 days without allowing cells to reach confluence. Cells were then harvested using TrypLE™ Express and plated onto mitomycin-c (Sigma-Aldrich Chemical Industries, USA) treated BEFs (2x10<sup>6</sup> cells/ 10 cm dish) at 5x10<sup>4</sup> cells per 10 cm dish. Mitomycin-c (Mit-C) inactivation and plating onto gelatine coated plates was conducted as follows. Confluent BEF cultures were washed with Dulbecco's phosphate buffered saline (DPBS) (Life Technologies, USA) and media was replaced with fibroblast growth media containing mitomycin-c (1 mg/ml). Cultures were incubated at 37°C, 5% CO<sub>2</sub>, in a humid atmosphere, for 4 hours, after which time media containing Mit-C was removed and cells were washed three times in DPBS to remove residual Mit-C. Mit-C inactivated cells were harvested by incubation with TrypLE™ Express for 10 minutes at 37°C, 5% CO<sub>2</sub>, in a humid atmosphere to detach adherent cells. Cells were collected and centrifuged at 400 xg for 5 minutes. Cells were resuspended in fibroblast growth media and plated onto 10 cm cell culture dishes that had been incubated with 10 ml 0.1% gelatine (in PBS) for 4 hours at 37°C prior to plating Mit-C inactivated BEFs.

#### **4.2.6 iPS Cell Culture**

This experiment was conducted three times with two technical replicates per treatment. Cells were fed daily by the replacement of 10 ml old media with 10 ml fresh iPS media described above. 20 μl of doxycycline was added to cells infected with the Tet-OSKM plasmid daily to induce transcription. Cultures were monitored daily for time to colony formation and the appearance of colonies. Cultures were grown for 21 days, after which colonies were counted to determine reprogramming efficiency, and were manually picked using a small gauge syringe and a 20 μl pipette. Colonies were washed in PBS (phosphate buffered saline) and frozen at -80°C until analysis. Control cultures of uninfected fibroblasts grown on MIT-C inactivated feeder layers were run in parallel with reprogramming experiments. These cells were treated identically to lentivirally transfected cells and were fed the same iPS media as lentivirally infected fibroblasts

A time line showing the process of lentiviral production and transfection and culture of lentiviral iPS cells is shown in Figure 4-1.

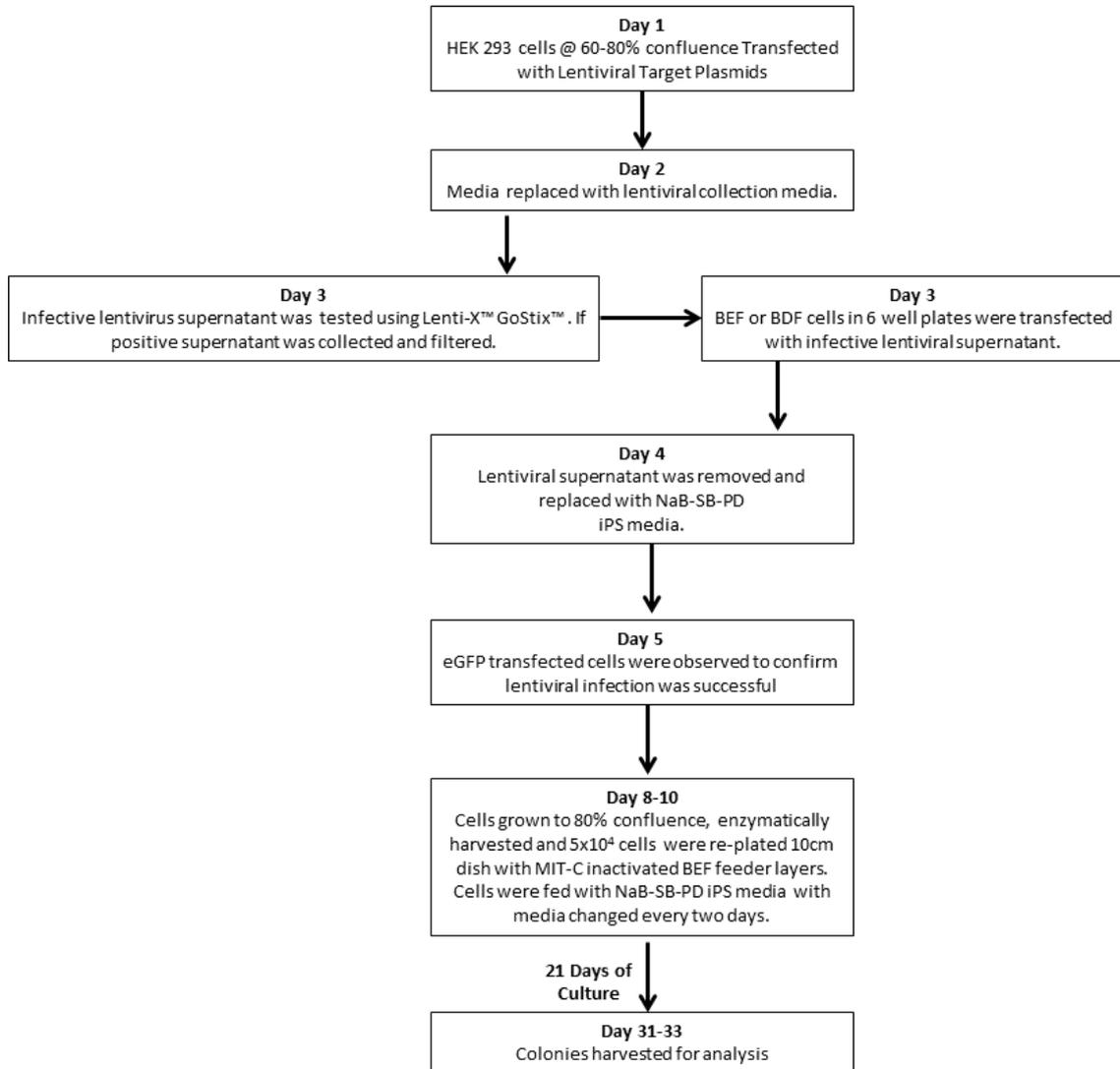


Figure 4-1. Timeline showing the process of lentiviral production and transfection and culture of lentiviral iPS cells in the experiments outlined in Chapter 4.

## 4.2.7 Analysis of Putative iPS Colonies

### 4.2.7.1 Colony Development

Putative iPS cultures were observed daily for colony formation, and the time to colony formation was recorded for each treatment. Photographs of colonies were taken using a Nikon Eclipse TE300 inverted microscope (Nikon, Japan) equipped with a coolLED PE light source (CoolLED, USA), and images were captured using ProgRes CapturePro 2.1 software (Jenoptik, Germany). Reprogramming efficiency of cultures was calculated after 21 days of culture and was determined by the total number of colonies observed divided by the total number of transfected cells initially plated.

### 4.2.7.2 Alkaline Phosphatase Staining

Alkaline phosphatase staining was carried out using Alkaline Phosphatase Live Stain kit (Life Technologies, USA) according to manufacturer's instructions. Basically, the growth media was removed and cells were washed with pre-warmed DMEM/F-12 for 2-3 minutes, media was aspirated and the wash was repeated. The 1x alkaline phosphatase (AP) stain was prepared by dilution of 500x stock solution in warm DMEM/F-12. The working solution was applied directly to the cell culture and cells were incubated for 20-30 minutes. After incubation, the live stain was aspirated and cells were washed twice with DMEM for 5 minutes per wash. Fresh DMEM/F-12 was added following the final wash and cells were photographed within 30 minutes using a Nikon Eclipse TE300 inverted microscope (Nikon, Japan) using a standard Fit-C filter (CoolLED, USA). Following visualisation, DMEM/F-12 was replaced with fresh iPS media and cells were returned to normal culture conditions.

### 4.2.7.2 PCR for Transgene Integration

For PCR reactions, genomic DNA was extracted from pooled, putative iPS colonies, using an Allprep RNA/DNA micro kit and QIAshredder (Qiagen USA) according to the manufactures instructions. Each PCR reaction contained 0.5 µg genomic DNA, 1 µl dNTPs (10 mM), 10 µl 5x Go Taq Reaction Buffer, 0.25 µl Go Taq (Life Technologies, USA), and 0.2 µM forward and reverse primer for Tet-OSKM, OSNL or OSKMNL constructs, made up to 50 µl with RNase/DNase free water. Primers for constructs are

shown in Table 4-3. PCR cycle conditions for transgene integration were, a denaturation step of 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, and finally an extension step at 72°C for 5 minutes. PCR products were run on a 1% agarose gel at 100V.

#### **4.2.7.2 Quantitative Real Time (qRT) PCR**

mRNA was extracted from pooled colonies (5 colonies from each treatment) using a Dynabeads® mRNA DIRECT™ Micro Kit (Life Technologies, USA) as per manufactures instructions. In short, 100 µl lysis buffer (provided) was added to each sample of cells, and cells were pipetted vigorously to aid in lysis. Samples were then transferred to microfuge tubes containing 20 µl of washed and resuspended Dynabeads® Oligo (dT)<sub>25</sub>. Samples were mixed for 5 minutes at room temperature on a roller to hybridise the Dynabeads® with mRNA. Samples were placed on a DynaMag™ unit (Life Technologies, USA) and supernatant was removed. The Dynabead®/mRNA samples were washed twice in Buffer A (supplied), by removing samples from magnet, adding 100 µl of buffer, pipetting to mix and then replacing on magnet to remove supernatant. Samples were the washed twice in Buffer B (supplied) and resuspended in ice cold 10 mM Tris-HCl (pH 7.5) and stored on ice until reverse transcription step.

cDNA was then produced from the resulting Dynabeads®/mRNA using the SuperScript™ III First-Strand Synthesis System (Life Technologies, USA ). Briefly, the Dynabead® solutions were placed on the DynaMag™ magnet and Tris-HCl was removed. 1 µl 50 µM oligo(dT)<sub>20</sub>, 1 µl 50 ng/µL random hexamers, 1 µl annealing buffer and 5µl of RNase/DNase free water, was added to each Dynabeads® pellet. The samples were then incubated in a thermal cycler at 65°C for 5 minutes, then immediately placed on ice for at least 1 minute. Tubes were briefly centrifuged to collect contents. 10 µl of 2x First-Strand Reaction Mix and 2 µl SuperScript™ III/RNase OUT Enzyme Mix was added to each tube. Samples were briefly centrifuged to mix and then briefly centrifuged to collect contents. Samples were incubated under the following conditions in a thermal cycler; 5 minutes at 25°C, 2 hours at 50°C, 5 minutes at 85°C. cDNA was stored at -20°C prior to quantitative Real Time-PCR (qRT-PCR).

Quantitative real time-PCR (qRT-PCR) reactions were carried out in iQ5 real time thermal cycler (Bio-Rad, USA). Each reaction contained 1 × IQ SYBR Green Supermix (Bio-Rad, USA), 0.5 µM each

forward and reverse primer, and 1  $\mu$ l cDNA (at 10 ng/ $\mu$ l) transcribed from Dynabead<sup>®</sup> /mRNA complex.

The reaction efficiencies for each primer set were determined by a standard curve produced using a serial dilution (100 ng, 20 ng, 4 ng, 0.8 ng and 0.16 ng of bovine fibroblast cDNA transcribed from mRNA), reactions were carried out in triplicate. Primer efficiencies are shown in Table 4-3. The reference gene *RPS26* was previously identified using the sheep GeNorm kit (PrimerDesign, UK). This housekeeper has been shown to be the best reference gene for the cell types used in this chapter in previous studies by McMillan (2012).

#### 4.2.8 Data Analysis

Differences in treatments means for the time to colony formation and colony numbers were analysed using ordinary one-way ANOVAs using GraphPad Prism<sup>®</sup> software (GraphPad Software, USA). Quantitative RT-PC  $C_t$  results were converted into gene expression data (fold change relative to the reference gene *RPS26*) using Microsoft Excel add-in, Genex, (Bio-Rad, USA).

Statistical analysis of gene expression data was conducted using two-way ANOVAs in GraphPad Prism<sup>®</sup> software (GraphPad Software, USA).  $P < 0.05$  was considered to be significant. All graphs were created using GraphPad Prism with error bars presented as means  $\pm$  SEM.

Table 4-2. Plasmids sourced from Addgene plasmid repository, for use in reprogramming experiments carried out in chapter 4.

Plasmid	Referred to as	Description	Addgene Plasmid Reference	Principle Investigation
<b>TetO-FUW-OSKM</b>	Tet-OSKM	Polycistronic plasmid for Tet-inducible expression of murine <i>POU5F1</i> , <i>SOX2</i> , <i>KLF4</i> and <i>Myc</i> for iPS cell generation	20321	(Carey et al., 2009)
<b>FUW-M2rtTA</b>	M2rtTA	Lentiviral plasmid expressing the reverse tetracycline transactivator for Doxycycline inducible control of expression	20324	(Hockemeyer et al., 2008)
<b>pEP4 E02S EN2L</b>	OSNL	Episomal vector expressing human <i>POU5F1</i> , <i>SOX2</i> , <i>NANOG</i> and <i>LIN28</i> for the production of iPS cells	20922	(Yu, J. et al., 2009)
<b>pEP4 E02S CK2M EN2L</b>	OSKMNL	Episomal vector containing human <i>POU5F1</i> , <i>SOX2</i> , <i>Lin 28</i> , <i>NANOG</i> , <i>KLF4</i> and <i>c-MYC</i> for the production of iPS cells	20924	(Yu, J. et al., 2009)
<b>psPAX2</b>	psPAX2	Empty vector backbone	12260	
<b>pMD2.G</b>	pMD2.G	Mammalian Expression ; Envelope	12259	
<b>PGK-H2BeGFP</b>	eGFP	Constitutive vector encoding for eGFP	21210	(Kita-Matsuo et al., 2009)

Chapter 4: Comparison of Reprogramming Factor Combinations in Producing Bovine iPS Cells

Table 4-3: Primers used in Chapter 4

Gene Construct	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Size (bp)	Reaction Efficiency (%)	Melt Peak (°C)	Specificity
<b>Pluripotency Genes</b>						
<i>POU5F1 (OCT4)</i>	AAGCGGACGAGTATCGAGAA	ACACTCGGACCACGTCTTTC	133	113.8	86	Bovine mRNA
<i>SOX2</i>	ATGAAGGAACACCCGGATTA	CATGTGCGCGTAGCTGTC	186	81.2	89	Bovine
<i>NANOG</i>	GTCCCGGTCAAGAAACAAAA	TCTGGAACCAGGTCTTCACC	163	106.1	87	Bovine mRNA
<i>c-MYC</i>	GGAAGAAATTCGAGCTGCTG	GTCGCAGATGAAGCTCTGGT	226	105.7	87	Bovine mRNA
<b>Lineage Markers</b>						
<i>TUBB3</i>	TGGAGCGCATCAGTGTCTAC	CAGTTGTTACCAGCCCCACT	172	99.3	87.5	Total
<i>NESTIN</i>	AAAGAAGGCTGGGATCCTGT	CCTCAAACCTTCCGACAGC	130	108.9	88	Total
<i>DESMIN</i>	GGGACATCCGTGCTCAGTAT	GTGGCGGTACTCCATCATCT	155	120.8	86.5	Total
<i>FoxA2</i>	ACCACTACGCCTTCAACCAC	GGGGTAGTGATCACCTGTT	134	85.8	86	Total
<b>Exogenous Constructs</b>						
<i>Tet-OSKM</i>	TGAGGCTACAGGGACACCTT	TCAACATCACCTGCTTGCTT	240			Exogenous/ Ectopic
<i>OSNL</i>	TGATTATGGCGGGACACCTG	TAGCCAGGTCCGAGGATCAA	113			Exogenous/ Ectopic
<i>OSKMNL</i>	TTGATCCTCGGACCTGGCTAA	CCACAGAACTCATAACGGCG	124			Exogenous/ Ectopic
<b>Reference Gene</b>						
<i>RPS26</i>	TCATTCGGAACATCGTAGAGG	CCTGACTACCTTGCTGTGAAT				Endogenous/ Reference Gene

## 4.3 Results

The effect of different cocktails of reprogramming factors on the generation of bovine iPS like cells was tested by lentiviral transduction of reprogramming constructs coding for three different combinations of transcription factors, Tet-OSKM, OSNL and OSKMNL into two cell types, bovine embryonic fibroblasts (BEFs) and bovine dermal fibroblasts (BDFs). This design featured two technical replicates for each treatment, with the entire experiment replicated three times.

### 4.3.1 Lentiviral Transduction Results in Gene Expression of Bovine Fibroblasts

Lentiviral cultures were tested for the presence of infective viral particles using Lenti-X™ GoStix™ (Clontech Laboratories, 2014). Lentiviral cultures that had successfully produced lentivirus resulted in the appearance of a positive test line, and a control line on the Lenti-X™ GoStix™ cassette. Only cultures that successfully produced lentivirus were used in reprogramming experiments. Some viral titres did appear low, with only a faint line appearing at the test line position as seen in Figure 4-2. This was still indicative of lentivirus being present, though at low titres, the resulting virus was still used in reprogramming experiments.

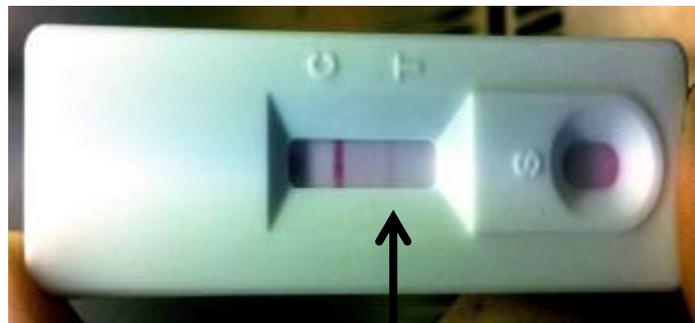


Figure 4-2. Lenti-X™ GoStix™ cassette loaded with lentiviral supernatant from OSNL lentiviral cultures. A faint line at the 'test position' indicates the presence of lentivirus.

To determine if lentiviral transduction resulted in gene expression of the transduced plasmid, a control GFP plasmid was lentivirally transduced into bovine fibroblasts. GFP expression was seen in transduced fibroblasts 3 days after transduction with the lentiviral preparations as shown in Figure 4-3 with the transfection efficiency for the GFP plasmid approximately 65%. Although an indirect measure of transfection efficiency, the expression of GFP following lentiviral transduction provided

evidence that the plasmids coding for reprogramming factors would also be introduced and expressed by fibroblast cells.

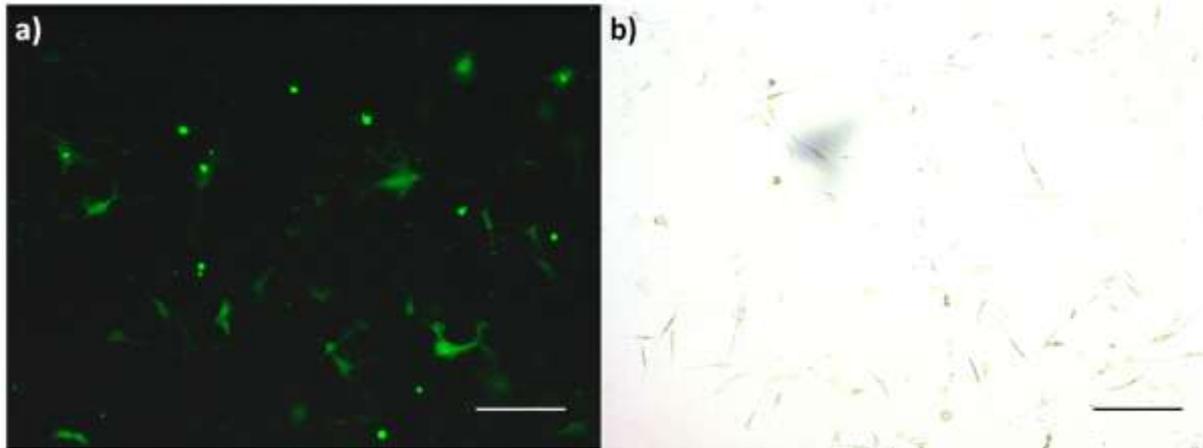


Figure 4-3. Expression of GFP in bovine fibroblast cells lentivirally transduced with eGFP plasmid (Addgene # 21210). a) fluorescent image for eGFP activity. b) Brightfield image of same field of cells. Scale bars represent 50  $\mu\text{m}$ .

#### **4.3.2 Different Combinations of Transcription Factors have no Effect on Colony Formation Kinetics but do have an Effect on Reprogramming Efficiency**

There was no significant effect of the combination of transcription factors on the time to colony formation. Additionally, there was no difference in the time to colony formation observed between the different cell types BEFs and BDFs. The average time to colony formation was approximately 12 days (Figure 4-4). No colonies were observed in the control cultures during 21 days of culture.

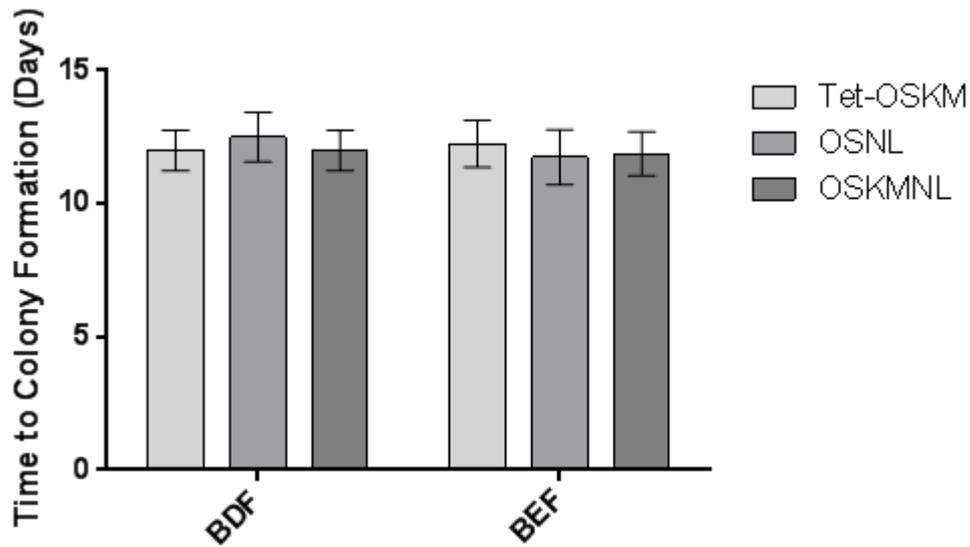


Figure 4-4: Time to colony formation (days) of putative bovine iPS like colonies produced from lentiviral infection of Bovine Embryonic Fibroblasts (BEFs) or Bovine Dermal Fibroblasts (BDFs) with reprogramming constructs coding for different combinations of transcription factors, Tet-OSKM, OSNL or OSKMNL. There were no differences in time to colony formation observed between treatments ( $P > 0.05$ ,  $n=6$ ). Error bars represent  $\pm$  SEM.

There were no statistically significant differences observed in the number of colonies observed per 10 cm dish after 21 days of culture. However, while not statistically significant, the pattern of the number of colonies produced was similar in both BDF and BEF transduced cultures, with OSNL transduction resulting in slightly higher numbers ( $P > 0.05$ ) than cultures transduced with OSKMNL or the Tet-OSKM combination of transcription factors (Figure 4-5). An average of 4.5 putative iPS colonies was observed per  $5 \times 10^4$  transduced cells cultured, giving a reprogramming efficiency of 0.009%.

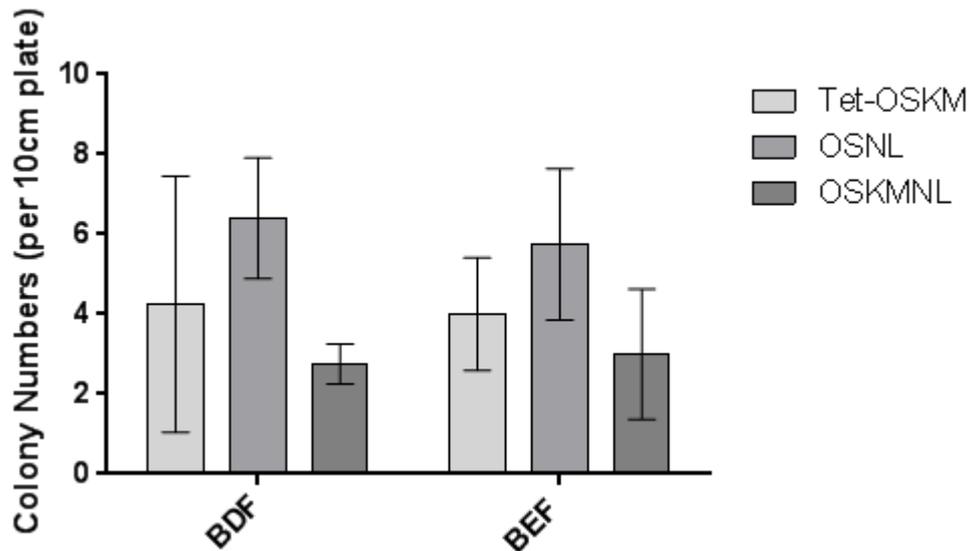
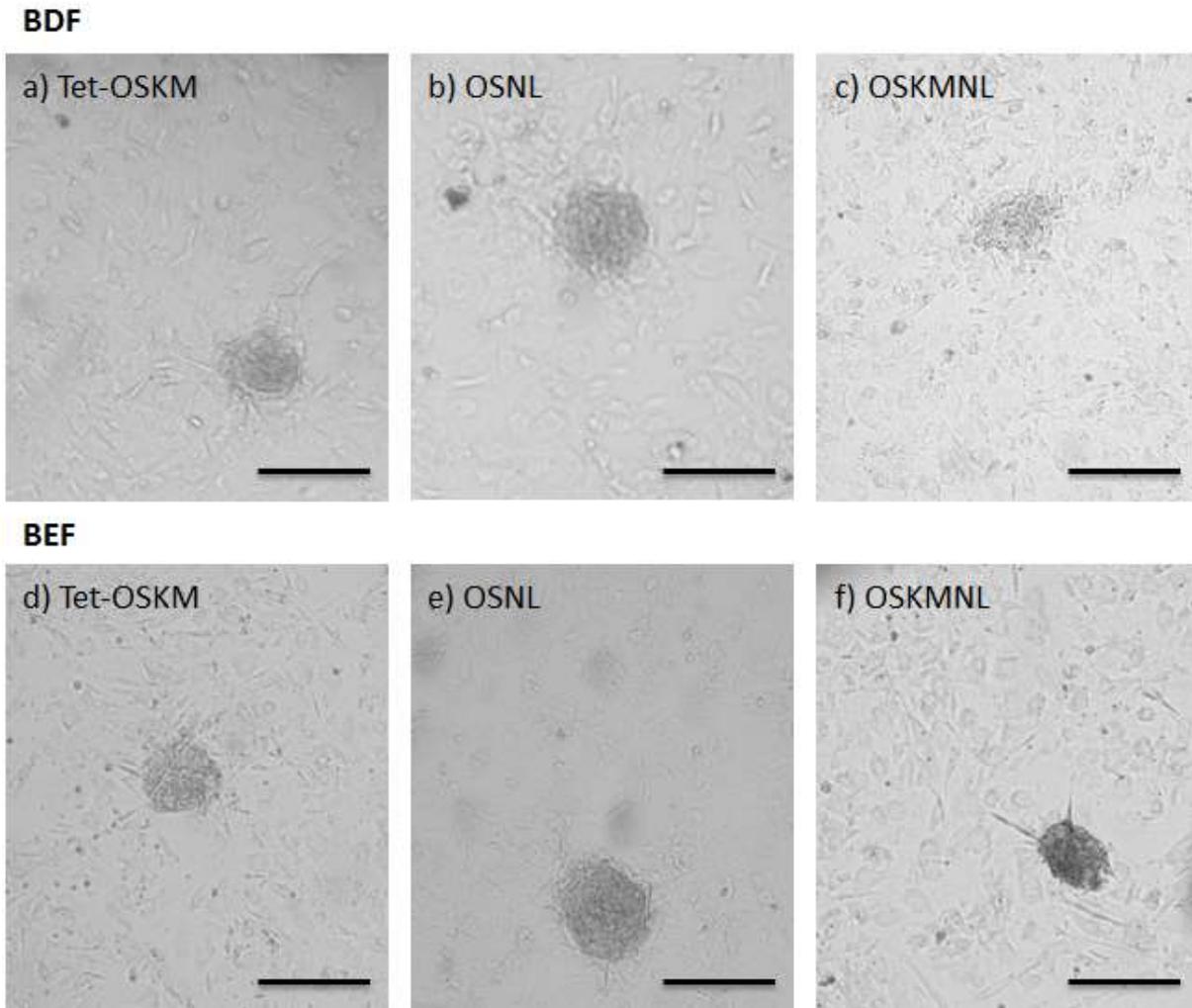


Figure 4-5: The average number of putative bovine iPS colonies observed per 10cm dish after 3 weeks of culture, for BDF or BEFs cultures lentivirally transduced with reprogramming constructs coding for different combinations of transcription factors, Tet-OSKM, OSNL or OSKMNL. There were no differences in the number of colonies observed after 21 days of culture between treatments ( $P > 0.05$ ,  $n = 6$ ). Error bars represent as  $\pm$  SEM.

#### 4.3.3. Reprogramming with a Different Combination of Transcription Factors has an Effect on Colony Morphology

Colonies displaying ES cell like morphology were derived from each treatment regardless of fibroblast type or reprogramming factor combination (Figure 4-6). Acceptable putative iPS colonies were morphologically classified as having tightly packed round cells with clearly defined colony borders. In contrast, colonies with poor morphology were irregularly shaped, had heterogeneous cell appearance and did not have clearly defined borders. The colony morphology of different treatments did appear to differ slightly between treatments, and also differed to a lesser degree within treatments. Colonies derived from Tet-OSKM and OSNL treatments (Figure 4-6 a, b, d, e) generally had better colony morphology than those derived from the OSKMNL combination of transcription factors (Figure 4-6 c, f). All colonies were relatively small and grew very slowly, when passaging was attempted the colonies ceased to expand; instead some appeared to differentiate, while others remained in a senescent like state. In addition, no colonies stained positively for alkaline phosphatase using the Alkaline Phosphatase Live Staining kit (Life Technologies, USA).



**Figure 4-6: Putative bovine iPS colonies representative of different treatments. a) BDF Tet-OSKM, b) BDF OSNL, c) BDF OSKMNL, d) BEF Tet-OSKM, e) BEF OSNL, f) BEF OSKMNL. Scale bars represent 100  $\mu$ m. N.B. No colonies were observed in control cultures during the 21 days of culture.**

PCR was performed on genomic DNA from transfected BEFs and BDFs with transgene specific primers to test integration of the transgenes from the Tet-OSKM, OSNL and OSKMNL vectors. Bands of the correct size were present in the DNA samples from all tested colonies, indicating integration of all transgenes into the host BEF and BDF genomes (Figure 4-7).



Figure 4-7: PCR for verification of integration of transgene from Tet-OSKM, OSNL or OSKMNL reprogramming constructs into host genome. PCR was conducted on genomic DNA from pooled colonies of BDFs and BEFs transfected with different constructs. +ve indicates positive plasmid control, -ve indicates genomic DNA from uninfected BEFs.

#### 4.3.4 Different Combinations of Transcription Factors have Cell-Type Dependent Effects on Pluripotency Gene Induction

The expression of pluripotency markers by putative bovine iPS colonies obtained from lentiviral infection of the three different combinations of transcription factors, Tet-OSKM, OSNL and OSKMNL was analysed by qRT-PCR (Figure 4-8a). For colonies derived from BDFs, *POU5F1* expression was highest in OSNL transduced colonies, followed by Tet-OSKM and then OSKMNL colonies (Figure 4-8a). *SOX2*, *c-MYC* and *NANOG* were only expressed in OSNL transduced colonies, other colonies did not express levels of *SOX2*, *c-MYC* or *NANOG* different to un-transfected BDFs (Figure 4-8b,c,d).

For colonies derived from BEFs, *POU5F1* expression was highest in OSNL transduced colonies followed by OSKMNL colonies (Figure 4-8a). Tet-OSKM colonies did not express *POU5F1* levels different from un-transfected BEFs. *SOX2* expression was also highest in OSNL transduced colonies, followed by OSKMNL colonies, while Tet-OSKM colonies did not express *SOX2* at levels statistically higher than un-transfected BEFs. *c-MYC* and *NANOG* were expressed at similar levels in OSNL and

OSKMNL transduced colonies, while Tet-OSKM colonies did not express *c-MYC* or *NANOG* at levels higher than un-transfected BEFs (Figure 4-8). BDF OSNL transduced colonies, expressed the highest levels of *POU5F1*, *SOX2* and *c-MYC* of all treatments, while BEF OSNL colonies expressed the highest levels of *NANOG* (Figure 4-8).

*POU5F1* and *SOX2* were present in all reprogramming constructs used for in this experiment; however, their levels of expression were different in different treatments. Furthermore, both *POU5F1* and *SOX2* expression appear to follow the same pattern of expression, with the highest expression in OSNL derived colonies, followed by BEF OSKMNL colonies with low or no expression in other cultures. *c-MYC* was only present in Tet-OSKM and OSKMNL reprogramming constructs, however, its expression was highest in OSNL transduced cultures and BEF OSKMNL cultures. *NANOG* expression was induced at higher levels when the transgene was present in the reprogramming construct.

Additionally the expression of all pluripotency markers appeared to be dependent on cell type, with BEF derived colonies expressing higher levels of *POU5F1* and *SOX2* than BDFs when transduced with OSNL or OSKMNL. BEFs also expressed higher levels of *c-MYC* than BDFs transduced with the OSKMNL reprogramming construct. Conversely, *NANOG* expression was higher in BDFs transduced with OSNL compared to BEFs transduced with the same construct, while BEFs transduced with the OSKMNL construct expressed higher *NANOG* levels than BDF OSKMNL transduced colonies.

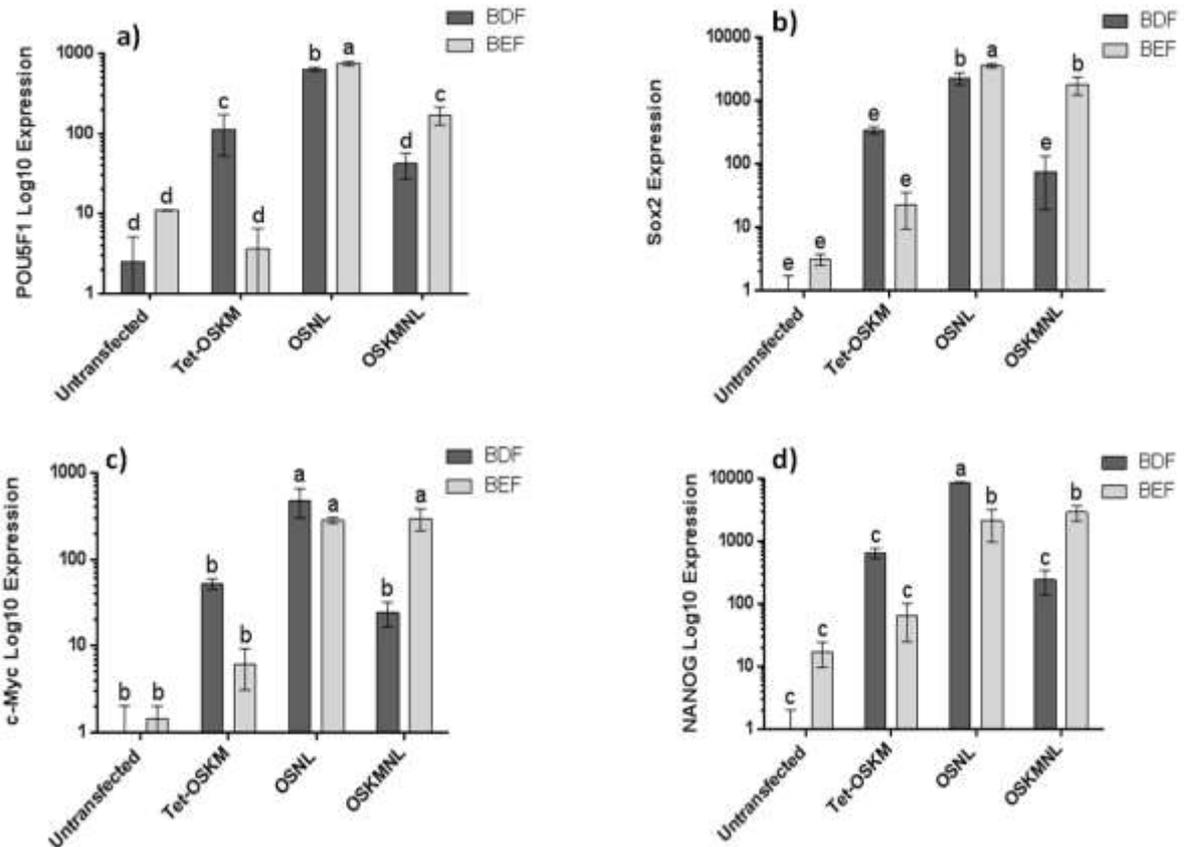


Figure 4-8. Log10 Expression of pluripotency markers relative to housekeeper gene *RPS26*, in putative bovine iPS colonies, produced by lentiviral transduction of reprogramming constructs coding for Tet-OSKM, OSNL or OSKMNL. (a) *POU5F1* (*OCT4*), (b) *SOX2*, (c) *c-MYC* and (d) *NANOG*. Different characters above treatment means indicate a significant difference between expression of pluripotency markers ( $P < 0.05$   $n=3$ ). Error bars represent as  $\pm$  SEM.

## 4.4 Discussion

Since the first reports of the successful production of induced pluripotent stem cells in 2006 (Takahashi & Yamanaka, 2006), there has been an interest in producing iPS cells from livestock species for use in transgenic breeding and disease models. However, the production of livestock iPS cells, particularly bovine iPS cells, has proven difficult to date. There have been very few successful accounts of generating bovine iPS cells, additionally, no robust or reproducible protocol has been established for bovine iPS cell derivation (Cao et al., 2012; Han et al., 2011; Sumer, Liu, Malaver-Ortega, et al., 2011).

Previous studies into the derivation of bovine iPS cells have primarily used the canonical reprogramming factors *OCT4*, *SOX2*, *KLF4* and *c-MYC* (*OSKM*) in polycistronic vectors (Huang, B. et

al., 2011) or as single factors introduced into fibroblast cells (Cao et al., 2012; Han et al., 2011; Sumer, Liu, Malaver-Ortega, et al., 2011). There is little known about the effects of different combinations of reprogramming factor cocktails in the production of bovine iPS cells, with the exception of the report that bovine iPS cells require the addition of exogenous NANOG in addition to exogenous OCT4, SOX2, KLF4 and c-MYC constructs to be reprogrammed (Sumer, Liu, Malaver-Ortega, et al., 2011). The results presented in this chapter show that the use of three different polycistronic vectors containing different cocktails of reprogramming factors can be used, with varying success, to elicit a reprogramming event that resulted in partially reprogrammed bovine iPS cells.

After completion of the experimental work on the thesis, a conceptual problem was discovered for some of the viruses used in this chapter. Two plasmids used for lentiviral production had been designed for episomal transfection, and not for lentiviral packaging but this had at first not been detected (Addgene Plasmid OSNL #20922 and OSKMNL #20924 (Yu, J. et al., 2009)). These plasmids do not have a lentiviral backbone containing *cis*-elements including LTR or *Psi* ( $\Psi$ ) sequences (Yu, J. et al., 2009) that are used for the encapsidation, reverse transcription and integration of the target sequence into the host genome (Tolmachov, Tolmachova, & Al-Allaf, 2011). However, numerous points of evidence have been presented in this chapter that suggest that lentivirus had been produced, although at a lower concentration than virus produced by lentiviral plasmids. Viral production had been confirmed by a non-quantitative dipstick test (pg. 124). BEF and BDF cell control cultures that were not infected with lentivirus showed no colony formation under the described growth conditions, indicating that the observed colonies in viral treatment groups were not unspecific cell aggregates as can appear in overgrown culture conditions. Furthermore, qRT-PCR analysis of the colonies confirmed that the expression of pluripotency markers *POU5F1*, *SOX2*, *c-MYC* and *NANOG* from OSNL and OSKMNL cultures was higher than Tet-OSKM cultures which were produced using canonical lentiviral plasmids. The primers used in this thesis were designed to be specific for mRNA by the inclusion of an intron (McMillan, 2012). qRT-PCR analysis of control fibroblasts showed that they did not express pluripotency markers at a significant level, indicating that the primers used for qRT-PCR analysis did not amplify genomic DNA, and that the expression of these markers was instead a result of the introduction of reprogramming factors. The combined evidence suggests that although the mechanism remains unclear at current, infective lentiviral particles had been produced from the plasmids used in this chapter.

A previous study conducted into the function of *cis*-regulatory elements in lentiviral plasmids showed that the deletion of large parts of genes including *gag*, believed to be responsible for packaging signals, did not reduce the production of lentiviral titres (Cui, Iwakuma, & Chang, 1999). Another study demonstrated that the encapsidation of the Epstein Barr virus, from which the OSNL and OSKMNL plasmids were derived, requires just one terminal repeat which does not have to be present as a *cis*-acting element for viral particles to be packaged (Zimmermann & Hammerschmidt, 1995). In fact, studies have demonstrated that viral sequences from the original Epstein Barr Virus are still present in derived vector (Cohen, 2000). It may be possible that genes, or parts of gene sequences from the Epstein Barr virus from which the OSNL and OSKMNL plasmids are derived, may still be present in the plasmid backbones, resulting in the ability of these plasmids to still be packed to produce a viral particle.

Alternatively, it may also be possible that the episomal plasmids had entered the host cells through residual Lipofectamine from the lentiviral production process that remained in the lentiviral collection media that was used to infect the host cells. Lipofection acts by cationic lipids forming complexes with DNA. This complex protects the DNA from nucleases and allows it to enter the cell (Kotzamanis, Abdulrazzak, Kotsinas, & Gorgoulis, 2011). Though the media containing the original Lipofectamine solution used to produce lentivirus in these experiments was removed and changed to collection media, the cells were not washed to prevent dislodging of the HEK293 cells, so it could be possible that some Lipofectamine remained and resulted in the lipofection of the episomal plasmids into the cells.

However, PCR analysis of genomic DNA from the colonies produced in these experiments indicated the presence of Tet-OSKM construct in the genomic DNA, and this was also the case for, OSNL and OSKMNL constructs. It is possible that as the OSNL and OSKMNL plasmids do not contain the LTR sequence necessary for integration into the host genome (Tolmachov et al., 2011), the PCR may instead be picking up residual plasmid DNA in the culture supernatant as described above. Episomal plasmid DNA is lost from the host cell by dilution as a result of cell division. However, studies have shown that episomal vectors are lost at a rate of 2-8% per cell division (Scilimenti & Calos, 1998). As the cell division and colony growth described in this chapter appear very slow, it cannot be ruled out at present that episomal plasmids remained in the cytoplasm of the cell, and when genomic DNA

was isolated for analysis, plasmid DNA may have also been isolated, resulting in the identification of the exogenous DNA in the host cell.

While the exact mechanisms of vector transmission remains unclear at present, and would have to be determined in further experiments, there remains little doubt that reprogramming factors were introduced into the fibroblasts, and that that the treatment led to colony formation and changes in pluripotency marker gene expression. In future work, it will be important to establish successful virus production and construct activation.

In the experiments described in this chapter, the use of the eGFP control was not used for a direct comparison of transfection efficiency between the control and the reprogramming lentiviral preparations. Instead it was used as an indirect control to determine if lentiviral transfection had been successful. As all lentiviruses produced in this chapter were tested with Lenti-X™ GoStix™ to confirm the presence of infective lentiviral particles, it was assumed if the infection of BEF and BDF cells with the eGFP lentivirus was successful the lentiviral preparations used for reprogramming would also be successfully transfected into the host cells as these transfections were conducted in parallel. In fact it is likely that the transfection efficiency of the eGFP lentivirus was not representative of the OSNL or OSKMNL lentiviruses due to the low level of virus produced by the transfection of these episomal plasmids and the inherent problems associated with them, as discussed above. At the time these experiments were conducted, target plasmids that contained a reporter gene for the direct analysis of transfection efficiency were not available for our use. Additionally, due to the small number of colonies produced in these experiments, staining for antibodies for the exogenous reprogramming plasmids was not considered as important as qRT-PCR analysis of pluripotency markers, and so colonies were all used for this purpose instead. Additionally, as these experiments were considered preliminary, determining the transfection efficiency of viruses was not considered as important as determining if reprogramming of the target cells was possible, and if so, if there was a difference in the total reprogramming efficiencies of using different combinations of reprogramming factors. If these experiments were to be explored further the use of lentiviral target plasmids with a reporter gene, or staining with antibodies for the exogenous transgenes could be used to determine transfection efficiency.

Both, BEFs and BDFs transfected with the three different constructs, Tet-OSKM, OSNL and OSKMNL, were able to produce colonies that displayed morphology different to original fibroblast cell type, and instead resembled embryonic stem (ES) cells. There was no significant difference in the time to colony formation of BEF or BDFs transfected with the different constructs suggesting that the use of different reprogramming factor cocktails does not influence reprogramming kinetics. Additionally there was no significant difference in the number of colonies observed in any treatment. However, while insignificant, OSNL cultures did produce slightly higher colony numbers than Tet-OSKM or OSKMNL transduced colonies in both BDF and BEF cultures. These results are preliminary and only six replicates were used, with further data obtained, the OSNL vector may be found to be superior in producing bovine iPS cells.

The colonies described in this chapter are not considered to be fully reprogrammed as they did not exhibit staining for alkaline phosphatase (AP) activity. However, it is possible that these colonies did express AP activity at one stage, but not at the time these colonies were stained. Future experiments could include conducting a time course for GFP expression of these colonies to determine when or if they express AP at different stages of reprogramming. Alkaline phosphatase activity is high in pluripotent stem cells and as such is a common test for pluripotency. However, the test is not considered to be highly stringent, as other non-pluripotent cell types can also express alkaline phosphatase activity (Stadtfeld & Hochedlinger, 2010). Alkaline phosphatase is involved in the phosphorylation of proteins necessary for pluripotency (Wang, Y.-C., Peterson, & Loring, 2014). There are some suggestions that protein phosphorylation acts directly on transcription factors OCT4, SOX2, KLF4 and Myc and may act to regulate pluripotency (Cai, N., Li, Qu, Liu, & Izpisua Belmonte, 2012). Alkaline phosphatase is found in high quantities in the cell membrane of pluripotent cells and is therefore used as a marker for pluripotency (Wang, Y.-C. et al., 2014). The lack of alkaline phosphatase staining in the putative bovine iPS colonies described here indicates incomplete reprogramming. Ideally, a line of embryonic stem cells would have been used as a positive control to confirm alkaline phosphatase activity was identified by the staining kit in parallel with all experiments. . However previous experiments as well as experiments outlined later in this thesis in chapters 6 and 7 detected alkaline phosphatase activity using the same kit. . As the putative iPS colonies produced in the experiments described in this chapter did not exhibit alkaline phosphatase activity, it was assumed that staining for cell surface antigen markers associated with pluripotency such as Tra-1-60 or SSEA4 would unlikely be positive. Staining for OCT4, SOX2 and KLF4 was not conducted in this line of investigation due to limited material; preference was instead given to collection of colonies for qRT-PCR analysis.

The putative iPS colonies produced from each treatment could not be successfully passaged. When manually picked and transferred to organ culture dishes seeded with Mit-C BEFS as feeders, the cells ceased to grow. Some colonies completely differentiated back to what resembled fibroblast cells and no longer resembled the round tightly packed cell morphology of ES-like cells. These results suggest that the cells were not fully reprogrammed and were instead in a partially reprogrammed state. Additionally, due to shortage of material, the formation of embryoid bodies (EBs) was not conducted in these experiments. However, as the colonies obtained in this experiment were clearly not fully reprogrammed; the differentiation of EBs into cell types from all three germ lineages was considered to be unlikely.

qRT-PCR analysis was conducted for expression of pluripotency markers using the housekeeper gene *RPS26*. This housekeeper was originally identified in previous work using the sheep GeNorm kit (PrimerDesign, UK) and was identified as the best of those tested. Previous studies have found *RPS26* to be an effective and reliable reference gene, and hence, no other housekeepers were used.

The primers for *POU5F1*, *SOX2*, *c-MYC* and *NANOG* used in this chapter were designed to be specific for bovine mRNA. BLASTs for the designed primers against genomic bovine DNA and the exogenous human and mouse constructs showed that all primers except the *SOX2* primer pairs did not amplify bovine genomic DNA, and none of the primer sets amplified human or mouse sequences (McMillan, 2012). Additionally, mRNA extraction from the putative bovine iPS colonies described in this chapter was conducted using the Dynabeads® mRNA DIRECT™ Micro Kit (Life Technologies, USA). Though the Dynabeads® kit is designed to only isolate mRNA, however, a very small possibility for genomic DNA contamination may still be possible (Life Technologies Inc, 2014). This was not considered to be a problem in the context of these experiments as un-transfected fibroblasts did not express pluripotency markers to a significant degree, suggesting that the expression of pluripotency markers by putative iPS colonies, as shown by qRT-PCR, was from mRNA and not from genomic DNA.

When colonies were analysed by qRT-PCR, only BDF or BEF OSNL and BEF OSKMNL transduced colonies expressed pluripotency markers *POU5F1*, *SOX2*, *c-MYC* and *NANOG*. In Tet-OSKM derived colonies, only BDF cultures expressed *POU5F1* at a higher level than un-transfected BDFs, furthermore, this was at a lower level than OSNL transfected colonies.

The first reports claiming to have produced bovine iPS cells suggested that NANOG was essential for the production of fully reprogrammed iPS cells (Sumer, Liu, Malaver-Ortega, et al., 2011). NANOG is a key factor in establishing pluripotency, and is frequently used to identify reprogrammed cells. In addition to the report by Sumer, Liu, Malaver-Ortega, et al. (2011) that proposed the importance of NANOG to bovine iPS cell generation, many other reports have been made reports claiming that the addition of NANOG aids in the reprogramming process (Hanna et al., 2009; Okita et al., 2007; Theunissen, Thorold et al., 2011). In contrast reports of bovine iPS cell generation without the addition of NANOG have also been made (Cao et al., 2012; Han et al., 2011). It was hypothesised that the addition of NANOG in reprogramming cocktails OSNL and OSKMNL would result in enhanced reprogramming compared to putative iPS cells produced by transduction of the Tet-OSKM construct.

The transduction of reprogramming constructs containing NANOG resulted in the increased expression of all pluripotency markers, including *NANOG*, with little to no expression of pluripotency markers seen in colonies transduced with Tet-OSKM. The expression of endogenous *NANOG* in the bovine iPS colonies transduced with reprogramming constructs containing NANOG, indicates that the addition of exogenous NANOG may be required to enhance reprogramming, with endogenous expression by these cells indicating that at least partial reprogramming has occurred. This is consistent with the claim by Sumer, Liu, Malaver-Ortega, et al. (2011), that NANOG is important for the reprogramming of bovine iPS cells. It has to be noted that in the experiments described in this chapter, cells only underwent partial reprogramming. The reprogramming conditions including media and supplements used in the experiments of this chapter differs from that used in the study that reported the addition of NANOG resulted in fully reprogrammed bovine iPS cells (Sumer, Liu, Malaver-Ortega, et al., 2011). The culture conditions described in the Sumer, Liu, Malaver-Ortega, et al. (2011) study did not successfully result in any reprogramming event in our hands (Data not shown). This provides evidence that NANOG may be essential for the reprogramming of bovine cells under certain circumstances. It should be highlighted that although production of bovine iPS cells have been reported, no further reports have been made reproducing these results. As none of the colonies derived from these experiments resulted in fully reprogrammed iPS cells, further studies into the use of these constructs in different conditions may give clearer results as to which combinations of reprogramming factors are most effective at producing bovine iPS cells.

Partially reprogrammed cells have been reported in numerous studies. However, currently it is unclear why these cells fail to undergo complete reprogramming. The reprogramming process is complex and as a result there are numerous aspects of the protocol that may result in the production of partially reprogrammed cells. Target cell type plays an important role in the success of reprogramming, with the differentiation status of the cells of particular importance. Different cell types including adult stem cells and different sub-populations of fibroblasts have been shown to be more amenable to reprogramming than others (Aasen et al., 2008; Byrne et al., 2009; Eminli et al., 2009; Niibe et al., 2011). These cell types are believed to be less differentiated than other cells and therefore easier to reprogram to a pluripotent state. Due to the ease of collection and culture of fibroblasts, these cells are commonly used to produce iPS cells in most species. However, there are differences in the differentiation potential types of fibroblasts used in reprogramming experiments. In this experiment there were no conclusive differences observed between the quality or efficiency of deriving iPS cells derived from adult (BDF) or embryonic (BEF) fibroblast. Adult bovine fibroblasts have been reprogrammed in previous studies (Huang, B. et al., 2011; Sumer, Liu, Malaver-Ortega, et al., 2011), however in the study by Huang, B. et al. (2011), embryonic fibroblasts were found to produce the highest number of colonies compared to foetal and adult fibroblasts.

The choice of vector can also significantly influence the reprogramming efficiency of iPS cells. In this chapter polycistronic lentiviral vectors were used to deliver different cocktails of reprogramming factors. Polycistronic vectors have successfully been used to reprogram human and mouse cells to pluripotency (Carey et al., 2009; Shao et al., 2009). A previous study reporting the generation of bovine iPS cells used a polycistronic vector in a non-viral reprogramming protocol (Huang, B. et al., 2011). Polycistronic vectors have the benefit of simplifying the reprogramming process by only requiring the addition of construct to cells to introduce all reprogramming factors, additionally the number of integration sites in the host genome is also limited (Stadtfield & Hochedlinger, 2010). Conversely, polycistronic vectors do result in significantly lower reprogramming efficiencies than the introduction of viral vectors for each reprogramming factor. The addition of factors as single vectors allows the stoichiometry of reprogramming factor expression to be altered, thereby improving reprogramming efficiency (Carey et al., 2011; Okita et al., 2008). The levels of reprogramming factors expressed as proteins may affect the reprogramming process if they are not in the correct ratios. However, the optimal expression of the different factors in different species and cell types has not been well documented (Carey et al., 2011). Due to a limited amount of material available no protein level analysis was carried out in these experiments.

There are also molecular aspects of cellular reprogramming that can influence its efficiency. DNA hyper-methylation can inhibit the reprogramming process by silencing the promoters of pluripotency genes (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2008). As a result transcription factor binding can be inhibited, thereby hampering reprogramming of somatic cells. For successful transcription factor binding it is necessary for pluripotency gene promoters to be demethylated (Sridharan et al., 2009). The structure of chromatin and histone acetylation is important also for the generation of iPS cells (Azuara et al., 2006; Bernstein et al., 2006). Histone deacetylation inhibitors, such as valproic acid and sodium butyrate have been implemented in the production of iPS cells to improve reprogramming efficiency (Huangfu et al., 2008; Liang et al., 2010; Mali et al., 2010).

Furthermore, the origin of transcription factors may also impact the efficiency of reprogramming of bovine cells. The transcription factors making up the Tet-OSKM construct described in this chapter were murine, while the OSNL and OSKMNL constructs were human sequences. The use of human or mouse factors to reprogram cells from different species has been well documented and is possible due to the high conservation of genes and their products between species. However, these factors do differ slightly between species, with human and bovine OCT4, SOX2, KLF4, c-MYC and NANOG proteins having 95%, 99%, 92%, 94% and 75% sequence similarities respectively (NCBI, 2014). Previous studies claiming to have produced bovine iPS cells have used both bovine origin transcription factors (Han et al., 2011; Huang, B. et al., 2011) and human transcription factors (Sumer, Liu, Malaver-Ortega, et al., 2011). Murine factors have been used to successfully reprogram cells from other livestock species including pigs (Ezashi et al., 2009) and sheep (Liu, J. et al., 2012), but have yet to be reported for reprogramming bovine cells. Though the majority of these factors are highly conserved it is possible that the resulting transcription factors may have a slightly different protein structures, and therefore have a lower binding affinity for the endogenous gene promoters, resulting in less efficient reprogramming. Though the conservation of NANOG between bovine and human sequences is lower than the other transcription factors, studies have shown that NANOG orthologs from different species are able to effectively reprogram somatic cells to pluripotency. This study demonstrated that avian NANOG was sufficient to establish pluripotency in mouse somatic cells, despite sharing only 13% sequence similarity with the endogenous protein (Theunissen, T et al., 2011). In the experiments described in this chapter the use of bovine transcription factor sequences for reprogramming may have resulted in the improved reprogramming, however, bovine transcription factors were not readily available for use, due to the time constraints of a PhD project it was decided that cloning bovine sequence constructs would not

be pursued. Additionally, the use of genetic constructs from the same species can result in difficulties differentiating between endogenous and transgene gene expression. Additionally, reprogramming constructs containing bovine factors are not easily accessible and can be difficult to produce. As previous studies have successfully produced iPS cells in livestock species using either mouse or human transcription factors it was decided to use a combination of these factors for the experiments of this thesis. Future reprogramming experiments could be improved by testing the efficiency of reprogramming by using bovine transcription factors to reprogram bovine somatic cells to pluripotency.

It is possible that the putative iPS cells described in this study did not undergo complete reprogramming due to one or more of these aspects of the reprogramming process. Further studies are needed to determine the causes of these partially reprogrammed cells. Although fully reprogrammed iPS cells were not produced as a part of this study, the partially reprogrammed cells derived offer a solid ground work for further study into the production of bovine iPS cells. There are two main areas of interest this current area of study will continue to address, the comparison of viral and non-viral reprogramming to produce bovine iPS cells, and the effect media conditions and small molecules on reprogramming.

The use of non-viral iPS generation is of importance for the potential use in clinical applications of the derived iPS cells. This is also important in the production of livestock iPS cells, especially for their potential use in transgenic and artificial breeding applications. Although the iPS media used in this study contains small molecules to enhance reprogramming efficiency by decreasing the time to colony formation (McMillan, 2012), other molecular pathways may be targeted to further increase the reprogramming efficiency. The use of the correct cocktail of small molecules may result in the successful production of bovine iPS cells by overcoming some of the barriers encountered in the reprogramming process described here.

## 4.5 Conclusion

At the current time there has been little success in the reproducible generation of fully reprogrammed bovine iPS cells. This chapter has demonstrated the production of partially reprogrammed bovine iPS cells by the lentiviral transduction of different reprogramming factor cocktails, Tet-OSKM, OSNL and OSKMNL, in an attempt to determine if one is more efficient at reprogramming bovine cells than another. Though the plasmids carrying OSNL and OSKMNL constructs were not of lentiviral origin, evidence has been presented in this chapter that infective lentiviral particles were produced using these episomal plasmids, and additionally, their delivery into the host fibroblast cells resulted in at least a partial reprogramming event. Results showed that all three constructs were capable of producing putative iPS colonies with ES cell like morphology, but only colonies derived using reprogramming constructs containing NANOG, OSNL and OSKMNL, expressed increased levels of pluripotency markers *POU5F1*, *SOX2*, *c-MYC* and *NANOG*. However, these cells could not be expanded in culture following passage, and did not stain positively for alkaline phosphatase activity. It was therefore concluded that the putative bovine iPS colonies produced in the experiments described in this chapter were only partially reprogrammed. Further study is needed to overcome problems associated with the reprogramming process, to attempt to create fully reprogrammed bovine iPS cells. The low efficiency by which even fully reprogrammed mouse and human iPS cells can be produced suggests that rare events are necessary to generate iPS cells. Different methods of reprogramming including viral and non-viral or non-integrating methods and the use of other small molecule combinations may improve reprogramming of bovine iPS cells.

## Chapter 5 : Comparison of Viral and Non-Viral reprogramming of Bovine Fibroblasts to Pluripotency

### 5.1 Introduction

The pioneering work conducted by Takahashi and Yamanaka (2006) to produce the first induced pluripotent stem cells (iPS cells), has since resulted in a vast amount of work into producing iPS cells from different species. The first iPS cells were generated from mouse embryonic fibroblasts using retroviral vectors coding for transcription factors OCT4 (*POU5F1*), SOX2, KLF4 and c-MYC (OSKM) (Takahashi & Yamanaka, 2006). iPS cells derived from human somatic cells were established shortly after, using lentiviral vectors with a both a slightly different combination of reprogramming factors OCT4 (*POU5F1*), SOX2, NANOG and LIN28 (OSNL) (Yu, Junying et al., 2007), and the original OSKM combination (Kazutoshi et al., 2007). One of the potential applications of iPS cells is their possible use in regenerative medicine, while in livestock their main potential lies in applications in transgenic breeding and artificial reproductive technologies. The use of iPS cells in these areas of application is complicated by the way in which traditional iPS cells are produced. The use of viral, integrative vectors results in permanent integrations of exogenous DNA into the host genome. Integrative vectors cause random insertional mutations, potentially causing gene disruption and subsequent tumorigenesis when cells are transplanted into the body (Kane, McRae, Denning, & Baker, 2008; Shevchenko, Medvedev, Mazurok, & Zakian, 2009). Hence iPS cells established through viral vectors are unable to be used in clinical applications. Consequently, to improve the suitability of iPS cells for use in regenerative medicine or in transgenic or artificial breeding, there is great interest in production of iPS cells free of genomic integration. Non- integrative and non-viral methods of cellular reprogramming have been used for the production of iPS cells from various species and cell types (Kane et al., 2008; Zhou, Y.-y. & Zeng, 2013). Each method of delivering reprogramming factors has advantages and disadvantages, which will be discussed further.

There are three viral methods of deriving iPS cells, retroviral vectors, used by Takahashi and Yamanaka (2006) in their preliminary experiments; lentiviral vectors, used for generating the first human iPS cells (Yu, Junying et al., 2007), and non-integrating viral vectors including adenoviruses (Zhou, W. & Freed, 2009). As stated above, the original work to establish iPS cells by Takahashi and

Yamanaka (2006) used retroviral vectors to introduce the four reprogramming factors OCT4 (*POU5F1*), SOX2, KLF4, c-MYC (OSKM) to murine fibroblasts. These initial retroviral vectors were derived from Molony murine leukemia viruses (MMLV), such as PMXs, and allowed the delivery and integration of transcription factors into the host genome of dividing cells (González et al., 2011; Takahashi, Okita, Nakagawa, & Yamanaka, 2007; Takahashi & Yamanaka, 2006).

The work conducted by Yu, Junying et al. (2007) to produce human iPS cells, used a lentiviral vector derived from the HIV virus to introduce the transcription factors OCT4 (*POU5F1*), SOX2, NANOG and LIN28 (OSNL). The group used a different combination of reprogramming factors to induce pluripotency due to the observation that the use of c-MYC, an oncogene, causes death and differentiation of human embryonic stem (ES) cells. Additionally, lentiviral vectors generally result in higher infection rates than retroviral vectors and, importantly, they are capable of infecting both dividing and non-dividing cells (O'Doherty et al., 2013). Moreover, the resulting reprogramming efficiencies of lentivirally derived iPS cells are comparable to retroviral vectors (Kane et al., 2008; Kazutoshi et al., 2007; Takahashi & Yamanaka, 2006; Yu, Junying et al., 2007). However, lentiviruses and the transgenes they carry are less effectively repressed in the host genome following reprogramming than retroviruses (Kane et al., 2008; Okita et al., 2007). Conversely, lentiviral vectors offer the option of inducible expression systems which are not available for retroviral vectors (Blelloch et al., 2007; Stadtfeld et al., 2008). As one of the important criteria of producing fully reprogrammed iPS cells is the complete silencing of transgene expression once cells are reprogrammed, lentiviral drug inducible systems are commonly used to control transcription. The Tet-inducible system, used most commonly, is controlled by doxycycline (DOX), with the addition of DOX turning on transgene transcription, while the absence halts transgene transcription. This method allows the transgenes to effectively be switched on and off, reducing the risk of continued transgene expression following complete reprogramming of cells. This method also allows for the selection of only fully reprogrammed cells. By removing DOX from culture and therefore switching off transgene expression, only cells with endogenous pluripotent gene expression will remain pluripotent (Stadtfeld et al., 2008).

Although the use of viral vectors results in relatively high reprogramming efficiencies, the integration of exogenous transcription factors into the host's genome increases the potential for insertional mutations, which have been shown to result in formation of tumours in chimeric animals (Okita et al., 2007). Even when transgene silencing occurs, there is still the potential for the transgene to become reactivated following differentiation, which may result in tumours in animals transplanted with iPS cells. It is thought the formation of tumours is most likely due to the expression of

oncogenes, including c-MYC, present in the reprogramming cocktail. The use of drug inducible systems has addressed this problem but the resulting iPS cells are still not safe enough for clinical applications (Brambrink et al., 2008).

The inherent risks associated with the use of integrative vectors have consequently led to the use of non-viral and non-integrative methods of transcription factor delivery. Some of the first attempts to produce iPS cells free of transgene integration used non-integrative adenoviruses. However, though adenovirus delivery of transcription factors was able to produce iPS cells free of transgene integration, extremely low reprogramming efficiencies (0.001-0.018%), and a high proportion of chromosomal abnormalities were reported (Stadtfield et al., 2008). The *piggyBac* transposon system has also been used to introduce reprogramming factors to cells, where, following integration into the host genome and subsequent reprogramming, the transgene can be completely excised (Chen, Y.-T. et al., 2010; Woltjen et al., 2009). iPS derivation by this method results in reprogramming efficiencies comparably lower than viral methods (approximately 0.1% compared to 0.1-1%) (Stadtfield & Hochedlinger, 2010). However, transposon mediated reprogramming is capable of delivering large gene constructs and polycistronic plasmids with a lesser effect on reprogramming efficiency than viral vectors (Chen, Y.-T. et al., 2010; Kaji et al., 2009; Patel & Yang, 2010). Transposons have been used to generate iPS cells from numerous species including humans (Chen, Y.-T. et al., 2010; Lacoste et al., 2009), mice (Kaji et al., 2009), horses (Nagy et al., 2011) and pigs (Kues, Wilfried et al., 2013; Woltjen et al., 2009). However, extensive screening is still required to ensure no transgenes remain following excision.

Episomal plasmids (episomes) are non-viral, non-integrating vectors, which are relatively simple to use and eliminate the need for consuming viral production before transduction of cells (González et al., 2011; Yu, J. et al., 2009). Episomal plasmids are capable of replicating in a host cell independently of the chromosomal DNA. Episomes can be delivered as single factors or as polycistronic plasmids with different combinations of the six reprogramming factors (OCT4 (*POU5F1*), SOX2, KLF4, c-MYC, NANOG and LIN28) on one plasmid. Episomal plasmids are commonly derived from the Epstein-Barr virus, specifically the oriP/EBNA1 (Epstein-Barr nuclear antigen-1) vector. These plasmids are capable of introducing transcription factors to cells without viral packaging, and are instead introduced by electroporation or Lipofectamine<sup>TM</sup> transfection. Most colonies produced using this method are reported to be free from transgene integration, additionally plasmids are lost from the cells through replication (Yu, J. et al., 2009). iPS cells derived using episomes possess similar

morphology, gene expression and differentiation potential to virally derived iPS cells. However the efficiency of producing these cells can be far lower than virally derived iPS cells, approximately 0.001% compared to 0.01-1% (Liu, Hua, Ye, Kim, Sharkis, & Jang, 2010; Stadtfeld & Hochedlinger, 2010; Yu, J. et al., 2009). The oncogene SV40 has been added to episomal plasmids to counteract cell toxicity and death caused by c-MYC. However, as SV40 is also an oncogene it may influence the safety of iPS cells derived using this plasmid (Jia et al., 2010). Most episomal vectors contain bacterial DNA in their backbone, posing a potential, though far lower, risk for integration and methylation of the host genome. Polycistronic episomal vectors can be relatively large, especially those containing all six reprogramming factors. It is thought that this size of the episome may have an impact on transfection efficiency and therefore impact reprogramming efficiency of iPS generation (Yu, J. et al., 2009). iPS cells derived from episomal plasmids are generally considered safe to use for clinical purposes, providing the cells used are thoroughly screened for transgene integration (Yu, J. et al., 2009).

Other non-viral methods of deriving iPS cells include minicircle vectors. These vectors are supercoiled DNA plasmids that have undergone processing to remove the bacterial backbone, and therefore contain no bacterial DNA (Jia et al., 2010). As a result minicircle vectors are far smaller in size than traditional episomal plasmids (approximately 3kb shorter), and consequently have higher transfection efficiencies. Additionally these vectors are reported to be expressed for longer in the cell due to decreased activation of exogenous silencing mechanisms (Chen, Z.-Y. Y., He, Ehrhardt, & Kay, 2003; Jia et al., 2010). Due to these attributes, minicircle vectors reportedly result in higher reprogramming efficiencies than episomal vectors (approximately 0.005% compared to 0.001%) (Jia et al., 2010; Okita et al., 2008; Stadtfeld & Hochedlinger, 2010; Yu, J. et al., 2009). Commercially available minicircles for iPS production are mostly polycistronic vectors encoding for the OSNL or OSKM factors, with an additional GFP reporter gene, minicircles for reprogramming are available commercially making them a desirable reprogramming tool.

mRNA, protein transduction and the supplementation of small molecules have also been used for cellular reprogramming. mRNA delivery completely eliminates the addition of exogenous DNA to a host cell, thereby removing the risk of integration into the host genome further. The pioneering study using synthetic mRNAs encoding for OCT4, SOX2, KLF4, c-MYC and LIN28 to generate human iPS cells, claimed the method was highly efficient with 2% of neonatal fibroblasts transfected producing iPS colonies in 17 days (Warren, L. et al., 2010). Although mRNA reprogramming is

appealing due to its high reprogramming efficiency and the availability to purchase the synthetic mRNAs commercially, successful reprogramming has only been reported in human cells when cultured with the media sold with the mRNA vectors. This limits the use of commercially available mRNAs in other species and cell types, and suggests the inclusion of small molecules in the proprietary media. The protocol for generating mRNA derived iPS cells is also complex, with the need for numerous transfections, which may increase the risk of tumours due to the high levels of c-MYC delivered to the cells (González et al., 2011).

Protein delivery negates the use of exogenous genetic material entirely. This method involves the delivery of reprogramming factors as proteins that are fused with peptides, including poly-A, to mediate transduction (Kim, D. et al., 2009; Zhou, H. et al., 2009). Although protein derived iPS cells have been successfully generated, they show extremely slow kinetics and relatively low reprogramming efficiencies similar to episomal plasmids (0.001%) (Stadtfeld & Hochedlinger, 2010). Additionally, the production and correct folding of these proteins that are required in large amounts is also difficult (González et al., 2011; Kim, D. et al., 2009).

The use of small molecules and chemicals that influence the same molecular pathways as some of the traditional Yamanaka factors has resulted in the ability to eliminate the introduction of some reprogramming factors in some instances. Using different combinations of small molecules such as histone methyltransferase (e.g. BIX-01294) allowed the reprogramming of neural progenitor cells with the addition of exogenous OCT4 (*POU5F1*) and KLF4 only (Shi, Yan et al., 2008). Furthermore a molecule capable of inhibiting transforming growth factor  $\beta$  (TGF- $\beta$ ) was shown to replace SOX2 in reprogramming (Ichida et al., 2009). However, the use of small molecules to replace reprogramming factors has been reported to result in decreased reprogramming efficiency (Ichida et al., 2009; Shi, Yan et al., 2008). Instead, small molecules are more commonly used in conjunction with reprogramming factors to improve efficiency of reprogramming. There are numerous small molecules and chemicals that have been shown to enhance reprogramming efficiency, and result in the production of stable transgene free iPS cells. The use of these molecules in reprogramming is considered safe as pathways that immediately affect reprogramming are targeted directly (O'Doherty et al., 2013).

The production of iPS cells from livestock has been far less successful than iPS cell generation from humans or mouse cells. The majority of iPS cells that have been produced from livestock species

have been reprogrammed using viral methods, mainly due to the superior reprogramming efficiency compared to non-viral or non-integrative techniques (Liu, J. et al., 2012; Ruan et al., 2011; Song et al., 2013; Sumer, Liu, Malaver-Ortega, et al., 2011). However, there have been reports of non-viral livestock iPS cell generation in pigs, cattle and horses (Huang, B. et al., 2011; Nagy et al., 2011; Woltjen et al., 2009).

The only report to produce putative, non-viral bovine iPS cells, used a poly-promoter plasmid with the bovine cDNAs for *POU5F1* (OCT4), *SOX2*, *KLF4* and *c-MYC*, controlled by their own independent promoter (Huang, B. et al., 2011). The study compared the production of bovine iPS cells using this vector from different cell lines (adult, embryonic and foetal fibroblasts) in addition to transfection with Lipofectamine™ or nucleofection. The study found that Lipofectamine™ transfection was generally superior to nucleofection, and that embryonic and foetal fibroblasts showed higher reprogramming efficiencies than adult fibroblasts. The study also compared the use of two different plasmids with reprogramming factors in a different order on the polycistronic cassette (OSKM and KMOS), and found that there was no difference in reprogramming efficiency. The study produced putative bovine iPS colonies with tightly packed dome shaped morphology with clear borders 10 days post transfection. Colonies expressed pluripotency markers, had normal karyotype and produced teratomas with cells representative of all three germ layers. However, these colonies were not deemed fully reprogrammed as they did not expand following passaging (Huang, B. et al., 2011). Table 5-1 shows the general characteristics of vectors used for reprogramming cells to pluripotency.

Chapter 5: Comparison of Viral and Non-Viral Reprogramming of Bovine Fibroblasts to Pluripotency

**Table 5-1: General characteristics of vectors used for reprogramming cells to pluripotency. Table indicates reports of methods used for deriving livestock iPS cells. Efficiencies are general and not specific to livestock generation.**

Vector	Efficiency	Integration into host genome	Use in Livestock iPS generation	Advantages	Disadvantages
Retrovirus	0.1% Murine 0.01% Human	Yes	Ovine (Liu, J. et al., 2012; Sartori et al., 2012) Bovine (Sumer, Liu, Malaver-Ortega, et al., 2011) Buffalo (Deng et al., 2012) Porcine (Park, K.-M. et al., 2013; Ruan et al., 2011)	High efficiency	Insertional mutations Unable to use drug inducible system Incomplete transgene silencing
Lentivirus	0.01-1%	Yes	Goat (Song et al., 2013) Bovine (Cao et al., 2012) Porcine (Ezashi et al., 2009)	High efficiency	Insertional mutations, Incomplete transgene silencing
Inducible Lentivirus	0.1-1%	Yes	Ovine (Li, Yang. et al., 2011) Porcine (Wu et al., 2009)	High efficiency Controlled transgene expression	Insertional mutations
Adenovirus	0.0001-0.0018%	No	No	No transgene integration	Extremely low transfection efficiency
Transposon/ PiggyBac System		Yes but excisable	Porcine (Kues, Wilfried et al., 2013; Woltjen et al., 2009) Equine (Nagy et al., 2011)	Full excision of transgene possible	Intensive screening of iPS cells required Lower reprogramming efficiency than viral systems
Episomes/ Plasmid	0.001%	No	Bovine (Huang, B. et al., 2011)	Generally no transgene integration	Low transfection efficiency. Occasional vector integration
Minicircle	0.005%	No	No	No transgene integration	Low transfection efficiency, but higher than plasmid.
mRNA	2%	No	No	No DNA introduced No transgene integration Highly efficient	Requires a complex protocol Only works on human cells in conjunction with commercially sold media

It is difficult to choose a reprogramming method that is suitable for all purposes. The lack of non-viral livestock iPS cells reflects the use of reprogramming vectors that traditionally offer higher reprogramming efficiencies such as retroviral and lentiviral vectors. The production of iPS cells has been centred around two approaches, the first, on production of iPS cells in an attempt to further understand reprogramming mechanisms, and the second to generate clinically applicable iPS cells. Although the focus of this thesis was primarily to produce bovine iPS cells in general, the end goal of this thesis was to produce bovine iPS cells that may be used for breeding applications including a potential use in germ cell transplantation, this has presented the need to produce livestock iPS cells free of exogenous transgenes.

To date, studies into the production of bovine iPS cells using either viral or non-viral vectors has not resulted in a reproducible or robust protocol for bovine iPS cell derivation (Cao et al., 2012; Li, Yang, et al., 2011; Sumer, Liu, Malaver-Ortega, et al., 2011). Additionally, the one study reporting non-viral bovine iPS cell generation did not result in fully reprogrammed cells (Huang, B. et al., 2011). Chapter 4 described attempts to produce bovine iPS cells using a lentiviral vector, and although partially reprogrammed iPS cells were produced, fully reprogrammed bovine iPS cells were not derived.

The first aim of this chapter was to compare the production of bovine iPS cells using viral and non-viral transfection. The effect of different combinations of reprogramming factors lentivirally transduced into bovine fibroblasts was previously tested in Chapter 4, however the effect of these combinations of transcription factors when non-virally transfected into somatic cells was not known. As such, the second aim of this chapter was to compare the reprogramming efficiency of viral and non-viral iPS cells produced using the different combinations of reprogramming factors previously tested in Chapter 4 (page 105). The reprogrammability of two different cell types, bovine dermal and embryonic fibroblasts, using lentiviral and non-viral vectors was also tested. The effect of reprogramming bovine cells with lentiviral or non-viral methods was compared through time to colony formation, number of colonies produced (reprogramming efficiency) and through characterisation of putative colonies produced in each treatment.

## 5.2 Materials and Methods

### 5.2.1 General

All experiments involving animals were approved and conducted according to guidelines given by the F D McMaster Laboratory Animal Ethics Committee for CSIRO Animal, Health and Food Sciences (AEC 11/20). Cell culture reagents were purchased from Life Technologies Corporation (USA), Sigma-Aldrich chemical industries (USA), and Stemgent (USA) where indicated. Plasmids used for reprogramming were obtained from plasmid repository Addgene. Plasmids used in chapter 5 are described in Table 5-3. Primers used in chapter 5 were designed using Primer3 software, and ordered through Biosearch Technologies, Inc. (USA). Primers used in Chapter 5 are shown in Table 5-2.

This experiment was repeated three times with two technical replicates for each treatment per experiment.

### 5.2.2 Derivation of Bovine Fibroblasts

Bovine dermal fibroblasts (BDFs) were isolated from skin samples from Angus male calves were taken during castration that was performed under general anaesthesia. Bovine embryonic fibroblasts (BEFs) were isolated from a Male *Bos Taurus* bovine foetus that was sourced from a local abattoir from slaughtered cattle. The foetus measured approximately 18cm crown to rump, giving an estimated age of 60-70 days gestation. Primary cell cultures were prepared as described in Chapter 4 (pg. 114). Cells were grown in fibroblast growth media (DMEM, 10% Foetal Bovine Serum (FBS), 2 mM GlutaMAX™, 100 µM non-essential amino acids (NNEA), with 1% anti-anti (10,000 U/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®) (all components from Life Technologies, USA), and were passaged every two to three days.

### 5.2.3 Production of bovine iPS cells

#### 5.2.3.1 Lentiviral production

All plasmids used for lentiviral production were obtained through the plasmid repository Addgene ([www.addgene.org](http://www.addgene.org)) and are listed in Table 5-3.

Lentivirus was produced using the Lipofectamine™ LTX system (Life Technologies, USA) as described in Chapter 4 (pg. 116). Basically HEK293 cells were transduced with 1 µg of the target plasmids (Tet-OSKM, OSNL, OSKMNL or M2rRTa), 1 µg pMD2.g and 2 µg psPAX2 according to manufacturer's instructions for the LTX Lipofectamine™ PLUS reagent kit. Lentiviral supernatants were collected after 48 hours, filtered through a 45 µm cell filter and lentiviral titres were tested using Lenti-X™ GoStix™ (Clontech Laboratories, 2014) as described in Chapter 4 (pg. 116). Lentiviral supernatants were then used for infection of BEF or BDF cells or snap frozen in liquid nitrogen and stored at -80°C until use.

#### 5.2.3.2 Lentiviral transfection and cell culture

5x10<sup>4</sup> BDFs and BEFs were transfected with prepared lentivirus carrying plasmids coding for Tet-OSKM, OSNL or OSKMNL as described in Chapter 4 (pg. 116). Following 24 hours of incubation in the viral supernatant, media was changed to iPS media (Minimum Essential Media Alpha (MEM-α), 20% FBS, 1% insulin transferrin-selenium (ITS), 2 mM GlutaMAX™, 100 µM NEAA, 50 U/mol penicillin, 50 mg/ml streptomycin, 0.1 mM β-mercaptoethanol (Sigma, USA), 4 ng/ml human leukemia inhibitory factor (LIF, Millipore, USA), and 10 ng/ml basic fibroblast growth factor (bFGF, Millipore, USA), and supplemented with 0.5 mM sodium butyrate (Sigma, USA), 0.5 µM PD0325901 (Stemgent, USA) and 2 µM SB431542 (Stemgent, USA). Once cells had become 80% confluent they were seeded at a density of 2x10<sup>4</sup> cells onto Mitomycin-C inactivated BEFs (2x10<sup>6</sup>/ 10cm dish) as described in chapter 4 (pg. 117). Following plating onto feeder layers transfected cells were cultured for 21 days with media changed every second day. 2 µg/ml doxycycline (DOX, Sigma- Aldrich Chemical Industries, USA) was added per 10 cm dish to the Tet-OSKM transduced cells daily to induce transcription.

### 5.2.3.3 Non-Viral Electroporation of Reprogramming Plasmids

Electroporation of reprogramming plasmids into BEFs and BDFs was conducted using the Neon™ Transfection System (Life Technologies, USA), according to the manufactures instructions. In brief, BEFs and BDFs were harvested using TrypLE™ Express (Life Technologies, USA) prior to electroporation.  $1 \times 10^6$  of each cell type were washed with dPBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Life Technologies, USA), the cells were then collected by centrifugation at 400 xg for 5 minutes. The cell pellet was taken up in the re-suspension buffer provided with the Neon Kit at a density of  $1 \times 10^7$  cells/ml. 2 µg of the reprogramming plasmid (Tet-OSKM with M2rtTA, OSNL or OSKMNL) was added to the cell suspension and then using the 10 µl Neon™ Pipette tip cells were electroporated at 1400 V, 20 Pulse width and 2 pulses. The cells were then transferred to equilibrated fibroblast growth media without antibiotics or antimycotics (DMEM, 10% Foetal Bovine Serum (FBS) (Life Technologies, USA), 2 mM GlutaMAX™, 100 µM non-essential amino acids (NNEA) (Life Technologies, USA)). This was repeated for the remaining reprogramming plasmids and the eGFP plasmid for both BEFs and BDFs. Cells were grown in culture for 24 hours, after which Anti-Anti (10,000 U/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, Life Technologies, USA) was added to each well. When cells reached 80-90% confluence after 2-3 days, they were enzymatically dissociated using TrypLE™ Express and plated at a rate of  $5 \times 10^4$  onto 10 cm gelatine coated tissue culture dishes containing Mit-C inactivated BEF feeder layers ( $2 \times 10^6$  feeders/dish). 2 µg/ml doxycycline was added to dishes with Tet-OSKM transfected cells to activate the expression of the transgene. Gelatine coating and inactivation of BEFs with Mit-C is described in Chapter 4 (pg. 117).

Following plating of transfected or infected cells onto Mit-C feeders, cells were grown for 21 days , with media changed every two days. Cells were observed daily for colony formation and the number of colonies per plate. Following culture of cells for 21 days, colonies were manually picked, washed with DPBS, and either frozen at  $-80^\circ\text{C}$  where they were stored before RNA extraction, or alternatively used to produce embryoid bodies. The timeline for lentiviral transfection and non-viral electroporation protocols are shown in Figure 5-1.

Control cultures of un-transfected bovine fibroblasts, lentiviral and non-viral iPS cultures were grown concurrently under the same conditions. This experiment was conducted three times with two technical replicates for each treatment in each repeated experiment.

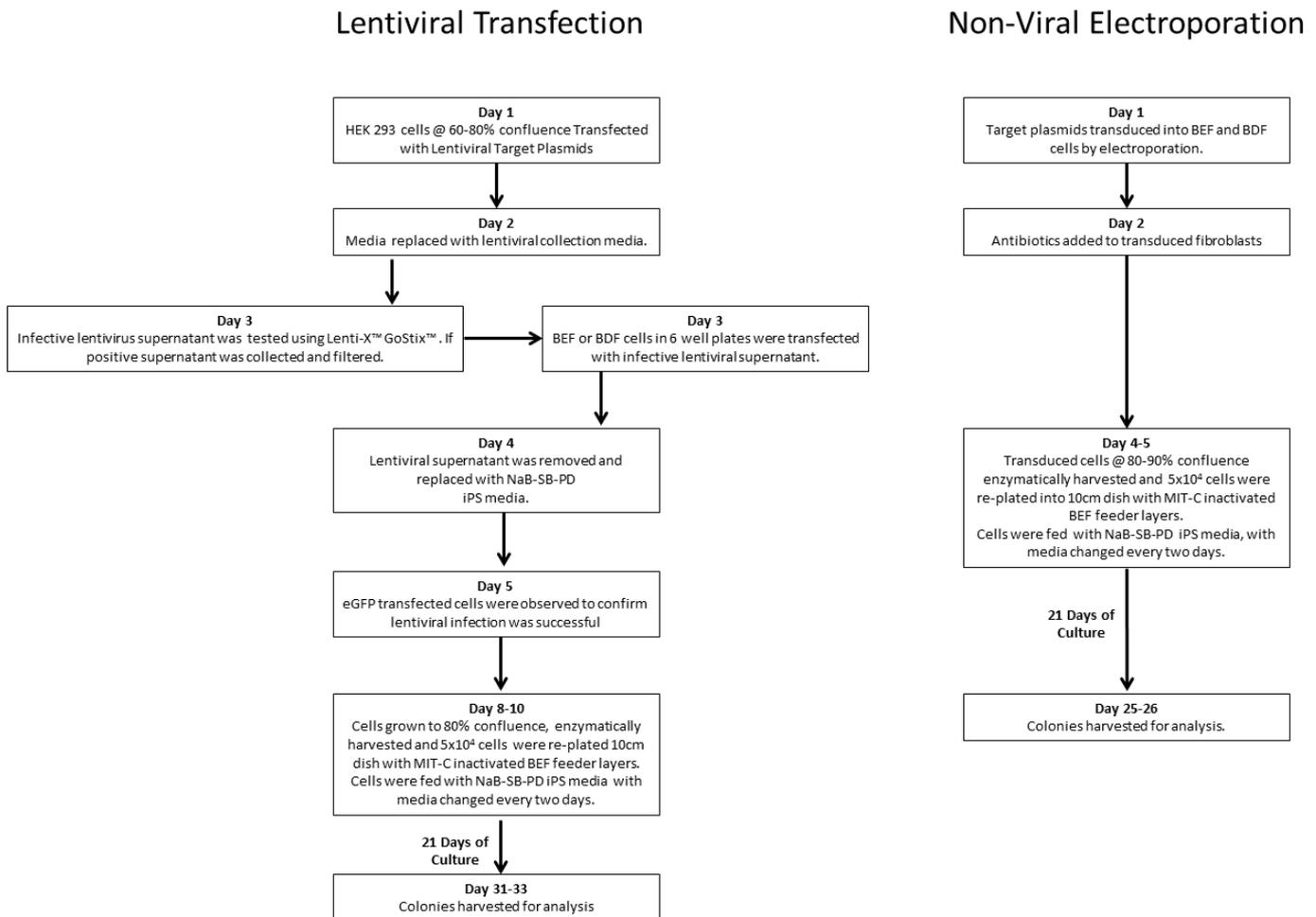


Figure 5-1. Timelines showing the processes of lentiviral and non-viral production of bovine iPS colonies in the experiments outlined in Chapter 5.

### 5.2.4 Embryoid Body (EB) Production

Colonies were manually picked after three weeks of growth, picked colonies were transferred to ultralow-adherent 6 cm cell culture dishes, and grown in iPS media without growth factors or small molecules (Minimum Essential Media Alpha (MEM- $\alpha$ ), 20% FBS, 1% insulin transferrin-selenium (ITS), 2 mM GlutaMAX™, 100  $\mu$ M NEAA, 50 U/mol penicillin, 50 mg/ml streptomycin, 0.1 mM  $\beta$ -mercaptoethanol (Sigma, USA)). Embryoid bodies were grown for two weeks, with media changed

every three days, after which the embryoid bodies were collected and stored at -80°C until qRT-PCR analysis was carried out.

### **5.2.5 Analysis of putative bovine iPS colonies**

Putative iPS cultures were observed daily for colony formation, and the time to colony formation was recorded for each treatment. Photographs of colonies were taken using a Nikon Eclipse TE300 inverted microscope (Nikon, Japan) equipped with a cooled *PE* light source (CoolLED, USA), and images were captured using ProgRes CapturePro 2.1 software (Jenoptik, Germany). Reprogramming efficiency of cultures was calculated after 21 days of culture and was determined by the total number of colonies observed divided by the total number of transfected cells initially plated.

Alkaline phosphatase live staining was carried out using the molecular probe kit from Life Technologies (USA) according to manufactures instructions. Basically, the growth media was removed and cells were washed with pre-warmed DMEM/F-12 for 2-3 minutes, media was aspirated and wash was repeated. The 1x alkaline phosphatase (AP) stain was prepared by dilution of 500x stock solution in warm DMEM/F-12. The working solution was applied directly to the cell culture and cells were incubated for 20-30 minutes. After incubation, the live stain was aspirated and cells were washed twice with DMEM for 5 minutes per wash. Fresh DMEM/F-12 was added following the final wash and cells were photographed within 30 minutes using a Nikon Eclipse TE300 inverted fluorescent microscope (Nikon, Japan) using a standard FITC filter (CoolLED, USA). Following visualisation, DMEM/F-12 was replaced with fresh iPS media and cells were returned to normal culture conditions.

Immunofluorescent staining for pluripotency markers SSEA4 and Tra-1-60 was carried out using the ES cell Characterisation kit (Millipore, USA). In short, cultures were incubated with 4 µg/ml mouse anti-Tra-1-60 IgM and mouse anti-SSEA4 IgG monoclonal antibodies in iPS media without LIF, bFGF and small molecules (Sodium butyrate (NaB), PD0325901 (PD), SB431542 (SB)) for 1 hour. Cultures were washed with warmed Dulbecco's Phosphate Buffered Saline (DPBS), followed by incubation with 2 µg/ml Alexa Fluor 488 goat anti-mouse IgM (Life Technologies) or 2 µg/ml Alexa Fluor 488 goat anti-mouse IgG (Life Technologies) in iPS media without LIF, bFGF or small molecules for one hour. Following incubation, cultures were washed with DPBS twice, and complete iPS media was

replaced. Cultures were observed on a Nikon Eclipse TE300 inverted microscope (Nikon, Japan) equipped with a cooled *PE* light source (CoolLED, USA), and images were captured using ProgRes CapturePro2.1 software (Jenoptik, Germany).

For PCR reactions, genomic DNA was extracted from pooled putative iPS colonies using an Allprep RNA/DNA micro kit (Qiagen USA). Each PCR reaction was carried out as described in Chapter 4 (pg. 119). Primers for plasmids are shown in Table 5-2.

### 5.2.6 mRNA Extraction and qRT-PCR analysis

For qRT-PCR reactions mRNA was extracted from pooled colonies using the Dynabeads<sup>®</sup> mRNA DIRECT<sup>™</sup> Micro Kit, and qRT-PCR reactions for expression of pluripotency and lineage markers were carried out as described previously in Chapter 4 (pg. 120). The reference gene *RPS26* was previously identified using the sheep GeNorm kit (PrimerDesign, UK). In previous studies by McMillan (2012), this housekeeper has been shown to be the best reference gene for the cell types used in this chapter. Primers used for qRT-PCR reactions are shown in Table 5-2.

### 5.2.7 Statistical Analysis

For results of time to colony formation and number of colonies produced, ordinary one way ANOVAs were conducted in GraphPad Prism to determine if group means differed significantly from one another ( $P < 0.05$ ).

qRT-PCR reactions were analysed in Excel add-in Genex, and Ct values from the raw analysis were converted into gene expression data as a fold change relative to reference gene *RPS26*. Statistical analysis of data was performed using GraphPad Prism<sup>®</sup> software (GraphPad Software, USA). For all results of qRT-PCR analysis, two-way ANOVAs with Tukey's multiple comparison tests were used to determine if statistical differences existed between group means, ( $P < 0.05$ ). Results are presented as means  $\pm$  SEM.

Table 5-2: Primers used in Chapter 5.

Gene Construct	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Size (bp)	Reaction Efficiency (%)	Melt Peak (°C)	Specificity
Pluripotency Genes						
<i>POU5F1 (OCT4)</i>	AAGCGGACGAGTATCGAGAA	ACACTCGGACCACGTCTTTC	133	113.8	86	Bovine mRNA
<i>SOX2</i>	ATGAAGGAACACCCGGATTA	CATGTGCGCGTAGCTGTC	186	81.2	89	Bovine
<i>NANOG</i>	GTCCCGGTCAAGAAACAAAA	TCTGGAACCAGGTCTTCACC	163	106.1	87	Bovine mRNA
<i>c-MYC</i>	GGAAGAAATTCGAGCTGCG	GTCGCAGATGAAGCTCTGGT	226	105.7	87	Bovine mRNA
Lineage Markers						
<i>TUBB3</i>	TGGAGCGCATCAGTGTCTAC	CAGTTGTTACCAGCCCCACT	172	99.3	87.5	
<i>NESTIN</i>	AAAGAAGGCTGGGATCCTGT	CCTCAAACCTTCCGACAGC	130	108.9	88	
<i>DESMIN</i>	GGGACATCCGTGCTCAGTAT	GTGGCGGTACTCCATCATCT	155	120.8	86.5	
<i>FoxA2</i>	ACCACTACGCCTTCAACCAC	GGGGTAGTGCATCACCTGTT	134	85.8	86	
Exogenous Constructs						
<i>Tet-OSKM</i>	TGAGGCTACAGGGACACCTT	TCAACATCACCTGCTTGCTT	240			Exogenous/ Ectopic
<i>OSNL</i>	TGATTATGGCGGGACACCTG	TAGCCAGGTCCGAGGATCAA	113			Exogenous/ Ectopic
<i>OSKMNL</i>	TTGATCCTCGGACCTGGCTAA	CCACAGAACTCATA CGGCG	124			Exogenous/ Ectopic
Reference Gene						
<i>RPS26</i>	TCATTCGGAACATCGTAGAGG	CCTGACTACCTTGCTGTGAAT				Endogenous/ Reference Gene

Table 5-3: Plasmids sourced from Addgene plasmid repository, for use in reprogramming experiments carried out in chapter 5.

Plasmid	Referred to as	Description	Addgene Plasmid Reference	Principle Investigation
<b>TetO-FUW-OSKM</b>	Tet-OSKM	Polycistronic plasmid for Tet- inducible expression of murine OCT4, SOX2, KLF4 and Myc for iPS cell generation	20321	(Carey et al., 2009)
<b>FUW-M2rtTA</b>	M2rtTA	Lentiviral plasmid expressing the reverse tetracycline transactivator for Doxycycline inducible control of expression	20324	(Hockemeyer et al., 2008)
<b>pEP4 E02S EN2L</b>	OSNL	Episomal vector expressing human OCT4, SOX2, NANOG and LIN28 for the production of iPS cells	20922	(Yu, J. et al., 2009)
<b>pEP4 E02S CK2M EN2L</b>	OSKMNL	Episomal vector containing human OCT4, SOX2, Lin 28, NANOG, KLF4 and c-MYC for the production of iPS cells	20924	(Yu, J. et al., 2009)
<b>psPAX2</b>		Empty vector backbone	12260	
<b>pMD2.G</b>		Mammalian Expression ; Envelope	12259	
<b>PGK-H2BeGFP</b>	eGFP	Constitutive vector encoding for eGFP	21210	(Kita-Matsuo et al., 2009)

## **5.3 Results**

To test the efficiency of bovine non-viral reprogramming, compared to viral reprogramming, a 3x2x2 factorial design was employed, where viral transduction and non-viral transfection of three different polycistronic plasmids coding for combinations of reprogramming factors, Tet-OSKM, OSNL or OSKMNL, was conducted in two different cell types, bovine embryonic fibroblasts (BEFs) or bovine dermal fibroblasts (BDFs). This design featured two technical replicates for each treatment, with the entire experiment replicated three times.

### **5.3.1 Electroporation and Lentiviral Transduction results in Gene Expression by Bovine Fibroblasts**

To determine if both lentiviral transduction and non-viral transfection by electroporation of was successful a control GFP plasmid was introduced to bovine fibroblasts using both methods. Cells transfected with either lentiviral GFP or by non-viral electroporation of the GFP plasmid expressed GFP as observed by fluorescent microscopy. The transfection efficiency for lentiviral transfection was lower than the efficiency of non-viral electroporation, as determined by the percent of GFP positive cells. The transfection efficiency eGFP by of lentiviral infection was similar to results observed in Chapter 4 of this thesis, with approximately 65% of cells observed GFP positive. Non-viral electroporation of the eGFP plasmid resulted in approximately 85% of cells observed GFP positive. Figure 5-2 shows the high transfection efficiency of non-viral electroporation delivery of the eGFP plasmid.

The expression of GFP following transfection by either method provided evidence that the plasmids coding for reprogramming factors would also be introduced and expressed by fibroblast cells. Interestingly, the transfected cells appeared to respond better to electroporation than lentiviral infection, and following electroporation became confluent faster than lentiviral infected cells

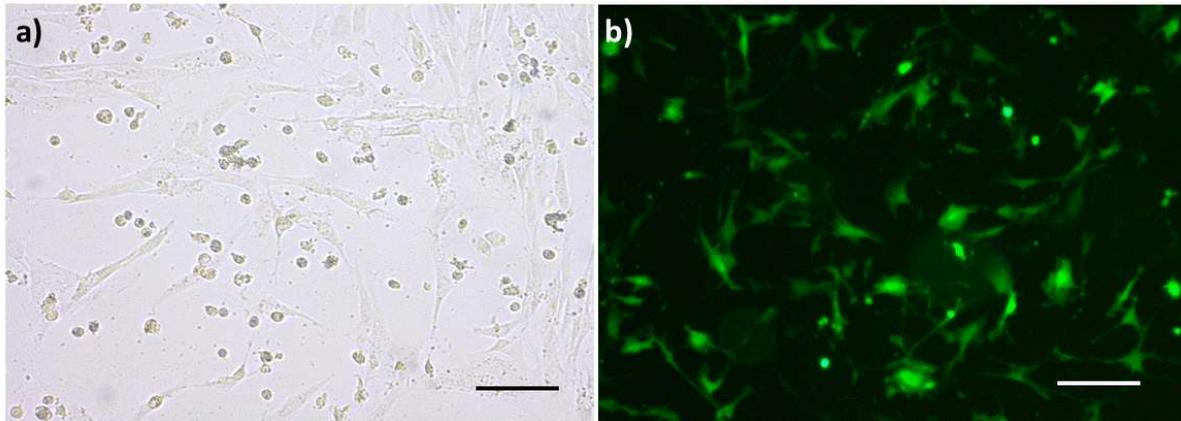


Figure 5-2. Expression of GFP protein by bovine fibroblast cells non-virally electroporated with the eGFP plasmid Scale bars represent 50  $\mu\text{m}$ .

### 5.3.2 Non-viral Reprogramming Increases Reprogramming Kinetics Compared to Lentiviral Reprogramming

The time colony formation was significantly less in cultures derived by non-viral reprogramming than by lentiviral derived cultures. Lentiviral cultures produced putative iPS colonies after an average of 12 days of culture, while non-viral cultures produced putative iPS colonies after an average of 10 days of culture ( $P < 0.05$ ). No colonies were observed in the control cultures during 21 days of culture. Figure 5-3a, shows the complete data set for the time to colony formation of all treatments. Figure 5-3b summarises the time to colony formation of BDF and BEF cells that were lentivirally transduced or non-virally transfected. This figure shows that there is a significantly lower time to colony formation when the same cell type is reprogrammed by non-viral electroporation, compared to lentiviral transduction ( $P < 0.05$ ). Furthermore, there was no difference in time to colony formation observed between different cell types that were reprogrammed using the same method ( $P > 0.05$ ). Figure 5-3c summarises the effect of time to colony formation of introducing different combinations of reprogramming factors by lentiviral transduction or non-viral electroporation. This figure shows that for the same combination of reprogramming factors, non-viral electroporation resulted in significantly lower time to colony formation than lentiviral transduction ( $P < 0.05$ ). There was no significant difference in the time to colony formation observed between the different combinations of reprogramming factors when introduced using the same method ( $P > 0.05$ ).

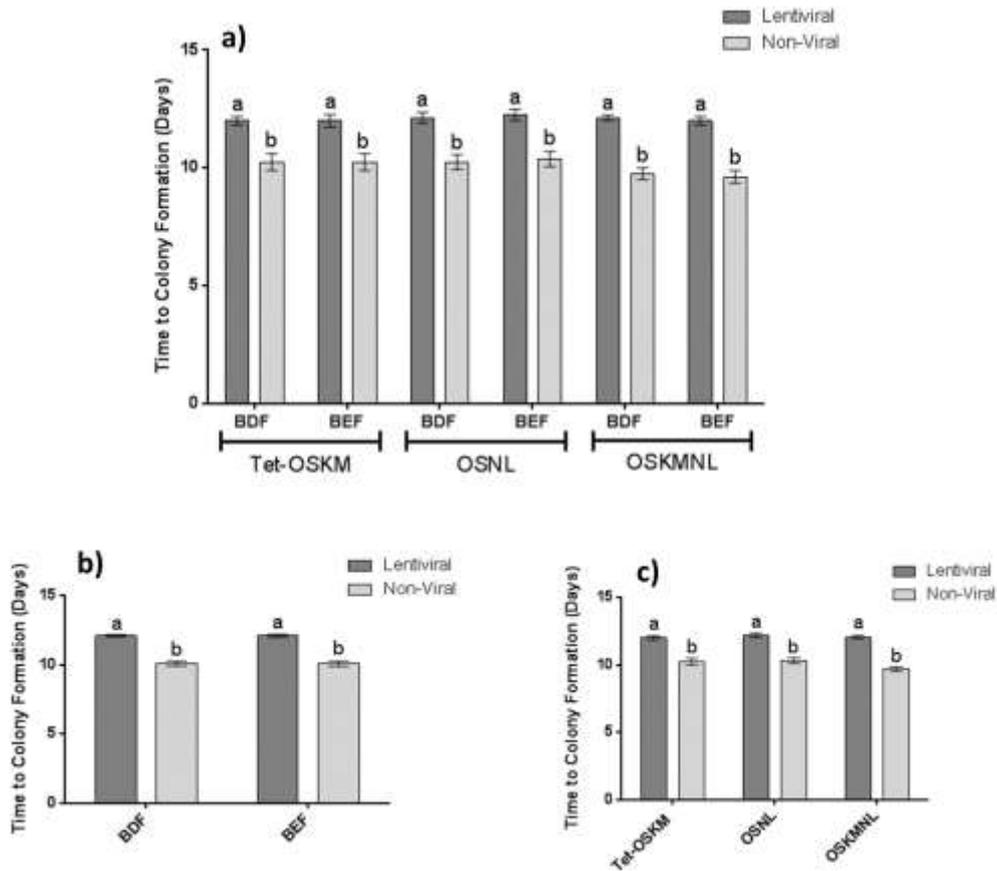


Figure 5-3. Time to formation of putative bovine iPS colonies (in days) of BEFs or BDFs transfected or infected with different combination of transcription factors; Tet-OSKM, OSNL or OSKMNL. a) complete data set for each treatment type showing the effect of cell type, combination of transcription factors and reprogramming method on the time to colony formation ( $P < 0.05$ ,  $n = 6$ ). b) Effect of cell type on time for colony formation of using lentiviral and non-viral reprogramming ( $P < 0.05$ ,  $n = 18$ ). c) Effect of combination of transcription factors on time to colony formation using lentiviral and non-viral reprogramming ( $P < 0.05$ ,  $n = 12$ ). Different characters above treatment means indicate a significant difference in observed colony number between treatments. Error bars are represented as  $\pm$  SEM.

### 5.3.2 Reprogramming Efficiency is not Affected by the use of Non-Viral or Lentiviral Reprogramming, but is Influenced by Cell Type and Choice of Transcription Factor Combination

The number of colonies observed per 10 cm dish after 21 days in culture was affected by cell type and the combination of reprogramming factors delivered by lentiviral transduction or non-viral electroporation. Figure 5-4a shows the complete data set for number of colonies produced for each treatment. Figure 5-4b summarises the effect of cell type on the number of colonies produced by lentiviral transduction or non-viral electroporation. This figure shows that while there is no significant difference in the number of colonies produced by lentiviral transduction or non-viral electroporation of BDFs, there was a pattern of a lower number of colonies produced by lentiviral

cultures. There was no difference observed between lentiviral and non-virally derived BEF iPS cultures. However, there was a pattern of BEF cultures producing lower colony numbers than BDF cultures. Though these numbers were not significantly different to BDF lentiviral cultures, they were significantly lower than BDF non-viral iPS cultures.

Figure 5-4c summarises the effect of the number of putative iPS colonies produced by different combinations of reprogramming factors introduced by lentiviral transduction or non-viral electroporation. This graph shows that there is no significant difference between the number of colonies produced by lentiviral or non-viral transfection of the same combination of transcription factors ( $P>0.05$ ). However, a pattern did emerge of fewer colonies produced in cultures lentivirally transduced or non-virally electroporated with the OSKMNL combination of transcription factors. Though OSKMNL non-viral cultures did not produce significantly lower colonies than other non-viral cultures, the number was notably lower. Furthermore, the number of colonies produced in lentiviral OSKMNL cultures was significantly less than lentiviral OSNL cultures ( $P<0.05$ ), and while not significantly less than lentiviral Tet-OSKM cultures, lentiviral OSKMNL cultures produced notably fewer colonies ( $P>0.05$ ) as was the pattern seen in non-viral OSKMNL cultures. A total average, from all treatments, of five putative iPS colonies, was observed per 10cm dish ( $P>0.05$ ,  $SEM=0.3$ ). An average reprogramming efficiency of 0.01% was recorded in this experiment.

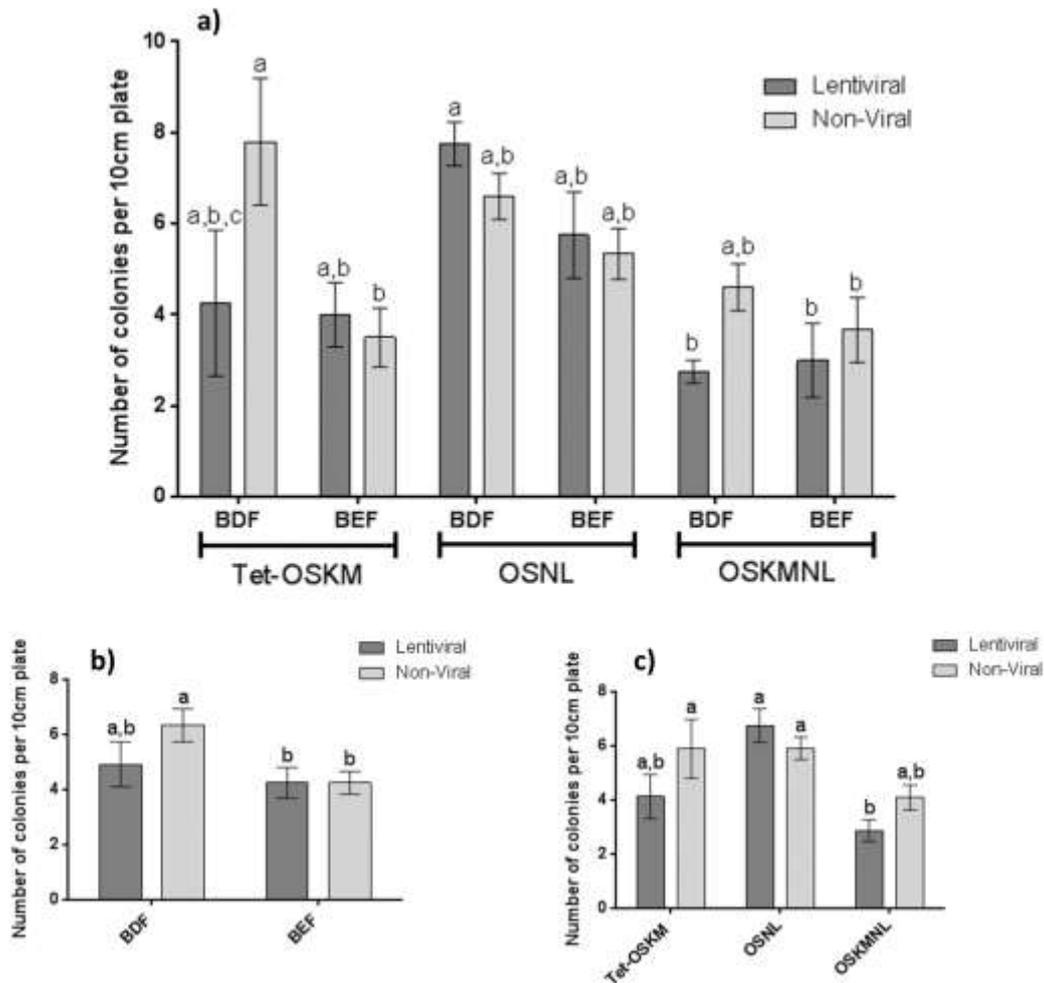
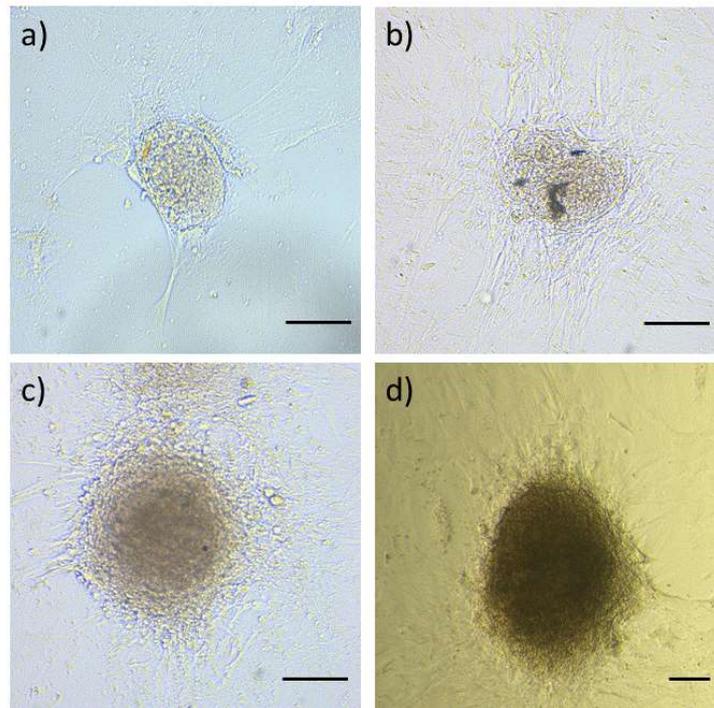


Figure 5-4. Comparison of number of putative bovine iPS colonies observed per 10cm dish after 21 days of culture for BEFs or BDFs transfected or infected with different combinations of transcription factors, Tet-OSKM, OSNL or OSKMNL. a) raw data for each treatment type showing the effect of cell type, combination of transcription factors and reprogramming method on the number of colonies produced (P<0.05, n=6). b) Effect of cell type using lentiviral or non-viral reprogramming on the average number of colonies produced per 10cm dish (P<0.05, n=18). c) Effect of combination of transcription factors on the average number of colonies produced by lentiviral or non-viral transfection (P<0.05, n=6). Different characters above treatment means indicate a significant difference in observed colony number between treatments, error bars are represented as  $\pm$  SEM.

### 5.3.3 Reprogramming Method Influences iPS Colony Morphology

Putative iPS colonies were observed daily to determine growth patterns. Lentivirally derived colonies appeared much smaller and grew far slower than those colonies derived using non-viral transfection of the reprogramming plasmids. Figure 5-5 shows examples of putative bovine iPS colonies from lentiviral and non-viral cultures. Lentivirally derived cultures formed small round colonies with ES like morphology consisting of small round cells, and were flatter than non-viral colonies (Figure 5-5a). Some colonies did not have well defined borders representative of good iPS morphology

(Figure 5-5b). Some lentiviral colonies ceased growing noticeably after about one week. Non-virally derived cells grew far larger than the lentivirally derived colonies and were composed of cells with ES like morphology around the borders while most appeared to become differentiated in the colony centre (Figure 5-5c). Between 14 and 21 days of culture some putative non-viral colonies developed into large dome shaped colonies, similar in appearance to embryoid bodies (Figure 5-5d).



**Figure 5-5. Colony morphology representative of Lentivirally and non-virally derived putative bovine iPS colonies. a)** Example of lentivirally derived colony composed of cells with ES cell like morphology. **b)** Lentiviral colony that does not display good iPS colony morphology, with un-clear borders. **c)** Example of putative bovine non-viral colony. Colony is composed of ES like cells around the perimeter of the colony, while the centre has begun to differentiate. **d)** Non-virally derived colony that has differentiated into a large embryoid body like structure following 21 days of culture. Scale bars represent 100  $\mu\text{m}$

Colonies were analysed for expression of alkaline phosphatase using live alkaline phosphatase stain (Life Technologies, USA), however, no colonies from any treatment stained positively for alkaline phosphatase expression. Immunohistochemistry staining was also carried out for pluripotency markers SSEA-4 and Tra-1-60; however no colonies stained positively for either marker.

Colonies were passed after 21 days of growth; however none continued to grow to any significant extent once passed, with most becoming immediately quiescent. Some colonies appeared to also

differentiated back to the original cell type with fibroblast like cells growing out from the passaged colonies.

### 5.3.4 Lentiviral Transduction Results in Transgene Integration into the Host Genome, While Non-Viral Electroporation does not

PCR analysis of transgene-specific primers showed that the transgenes from all three reprogramming plasmids had successfully integrated into the host genome in the lentivirally derived iPS colonies (BDF-L or BEF-L). There was no integration of DNA in the colonies derived using episomal transfection of the transgene (BDF-N or BEF-N) (Figure 5-6).

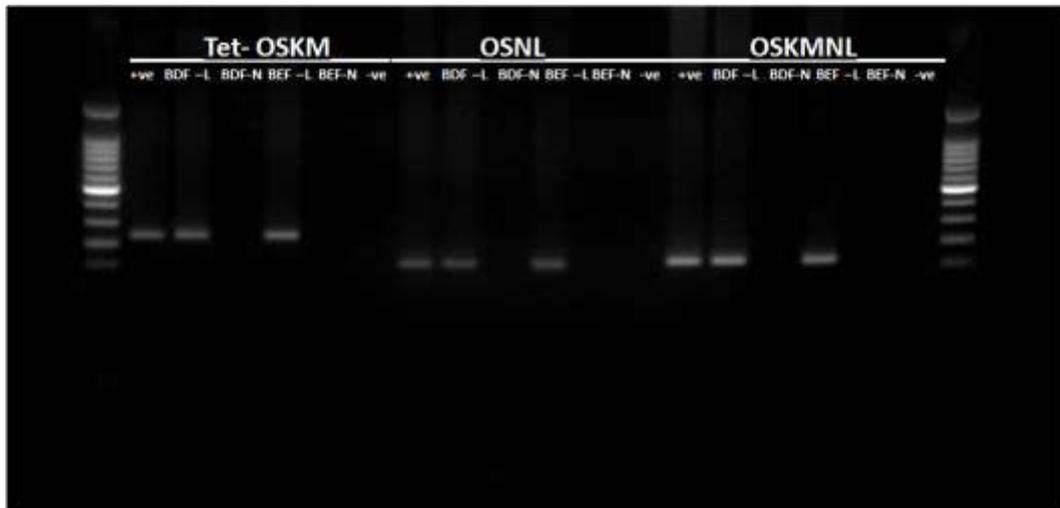


Figure 5-6: Gel showing integration of transgene in host genome of bovine embryonic stem cells for lentivirally transduced cells and non-virally transfected cells. Integration is absent in BEFs and BDFs transfected with Episomal plasmids (BDF-N or BEF-N), but is present in lentivirally transduced cells (BDF-L or BEF-L). +ve indicates the positive control plasmid, -ve indicates the negative control where the OCT4 plasmid was used in PCR rather than DNA from transfected cells.

### 5.3.5 Expression of Pluripotency Markers Differs Between Lentiviral and Non-Viral iPS Cultures

After 21 days of culture, colonies were manually picked and five pooled colonies from each treatment were analysed for expression of pluripotency markers *POU5F1* (OCT4), *SOX2*, *c-MYC* and *NANOG* using qRT-PCR. There was no clear pattern of the effect of pluripotency marker expression by lentiviral or non-virally derived putative bovine iPS colonies (Figure 5-7a)

All colonies derived from the either lentiviral or non-viral reprogramming of BDF or BEFs expressed higher levels of *POU5F1* than the un-transfected cell types (Figure 5-7a, b). Reprogramming method did not seem to affect expression of *POU5F1* in BDF derived cultures (Figure 5-7a). *POU5F1* expression increased in Tet-OSKM and OSNL non-viral colonies, but decreased in OSKMNL non-viral colonies compared to lentiviral transduction of the same combination of transcription factors. In BDF derived cultures the highest *POU5F1* expression was observed in Tet-OSKM non-viral colonies. Lower levels of *POU5F1* expression were observed in Tet-OSKM lentiviral colonies and OSNL non-viral colonies, with slightly lower levels of *POU5F1* expression by OSKMNL lentiviral colonies. OSKMNL non-viral and OSNL lentiviral colonies expressed the lowest levels of *POU5F1*. Similar to BDF derived cultures, in BEF cultures, there was no clear pattern of *POU5F1* expression by lentiviral or non-virally derived colonies (Figure 5-7a). Tet-OSKM non-viral colonies expressed higher levels than Tet-OSKM lentiviral colonies, while OSNL and OSKMNL non-viral colonies expressed lower levels than the lentiviral derived colonies (Figure 5-7a). The highest *POU5F1* expression in BEF cultures was observed in Tet-OSKM non-viral colonies, with lower levels of expression by OSNL lentiviral colonies, followed by OSNL non-viral colonies. OSKMNL lentiviral colonies expressed lower levels still, followed by Tet-OSKM lentiviral and OSKMNL non-viral colonies which expressed similar levels of *POU5F1* (Figure 5-7b).

There was no obvious pattern of effect of reprogramming method on *SOX2* expression (Figure 5-7c, d). *SOX2* was expressed by most iPS colonies except BDF OSKMNL non-viral colonies, and BEF Tet-OSKM lentiviral colonies. In BDF derived colonies OSKMNL lentiviral cultures expressed the highest levels of *SOX2*, followed by lentiviral or non-viral Tet-OSKM and OSNL non-viral colonies. OSNL lentiviral colonies expressed lower levels of *SOX2* than these treatments (Figure 5-7c). In BEF derived cultures, the highest levels of *SOX2* expression was also observed in OSKMNL lentiviral cultures. Lower levels of expression were observed in OSNL lentiviral colonies, followed by Tet-OSKM and

OSNL non-viral cultures. The lowest expression of *SOX2* was observed in OSKMNL non-viral cultures (Figure 5-7d).

Again, there was no clear pattern of effect of reprogramming method on *c-MYC* expression. However, all non-virally derived cultures, except BDF OSKMNL colonies, expressed higher *c-MYC* than lentiviral colonies produced by the same combination of reprogramming factors. *c-MYC* expression was observed in all cultures except BEF Tet-OSKM lentivirally derived colonies (Figure 5-7e, f). In BDF derived cultures, as in *SOX2* expression, the highest levels of *c-MYC* expression were also observed in OSKMNL lentiviral cultures. Lower levels of *c-MYC* expression were observed in OSNL non-viral colonies, followed by Tet-OSKM and OSKMNL non-viral colonies. Tet-OSKM lentiviral colonies expressed lower levels still, followed by OSNL lentiviral colonies (Figure 5-7e). In BEF derived iPS cultures, the highest levels of *c-MYC* expression was observed in OSKMNL non-viral colonies, followed by OSNL non-viral colonies. OSNL and OSKMNL lentiviral colonies expressed lower levels of *c-MYC*. While Tet-OSKM non-viral followed by lentiviral colonies expressing the lowest levels of *c-MYC* (Figure 5-7f).

Again, there was no obvious pattern of effect of reprogramming method on the expression of *NANOG*. All cultures except BEF Tet-OSKM lentiviral colonies expressed *NANOG* (Figure 5-7g, h). Additionally, *NANOG* was not consistently observed at higher levels in cultures derived from reprogramming factor combinations containing *NANOG*. In BDF derived iPS cultures *NANOG* was once again expressed at the highest levels by OSKMNL lentiviral colonies. Lower *NANOG* levels were expressed by OSNL non-viral colonies, followed by Tet-OSKM non-viral and then OSKMNL non-viral colonies. Tet-OSKM and OSNL lentiviral colonies expressed the lowest *NANOG* levels (Figure 5-7g). In BEF derived cultures OSNL lentiviral colonies expressed the highest *NANOG* levels. Lower levels were expressed by OSKMNL lentiviral and Tet-OSKM non-viral colonies. The lowest *NANOG* levels were observed in OSKMNL non-viral and OSNL non-viral cultures (Figure 5-7h).

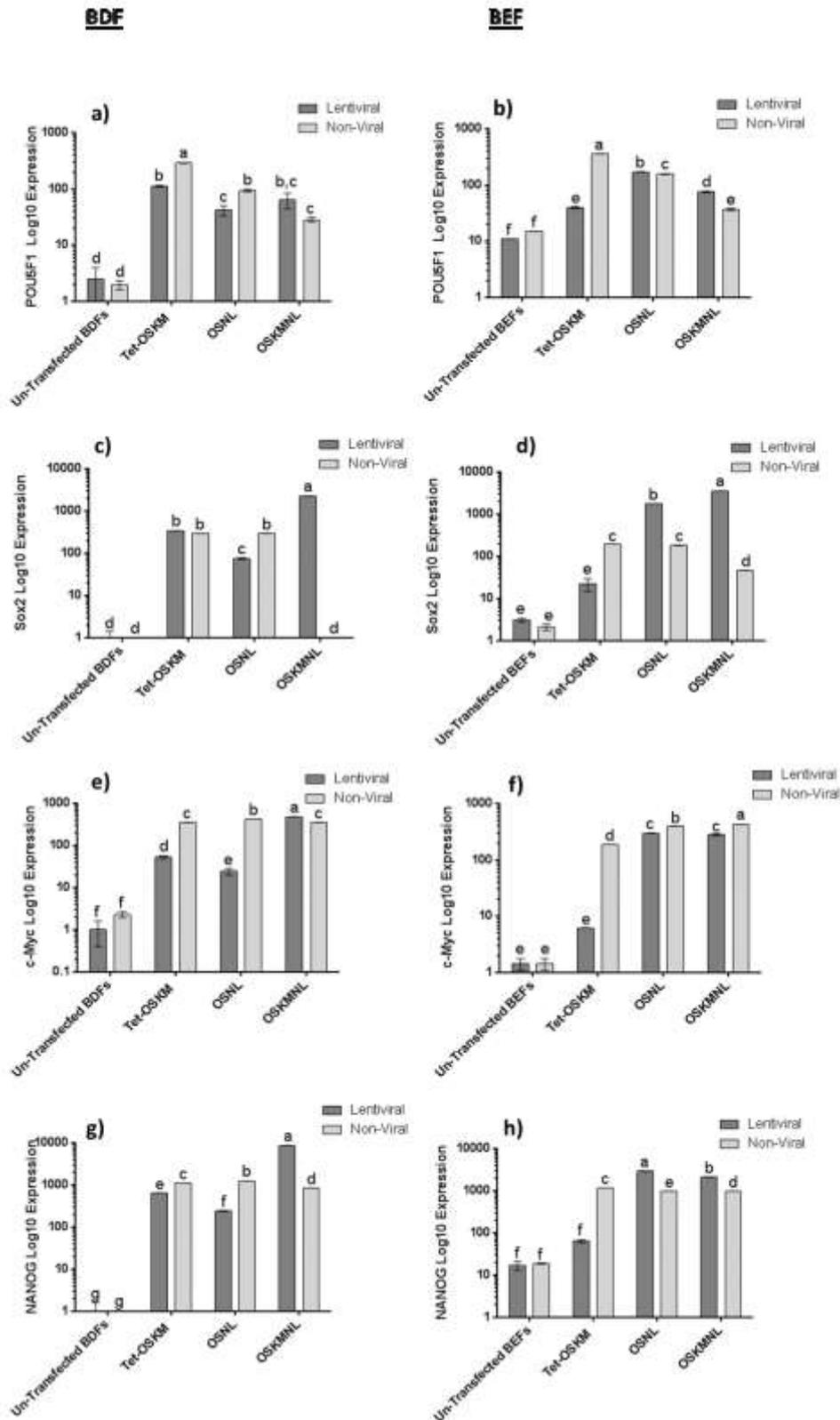


Figure 5-7: Log<sub>10</sub> Expression of pluripotency markers, relative to housekeeper gene *RPS26*, of putative lentiviral and non-viral bovine iPS colonies, derived by transfection of BEFs or BDFs with transcription factor combinations Tet-OSKM, OSNL or OSKMNL s. a) *POU5F1* expression by BDF derived bovine iPS colonies, b) *POU5F1* expression by BEF derived bovine iPS colonies, c) *SOX2* expression by BDF derived bovine iPS colonies, d) *SOX2* expression by BEF derived bovine iPS colonies, e) *c-MYC* expression by BDF derived bovine iPS colonies, f) *c-MYC* expression by BEF derived bovine iPS colonies, g) *NANOG* expression by BDF derived bovine iPS colonies, h) *NANOG* expression by BEF derived bovine iPS colonies. Different characters above treatment means indicate a significant difference in the expression of pluripotency markers ( $P < 0.05$ ,  $n=3$ ), error bars are represented as  $\pm$  SEM.

### 5.3.6 Some EBs Derived from Putative Non-Viral Bovine iPS Colonies Express Markers from all Three Germ Lineages

In order to derive embryoid bodies (EBs), some colonies were picked and transferred to low adherent cell culture dishes and grown in iPS media without growth factors or small molecules. Embryoid bodies were grown for two weeks with media changed every three days. EBs were then collected and analysed for lineage markers, *Tubb3* and *Nestin* (Ectoderm), *Desmin* (Mesoderm) and *FoxA2* (Endoderm) by qRT-PCR (Figure 5-8).

No EBs derived from lentiviral iPS colonies expressed markers from all three germ lineages. The only EB treatments to express markers from all three germ layers were derived from BDF OSNL non-viral and BEF OSKMNL non-viral iPS colonies. These EBs expressed high levels of *Tubb3*, *Desmin* and *FoxA2* (Figure 5-8). BDF Tet-OSKM lentivirally derived EBs expressed markers from the mesoderm and endoderm germ layers, with the highest *Desmin* and *FoxA2* expression of BDF derived cultures (Figure 5-8e, g). EBs derived from BDF OSNL lentiviral iPS colonies expressed both ectoderm markers *Tubb3* and *Nestin* (Figure 5-8a, c). Similarly, EBs derived from lentiviral BEF OSNL iPS colonies also expressed *Tubb3*, but did not show *Nestin* expression (Figure 5-8b, d). EBs derived from BEF OSNL non-viral colonies expressed markers from the ectoderm and mesoderm (Figure 5-8f, h). EBs derived from non-viral Tet-OSKM BEF iPS colonies also expressed low levels of *FoxA2* (Figure 5-8h).

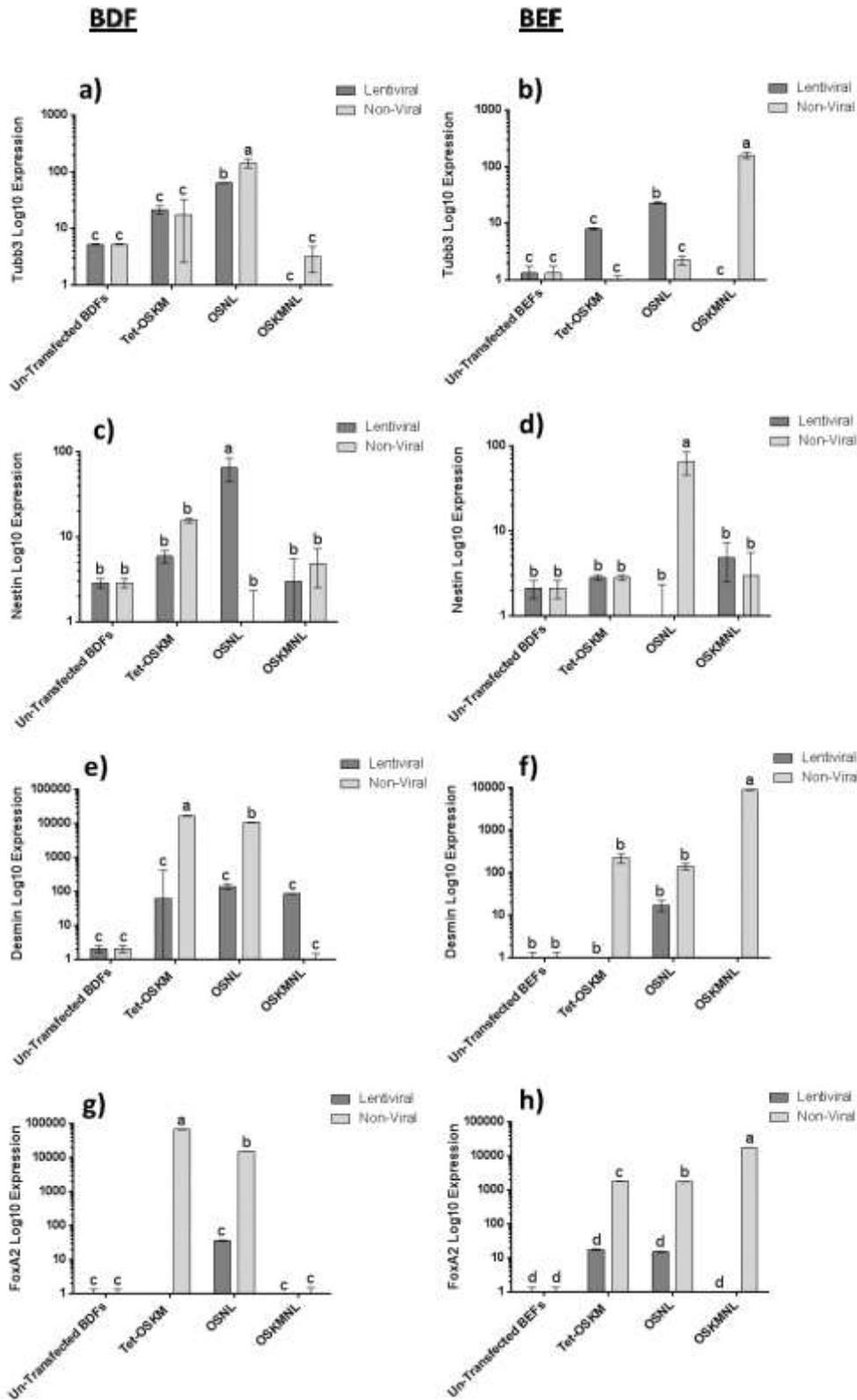


Figure 5-8. Log<sub>10</sub> Expression, relative to housekeeper gene *RPS26*, of lineage markers *Tubb3* (Ectoderm), *Nestin* (Ectoderm), *Desmin* (Mesoderm), *FoxA2* (Endoderm) expressed by embryoid bodies derived from lentiviral or non-viral transfection of BDF or BEFs with different combinations of transcription factors, Tet-OSKM, OSNL and OSKMNL. a) *Tubb3* expression by BDF derived iPS colonies, b) *Tubb3* expression by BEF derived iPS colonies, c) *Nestin* expression by BDF derived iPS colonies, d) *Nestin* expression by BEF derived iPS colonies, e) *Desmin* expression by BDF derived iPS colonies, f) *Desmin* expression by BEF derived iPS colonies, g) *FoxA2* expression by BDF derived iPS colonies, h) *FoxA2* expression by BEF derived iPS colonies. Different characters above treatment means indicate a significant difference in the expression of lineage makers ( $P < 0.05$ ,  $n = 3$ ). Error bars are represented as  $\pm$  SEM.

## **5.4 Discussion**

Since the initial production of induced pluripotent stem (iPS) cells by retroviral transduction, numerous studies have been conducted to improve the efficiency of producing iPS cells that may be used in clinical applications. Attempts have been made to move away from reprogramming methods that use viral or integrative methods, with non-viral, non-integrating methods of reprogramming deemed desirable. iPS cells by non-integrating methods are free from exogenous DNA and transgene integration and can therefore be potentially used in clinical applications. The production of livestock iPS cell has been more difficult than attempts to derive pluripotent cells from humans and mice. However, efforts have still been made to produce livestock iPS cells without permanent genomic alterations using non-viral methods (Huang, B. et al., 2011; Kues, Wilfried et al., 2013; Woltjen et al., 2009).

This chapter compares the production of putative bovine iPS cells using an integrative lentiviral vector and the non-viral, non-integrative electroporation of episomal plasmids. As discussed previously in Chapter 4, the OSNL and OSKMNL plasmids used to produce lentivirus in this chapter were not genuine lentiviral plasmids. However, evidence has been given both in this chapter and in Chapter 4 that lentivirus was in fact produced and these reprogramming constructs were delivered to the host cells where they were integrated into the host genome and expressed to result in reprogramming of the somatic cells to a partially reprogrammed state. This evidence included the positive assay tests of lentiviral titre by use of Lenti-X™ GoStix™, typical iPS colony morphology, the expression of pluripotency markers as shown by qRT-PCR analysis, and the integration of exogenous DNA into the host genome as shown by PCR of genomic DNA. The reasons these results may have been seen are discussed further in Chapter 4.

The results presented in this chapter demonstrate that non-virally derived bovine iPS colonies appear in culture two days faster than lentiviral method (10 days compared to 12 days). Cell type or the combination of transcription factors did not affect the time to colony formation in this instance. This suggests that cell type or combination of transcription factors, and therefore combination of reprogramming factors, did not affect reprogramming kinetics in these experiments. Mátrai, Chuah, and VandenDriessche (2010) report that low viral titre can result in low reprogramming efficiencies and slower reprogramming kinetics. The reliable production of high concentrations of lentivirus was difficult in these experiments without the equipment to concentrate virus to produce high

concentration titres. The low viral titre and therefore low transduction of viral particles may have caused the comparably longer time to colony formation, of lentiviral iPS cells. It is possible that reprogramming with episomal plasmids resulted in increased delivery of transgenes to the cells; thereby initiating reprogramming faster. This may explain why the colonies emerge faster and grow larger. The cells also appeared to respond better to electroporation than viral infection and following electroporation became confluent faster than lentiviral infected cells. As the non-viral cells recovered faster following the addition of the transgene, they may have begun dividing sooner to produce colonies in a shorter amount of time. However as the plasmids are not integrated into the host genome they are lost after numerous cell cycles, with plasmids derived from the Epstein Barr virus generally lost, or expression has decreased significantly after 7-14 days in the host cell (Einav et al., 2003). Cells may not have been fully reprogrammed after this time and began to differentiate, giving them an embryoid body like appearance. Similarly it is likely lentiviral iPS cells were not fully reprogrammed and therefore did not grow quickly and instead soon ceased to grow, perhaps becoming senescent, a process which can be triggered by the reprogramming process in which a permanent state of cell cycle arrest occurs (Campisi & d'Adda di Fagagna, 2007) (Banito, A. & Gil, 2010; Campisi & d'Adda di Fagagna, 2007).

The number of putative bovine iPS colonies produced after 21 days of culture was influenced more by cell type and combination of transcription factors used than by lentiviral or non-viral reprogramming methods. BDF non-viral iPS cultures produced higher numbers of colonies than BEF lentiviral or non-viral colonies. This is in contrast to previous claims that bovine embryonic or foetal fibroblasts are more easily reprogrammed than adult dermal fibroblast (Huang, B. et al., 2011). Additionally, although not significant, a pattern was observed where a lower number of colonies were produced by OSKMNL cultures than Tet-OSKM or OSNL cultures. This suggests that reprogramming using the polycistronic OSKMNL vector, by either lentivirus or electroporation, is less effective at producing iPS colonies than Tet-OSKM or OSNL cultures. This may be due to the large size of the OSKMNL construct (16500kb (Yu, J. et al., 2009)) compared to OSNL (15400kb (Yu, J. et al., 2009)) and Tet-OSKM (13376kb (Carey et al., 2009)), reducing transfection efficiency thereby decreasing reprogramming efficiency (Ribeiro et al., 2012). Previous studies have demonstrated that the transduction of smaller plasmids can increase the intracellular plasmid copy number by up to 2.6-fold. Additionally the number of cells expressing the proteins from the transduced plasmids can be twice the number compared to those transduced with larger plasmids (Ribeiro et al., 2012).

iPS colonies produced using electroporation of episomal plasmids did not show integration into the host genome, while those produced using lentiviral methods did show integration. This provides evidence that non-viral electroporation did not result in the permanent integration of transgenes into the host genome. As with the use of any iPS cells for clinical purposes, it is important that non-viral bovine iPS cells be free of transgenic integrations to lower the risk of tumorigenesis (Cela, 2009; Okita et al., 2008). Interestingly, although the Tet-OSKM plasmid used in these experiments is designed to be used in a lentiviral vector, and therefore contains a lentiviral backbone, integration of this plasmid into the host genome was not seen in the non-virally transfected cells that were analysed. It is important to note that as all the plasmids used in these experiments are DNA based there is still a potential, though lower risk, for plasmids to be integrated into the host cells genome (Stadtfeld et al., 2008; Zhou, W. & Freed, 2009). The producers (Yu, Junying et al., 2007) of the episomal plasmids OSNL and OSKMNL used in this chapter (Addgene plasmids 20922, and 20924 respectively), report no integration into the genome of host cells after extensive screening (Yu, J. et al., 2009). Although unexpected, the episomal plasmids showed integrating into the host genome in the experiment outlines in this chapter, confirming that it is possible for these plasmids to integrate. This unusual result has been discussed in length in Chapter 4. Additionally, although it was not seen in these experiments, it may be possible for these plasmids to integrate into the host genome when non-viral reprogramming methods are used. The results presented in this chapter indicate that putative bovine iPS cells were created without genetic integration into the host genome.

Both BEF and BDFs transfected non-virally, or with lentivirus coding for one of three of the combinations of transcription factors used in this experiment, were able to produce colonies that showed embryonic stem (ES) cell-like morphology. However, colony morphology did differ between viral and non-viral iPS colonies. Non-viral colonies generally grew faster and larger than the viral colonies and some developed into large dome shaped EB like structures. The viral iPS colonies remained smaller than the non-viral colonies and ceased growing noticeably after about one week. These colonies were flatter in appearance than the non-viral colonies and resembled early mouse iPS colonies.

No colonies produced in this experiment were considered fully reprogrammed as they did not stain for alkaline phosphatase activity or ES cell markers SSEA-4 or TRA 1-60. Protein expression of pluripotency makers by antibody staining was considered in this line of experiments; however it was unsuccessful due to technical difficulties associated with the inability of the colonies to adhere to

chamber slides for the staining protocol. The expression of pluripotency markers determined by qRT-PCR analysis was believed to be sufficient due to these cells only being described as partially reprogrammed.

When colonies were passaged they did not continue to grow, indicating these cells may have become senescent. As part of the reprogramming process pathways that lead to senescence and apoptosis of cells are triggered. Studies have found that inhibition of these responses can result in increased reprogramming efficiency and speed of reprogramming. It has been shown that manipulation of these processes can be achieved by introducing SV40 large T antigen (SV40 LT) and/or hTERT to somatic cells along with reprogramming factors. The addition of these additional factors has been shown to improve reprogramming of human fibroblast cells (Park, I.-H. et al., 2008). Additionally, as p53 has been shown to prevent reprogramming of cells with DNA damage, knockdown of p53 has also been shown to increase reprogramming efficiency of human and mouse cells in numerous studies (Banito, Ana et al., 2009; Marión et al., 2009; Zhao, Yang et al., 2008). Furthermore the addition of antioxidants such as vitamin C to cells can increase the reprogramming efficiency by transiently inhibiting senescence caused by the introduction of reprogramming factors (Banito, A. & Gil, 2010; Esteban et al., 2010). It is believed that cells that are older, such as adult fibroblasts, are closer to the onset of senescence making them more difficult to reprogram (Li, W. et al., 2009). In this experiment, there was no observable effect of cell type on time to reprogramming. However, there was an effect of cell type on the number of colonies produced. BEF lentiviral and non-viral cultures produced significantly fewer colonies than BDF non-viral colonies, and though not significant, notably lower numbers of colonies than BDF lentiviral cultures. This is in contrast to previous reports that younger cells may be easier to reprogram. However, as no colonies could be described as being fully reprogrammed it is difficult to determine if, in the event of complete reprogramming, BEF cultures would be more efficiently reprogrammed than BDF cultures.

Most treatments produced putative bovine iPS colonies that expressed pluripotency markers *POU5F1*, *SOX2*, *c-MYC* and *NANOG* at a higher level than the un-transfected cell type. This provided evidence for the pluripotent status. It was interesting that the BEF Tet-OSKM colonies did not express *SOX2*, *c-MYC*, or *NANOG* levels different to the transfected cell type. This is in contrast to previous experiments that used bovine foetal or embryonic fibroblasts to derive bovine iPS cells using the OSKM combination of transcription factors (Han et al., 2011). Different reprogramming and media protocols were used in this experiment; this may have influenced the ability to reprogram

bovine cells with the OSKM combination of transcription factors. Additionally, only 15 colonies were analysed for gene expression from each treatment. It is possible that other colonies from Tet-OSKM cultures that were not analysed did express pluripotency makers.

Additionally, optimal levels of expression of pluripotency markers in bovine ES cells or iPS cells have not been described. It is therefore difficult to assess pluripotency according to the levels of expression of these factors. Reports have shown that high OCT4 levels in excess of other factors results in fully reprogrammed mouse iPS cells (Tiemann et al., 2011). While other reports claim that high OCT4, KLF4 and c-MYC and low SOX2 expression results in the highest quality iPS cells (Nagamatsu et al., 2012). Though the putative bovine iPS cells described in this chapter expressed pluripotency markers, they may not have been at the optimal ratios to induce complete reprogramming.

Interestingly most colonies analysed express increased levels of endogenous *NANOG*. *NANOG* is expressed only weakly or not at all in partially reprogrammed iPS cells and as such is often used as a screening tool to isolate fully reprogrammed iPS cells (Okita et al., 2007; Silva et al., 2008; Silva, J. et al., 2009; Sridharan et al., 2009; Takahashi & Yamanaka, 2006). *NANOG* is also important in converting partially reprogrammed cells to fully pluripotent state (Silva, J. et al., 2009). Perhaps the levels of *NANOG* expression are not high enough in these cells to result in fully reprogrammed iPS cells. Alternatively, there may be other factors that are blocking the reprogramming process.

Although most iPS cultures expressed pluripotency markers, embryoid bodies (EBs) that expressed markers for all three germ layers, were only produced from BDF OSNL and BEF OSKMNL non-viral iPS colonies. Furthermore, the cultures that produced EBs with pluripotent characteristics did not express the highest levels of pluripotency markers. Instead BDF OSNL cultures consistently expressed the second highest levels of markers, while BEF OSKMNL cultures expressed relatively low levels of pluripotency markers. This further highlights the difficulties in assessing pluripotency based on the expression levels of pluripotency markers.

It is interesting that no lentiviral cultures were able to produce EBs that expressed markers from all three germ lineages as viral transduction is generally considered to be more efficient at producing iPS colonies than non-viral methods (O'Doherty et al., 2013). Additionally, the production of bovine

EBs capable of differentiating into cell types from all three germ lineages have previously been derived from virally derived iPS colonies (Cao et al., 2012; Han et al., 2011; Sumer, Liu, Malaver-Ortega, et al., 2011). The results from this chapter show that only non-viral BDF OSNL and BEF OSKMNL cultures produced EBs that were potentially pluripotent. EBs produced from lentiviral transduced iPS cells and other non-viral cultures were not pluripotent. It is possible that the iPS colonies that were used to produce these embryoid bodies may have had pluripotent potential at one stage, but may have lost pluripotency by the time EBs were produced. Alternatively the cells may have never been fully pluripotent despite their expression of pluripotency markers.

It is also interesting that the two cultures that produced EBs that expressed markers from all three germ lineages were reprogrammed using transcription factor combinations including NANOG. This supports the finding by Sumer, Liu, Malaver-Ortega, et al. (2011), that NANOG is required for producing bovine iPS cells. Additionally, reports have been made that reprogramming with a combination of six transcription factors can extensively improve human iPS cell reprogramming compared to the OSNL transcription factor cocktail (Liao et al., 2008). Although lower reprogramming efficiency was observed in OSKMNL cultures in this experiment, it is possible that BEF-OSKMNL iPS colonies described here were more fully reprogrammed than other BEF derived cells, as the colonies were able to produce EBs with pluripotent characteristics.

In either case, the iPS colonies that were produced in these experiments using non-viral electroporation were not fully reprogrammed. Although most treatments produced putative iPS colonies that expressed pluripotency markers, they did not exhibit alkaline phosphatase activity or pluripotency markers SSEA4 or TRA1-60 and were not able to be passaged. This indicated that colonies were not fully reprogrammed, and were instead to some extent partially reprogrammed. Some potential causes of partially reprogrammed cells are explained in Chapter 4. Reports have shown that cells that are partially reprogrammed may be converted to a fully reprogrammed state through the use of small molecules to assist in reprogramming and improve reprogramming efficiency (Mikkelsen et al., 2008). The cells described in this chapter and in chapter 4 were supplemented with a three molecule combination of small molecules, sodium butyrate (NaB) a HDAC inhibitor, PD0325901, a MEK inhibitor and SB431542, a TGF $\beta$  inhibitor. This combination of small molecules has previously been shown to increase the efficiency of reprogramming bovine cells to pluripotency through significantly decreasing the time to colony formation (McMillan, 2012). It is clear that although this combination of small molecule enhances reprogramming it does not result in

the production of fully reprogrammed bovine iPS cells. It is possible that different small molecule combinations may be used to enhance reprogramming further and potentially produce fully reprogrammed bovine iPS cells.

The results presented in this chapter indicate that non-viral transfection of bovine fibroblasts, may be more efficient at producing more fully reprogrammed cells than lentiviral transduced cells, as only non-virally derived EBs expressed markers from all three germ lineages. This result is contrary to other reports, that lentiviral transduction is more efficient than non-viral methods at producing iPS cells (González et al., 2011; Kane et al., 2008; O'Doherty et al., 2013). However, it is important to note that not all non-viral cultures produced EBs that expressed markers from all three germ lineages. Nevertheless, these results are encouraging and suggest that with optimised culture conditions, it may be possible to produce fully reprogrammed bovine iPS cells using non-integrating methods.

The addition of other small molecules or different small molecule combinations to iPS media has the potential to enhance the reprogramming process, and may result in the complete reprogramming of bovine somatic cells to pluripotency.

## **5.5 Conclusion**

The production of non-viral iPS cells without genomic modifications has generated immense interest in the field of stem cell biology due to the potential use of these cells in clinical applications.

Although the production of livestock, in particular bovine iPS cells has proven difficult, attempts to produce non-viral iPS cells from these species have been made. The production of non-viral livestock iPS cells offers the possibility of using iPS cells in transgenic breeding application in these animals.

This chapter describes the production of putative non-viral bovine iPS cells through the use of episomal plasmids and a minicircle vector. Contrary to current reports, in this chapter it has been demonstrated that the use of non-viral electroporation of reprogramming constructs may be more effective at producing bovine iPS cells than lentiviral reprogramming, at least under the conditions described here. However, the putative iPS cells produced using lentiviral transduction or non-viral electroporation are believed to be only partially reprogrammed. The work presented in this chapter has provided encouraging results that non-viral bovine iPS cells may be produced with further

optimisation of reprogramming conditions. It is hypothesised that the addition of small molecules or different media combinations could result in the full reprogramming of bovine somatic cells to pluripotency, or at least enhances the reprogramming of these cells.

## **Chapter 6 : Effect of Small Molecules on Bovine iPS production**

### **6.1 Introduction**

Since the first reports of reprogramming somatic cells to a pluripotent state were made (Takahashi & Yamanaka, 2006), the field of iPS generation has made major progress toward producing iPS cells from different species and creating safer methods of producing iPS cells for potential use in clinical applications. However, the production of iPS cell still has inherent problems. Though non-viral reprogramming has addressed problems of insertional mutagenesis caused by integrating vectors, the inherently problematic use of oncogene transcription factors including c-MYC is still common. Additionally, the reprogramming efficiency of non-viral methods is far lower than viral reprogramming. Furthermore the derivation of partially reprogrammed iPS colonies and slow speed of generating fully reprogrammed iPS cells, also inhibits this technology. The use of small molecules to enhance the reprogramming process has provided hope to potentially alleviate these problems by increasing reprogramming efficiency and replacing traditional reprogramming factors (Efe & Ding, 2011; O'Doherty et al., 2013; Sumer et al., 2010).

Increased understanding of the molecular mechanisms and pathways involved in reprogramming and transcriptional regulation of reprogramming factors has resulted in the ability to modulate and improve the reprogramming process by the use of small molecules. Small molecules have the benefit of enhancing reprogramming in several ways. Small molecules that act as epigenetic modifiers improve reprogramming efficiency through affecting cellular plasticity, alternatively small molecule compounds can act to modulate a pathway specific for reprogramming, allowing them to replace transcription factors that would normally act on the pathway (Efe & Ding, 2011). Small molecules have been reported to functionally replace traditional reprogramming factors including SOX2 (Ichida et al., 2009; Li, W. et al., 2009; Li, Wenlin et al., 2009) in murine and human cells, KLF4 and c-MYC (Shi, Y., Despons, Do, Hahm, & Schöler, 2008; Wernig, Meissner, Cassady, & Jaenisch) in murine cells. Several pathways including the Wnt-  $\beta$ -catenin, calcium-cAMP, MEK/ERK and TGF- $\beta$  pathways, have been identified as being involved in the reprogramming process. Small molecules that illicit effects on these pathways have been found to improve reprogramming efficiency and enhance the iPS cells produced (Efe & Ding, 2011; Sumer et al., 2010).

The mitogen-activated protein kinase (MEK/ERK) pathway, is believed to regulate the activity of c-MYC along with other transcription factors including NANOG (Wu, Bi et al., 2013). This pathway is capable of activating lineage committed differentiation of iPS cells (Barbosa et al., 2012; Kunath et al., 2007; Li, J. et al., 2007; Zhang, X. et al., 2013) and inhibition of the ERK pathway results in self-renewal of both mouse and human ES cells (Wu, B., Li, W., Wang, L., Liu, Z., & Zhao, X., 2013). The MEK/ERK pathway can be inhibited through the use of small molecule MEK inhibitors including PD0325901 or PD98059 or the ERK inhibitor PD184352. These molecules promote cellular reprogramming and self-renewal by blocking the signals of the MEK pathway, thereby inhibiting the differentiation of iPS cells (Li, W. et al., 2009; Ying, Q.-L. et al., 2008). The small molecule PD184352 has been found to enhance pluripotency in mouse ES cells (Ying, Q.-L. et al., 2008). Additionally, iPS cell generation can be enhanced with the addition of MEK inhibitor PD0325901, with the effects of the molecule believed to be most important during the later stages of reprogramming to promote growth of iPS cells (Shi, Yan et al., 2008).

Inhibition of the transforming growth factor  $\beta$  (TGF- $\beta$ ) pathway has also been found to influence reprogramming efficiency. In murine cells the inhibition of this pathway is believed to affect reprogramming in two ways, first through the up-regulation of NANOG (Ichida et al., 2009), and secondly through facilitating the mesenchymal to epithelial transition (MET) pathway (Zhou, H. & Ding, 2010). For correct differentiation of cells to occur during early embryonic development, it is essential that cells undergo an epithelial to mesenchymal transition (EMT) (Chaffer et al., 2007). The opposite transition of mesenchymal to epithelial lineage has been shown to result in the reprogramming of somatic fibroblasts to iPS cells (Li, R. et al., 2010). It is believed that this transition occurs by suppressing pro-EMT signals and results in the activation of epithelial signals inside the cells. Transcription of SOX2 and OCT4 suppresses the EMT mediator, Snail, while KLF4 induces the expression of epithelial genes, and c-MYC results in the down regulation of products of the transforming growth factor  $\beta$  (TGF- $\beta$ ) pathway, TGF- $\beta$ 1 and TGF- $\beta$  receptor 2 (Li, R. et al., 2010). The inhibition of TGF- $\beta$  with small molecules such as SB431542 and AM-83-01 has been shown to result in improved reprogramming efficiency of murine iPS cells (Ladewig, Mertens, Kesavan, Doerr, & Poppe..., 2012; Li, W. et al., 2011; Li, W. et al., 2009). Additionally, reports have shown that by targeting the TGF- $\beta$  pathway it is possible to produce murine iPS cells without KLF4 and c-MYC, or SOX2 and c-MYC (Ichida et al., 2009; Li, W. et al., 2009; Maherali & Hochedlinger, 2009). Reports have also been made that inhibition of AMI-5 mediated protein arginine methyltransferase expression by AM-83-01, which also targets the TGF- $\beta$  pathway, can result in cellular reprogramming of mouse fibroblasts with transduction of OCT4 only (Yuan, Xu. et al., 2011).

Another class of small molecules acts by affecting the epigenetics of somatic cells to enhance plasticity and improve reprogramming. The regulation of expression and suppression of genes is primarily determined by the structure of chromatin, in particular the acetylation or deacetylation of lysine in the tails of the histone cores. Acetylation and deacetylation is controlled by histone deacetylases (HDACs) and histone acetyltransferases (HATs) respectively (Marks et al., 2004; Monneret, 2005). It is thought that the reprogramming of somatic cells to pluripotency requires the modification of the epigenetic chromatin structure that is normally stable in somatic cells (Zuccotti, Garagna, & Redi, 2000). In order to facilitate the process of modification of chromatin structure, specifically the acetylation of histones, HDAC inhibitors can be used to return the genome to a transcriptionally permissive state (Zhao, J. et al., 2010). Butyrate is a naturally occurring short chain fatty acid that acts as a histone deacetylase (HDAC) inhibitor, to support the self-renewal of human and mouse embryonic stem (ES) cells and iPS cells (Liang et al., 2010; Ware et al., 2009). Butyrate has been shown to not only increase the number of fully reprogrammed iPS colonies, but to also decrease the number of partially reprogrammed iPS cells derived (Liang et al., 2010). It is thought that the action of butyrate is mediated by c-MYC, and has the largest effect when added at the beginning of reprogramming. Butyrate is able to cause the up-regulation of pluripotency genes during reprogramming (Liang et al., 2010). Conversely high concentrations of butyrate have been shown to induce differentiation in pluripotent cells (Newmark, Lupton, & Young, 1994). Butyrate appears to facilitate reprogramming of mouse iPS cells when applied at a range of 0.5-1mM (Liang et al., 2010).

Valproic acid (VPA) is another HDAC inhibitor and is generally considered to be the most potent HDAC inhibitor (Kim, Y. et al., 2011). Studies have shown VPA is capable of improving reprogramming efficiency of human and mouse cells by more than 100 fold compared to reprogramming with no small molecule supplementation (Huangfu et al., 2008), with strong effects on porcine improving the development of porcine embryos in vitro (Kim, Y. J., Ahn, K. S., Kim, M., & Shim, H., 2011). VPA also shows more potent activity on reprogramming than similar small molecules including 5'-azacytidine, a DNA methyltransferase inhibitor, which results in an increase in efficiency of reprogramming of only 10-fold (Huangfu et al., 2008; Kim, Y. et al., 2011).

In addition to the ability of VPA to improve reprogramming efficiency, it has also been reported to negate the use of some exogenous reprogramming factors. The production of retroviral human iPS

cells with the combination of three transcription factors OCT4 (*POU5F1*), SOX2 and KLF4, in conjunction with VPA supplementation, has been shown to result in the highly efficient derivation of iPS cells (approximately 1% reprogramming efficiency), that were also able to contribute to germline transmission (Huangfu et al., 2008). iPS cells generated with only OCT4 and SOX2 has also been reported by treatment of human fibroblasts with VPA during the reprogramming process, although reprogramming efficiency was far lower (approximately 0.001%) (Huangfu et al., 2008). The supplementation of VPA has successfully produced iPS cells without then need for exogenous oncogenes. However, it has been shown that VPA alone is not sufficient to reprogram cells to pluripotency (Huangfu et al., 2008). It is also possible that treatment with VPA increases the expression of endogenous KLF4 and c-MYC, thereby rendering the transfection of exogenous transgenes unnecessary (Stadtfield & Hochedlinger, 2010). It has also been proposed that VPA not only affects the epigenetics of somatic cells making them more amenable to reprogramming, but also enhances pluripotency by direct activation of OCT4 transcription and by repression genes involved in differentiation (Teng et al., 2010).

However, it has also been reported that VPA supplementation does not work to improve reprogramming efficiency, but instead results in the suppression of growth on un-reprogrammed cells (Yusa, Rad, Takeda, & Bradley, 2009). This study showed that the supplementation of VPA in murine iPS cultures produced colonies that were indistinguishable from ES cells, being more spherical in morphology, but also smaller than colonies that were not treated with VPA. This study concluded that thus, VPA appears to suppress un-reprogrammed cell growth and facilitates the growth and expansion of reprogrammed cells.

Synergistic effects from targeting multiple pathways involved in reprogramming have been shown to dramatically enhance reprogramming efficiency. The dual combination of MEK inhibitor PD0325901 and TGF- $\beta$  inhibitor SB431542 has been shown to considerably improve the generation of human iPS cells (more than 100 fold) with efficiencies of over 1% recorded (Lin et al., 2009). This increase is believed to be due to enhancing the MET pathway (Lin et al., 2009). Furthermore, the addition of Thiazovivin, a small molecule that directly targets Rho-associated kinase (ROCK), to the PD0325901 and SB431542 small molecule combination, has reportedly improved reprogramming efficiency by over 200 fold (Xu, Y. et al., 2010). The addition of the combination of PD032901, SB431542 and HDAC inhibitor sodium butyrate (NaB), facilitates more efficient reprogramming of human iPS cells through faster reprogramming of cells and by significantly decreasing the numbers of partially reprogrammed colonies when compared to reprogramming with transcription factors alone (Zhang, Zhonghui et al., 2011). The combination of small molecule MEK inhibitor PD0325901, and CHIR99021

targeting glycogen synthase kinase 3 (GSK3), is a commonly used small molecule combination known as 2i. 2i media has been shown to significantly improve reprogramming in numerous species including murine (Silva et al., 2008) bovine (Huang, B. et al., 2011) and porcine (Esteban et al., 2009). In each cocktail of small molecules, the efficiency of reprogramming was increased with each additional small molecule added; indicating synergistic effects small molecules have on reprogramming of iPS cells.

To date the use of small molecules in reprogramming of livestock cells to a pluripotent state has yet to be fully explored. Some reports of livestock iPS cell generation have implemented small molecule supplementation to enhance reprogramming (Huang, B. et al., 2011; Nagy et al., 2011), however, most reports of iPS cell generation from livestock species have been made without the use of small molecules. However, it is important to note that these protocols have not necessarily resulted in fully reprogrammed iPS cells to the extent expected from mouse and human cells. In the first reports of derivation of equine iPS cells a combination of five small molecules, MEK inhibitor PD0325901, TGF- $\beta$  inhibitor A83-01, Thiazovivin targeting ROCK, and ALK receptor inhibitor SB431542, were used (Nagy et al., 2011). As this was the first report of successful equine iPS cell generation, and results have yet to be replicated independently, it is not clear if the combination improves reprogramming efficiency or if these molecules are in fact necessary for reprogramming to occur.

As stated previously 2i media (PD0325901 and CHIR99021) has been used to derive iPS cells from porcine and bovine species (Esteban et al., 2009; Huang, B. et al., 2011). 2i media has been reported to promote the complete reprogramming of porcine iPS cells through enhancing cell compaction and proliferation (Esteban et al., 2010). Huang, B. et al. (2011) reported the non-viral derivation of bovine iPS cells was enhanced through the addition of 2i media, with the expression of pluripotency markers only occurring when cells were supplemented with 2i small molecules. However, the cells that were produced in this study were not fully reprogrammed and could not be expanded in culture. Conversely other reports of bovine iPS cell derivation have not used any small molecule supplementation (Sumer, Liu, Malaver-Ortega, et al., 2011). Considering the difficulties experienced in producing fully reprogrammed iPS cells from livestock species it is reasonable to assume that the supplementation of these cells with small molecule combinations may have profound effects on the reprogramming process, and could potentially lead to the derivation of fully reprogrammed livestock iPS cells.

The effect of some different combinations of small molecules, including the use of 2i media has previously been tested for reprogramming bovine cells to pluripotency at the F D McMaster laboratory, Armidale, NSW (McMillan, 2012). This study found that the combination of three small molecules sodium butyrate (NaB) a histone deacetylase (HDAC) inhibitor, TGF- $\beta$  inhibitor SB431542(SB) and MEK inhibitor PD0325901 (PD) (NaB-SB-PD), originally described by Zhang, Zhonghui et al. (2011) has the largest effect of combinations tested on improving reprogramming of these cells. The NaB-SB-PD cocktail of small molecules was shown to accelerate the kinetics of bovine iPS production with putative colonies observed after an average of 12 days, compared to 21 days after lentiviral transduction. The colonies produced with the NaB-SB-PD supplementation also stained positively for alkaline phosphatase (McMillan, 2012). However, the colonies reported here were not fully reprogrammed and could not be expanded following passage.

The first aim of this chapter was to determine if the addition of valproic acid, known to significantly improve reprogramming of murine and human iPS cells, to the existing cocktail of small molecules, NaB-SB-PD, enhanced reprogramming of bovine iPS cells. This was determined by comparing time to colony formation, reprogramming efficiency and characteristics of putative bovine iPS colonies for those supplemented with NaB-SB-PD combination and with the addition of VPA, VPA-NaB-SB-PD combination. The second aim of this chapter was to determine the effects on reprogramming of adding the small molecules VPA, NaB, PD0325901 and SB431542 individually, and in different combinations. This was determined in the same way as described above.

## **6.2 Materials and Methods**

### **6.2.1 General**

All experiments involving animals were approved and conducted under the F D McMaster Laboratory Animal Ethics Committee for CSIRO Animal, Health and Food Sciences (AEC 11/20). Cell culture reagents were purchased from Life Technologies Corporation (USA), Sigma-Aldridge Chemical Industries (USA), and Stemgent (USA). Primers used in Chapter 6 were designed using Primer3 software, and ordered through Biosearch Technologies, Inc. Primers used in Chapter 6 are

shown in Table 6-1. This experiment was repeated three times with two technical replicates for each treatment per experiment.

### 6.2.2 Derivation of Bovine Fibroblasts

Bovine dermal fibroblasts (BDFs) were isolated from skin samples from Angus male calves taken during castration that was performed under general anaesthesia. Bovine embryonic fibroblasts (BEFs) were isolated from a Male *Bos taurus* bovine foetus that was sourced from a local abattoir from slaughtered cattle. The foetus measured approximately 18cm crown to rump, giving an estimated age of 60-70 days gestation. Primary cell cultures were prepared as described in Chapter 4 (pg. 114). Fibroblast cells were grown in fibroblast growth media (DMEM, 10% Foetal Bovine Serum (FBS), 2 mM GlutaMAX™, 100 µM non-essential amino acids (NNEA), with 1% anti-anti (10,000 U/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®) (all components from Life Technologies, USA), and were passaged every two to three days.

### 6.2.3 Non-Viral Reprogramming of Fibroblasts

BEF and BDF cells were non-virally transfected with one of three reprogramming constructs coding for different combinations of transcription factors described in Table 6-2, Tet-OSKM (Addgene plasmid # 20321 (Carey et al., 2009)) with tetracycline transactivator M2rtTA (Addgene plasmid #20324 (Hockemeyer et al., 2008)), OSNL (Addgene plasmid #20922 (Yu, J. et al., 2009)) or OSKMNL (Addgene plasmid # 20924 (Yu, J. et al., 2009)). Transfection was achieved using a Neon™ Transfection System (Life Technologies), according to the manufactures instructions outlined in Chapter 5 (pg. 152). Once electroporated cells had reached 80% confluence (after approximately 3 days) they were harvested enzymatically using TrypLE™ Express and plated at a rate of  $5 \times 10^4$  onto 10 cm gelatine coated tissue culture dishes containing Mitomycin-C (Mit-C) inactivated BEF feeder layers ( $2 \times 10^6$  feeders/dish) as described in Chapter 4 (pg. 117). Doxycycline (2 µg/ml) was added to dishes with Tet-OSKM transfected cells to activate the expression of the transgene. Cultures were fed with iPS media (Minimum Essential Media Alpha (MEM-α), 20% FBS, 1% insulin transferrin-selenium (ITS), 2 mM GlutaMAX™, 100 µM NEAA, 50 U/mol penicillin, 50 mg/ml streptomycin, 0.1 mM β-mercaptoethanol (Sigma, USA), 4 ng/ml human leukemia inhibitory factor (LIF, Millipore, USA), and 10 ng/ml basic fibroblast growth factor (bFGF, Millipore, USA). Putative iPS cultures were

supplemented with the small molecule, sodium butyrate (NaB) (0.5 mM, Sigma, USA), PD0325901 (PD) (0.5  $\mu$ M, Stemgent, USA), SB431542 (SB) (2  $\mu$ M, Stemgent, USA) and valproic acid (VPA) (1 mM, Sigma, USA), in the combinations listed in Table 6-3 and Table 6-4.

Control cultures of un-transfected bovine fibroblasts and non-viral iPS cultures were grown in parallel under the same conditions for 21 days with media changed every two days.

### 6.2.5 Embryoid Body (EB) Production

Following the culture of iPS colonies for 21 days, colonies were picked, washed in PBS, and frozen at  $-80^{\circ}\text{C}$  for later RNA isolation for qRT-PCR analysis, or used to derive embryoid bodies. In short colonies used to derive embryoid bodies were manually picked and transferred to ultra-low-adherent 6cm cell culture dishes, and grown in iPS media without growth factors or small molecules (Minimum Essential Media Alpha (MEM- $\alpha$ ), 20% FBS, 1% insulin transferrin-selenium (ITS), 2 mM GlutaMAX<sup>TM</sup>, 100  $\mu$ M non-essential amino acids (NNEA), 50 U/mol penicillin, 50 mg/ml streptomycin, 0.1 mM  $\beta$ -mercaptoethanol (Sigma, USA)). Embryoid bodies were grown for 14 days, with media changed every three days, after which the EBs were harvested and stored at  $-80^{\circ}\text{C}$  until qRT-PCR analysis was carried out.

### 6.2.6 Analysis of Putative Bovine iPS Colonies

The iPS cultures from both experiments were observed daily for colony formation, and the time to colony formation was recorded for each treatment. Following the culture of iPS cells for 21 days, colony numbers were recorded per 10cm plate, and the reprogramming efficiency for each treatment was determined by the total number of putative colonies produced as a percent of the total number of initially plated transfected cells. Photographs of developing colonies were taken using a Nikon Eclipse TE300 inverted microscope (Nikon, Japan) equipped with a cooled *PE* light source (COOLED, USA), and images were captured using ProgRes CapturePro 2.1 software (Jenoptik, Germany).

Colonies were stained for alkaline phosphatase activity using the alkaline phosphatase live stain (Life Technologies, USA) according to manufactures instructions outlined in Chapter 4 (pg. 119). Stained

cells were visualised using an inverted fluorescent microscope (Nikon, Japan) using a standard FITC filter. Immunofluorescent staining for pluripotency markers SSEA4 and Tra-1-60 was carried out using the ES Cell Characterisation kit (Millipore, USA) as described in Chapter 5 (pg. 154).

### 6.2.6 mRNA Extraction and qRT-PCR analysis

mRNA was extracted from 5 pooled colonies or embryoid bodies for each treatment using Dynabeads® mRNA DIRECT™ Micro Kit according to the manufactures instructions (Life Technologies, USA) described in Chapter 4 (pg. 120). cDNA synthesis was carried out using the SuperScript™ III First-Strand Synthesis System (Life Technologies, USA) described in Chapter 4 (pg. 120). qRT-PCR reactions for expression of pluripotency and lineage markers were carried with the out as described previously in Chapter 4 (pg. 120). The reference gene *RPS26* was previously identified as the best housekeeper for the cells used in this chapter using the sheep GeNorm kit (PrimerDesign, UK) (McMillan, 2012). Primers used for qRT-PCR reactions are described in Table 6-1.

### 6.2.7 Statistical Analysis

qRT-PCR reactions were analysed in Microsoft Excel add-in Genex, Ct values from the raw analysis were converted into gene expression data as a fold change relative to reference gene *RPS26*. Statistical analysis of data was performed using GraphPad Prism® software (GraphPad Software, USA). For all results of qRT-PCR analysis two-way ANOVAs with Tukey's multiple comparison tests were used to determine if statistically significant differences existed between group means, ( $P < 0.05$ ). Results are presented as means  $\pm$  SEM. For results of time to colony formation and number of colonies T-Tests were conducted in GraphPad Prism® with a Welch's correction to determine if there were statistically significant differences in treatment means ( $P < 0.05$ ). The reprogramming efficiency of cultures was calculated after 21 days of culture and was determined by the total number of colonies observed divided by the total number of transfected cells initially plated ( $5 \times 10^4$  per dish).

Table 6-1. Primers used in Chapter 6.

Gene Construct	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Size (bp)	Reaction Efficiency (%)	Melt Peak (°C)	Specificity
<b>Pluripotency Genes</b>						
<i>POU5F1</i> (OCT4)	AAGCGGACGAGTATCGAGAA	ACACTCGGACCACGTCTTTC	133	113.8	86	Bovine mRNA
<i>SOX2</i>	ATGAAGGAACACCCGGATTA	CATGTGCGCGTAGCTGTC	186	81.2	89	Bovine
<i>NANOG</i>	GTCCCGGTCAAGAAACAAAA	TCTGGAACCAGGTCTTCACC	163	106.1	87	Bovine mRNA
<i>c-MYC</i>	GGAAGAAATTCGAGCTGCG	GTCGCAGATGAAGCTCTGGT	226	105.7	87	Bovine mRNA
<b>Lineage Markers</b>						
<i>TUBB3</i>	TGGAGCGCATCAGTGTCTAC	CAGTTGTTACCAGCCCCACT	172	99.3	87.5	Endogenous
<i>NESTIN</i>	AAAGAAGGCTGGGATCCTGT	CCTCAAACCTTCCGACAGC	130	108.9	88	Endogenous
<i>DESMIN</i>	GGGACATCCGTGCTCAGTAT	GTGGCGGTACTCCATCATCT	155	120.8	86.5	Endogenous
<i>FoxA2</i>	ACCACTACGCCTTCAACCAC	GGGGTAGTGCATCACCTGTT	134	85.8	86	Endogenous
<b>Reference Gene</b>						
<i>RPS26</i>	TCATTCGGAACATCGTAGAGG	CCTGACTACCTTGCTGTGAAT				Endogenous/ Reference Gene

Table 6-2. Plasmids sourced from Addgene plasmid repository, for use in reprogramming experiments carried out in chapter 6.

Plasmid	Referred to as	Description	Addgene Plasmid Reference	Principle Investigation
<b>TetO-FUW-OSKM</b>	Tet-OSKM	Polycistronic plasmid for Tet-inducible expression of murine <i>POU5F1</i> , <i>SOX2</i> , <i>KLF4</i> and <i>Myc</i> for iPS cell generation	20321	(Carey et al., 2009)
<b>FUW-M2rtTA</b>	M2rtTA	Lentiviral plasmid expressing the reverse tetracycline transactivator for Doxycycline inducible control of expression	20324	(Hockemeyer et al., 2008)
<b>pEP4 E02S EN2L</b>	OSNL	Episomal vector expressing human <i>POU5F1</i> , <i>SOX2</i> , <i>NANOG</i> and <i>LIN28</i> for the production of iPS cells	20922	(Yu, J. et al., 2009)
<b>pEP4 E02S CK2M EN2L</b>	OSKMNL	Episomal vector containing human <i>POU5F1</i> , <i>SOX2</i> , <i>Lin 28</i> , <i>NANOG</i> , <i>KLF4</i> and <i>c-MYC</i> for the production of iPS cells	20924	(Yu, J. et al., 2009)
<b>psPAX2</b>	psPAX2	Empty vector backbone	12260	
<b>pMD2.G</b>	pMD2.G	Mammalian Expression ; Envelope	12259	
<b>PGK-H2BeGFP</b>	eGFP	Constitutive vector encoding for eGFP	21210	(Kita-Matsuo et al., 2009)

## 6.3 Results

### Experiment 1- The Effect of the addition of VPA to small molecule cocktail NaB-SB-PD.

To determine the effect of valproic acid (VPA) supplementation in addition to the currently used small molecule combination of NaB-SB-PD on reprogramming bovine cells to a pluripotent state, , , a 3x2x2 factorial design was employed. Bovine dermal fibroblasts (BDFs) and bovine embryonic fibroblasts (BEFs) were non-virally transfected with one of three reprogramming constructs coding for transcription factor combinations Tet-OSKM, OSNL or OSKMNL. iPS cultures were grown for 21 days with supplementation of small molecule combinations NaB-SB-PD or VPA-NaB-SB-PD (Table 6-3). This design featured two technical replicates for each treatment, with the entire experiment replicated three times.

Table 6-3. Small molecule combinations tested in experiment 1.

Experiment 1	Small Molecule Combinations
Treatment 1 (control)	NaB SB431542 PD0325901
Treatment 2 (VPA)	VPA NaB SB431542 PD0325901

#### 6.3.1 Valproic Acid Accelerates Reprogramming Kinetics and Improves Reprogramming Efficiency

The time to colony formation was significantly lower in cultures supplemented with VPA in addition to the combination of three small molecules NaB-SB-PD. Colonies appeared on average after 10 days of culture when supplemented with the VPA-NaB-SB-PD small molecule combination, as opposed to 12 days in cultures supplemented with the NaB-SB-PD combination alone (Figure 6-1). There was no effect of cell type observed on the time to colony formation. There was no difference in time to colony formation of BDF and BEF cultures supplemented with the same small molecule combination (Figure 6-1b). There was no difference in time to colony formation observed between cultures transfected with different combinations of transcription factors when cultures were supplemented

with the same small molecule combination (Figure 6-1c). No colonies were observed in control cultures.

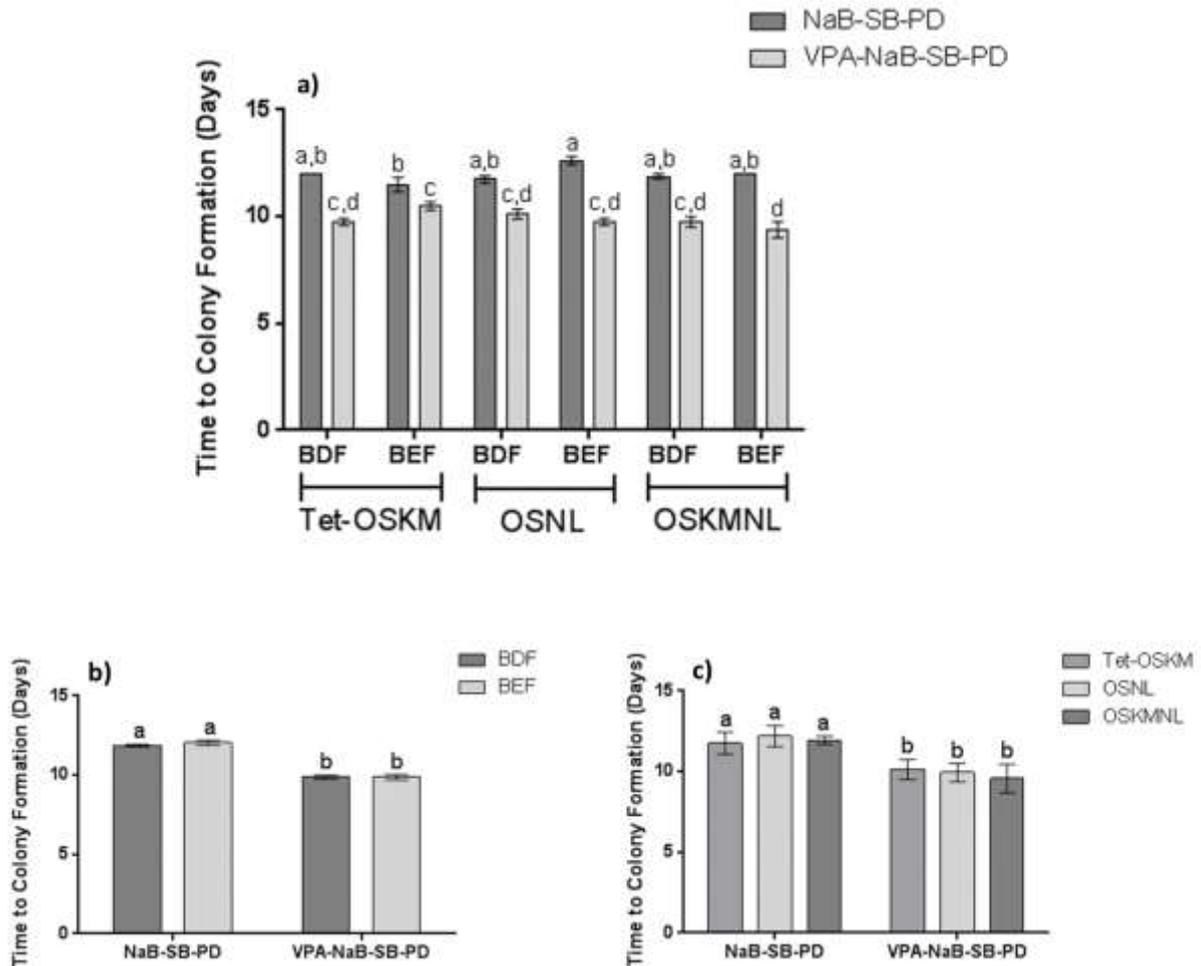


Figure 6-1. Time to colony formation of colonies supplemented with small molecule combinations NaB-SB-PD compared to colonies supplemented with VPA-NaB-SB-PD. a) Time to colony formation of BDF or BEFs transfected with different combination of transcription factors Tet-OSKM, OSNL or OSKMNL, supplemented with small molecule combinations NaB-SB-PD or VPA-NaB-SB-PD ( $P < 0.05$ ,  $n = 6$ ). b) The effect of small molecule combinations NaB-SB-PD and VPA-NaB-SB-PD time to colony formation in two cell types BDF and BEFs ( $P < 0.05$ ,  $n = 18$ ). c) Effect of small molecule combinations NaB-SB-PD and VPA-NaB-SB-PD on time to colony formation of cultures transfected with different combination of transcription factors, Tet-OSKM, OSNL and OSKMNL ( $P < 0.05$ ,  $n = 12$ ). \*VPA= Valproic acid (1 mM), NaB= Sodium butyrate (0.5 mM), SB= SB431542 (2  $\mu$ M), PD= PD0325901 (0.5  $\mu$ M), BDF= bovine dermal fibroblasts, BEF= bovine embryonic fibroblasts. Different characters above treatment mean indicate a significant difference between treatments. Error bars are represented as  $\pm$  SEM.

The number of putative iPS colonies observed after 21 days of culture was significantly higher in all cultures supplemented with the small molecule cocktail containing VPA compared to cultures supplemented with the NaB-SB-PD small molecule combination alone (Figure 6-2). An average of five colonies were present in NaB-SB-PD cultures following 21 days of culture, while an average of 26 colonies were present in cultures supplemented with VPA-NaB-SB-PD.

There was no effect observed of cell type on the number of colonies produced by treatments supplemented with either the VPA-NaB-SB-PD or NaB-SB-PD small molecule combinations. BDF and BEF cultures both produced significantly higher numbers of colonies when supplemented with VPA-NaB-SB-PD than when supplemented with NaB-SB-PD alone (Figure 6-2b).

Additionally, there were significantly fewer colonies observed in OSKMNL transfected cultures than Tet-OSKM or OSNL treatments when supplemented with the VPA-NaB-SB-PD small molecule cocktail. Furthermore, although not significantly different, the same trend was observed in the treatments supplemented with the three small molecule combination, NaB-SB-PD (Figure 6-2c). There were no observable differences in the numbers of colonies produced by Tet-OSKM and OSNL cultures when supplemented with the same small molecule combinations. The reprogramming efficiency, as calculated by the total number of putative colonies observed per treatment, was 0.034% in cultures supplemented with VPA-NaB-SB-PD, and 0.008% in cultures supplemented with NaB-SB-PD alone. Furthermore the reprogramming efficiency of when accounting for only Tet-OSKM and OSNL cultures supplemented with VPA was approximately 0.062%, with an average of 31 colonies produced per dish in these treatments.

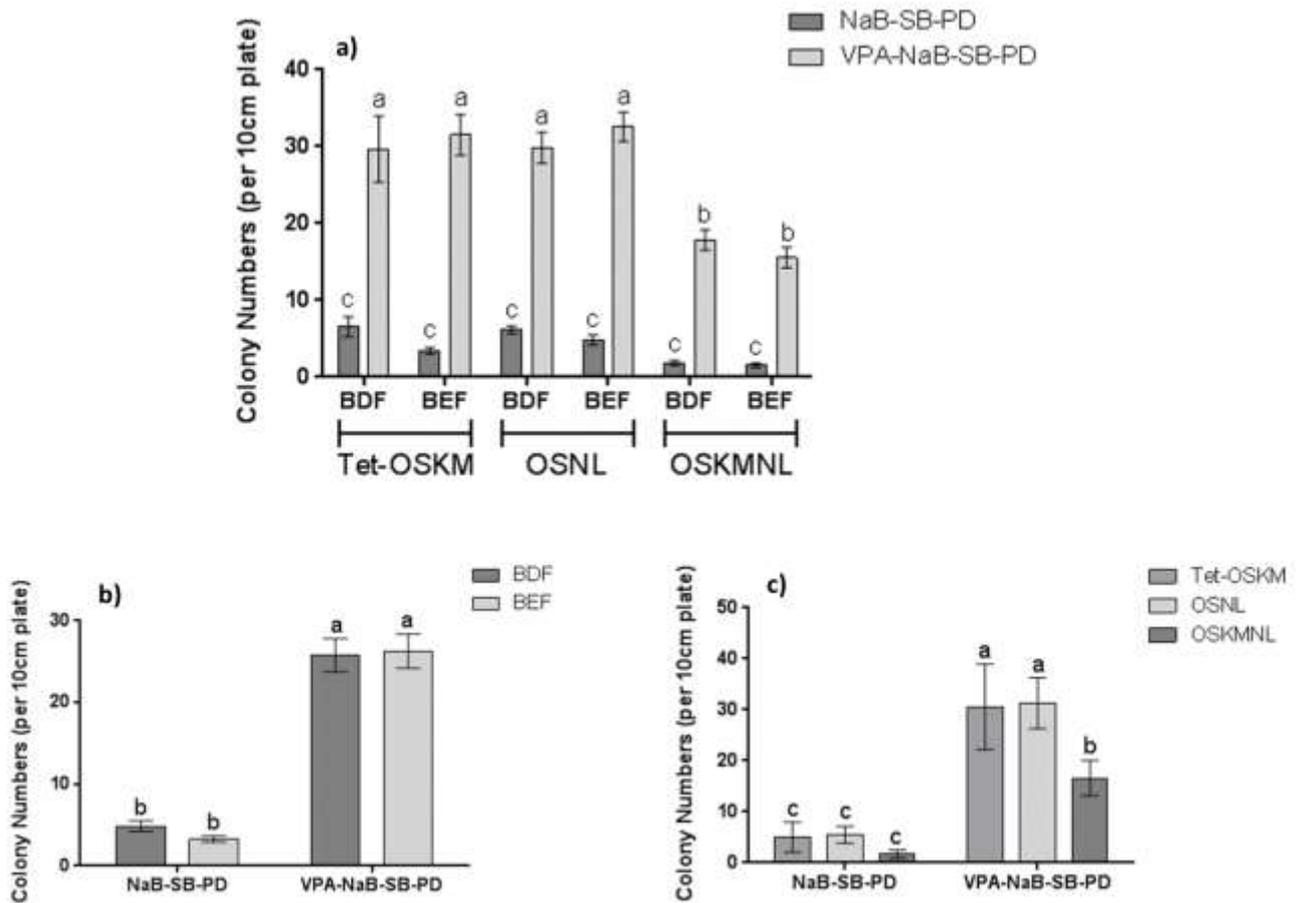
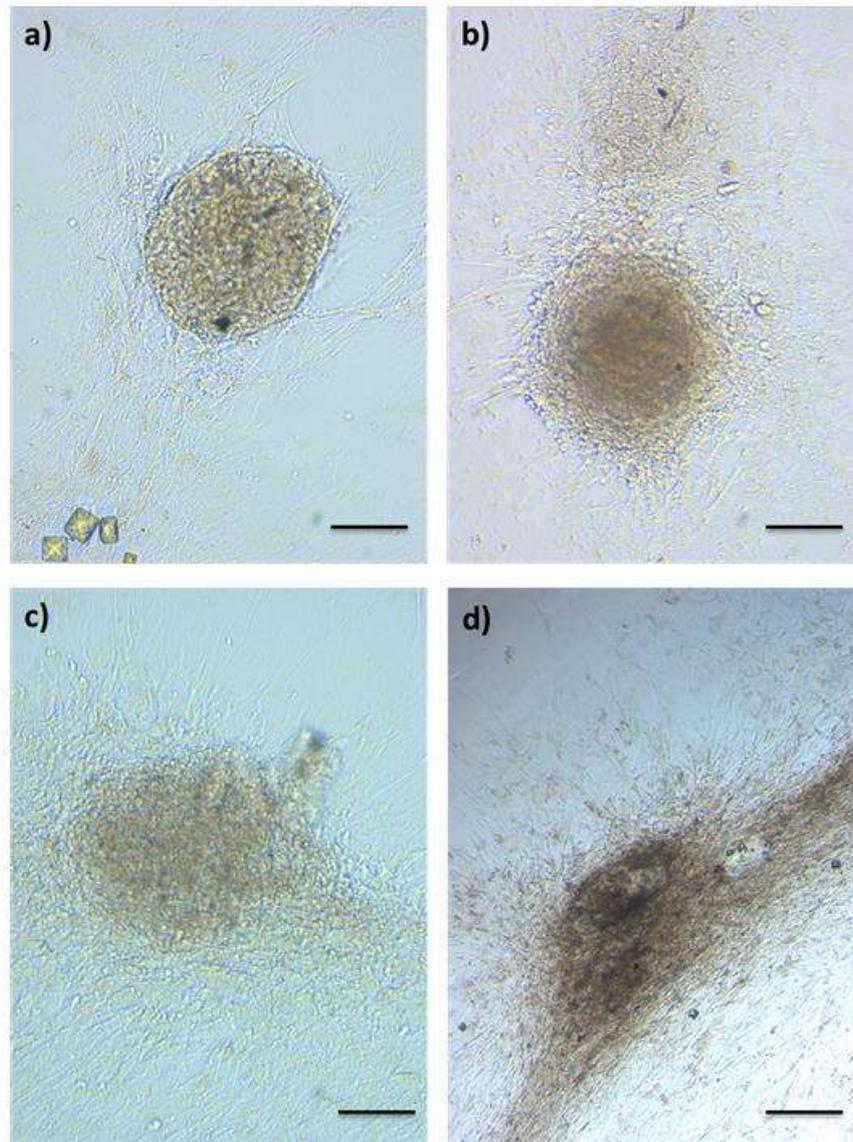


Figure 6-2. Number of putative non-viral bovine iPS colonies produced by supplementation with small molecule combinations NaB-SB-PD and VPA-NaB-SB-PD. a) Average number of colonies produced by transfection of BDF or BEFs with different combination of transcription factors, Tet-OSKM, OSNL or OSKMNL with iPS cultures supplemented with small molecule combinations NaB-SB-PD or VPA-NaB-SB-PD ( $P < 0.05$ ,  $n = 6$ ). b) The effect of small molecule combinations NaB-SB-PD and VPA-NaB-SB-PD the average number of colonies produced by transfection of two cell types BDF and BEFs ( $P < 0.05$ ,  $n = 18$ ). c) Effect of small molecule combinations NaB-SB-PD and VPA-NaB-SB-PD on the average number of colonies produced from cultures transfected with a different combination of transcription factors, Tet-OSKM, OSNL or OSKMNL ( $P < 0.05$ ,  $n = 12$ ). \*VPA= Valproic acid (1 mM), NaB= Sodium butyrate (0.5 mM), SB= SB431542 (2  $\mu$ M), PD= PD0325901 (0.5  $\mu$ M), BDF= bovine dermal fibroblasts, BEF= bovine embryonic fibroblasts. A different character above treatment means indicates a significant difference between treatments. Error bars are represented as  $\pm$  SEM

### **6.3.2. Colony Morphology is Improved by the Addition of Valproic Acid**

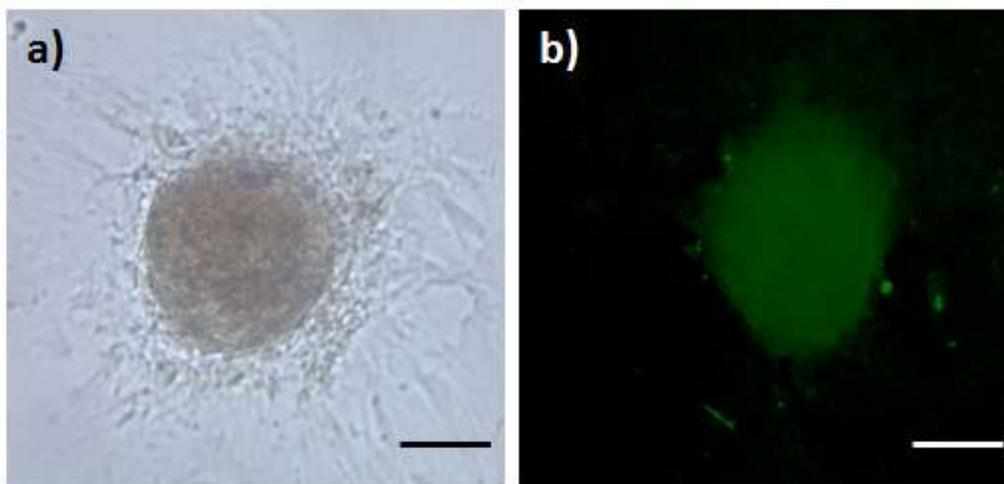
Colony morphology appeared to improve with the addition of VPA in the small molecule cocktail. Cultures supplemented with the VPA-NaB-SB-PD small molecule cocktail produced colonies composed of cells that were in general, more tightly packed and formed colonies that were larger and domed shaped with clear borders. Figure 6-3a shows a colony displaying typically good morphology. Some colonies in both small molecule treatments showed areas of differentiation, generally in the colony centre, though some cells around the colony edge still appeared to have ES cell like morphology, and hence these colonies were deemed as having good morphology. Figure 6-3b shows a colony that had begun to differentiate, with darker differentiated cells in the middle of the colony and ES like cells around the colony edges. Cultures supplemented with the NaB-SB-PD combination of small molecules also produced colonies with typically good iPS morphology, though a higher proportion of colonies were comprised of cells that were less tightly packed or did not have clear colony borders (Figure 6-3c). Additionally some colonies in cultures supplemented with either VPA-NaB-SB-PD or NaB-SB-PD became differentiated and grew into large embryoid body like structures after approximately 14 days of culture (Figure 6-3d). In cultures supplemented with the small molecule combination VPA-NaB-SB-PD, approximately 73% of colonies had good morphology (example Figure 6-3a and b) while 27% of colonies had poor morphology or became differentiated (example Figure 6-3c and d). In contrast, in cultures supplemented with the small molecule combination NaB-SB-PD, 54% of colonies displayed good morphology while 46% had poor colony morphology or became differentiated.



**Figure 6-3.** a) Example of typically good colony morphology observed at higher frequencies in cultures supplemented with the VPA-NaB-SB-PD small molecule combination. b) Colony showing typical differentiation in the centre, ES like cells were still observed around the edges of the colony. c) Colony with poorer morphology, cells are less tightly packed and the colony boarder is not clear. d) Colony with poor morphology that has become overgrown and differentiated. Scale bars represent 100  $\mu\text{m}$ .

### **6.3.3. Putative iPS Colonies Express Alkaline Phosphatase Activity when Supplemented with Valproic Acid**

Some colonies supplemented with VPA-NaB-SB-PD stained positively for alkaline phosphatase live staining at 12 days of culture. Alkaline phosphatase activity was not observed in cultures supplemented with the NaB-SB-PD small molecule combination. No colonies stained positively for pluripotency markers SSEA4 and Tra-1-60.



**Figure 6-4.** Colonies supplemented with the VPA-NaB-SB-PD small molecule combination stain positive for alkaline phosphatase using the alkaline phosphatase live staining kit (Life Technologies). a) Bright field image of alkaline phosphatase positive colony. b) Fluorescent image of colony showing alkaline phosphatase live staining. Scale bars represent 100  $\mu\text{m}$ .

#### **6.3.4. VPA Supplementation does not Consistently Increase the Expression of Pluripotency Markers**

Pooled colonies obtained from all treatments were analysed for expression of pluripotency markers *POU5F1*, *SOX2*, *c-MYC* and *NANOG* using qRT-PCR. Most iPS colonies, regardless of small molecule cocktail combination, fibroblast type, or combination of transcription factors transfected, expressed significantly increased levels of all pluripotency markers analysed compared to the un-transfected cell type. Furthermore, the level of expression of pluripotency markers was not consistently higher in either the VPA-NaB-SB-PD or NaB-SB-PD treatments Figure 6-5.

In BDF cultures, there was no clear pattern of influence of small molecule cocktail on *POU5F1* expression. BDF Tet-OSKM derived colonies supplemented with either small molecule combination, and OSNL cultures supplemented with VPA-NaB-SB-PD, expressed the highest levels of *POU5F1*. BDF OSKMNL cultures supplemented with NaB-SB-PD also expressed *POU5F1*, though at lower levels. Conversely, BDF OSNL cultures supplemented with NaB-SB-PD and OSKMNL cultures supplemented with VPA-NaB-SB-PD did not express *POU5F1* at levels higher than un-transfected BDFs (Figure 6-5a). In BEF cultures, all treatments expressed *POU5F1* at levels higher than un-transfected BEFs. All BEF treatments supplemented with the small molecule cocktail, VPA-NaB-SB-PD, expressed higher levels of *POU5F1* than cultures supplemented with the small molecule combination NaB-SB-PD. The

highest levels of expression of *POU5F1* occurred in BEF OSNL cultures supplemented with VPA-NaB-SB-PD. These treatments expressed *POU5F1* at levels 5 fold higher than the highest *POU5F1* expressing BDF treatment. BEF OSKMNL cultures supplemented with VPA-NaB-SB-PD expressed the second highest levels of *POU5F1*, followed by BEF OSNL cultures supplemented with NaB-SB-PD. Lower levels of *POU5F1* were expressed by BEF Tet-OSKM treatments supplemented with VPA-NaB-SB-PD, while BEF Tet-OSKM and BEF OSKMNL cultures supplemented with NaB-SB-PD expressed the lowest levels of *POU5F1* (Figure 6-5b).

Similarly, *SOX2* expression was not consistently influenced by the different small molecule cocktails. In BDF cultures OSNL colonies supplemented with VPA-NaB-SB-PD expressed the highest *SOX2* expression of any BDF derived treatment group, additionally this treatment expressed approximately 11 fold higher levels of *SOX2* than the same treatment supplemented with the NaB-SB-PD combination. Conversely, Tet-OSKM colonies expressed significantly higher *SOX2* levels when supplemented with NaB-SB-PD alone than those supplemented with additional VPA. OSKMNL cultures and OSNL cultures supplemented with NaB-SB-PD did not express *SOX2* at levels higher than the un-transfected cell types (Figure 6-5c). In BEF cultures only Tet-OSKM colonies supplemented with VPA-NaB-SB-PD and OSNL cultures supplemented with the NaB-SB-PD cocktail expressed *SOX2* at levels higher than un-transfected BEFs (Figure 6-5d).

*c-MYC* expression appeared to be less strongly influenced by the combination of small molecules supplemented than the other pluripotency markers analysed. In BDF cultures, no treatments expressed levels of *c-MYC* significantly higher than the un-transfected cells and overall expression reached only low levels above basal expression levels in untransformed BDFs (Figure 6-5e). In BEF cultures, OSNL cultures supplemented with VPA-NaB-SB-PD and OSKMNL colonies supplemented with NaB-SB-PD expressed *c-MYC* at significantly higher levels than the corresponding treatment group supplemented with the small molecule combination NaB-SB-PD, and compared to un-transfected BEFs (Figure 6-5f).

Supplementation of VPA-NaB-SB-PD appeared to increase the expression of *NANOG* in BDF and BEF treatments compared to those supplemented with NaB-PD-SB. In BDF cultures, all treatments expressed *NANOG* at robustly higher levels than the un-transfected BDFs. BDF Tet-OSKM and OSNL treatments supplemented with VPA-NaB-SB-PD expressed higher levels of *NANOG* than those supplemented with NaB-SB-PD alone. Additionally, BDF OSNL cultures expressed the highest levels

of *NANOG* of any BDF or BEF treatment. BDF OSKMNL cultures expressed *NANOG* levels higher than the un-transfected BDFs, but there was no significant difference between treatments supplemented with the different small molecule combinations (Figure 6-5g). In BEF cultures all treatments except Tet-OSKM colonies supplemented with the NaB-SB-PD small molecule cocktail expressed *NANOG* at higher levels than the transfected cell type. BEF OSKMNL colonies supplemented with VPA- NaB-SB-PD expressed the highest levels of *NANOG* followed by OSNL colonies supplemented with VPA- NaB-SB-PD. Other treatments expressed similar levels of *NANOG* (Figure 6-5h).

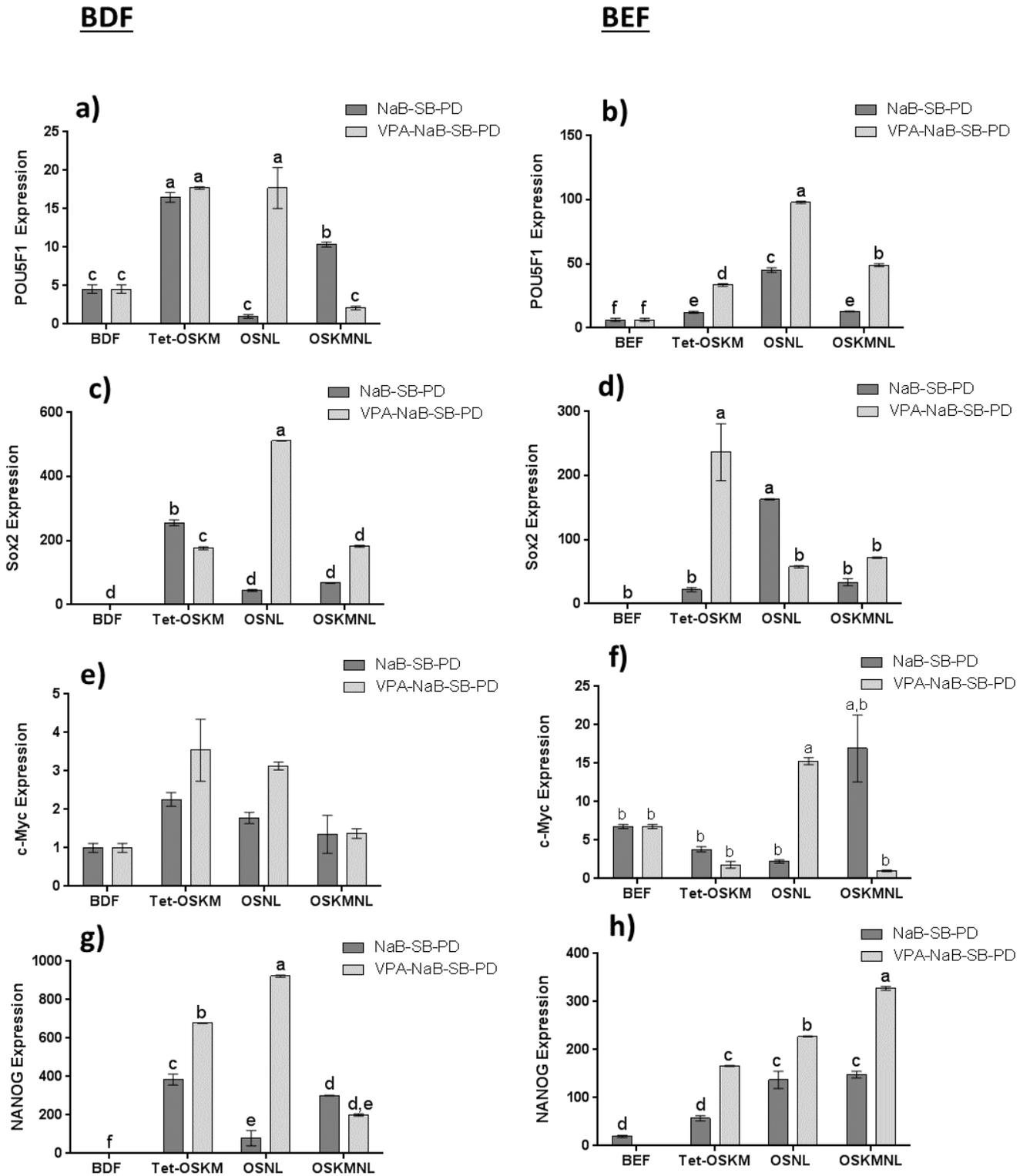
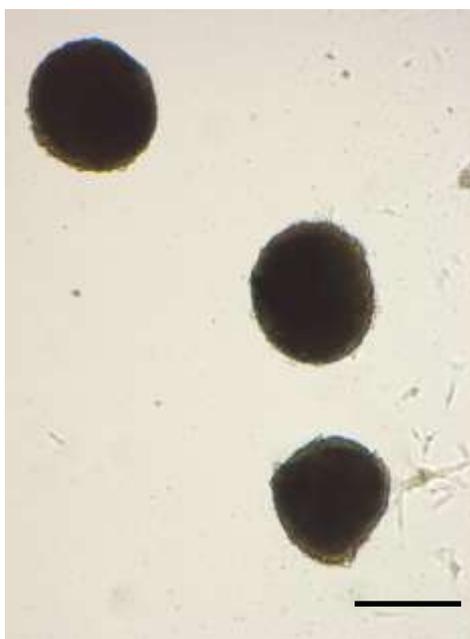


Figure 6-5. Expression of pluripotency markers, by BDF (a, c, e) or BEF (b, d, f) putative iPS cultures, transformed by transfection with Tet-OSKM, OSNL or OSKMNL, and supplemented with small molecule cocktails VPA-NaB-SB-PD or NaB-SB-PD. Gene expression relative to housekeeper gene *RPS26*, is shown for a) and b) *POU5F1*, c) and d) *SOX2*, e) and f) *c-MYC* and g) and h) *NANOG*. \*VPA= Valproic acid (1 mM), NaB= Sodium butyrate (0.5 mM), SB= SB431542 (2  $\mu$ M), PD= PD0325901 (0.5  $\mu$ M), BDF= bovine dermal fibroblasts, BEF= bovine embryonic fibroblasts. Different characters above treatments means indicates a significant difference in expression between treatments ( $P < 0.05$ ,  $n=3$  Error bars are represented as  $\pm$  SEM).

### 6.3.5. Embryoid Bodies Express Makers from all Three Germ Lineages.

Embryoid bodies (EBs) were derived from colonies that were manually picked from all treatment groups. Embryoid bodies were grown in suspension culture for two weeks in the absence of growth factors or small molecules to encourage random differentiation. EBs derived from all treatments displayed typical morphology, growing into large spherical dome like aggregates. Figure 6-6 shows the typical morphology of embryoid bodies produced in this experiment.



**Figure 6-6. Photograph of embryoid bodies' representative of those formed from colonies derived by supplementation with NaB-SB-PD or VPA-NaB-SB-PD. Scale bar represents 100  $\mu$ m.**

After EBs were cultured for two weeks they were harvested and analysed for lineage makers *Tubb3* and *Nestin* (Ectoderm), *Desmin* (Mesoderm) and *FoxA2* (Endoderm) by qRT-PCR (Figure 6-7). BDF and BEF Tet-OSKM and OSNL cultures that were originally supplemented with VPA-NaB-SB-PD all produced embryoid bodies that expressed markers from all three germ lineages. No treatments supplemented with the NaB-SB-PD small molecule combination were able to produce embryoid bodies that expressed all three germ lineages (Figure 6-7).

EBs derived from all treatments supplemented with VPA-NaB-SB-PD, except OSKMNL transfected cultures, expressed higher levels of ectoderm marker *Tubb3*, than treatments supplemented with NaB-SB-PD alone (Figure 6-7a,b). The level of *Tubb3* expression in EBs derived from OSKMNL cultures was not significantly different for either small molecule cocktails. However, while those derived from BEFs showed lower *Tubb3* expression than the un-transfected BEFs, those derived from BDFs showed statistically higher levels than un-transfected BDFs. Interestingly, EBs derived from BEF cultures supplemented with NaB-SB-PD small molecules generally expressed lower levels of *Tubb3* than the un-transfected cell type. This effect of NaB-SB-PD was also seen in EBs derived from BDF Tet-OSKM and OSNL cultures (Figure 6-7b).

In EBs derived from both BDF and BEF cultures, all treatment groups expressed significantly lower levels of ectoderm marker *Nestin* than the un-transfected cell type. EBs derived from BDF OSNL cultures supplemented with either small molecule cocktail and Tet-OSKM cultures supplemented with VPA-NaB-SB-PD expressed low levels of *Nestin* (Figure 6-7c). EBs derived from BEF Tet-OSKM and OSNL treatments supplemented with VPA-NaB-SB-PD expressed low levels of *Nestin*, though these were higher than the expression levels of EBs derived from the same reprogramming plasmid treatments supplemented with NaB-SB-PD (Figure 6-7d). No *Nestin* expression was observed in OSKMNL derived EBs.

EBs derived from cultures supplemented with VPA-NaB-SB-PD generally expressed higher levels of the mesoderm marker *Desmin*. Additionally all EBs expressed *Desmin* at higher levels than the un-transfected cell type, except EBs derived from BEF OSKMNL colonies supplemented with NaB-SB-PD. In BDF cultures EBs derived from Tet-OSKM and OSNL colonies supplemented with VPA-NaB-SB-PD expressed higher levels of *Desmin* than those derived from NaB-SB-PD supplementation. OSKMNL derived EBs did not express different levels of *Desmin* regardless of their initial small molecule supplementation (Figure 6-7e). In BEF cultures all treatments supplemented with VPA- NaB-SB-PD produced EBs that expressed higher levels of *Desmin* than those derived from NaB-SB-PD supplemented colonies. However, OSKMNL derived colonies expressed several fold lower *Desmin* levels than Tet-OSKM or OSNL cultures, which in turn expressed overall the highest levels of *Desmin* (Figure 6-7f).

The expression of endoderm marker *FoxA2* appeared to be consistently influenced by initial small molecule supplementation. EBs derived from BDF Tet-OSKM and OSNL supplemented with VPA-

NaB-SB-PD expressed higher levels of *FoxA2* than those derived from NaB-SB-PD supplemented colonies. EBs derived from OSKMNL colonies did not express *FoxA2*. In BEF derived EBs, those derived from colonies initially supplemented with VPA- NaB-SB-PD expressed higher levels of *FoxA2* than those derived from NaB-SB-PD supplemented colonies (Figure 6-7g). In Both BDF and BEF derived EBs, those derived from Tet-OSKM colonies expressed the highest *FoxA2* levels (Figure 6-7g,h).

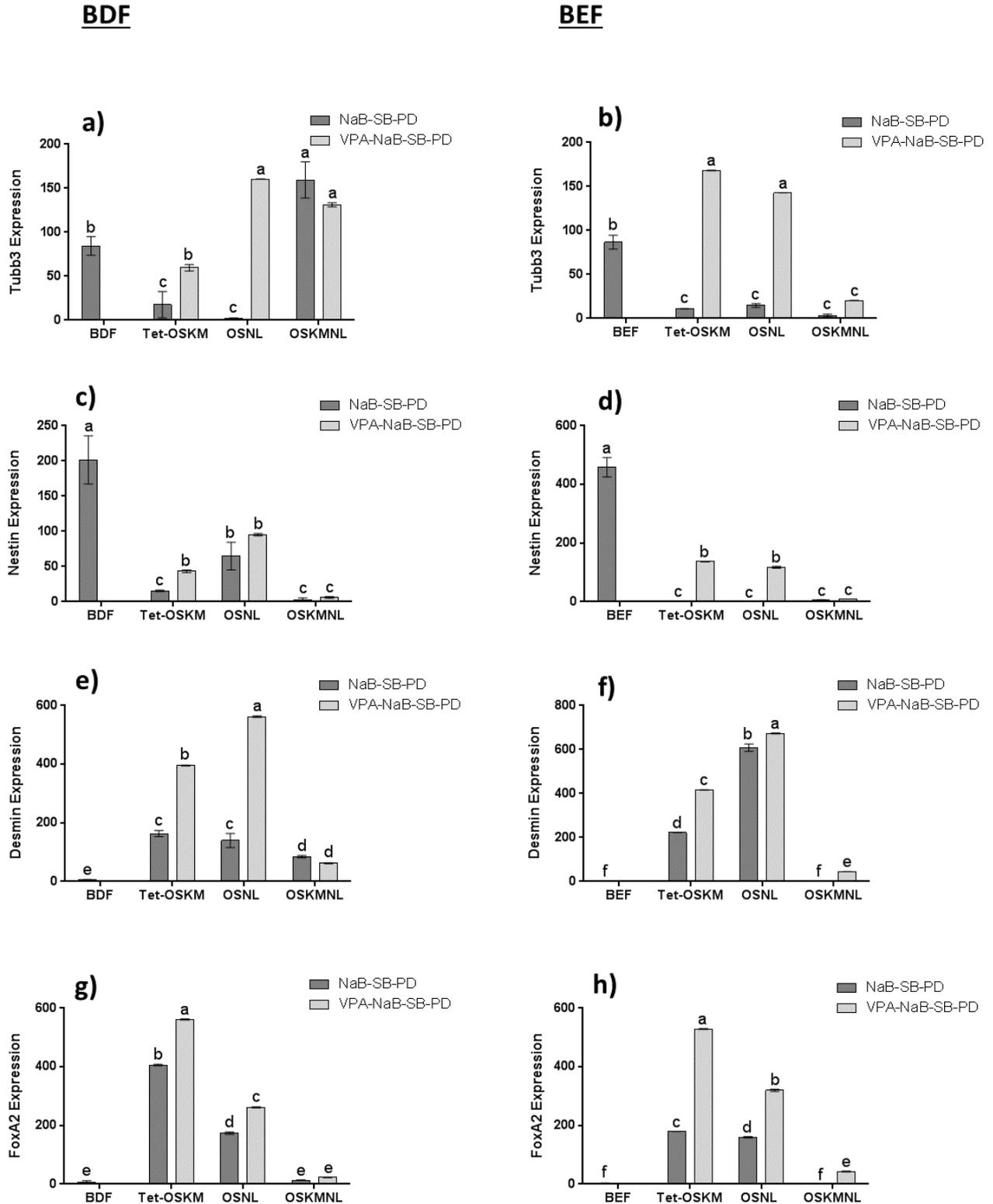


Figure 6-7. Expression of lineage markers by EBs derived from putative BDF (a, c, e) or BEF (b, d, f) iPS cultures, transformed by transfection with Tet-OSKM, OSNL or OSKMNL, that were initially supplemented with small molecule cocktails VPA-NaB-SB-PD or NaB-SB-PD. Gene expression relative to housekeeper gene *RPS26*, is shown for a) and b) *Tubb3*, c) and d) *Nestin*, e) and f) *Desmin* and g) and h) *FoxA2*. \*VPA= Valproic acid (1 mM), NaB= Sodium butyrate (0.5 mM), SB= SB431542 (2  $\mu$ M), PD= PD0325901 (0.5  $\mu$ M), BDF= bovine dermal fibroblasts, BEF= bovine embryonic fibroblasts. Different characters above treatments means indicates a significant difference in expression between treatments ( $P < 0.05$ ,  $n=3$  Error bars are represented as  $\pm$  SEM).

## **Experiment 2- Effect of Individual and Combinations of Small Molecules on Bovine iPS Cell Generation**

To further understand the interactions of small molecules in cellular reprogramming, a large set of different combinations of small molecules were investigated for their effects on reprogramming bovine cells to pluripotency as shown in Table 6-4.

In the following experiment only cell type, BEFs, were reprogrammed using one transcription factor combination, Tet-OSKM. This construct was chosen for comparison with the original work conducted by McMillan (2012) of the effects of small molecules on the reprogramming of bovine iPS cells.

The previous experiment described in this chapter showed that HDAC inhibitor valproic acid (VPA) significantly enhanced the reprogramming of bovine iPS cells. To determine if there was a difference in reprogramming efficiency of bovine iPS cells, cultures of BEFs transfected with the Tet-OSKM reprogramming construct were supplemented with four small molecules, HDAC inhibitors sodium butyrate (NaB) and valproic acid (VPA), TGF- $\beta$  inhibitor SB431542 (SB), MEK inhibitor PD0325901 (PD), either individually or in different combinations. As a control, some cultures were also grown in the absence of any small molecules (iPS media alone). As a negative control, transfected fibroblasts were also grown in the same conditions and supplemented with the different small molecule combinations. No colonies were observed in these cultures. This experiment was repeated three times, with two technical replicates for each different combination of small molecules in each repeated experiment.

Table 6-4. Small molecule combinations tested in experiment 2.

Experiment 2	Small Molecule Combinations
iPS Media	None
Individual Small Molecules	NaB SB431542 PD0325901 VPA
2 Molecule Combinations	NaB-SB NaB-PD SB-PD VPA-NaB VPA-SB VPA-PD
3 Molecule Combinations	NaB-SB-PD VPA-NaB-SB VPA-NaB-PD VPA-SB-PD
4 Molecule Combination	VPA-NaB-SB-PD

### 6.3.6 Supplementation of Different Combinations of Small Molecule Influences Reprogramming Kinetics and Efficiency

The supplementation of the small molecules VPA, NaB, SB or PD individually or in different combinations with each other, influenced the time to colony formation of putative bovine iPS colonies (Figure 6-8). The time to colony formation of cultures not supplemented with small molecules was on average 21 days. The individual supplementation of any of the small molecules tested, except NaB, resulted in a decreased time to colony formation compared to iPS cultures without small molecule supplementation. Colonies were observed following an average of 19 days in culture when supplemented with NaB, which was not statistically different to cultures grown without small molecule supplementation. Cultures supplemented with either SB or PD small molecule alone produced iPS like colonies following an average of 14 days of culture. Cultures supplemented with VPA alone produced colonies significantly faster than SB or PD supplemented cultures, with colonies observed after 9 days of culture (Figure 6-8).

The time to colony formation was decreased further with the supplementation of a combination of two small molecules, except the combination of NaB-SB, which did not produce colonies quicker than NaB supplementation alone, and interestingly this time was longer than for SB alone. Supplementation with NaB-PD or SB-PD resulted in decreased time to colony formation compared to

SB or PD supplementation alone, with colonies observed after 11 days, but this change was not statistically significant. The supplementation of VPA in combination with any other small molecule decreased the time to colony formation further, with all treatments supplemented with VPA, producing colonies after an average of 9 days of culture. The supplementation of NaB-SB-PD produced colonies after an average of 11 days of culture. The supplementation of three or more small molecules including VPA did not decrease the time to colony formation further than the addition of VPA alone or in combination with one other small molecule (Figure 6-8).

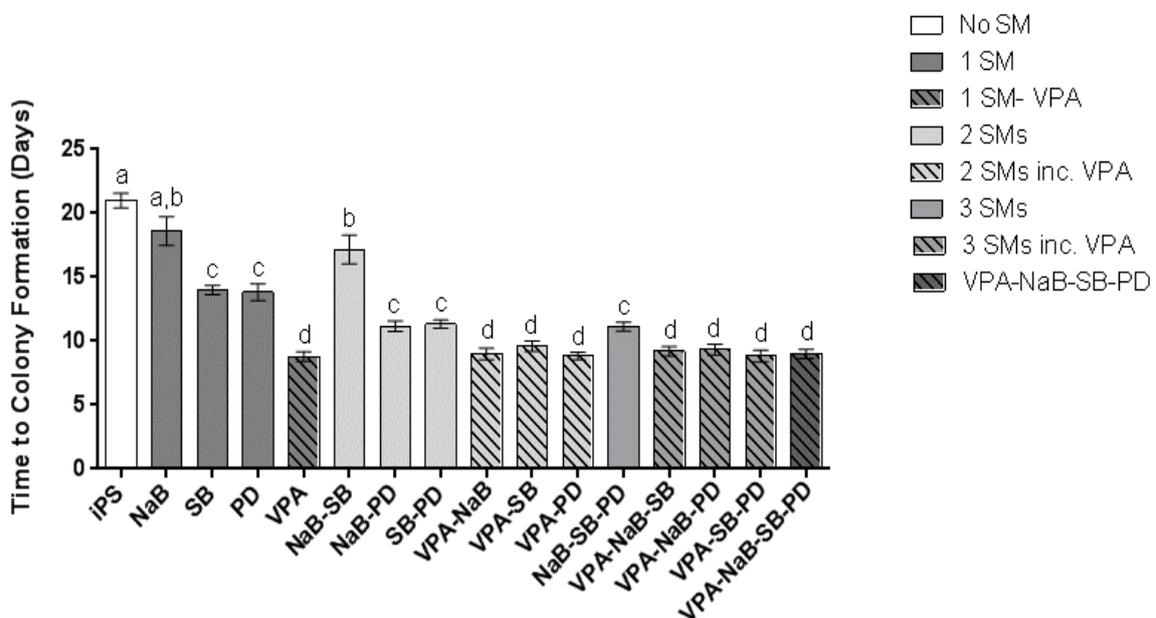


Figure 6-8. Time to colony formation of putative non-viral bovine iPS cultures supplemented with different combinations of the small molecules Valproic acid (VPA (1 mM)), Sodium Butyrate (NaB 0.5 mM)), SB431542 (SB (2  $\mu$ M)) and PD0325901 (PD (0.5  $\mu$ M)). Legend shows treatments divided into different numbers of small molecules supplemented in combination with each other, diagonal lines highlight treatments with VPA supplementation. Different letters above treatments indicates they are significantly different from one another ( $P < 0.05$ ,  $n=6$ ). Error bars are represented as  $\pm$  SEM.

The average numbers of putative iPS colonies produced following 21 days of culture was also influenced by the combination of small molecules supplemented. There were a significantly higher number of colonies observed in cultures supplemented with any combination of small molecules containing VPA (Figure 6-9). An average of 21 colonies were observed in VPA supplemented cultures, equating to a reprogramming efficiency of 0.042%, compared to an average of 4 colonies in cultures supplemented with small molecules not including VPA, equating to a reprogramming efficiency of

0.008%. Cultures supplemented with VPA alone or in combination with one other small molecule, produced significantly higher numbers of colonies than treatments grown without VPA supplementation. Additionally, the combination of VPA and PD resulted in a higher number of colonies than the combination of VPA with either NaB or SB. Furthermore, the supplementation of iPS cultures with VPA in addition to either PD alone, or in combination with two or more other small molecules, produced significantly higher numbers of colonies than other treatments, with an average of 31 colonies, and a reprogramming efficiency of 0.062%, observed after 21 days ( $P < 0.05$ ).

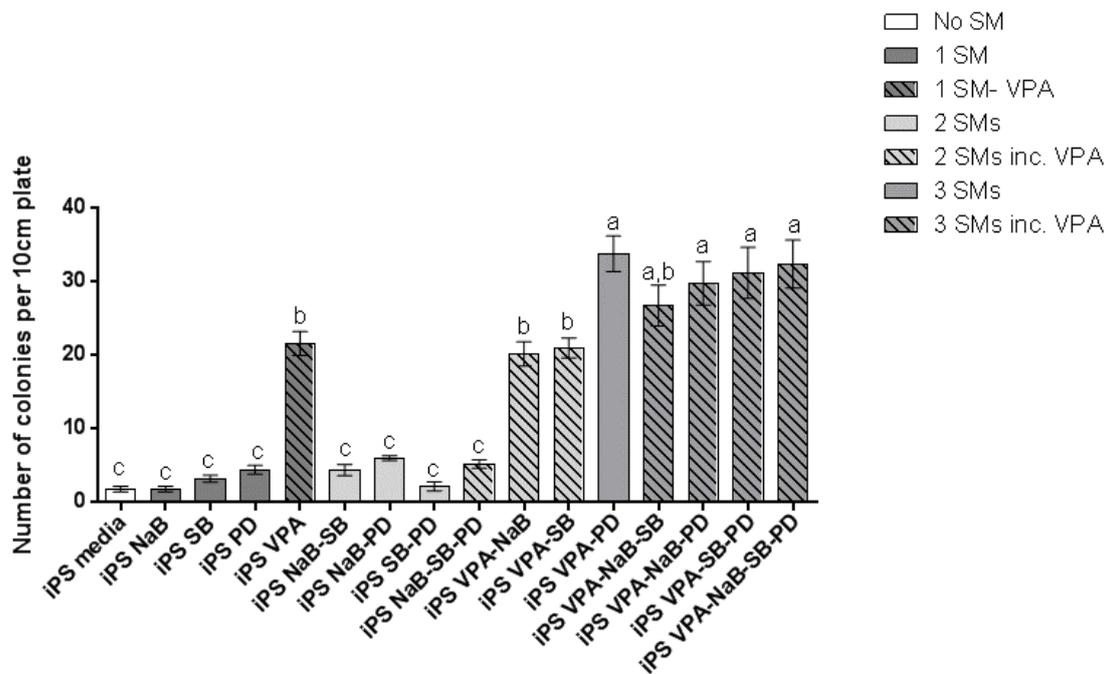


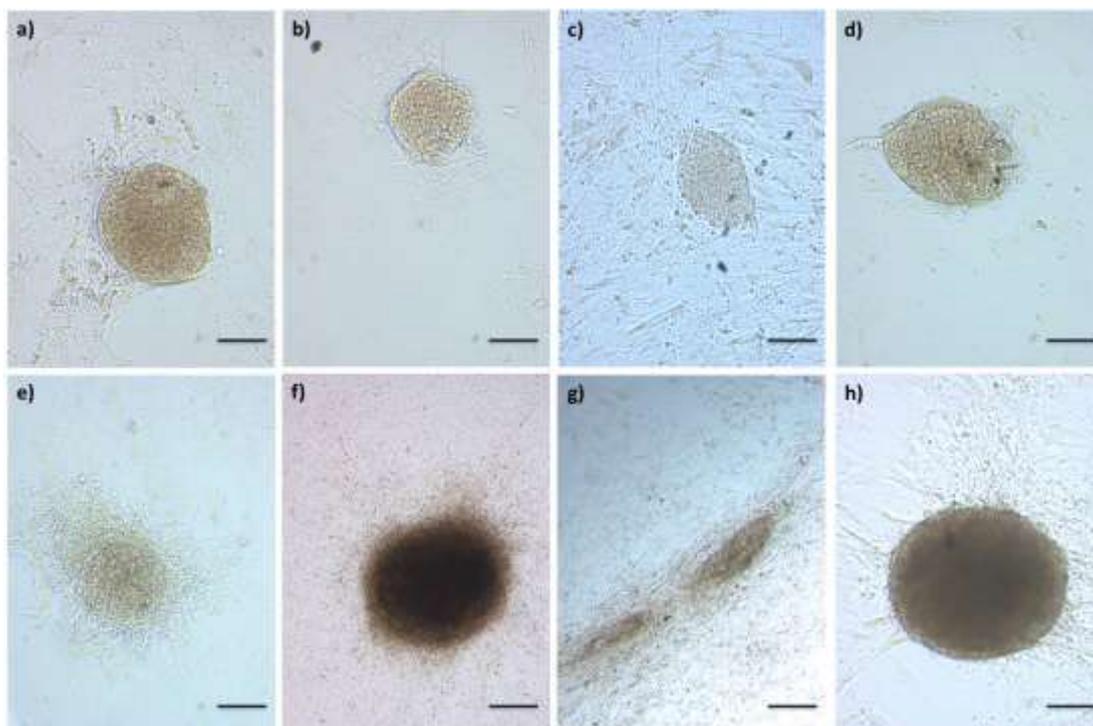
Figure 6-9. Average number of putative bovine iPS colonies observed per 10 dish of cultures supplemented with different combinations of the small molecules. Supplementation of small molecules occurred at the following concentrations; Valproic Acid (VPA (1 mM)), Sodium Butyrate (NaB 0.5 mM), SB431542 (SB (2  $\mu$ M)) and PD0325901 (PD (0.5  $\mu$ M)). Legend shows treatments divided into different numbers of small molecules supplemented in combination with each other, diagonal lines highlight treatments with VPA supplementation. Different letters above treatments indicates they are significantly different from one another ( $P < 0.05$ ,  $n=6$ ). Error bars are represented as  $\pm$  SEM.

### 6.3.7 Supplementation of Different Combinations of Small Molecules Effects Colony Morphology

There was a difference in colony morphology observed between treatments supplemented with different combinations of small molecules. Cultures supplemented with small molecule

combinations, VPA-SB, VPA-PD, NaB-SB-PD and VPA-NaB-SB-PD more consistently produced colonies with good colony morphology than any other small molecule treatments. Between 50-75% of colonies in these treatments had good colony morphology and appeared similar to the colonies in Figure 6-10 a, b, and d. These colonies had clear borders and were comprised of tightly packed round ES like cells. Some colonies appeared smaller but were clearly comprised of tightly packed round cells that resembled ES like cells similar to those shown in Figure 6-10c. Some colonies that had begun to differentiate but still had clear borders and contained ES like cells around the perimeter of the colony were still considered to have good colony morphology (Figure 6-10d).

Conversely, Figure 6-10 e, f, g and h show examples of colonies with poor morphology. These colonies did not have clearly defined borders (Figure 6-10e), or became fully differentiated (Figure 6-10f, g). Some colonies grew into large embryoid body like structures (Figure 6-10h). Un-supplemented iPS cultures or cultures which were supplemented with only one small molecule, or with the VPA-NaB in combination, displayed higher proportions of colonies with poor colony morphology than other supplementation treatments. Without supplementation of small molecules fewer colonies with good morphology were produced, with only 20% of colonies exhibiting good morphology, while most colonies observed had morphologies similar to that shown in Figure 6-10e, where clear colony borders were not present. The supplementation of one small molecule in iPS cultures resulted in between approximately 30-40% of colonies having good morphology (NaB 31%, SB 40%, PD, 34%, VPA, 30%). Cultures supplemented with NaB or VPA alone or in combination as VPA-NaB produced a high proportion of colonies that became differentiated, similar to those shown in Figure 6-10 f, g and h.



**Figure 6-10.** Examples of colony morphology observed in putative bovine iPS cultures supplemented with different combinations of small molecules. Figures 10 a-d show examples of good colony morphology, typically observed in iPS cultures supplemented with VPA-SB, VPA-PD, NaB-SB-PD and VPA-NaB-SB-PD a, b) photographs of colonies with good morphology, colonies have clear borders and are comprised of tightly packed round ES like cells. c) small/young colony comprised of round ES like cells. d) Colony that has begun to differentiate in the centre but still has ES like cells around the colony perimeter. Figures 10 e-h show examples of colonies with poor morphology typically observed in cultures not supplemented with small molecules, or supplemented with only one small molecule or with NaB-VPA in combination. e) Poor colony morphology, colony does not have clear borders. f) Colony that has differentiated. g) Differentiated colony typically observed around plate edges, shows large areas of differentiation. h) Colony displaying embryoid body-like appearance. Scale Bars represent 100  $\mu\text{m}$ .

Only colonies supplemented with the VPA-NaB-SB-PD small molecule combination showed positive staining for alkaline phosphatase activity. No treatments resulted in colonies that stained positively for SSEA4 or Tra-1-60. Additionally, when colonies were passaged they did not expand in culture.

### 6.3.8 The Combination of Four Small Molecules VPA-NaB-SB-PD, Consistently Results in Increased Expression of Pluripotency Markers

Following 21 days of culture, putative bovine iPS colonies were manually picked for analysis of pluripotency marker expression by qRT-PCR. Cultures supplemented with the small molecule combination VPA-NaB-SB-PD showed the highest expression of *POU5F1*, followed by cultures supplemented with VPA-SB (Figure 6-11a). An increase in *POU5F1* expression of almost 3 fold was observed in cultures supplemented with VPA-NaB-SB-PD compared to those supplemented with

VPA-SB. Interestingly, *POU5F1* expression was lower in cultures supplemented with VPA-NaB-SB or VPA-SB-PD than in those supplemented with VPA-SB alone ( $P < 0.05$ ) (Figure 6-10a). Cultures supplemented with no small molecules (iPS) or with NaB, PD or VPA alone, showed no statistically significant difference in expression of *POU5F1* compared to the un-transfected BEFs ( $P > 0.05$ ). Conversely, supplementation with SB alone resulted in the third highest expression of *POU5F1* of all treatments. *POU5F1* expression did not seem to increase consistently with supplementation of an increased number of small molecules, with the exception of the four molecule combination of VPA-NaB-SB-PD which expressed the highest levels of *POU5F1* of any treatment.

When cultures were supplemented with at least one small molecule, *SOX2* expression increased significantly compared to the un-transfected BEFs, and SB had the highest effect of the single small molecule treatments ( $P < 0.05$ ) (Figure 6-11b). Again, the highest expression of *SOX2* occurred in cultures supplemented with the combination of four small molecules VPA-NaB-SB-PD. *SOX2* expression did not increase consistently with supplementation of increasing numbers of small molecules or with the addition of VPA. The lowest *SOX2* expression was observed in cultures supplemented with NaB or PD alone, or with VPA-NaB-PD or VPA-SB-PD.

*NANOG* expression was significantly higher in cultures supplemented with VPA-NaB-SB-PD, NaB-SB-PD, VPA-PD, VPA-SB, VPA-NaB or SB alone, than in un-transfected BEFs. Similar to *POU5F1* and *SOX2* expression, *NANOG* expression was also highest in cultures supplemented with VPA-NaB-SB-PD ( $P < 0.05$ ) (Figure 6-11c). The second highest expression of *NANOG* was observed in colonies supplemented with SB alone, followed by those supplemented with VPA-SB.

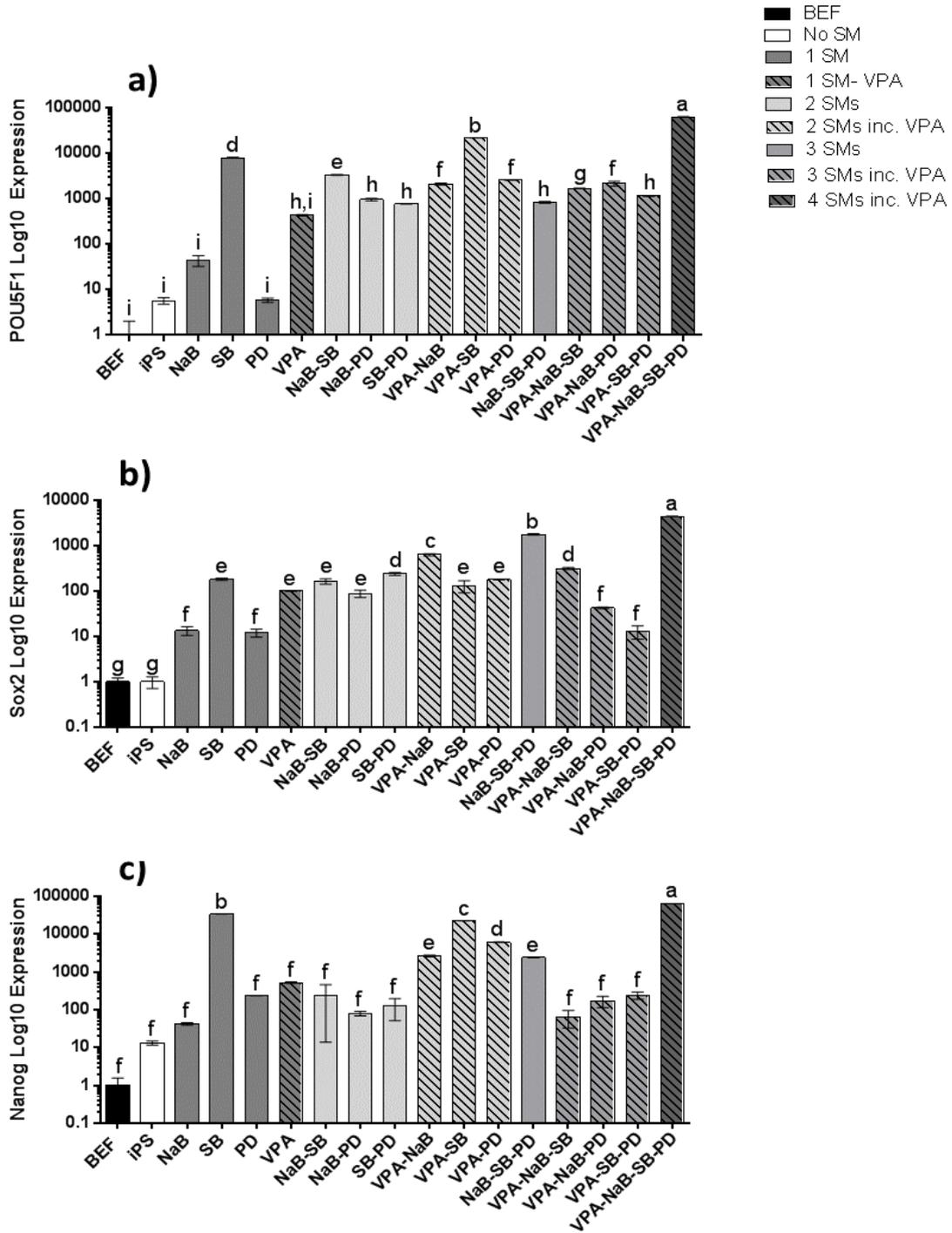
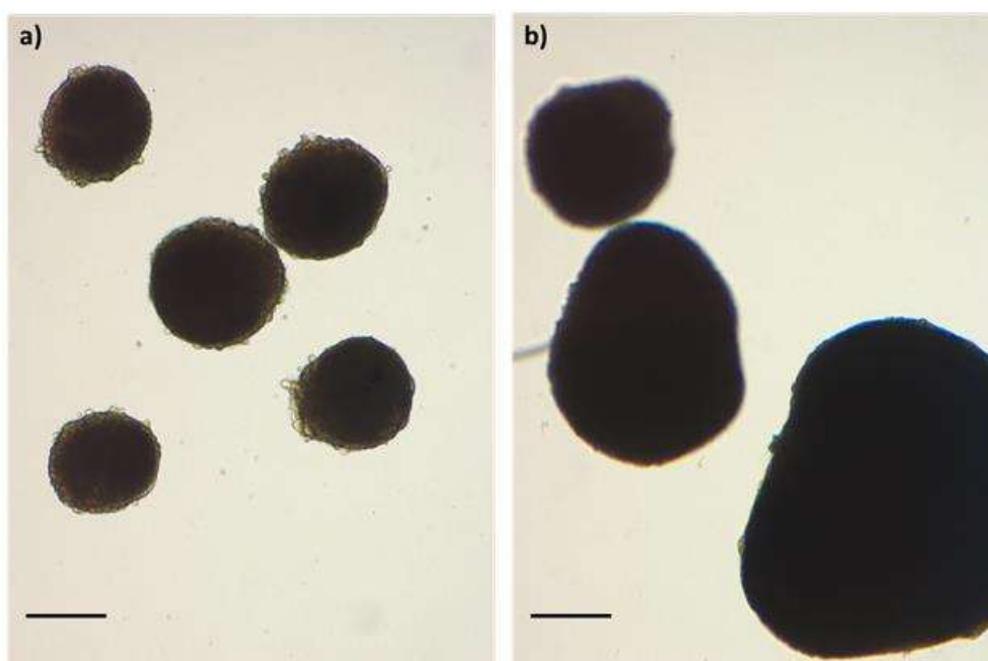


Figure 6-11. Log10 expression of Pluripotency Markers a) *POU5F1*, b) *SOX2* and c) *NANOG* by putative bovine iPS colonies after 21 days of culture. Legend shows treatments divided into different numbers of small molecules supplemented in combination with each other, diagonal lines highlight treatments with VPA supplementation. Different characters above treatments indicates a significant difference in the expression of pluripotency marker ( $P < 0.05$ ,  $n=3$ ). Error bars are represented as  $\pm$  SEM.

### 6.3.8 Embryoid Bodies Derived from iPS Colonies Supplemented with Four Small Molecules VPA-NaB-SB-PD, Expressed Markers from all Three Germ Lineages

Following 21 days of culture, putative bovine iPS colonies were manually picked and transferred to low adherent cell culture dishes and grown in the absence of growth factors or small molecules to derive embryoid bodies. All treatments successfully produced embryoid bodies with typical large spherical morphology as shown in Figure 6-12.



**Figure 6-12.** Photographs showing typical embryoid body morphology after a) 7 days and b) 14 days of culture. Scale bars represent 100  $\mu\text{m}$ .

Embryoid bodies were analysed by qRT-PCR for expression of lineage markers *Tubb3* (ectoderm), *Desmin* (mesoderm) and *FoxA2* (endoderm). Embryoid bodies derived from all treatments supplemented with VPA, except VPA-SB, showed expression of all three lineage markers. All three lineage markers were also expressed by EBs derived from cultures supplemented with VPA alone, NaB-PD and NaB-SB-PD small molecule combinations. Embryoid bodies derived from VPA-NaB-SB-PD treatments consistently showed high expression of all three lineage markers (Figure 6-13).

All treatments showed expression of ectoderm marker *Tubb3* which was also expressed by un-transfected BEFs, but was expressed at the highest levels in VPA-SB-PD cultures. Cultures supplemented with iPS media with no small molecule supplementation, or NaB, SB or PD alone, or with NaB-PD, SB-PD combinations expressed *Tubb3* at lower levels than the un-transfected BEFs (Figure 6-13a).

Expression of mesoderm marker *Desmin* was observed in embryoid bodies derived from all cultures except treatment groups with either NaB or SB alone, and for VPA-SB. *Desmin* was also expressed by EBs derived from colonies grown without any small molecule supplementation (iPS) (Figure 6-13b). The highest levels of *Desmin* were expressed in EBs derived from cultures supplemented with NaB-SB-PD. The addition of VPA to iPS cultures did not necessarily increase the levels of *Desmin* expression by EBs.

Endoderm marker *FoxA2* was expressed by EBs derived from cultures supplemented with VPA alone, SB-PD, VPA-NaB, VPA-PD, NaB-SB-PD, VPA, NaB-SB, VPA-NaB-PD, VPA-SB-PD and VPA-NaB-SB-PD (Figure 6-13c). Only very low levels of *FoxA2* expression were observed in EBs derived from cultures supplemented with VPA alone. Of the two-molecule combinations, VPA-NaB and VPA-SB had higher expression levels than NaB-PD, but lower levels than VPA-PD. The three-small molecule combinations resulted in higher expression levels of *FoxA2* than any two-molecule combination, with the exception of VPA-SB-PD, which was as low as NaB-PD. EBs derived from treatments supplemented with VPA-NaB-SB-PD expressed the highest levels of *FoxA2*.

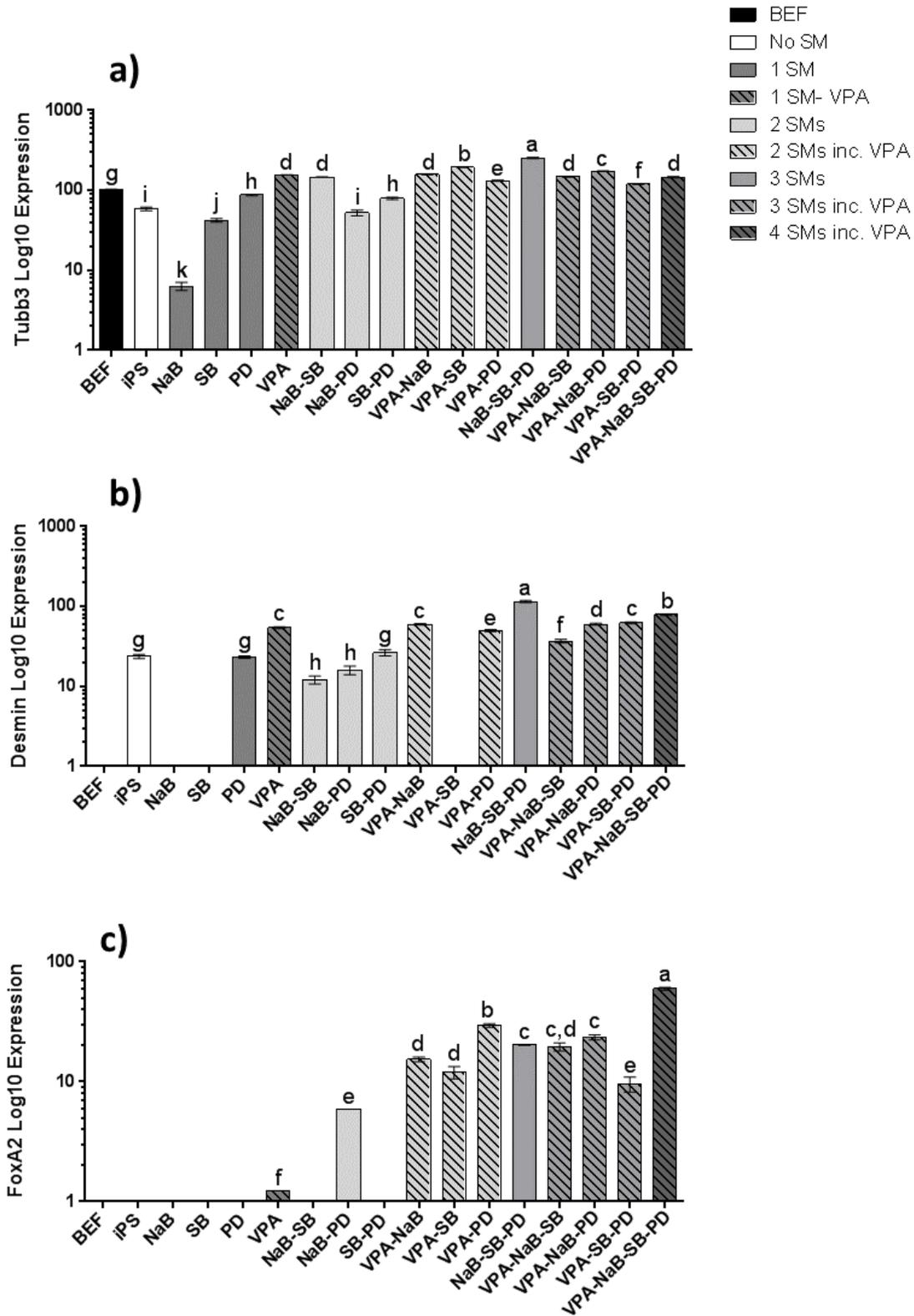


Figure 6-13. Log 10 expressions of lineage markers a) *Tubb3*, b) *Desmin* and c) *FoxA2*, by embryoid bodies derived from putative bovine iPS cultures. Legend shows treatments divided into different numbers of small molecules supplemented in combination with each other, diagonal lines highlight treatments with VPA supplementation. Different characters above treatments indicates a significant difference in the expression of pluripotency marker ( $P < 0.05$ ,  $n=3$ ). Error bars are represented as  $\pm$  SEM.

## **6.4 Discussion**

The generation of induced pluripotent stem (iPS) cells from livestock has proven difficult to date. The use of small molecules that target pathways involved in the reprogramming process has provided a means to enhance reprogramming of cells, by improving reprogramming efficiency, converting partially reprogrammed cells to a fully reprogrammed state and increasing reprogramming kinetics. Previous experiments outlined in this thesis have used a small molecule combination of HDAC inhibitor sodium butyrate (NaB), TGF- $\beta$  inhibitor SB431542 (SB), and MEK inhibitor PD0325901 (PD). This combination of small molecules has previously been reported to increase the kinetics of bovine iPS cell reprogramming by an average of 9 days compared to standard iPS media alone (McMillan, 2012). This combination of small molecules has also been implemented in human retroviral iPS cell generation and has been shown to dramatically improve reprogramming efficiency of human hepatocytes with an efficiency of 0.93% reported compared to normal retroviral efficiencies of up to 0.05% (Zhang, Zhonghui et al., 2011). The NaB-SB-PD combination of small molecules has been used throughout this thesis due to its ability to accelerate kinetics of bovine iPS generation and has resulted in the production of partially reprogrammed viral and non-viral bovine iPS cells that exhibit some characteristics of pluripotency (Chapter 4 and 5).

There have been numerous reports that the supplementation of another HDAC inhibitor, valproic acid (VPA), is capable of substantially increasing reprogramming efficiency. The production of human iPS cells has been improved 40-50 fold with the addition of VPA (Huangfu et al., 2008), while other reports claim to have improved reprogramming efficiency by more than 100 fold depending on cell type (Huangfu et al., 2008). VPA is believed to be the most potent HDAC inhibitor, improving reprogramming efficiency more greatly than similar molecules (Kim, Y. et al., 2011). The first experiment of this chapter aimed to determine if the addition of VPA to the current small molecule cocktail of NaB-SB-PD would improve reprogramming of bovine iPS cells.

When valproic acid was added in combination with the NaB-SB-PD small molecule cocktail, a significant decrease in time to colony formation was observed in all treatments compared to those cultures supplemented with NaB-SB-PD alone. The increase in reprogramming kinetics observed in this experiment is in agreement with other studies that have shown acceleration in reprogramming kinetics with the addition of VPA (Yang, Y. et al., 2012; Zhang, Y., Li, Laurent, & Ding, 2012). The addition of VPA to the NaB-SB-PD small molecule cocktail also resulted in a large increase in the

number of colonies observed after 21 days of culture. This is in agreement with studies reporting that VPA increases reprogramming efficiency.

Cultures supplemented with VPA-NaB-SB-PD produced 6.5 fold more colonies than treatments supplemented with NaB-SB-PD alone, indicating the efficiency of VPA supplementation in bovine iPS reprogramming. Additionally, both for cultures supplemented VPA-NaB-SB-PD or NaB-SB-PD, transfection with Tet-OSKM or OSNL produced more colonies than cultures transfected with the OSKMNL combination of transcription factors. The addition of VPA also increased the numbers of colonies observed in OSKMNL cultures, but not to the same extent as Tet-OSKM or OSNL colonies (0.034% for OSKMNL compared to 0.062% for Tet-OSKM or OSNL). This is consistent with previous results of this thesis that showed OSKMNL cultures had lower reprogramming efficiencies than Tet-OSKM or OSNL cultures (Chapter 5, pg. 160). This is thought to be due to the large size of the reprogramming construct restricting plasmid uptake by host cells (Ribeiro et al., 2012). It should be noted that colonies produced in this experiment are not considered to be fully reprogrammed. As such, the reprogramming efficiency calculated in this instance was determined using the number of *all* colonies observed. This is in contrast to other methods of calculating reprogramming efficiency where only fully reprogrammed colonies as determined by NANOG or SSEA or Tra 1-60 expression are used to determine the efficiency of reprogramming (Huangfu et al., 2008; Zhang, Zhonghui et al., 2011). Therefore these efficiencies should not be compared to other reports but only considered in the context of this thesis.

This chapter did not provide evidence for difference in the reprogrammability of the two cell types, bovine dermal or embryonic fibroblasts. There was no difference in the time to colony formation or the number of colonies observed between BDFs and BEFs cultures supplemented with the same small molecule cocktail. This corresponds with results from other chapters (Chapter 4 and 5) and suggests that age of cells may not affect the reprogrammability of bovine iPS cells, and is possibly an indicator for embryonic fibroblasts of bovine embryos being further differentiated than those of other species, in particular murine. However, as the iPS cells described here are not fully reprogrammed it is difficult to tell if complete reprogramming under the ideal culture conditions would be more efficient for BDF or BEF cells.

Colony morphology appeared to improve with the addition of VPA, with more colonies in cultures treated with VPA displaying 'good' quality colony morphology compared to cultures supplemented

with the NaB-SB-PD combination. Cultures supplemented with the VPA-NaB-SB-PD cocktail also showed positive alkaline phosphatase staining, while cultures supplemented with the NaB-SB-PD small molecule cocktail did not. This indicates that cultures supplemented with VPA were more fully reprogrammed than those that were not exposed to VPA. However, alkaline phosphatase is not considered to be a stringent test of pluripotency, and as colonies supplemented with VPA did not also exhibit staining for SSEA4 or Tra 1-60, they are still not considered to be fully reprogrammed. It should be noted that the previous work conducted to produce lentiviral bovine iPS cells using the NaB-SB-PD small molecule cocktail did report positive alkaline phosphatase staining of some colonies. However, alkaline phosphatase activity was not observed in any cultures supplemented with NaB-SB-PD in the experiments in this chapter or previous experiments (Chapter 4 and 5). The reasons for the lack of alkaline phosphatase in NaB-SB-PD cultures are therefore unclear.

In BEF cultures, supplementation with the VPA-NaB-SB-PD small molecule cocktail resulted in higher expression of pluripotency markers *POU5F1*, and *NANOG* compared to the equivalent treatment supplemented with NaB-SB-PD. This pattern was not observed in BDF cultures, and therefore the effects of VPA may have more influence on some cell types than others. The effects on *SOX2* and *c-MYC* expression were less clear, with some VPA supplemented colonies expressing higher levels than the NaB-SB-PD treatments and some expressing lower levels. VPA has been shown to have a direct effect on the expression of *OCT4* by enhancing *POU5F1* promoter activity (Teng et al., 2010). This may explain the increase of *POU5F1* expression in cultures supplemented with VPA. *OCT4* is arguably the most important transcription factor for inducing and maintaining pluripotency, with reports that high levels of *OCT4/POU5F1* expression are essential for reprogramming (Shi, G. & Jin, 2010; Tiemann et al., 2011). Given this, it is reasonable to conclude that one mechanism that VPA enhances reprogramming of putative bovine iPS cells is by increasing expression of *POU5F1*.

As the ideal levels of pluripotency marker expression are not known for bovine cells, and are not well documented for other species it is difficult to decide whether one treatment expressed an optimal or better ratio of markers compared to another. Reports of pluripotency marker expression by iPS cells from other species suggest that *OCT4* expression should be high, and *SOX2* and *KLF4* low (Tiemann et al., 2011), while others claim the most efficient ratio is high levels of *OCT4*, low *SOX2*, high *KLF4* and *c-MYC* (Nagamatsu et al., 2012). It is reasonable to assume that cultures in this experiment with high *POU5F1* expression are probably more likely to be more completely reprogrammed than those with low levels. Without knowing the ideal expression levels of

pluripotency markers for bovine cells, the expression of pluripotency markers at higher levels than the un-transfected cell type remains a reasonable way to determine if a reprogramming event has taken place.

Cultures supplemented with VPA-NaB-SB-PD were able to produce embryoid bodies that expressed markers from all three germ lineages. In the first experiment of this chapter, EBs derived from colonies supplemented with NaB-SB-PD did not express markers from all three lineages. However, in the second experiment outlined in this chapter NaB-SB-PD supplementation allowed production of EBs that expressed markers from all three germ lineages. This is consistent with previous results in Chapter 5 (pg. 168) of this thesis, and suggests that the supplementation of NaB- SB-PD supports generation of cells with pluripotent potential. However, supplementation with VPA does appear to increase the expression of pluripotency markers compared to those derived from cultures supplemented with NaB-SB-PD. The results of this first experiment confirm the hypothesis that the addition of VPA to bovine iPS cultures enhances reprogramming of cells as seen by an increase in reprogramming kinetics, large increase in colony numbers, increased expression of *POU5F1* compared to colonies produced without exposure to VPA, and presence of alkaline phosphatase activity.

The effect on reprogramming of the small molecules VPA, NaB, SB431542 and PD0325901 when used individually or in different combinations was previously unclear. The second experiment described in this chapter aimed to determine the effect supplementation of small molecules individually and in different combinations had on reprogramming bovine iPS cells. BEFs non-virally transfected with the Tet-OSKM reprogramming construct were supplemented with small molecules either individually or in combinations shown in

Table 6-4. The cell type and reprogramming construct used for this experiment were chosen to allow direct comparison with work done previously on effect of reprogramming bovine iPS cells with NaB-SB-PD small molecule cocktail where the same cell type and reprogramming factor combination were used for reprogramming (McMillan, 2012).

Previous studies of human and mouse iPS cell generation have reported the use of both single small molecules and a number of different combinations of small molecules to enhance reprogramming efficiency of iPS cell generation from different somatic cell types. Generally it has been observed that the greatest effects on reprogramming can be seen when small molecules are used in combination, with the more small molecules supplemented, the stronger effect on reprogramming (Lin et al., 2009; Zhang, Zhonghui et al., 2011).

In this study, time to colony formation decreased with the addition of VPA either individually or in any combination with other the small molecules tested here. No difference in time to colony formation was observed between treatments supplemented with any combination of small molecules containing VPA. This indicates the potent effect of VPA on cellular reprogramming. The addition of all other small molecules either individually or in combination (except NaB alone) also resulted in a decrease in time to colony formation compared to cultures supplemented with iPS media without small molecules. This indicates the importance of the target pathways MEK (PD) and TGF- $\beta$  (SB) and the modification of chromatin structure (NaB and VPA) on the reprogramming of bovine iPS cells. It is interesting that the addition of NaB alone did not result in increased reprogramming kinetics, as a previous study producing mouse iPS cells reported that using butyrate at rates of 0.5-1mM showed accelerated reprogramming (2-3 days faster). Butyrate was used at 0.5mM in the experiments outlined in this chapter so would have been expected to accelerate kinetics of reprogramming. However, the effects of butyrate concentration on bovine cells are not documented and higher or lower concentrations may be required to observe enhanced reprogramming. The results presented in this chapter indicate that VPA increases the kinetics of reprogramming more than other small molecules used either individually or in combination with others. Huangfu et al. (2008) suggest that VPA may have other modes of action in addition to HDAC inhibitor, which may account for the acceleration of reprogramming kinetics and increased reprogramming seen in cultures are supplemented with VPA compared to NaB. Although the average time to colony formation was slightly lower than previously observed for the same treatments in the previous experiment, the difference was not statically different ( $P>0.05$ ).

The reprogramming efficiency, measured as the total number of colonies produced compared to the original number of cells transduced, was highest in any treatments supplemented with VPA. The number of colonies produced by cultures supplemented with small molecule cocktails not containing VPA did not compared to differ cultures derived without the supplementation of small molecules. This result is in contrast to reports that claim the addition of one or more small molecules enhance reprogramming efficiency. Liang et al. (2010) reported that the addition of butyrate alone can improve reprogramming efficiency of mouse iPS cells by 7 fold. It has also been reported that the combination of SB-PD results in more than a 200 fold increase in reprogramming efficiency of human iPS cells (Lin et al., 2009). Zhang, Y. et al. (2012) reported that efficiency was improved by adding NaB or VPA alone or SB-PD in combination, although at lower levels that the NaB-SB-PD combination used here. In this chapter the number of colonies observed in cultures treated with VPA individually, or in any combination with other small molecules, was far higher than the number of colonies observed in cultures treated with other small molecules. The highest numbers of colonies were observed in cultures supplemented with VPA in combination with PD or at least two other small molecules. This is in agreement with the reports that reprogramming is enhanced further with supplementation of each additional small molecule (Huangfu et al., 2008). It is interesting that the combination of two small molecules VPA-PD resulted in the same high number of colonies generated as those supplemented with VPA in combination with two or three small molecules. The addition of VPA-PD may be sufficient for enhancing reprogramming without the addition of other small molecules. These results indicate that the derivation of bovine iPS cells may be more reliant on the modification of chromatin by histone acetylation rather than activation of MEK and TGF- $\beta$  pathways which have been found to be sufficient to enhance reprogramming in other cell types from other species. However it is clear that the addition of small molecules targeting the MEK and TGF- $\beta$  pathways enhances reprogramming in combination with NaB or VPA as HDAC inhibitors.

As was observed in the first experiment described in this chapter, colony morphology appeared to be improved in cultures supplemented with VPA, providing VPA was added in combination with at least one other small molecule that targeted a different pathway involved in reprogramming. The combination of both HDAC inhibitors alone, VPA-NaB, resulted in a large proportion of colonies with poor colony morphology, with large irregularly shaped, differentiated colonies commonly observed. HDAC inhibitors have been found to induce differentiation of cells when added at high levels (NaB >1mM) (Newmark et al., 1994). Colonies that were differentiated occurred more frequently in cultures that were supplemented with VPA or NaB alone or in combination with each other without

another small molecule that targets an alternative pathway. It is possible that the histone acetylation that is facilitated by VPA and NaB causes differentiation by increasing the flexibility of the chromatin structure, thereby making genes easier to switch on and off. This may result in the spontaneous activation of genes that cause differentiation of iPS cells (Liang et al., 2010). To determine if histone acetylation was facilitated by the supplementation of NaB or VPA or a combination of both, the levels of the histone H3K9ac levels could be analysed. H3K9ac is found at actively transcribed promoters, indicating when a gene is switched on for transcription. Pluripotent cell genomes are characterised by a unique epigenetic signature, studies have shown that there is a distinct link between the potency of cells and histone modification (Hezroni, Tzchori, Davidi, Mattout, & Biran, 2011). The study demonstrated that more potent cells had higher levels of H3K9ac, while less potent cells had significantly reduced levels. Additionally, it was shown that the treatment of less potent cells with HDAC inhibitors increased not only their potency but also H3K9ac levels (Hezroni et al., 2011). H3K9ac levels of colonies produced in this line of experiments could be analysed to determine if levels are increased more by the supplementation of VPA, NaB or a combination of both. This may give a further insight into the process these small molecules enhance reprogramming of these cells.

In addition to the role of VPA in reprogramming iPS cells, it has also been shown to be effective in treating acute myeloid leukemia by inducing differentiation and apoptosis of cancer cells and promoting the growth of normal stem cells (Bug et al., 2005; Teng et al., 2010). iPS cells and cancer cells share many characteristics; it is possible that, in cells which are not fully reprogrammed, supplementation with VPA may cause differentiation and apoptosis similar to the mechanism observed in cancer cells.

The supplementation of HDAC inhibitors is believed to be most useful at the beginning of reprogramming due to their effects on chromatin structure (Liang et al., 2010). The supplementation of NaB and VPA during the entire reprogramming process of this experiment potentially resulted in the maintenance of chromatin remodelling during this entire period. This may have effected correct gene regulation in the putative iPS cells and may have led to increased levels of differentiation or incomplete reprogramming. When VPA was added in combination with at least one other small molecule with a different mode of action (SB or PD) colony morphology was greatly improved. The supplementation of other small molecules in combination with either HDAC inhibitor NaB or VPA may delay iPS cell differentiation through activation or inhibition of pathways to maintain

pluripotency. This may explain why when NaB or VPA were used in combination with SB and/or PD poor colony morphology or a large number of differentiated colonies were not observed at high proportions. This highlights the importance of using these small molecules in combination to enhance reprogramming of iPS cells by targeting numerous pathways involved in reprogramming.

As discussed earlier, high expression of OCT4 (*POU5F1*) is necessary for reprogramming of somatic cells to pluripotency (Nagamatsu et al., 2012; Shi, G. & Jin, 2010; Tiemann et al., 2011). The highest expression of *POU5F1* was seen in colonies supplemented with the combination of four small molecules VPA-NaB-SB-PD. This indicates that VPA and NaB may have a synergistic effect on the expression of *POU5F1* which may be mediated by the inhibition of the MEK and TGF- $\beta$  pathways. The expression of *SOX2* was also highest in the VPA-NaB-SB-PD supplemented colonies, although these levels were moderate compared to *POU5F1* expression levels. The endogenous expression of *NANOG* is essential for the reprogramming of iPS cells, and high expression of *NANOG* has been used to identify and isolate fully reprogrammed colonies (Silva, J. et al., 2009; Theunissen, Thorold et al., 2011). Similar to *POU5F1* and *SOX2* expression, the expression of *NANOG* was highest in colonies supplemented with the small molecule combination VPA-NaB-SB-PD. The level of *NANOG* expression observed in this treatment was similar to the high level of *POU5F1* expression seen for the same treatment. These results indicate that the supplementation of four small molecules in combination, VPA-NaB-SB-PD, resulted in putative bovine iPS colonies that expressed high levels of *POU5F1* and *NANOG* and low to moderate levels of *SOX2*. These expression levels conform to the pattern of marker expression believed to be optimal for the derivation of murine iPS cells (Tiemann et al., 2011). However, it is important to note that no colonies produced from any treatment described in this chapter were considered to be fully reprogrammed; it is possible the expression of pluripotency markers was not optimal or that there were other roadblocks preventing the complete reprogramming of the putative iPS cells described here.

It is clear that the combination of VPA-NaB-SB-PD has a dramatic effect on the expression of pluripotency makers in putative iPS colonies, yet it is less clear what effects individual small molecules or small molecules in different combinations have on expression of *POU5F1*, *SOX2* and *NANOG*. From this line of experiments no clear patterns of expression were evident between different markers for the same treatment. It is interesting to note the high expression of *POU5F1* and *NANOG* by iPS colonies supplemented with SB431542 alone. SB431542 is a TGF- $\beta$  inhibitor, and TGF- $\beta$  and similar molecules play important roles in self-renewal and differentiation of stem cells.

TGF- $\beta$  acts to initiate epithelial-mesenchymal transition (EMT) which occurs in embryonic development. The opposite process of mesenchymal-epithelial transition (MET) is essential for the reprogramming of somatic cells to pluripotency (Watabe & Miyazono, 2009), consequently, inhibiting TGF- $\beta$  enhances cellular reprogramming (Lin et al., 2009). As an end point of MET, E-Cadherin has been shown to be down regulated and is believed to regulate NANOG expression, which in turn effects OCT4 expression (Chou, Y.-F. et al., 2008; Thiery & Sleeman, 2006). These processes may explain how SB supplementation resulted in increased *POU5F1* and *NANOG* expression. However, it is unclear why the effect of SB on the expression of these two markers appears to decrease when in combination with other small molecules. NaB is thought to effect the expression of c-MYC, but due to limited material c-MYC expression was not analysed. It would be interesting to analyse the effects of the different small molecules on other pluripotency markers including KLF4 and c-MYC in future experiments. Further experiments could analyse miRNA or protein production associated known pluripotency pathways. Assays to detect proteins and miRNA associated with these pathways are commercially available and easy to use, making them an attractive option to determine the effect of small molecules on cellular pathways (QIAGEN, 2014).

As both VPA and NaB are HDAC inhibitors, it was of interest to determine whether both molecules were necessary for reprogramming. It was shown that VPA supplemented individually was able to accelerate kinetics of reprogramming and increase reprogramming efficiency. However, the expression of pluripotency markers in colonies that were supplemented with VPA-NaB-SB-PD was higher than colonies supplemented with VPA-SB-PD or NaB-SB-PD, suggesting that VPA-NaB may have a synergistic effect on the reprogramming of cells.

Treatments supplemented with a combination of at least three small molecules consistently produced embryoid bodies that expressed markers from all three germ lineages. This suggests that the supplementation of a combination of small molecules contributes to the pluripotency status of putative bovine iPS cells. This is in agreement with previous studies using the NaB-SB-PD combination, where EBs stained positively for markers from all three lineages (Zhang, Zhonghui et al., 2011). In contrast to reports that butyrate treatment results in pluripotent cells that can produce teratomas expressing markers for all three lineages (Liang et al., 2010), NaB treatment did not result in expression of mesoderm or endoderm marker in the experiments outlined in this chapter. However, the reports of NaB improving cellular reprogramming were made by studies on mouse and human iPS cell and EB generation, where the production of fully reprogrammed iPS cells has been

well documented. Although expression of lineage markers is a less stringent test for pluripotency than teratoma formation or the most stringent test, tetraploid complementation, due to time and material constraints teratomas assays could not be conducted in the experiments outlined in this thesis.

## **6.5 Conclusion**

Bovine iPS cell generation has proven difficult using standard iPS culture techniques. The recent advances that small molecule supplementation has had on the generation of iPS cells, provides encouragement that the manipulation of pathways involved in reprogramming may be used to generate fully reprogrammed bovine iPS cells.

Using a combination of four small molecules valproic acid (VPA), sodium butyrate (NaB), SB431542 (SB) and PD0325901 (PD), putative bovine iPS colonies were generated that exhibited embryonic stem cell like morphology, stained positively for Alkaline Phosphatase and expressed high levels of pluripotency markers. Colonies derived cultures supplemented with VPA-NaB-SB-PD were able to produce embryoid bodies that expressed markers from all three germ lineages. This combination was found to be more efficient at reprogramming bovine cells than the NaB-SB-PD combination used previously in this thesis.

Additionally, the effects different small molecules had on reprogramming of bovine iPS cells when cultures were supplemented with individual molecules or small molecules in combination with each other was also tested. It was found that VPA has the greatest effect on accelerating kinetics of reprogramming and improving reprogramming efficiency of the four molecules tested. However, the greatest improvements in reprogramming were seen when VPA was supplemented in combination with other small molecules. This study indicated the importance of histone deacetylation in combination with other molecular pathways MEK and TGF- $\beta$  on the production of bovine iPS cells. However, the cells produced by supplementation with this combination of small molecules were not considered to be fully reprogrammed as they could not be passaged, and did not stain for SSEA4 or Tra-1-60. However these putative bovine iPS colonies did exhibit characteristics similar to conventional embryonic stem cells, indicating these cells were most likely in a partially reprogrammed state.

These results are encouraging and have provided groundwork for finding a combination of small molecules that may be used to produce more fully reprogrammed bovine iPS cells. As embryonic stem cells have yet to be successfully isolated and cultured from the bovine, it is difficult to determine which pathways are integral to maintaining a pluripotent state in bovine cells. Further analysis of these pathways and interactions in bovine stem cells would provide more insight into which pathways should be targeted to enhance reprogramming in bovine cells.

## **Chapter 7 : Production of Bovine iPS Cells by Non-Viral Minicircle Transfection**

### **7.1 Introduction**

Induced pluripotent stem (iPS) cells are of great interest in the livestock industry for their potential applications in the production of transgenic livestock and for use in reproductive applications. Recent advances in cellular reprogramming methodology have resulted in the ability to produce iPS cells from species, including livestock that were once difficult to reprogram. However, bovine iPS cell generation has been difficult, with few studies successfully reporting the production of fully reprogrammed bovine iPS cells and no robust or reproducible protocol for the production of these cells yet to be established (Cao et al., 2012; Huang, B. et al., 2011; Sumer, Liu, Malaver-Ortega, et al., 2011).

For iPS cells to be used safely in clinical applications, reprogramming must occur without transgene integration to ensure the risk of tumorigenesis is reduced (Cela, 2009). The use of non-viral, non-integrating vectors for the production of iPS cells has become more popular since the inception of iPS cell generation. These non-integrating vectors can be used to generate iPS cells that can potentially be used for clinical purposes. However, the inherent problem associated with non-viral vectors, of significantly lower reprogramming efficiencies than viral methods, has limited the adoption of these methods (Jia et al., 2010). To date only one investigation into the production of non-viral bovine iPS cells has been reported, with the resulting cells described as being only partially reprogrammed (Huang, B. et al., 2011). Recent advances in the production of non-viral vectors and the use of small molecules and growth factors to enhance reprogramming has improved the prospects of non-viral iPS derivation and may be used to improve the reprogramming of bovine cells.

The availability of commercially available non-viral, non-integrating minicircle vectors presents a potentially easy and efficient method for the production of iPS cells without transgene integration. Minicircle vectors are supercoiled DNA plasmids that lack a bacterial backbone through removal by PhiC31-mediated intramolecular combination. Minicircle plasmids contain no bacterial DNA and therefore lack a bacterial origin of replication and antibiotic resistance genes, and as such are far smaller than traditional episomal vectors (approximately 3kb shorter) (Chen, Z.-Y. Y. et al., 2003; Jia

et al., 2010). It is claimed that minicircle vectors have a far higher transfection rate compared to larger episomal vectors, likely due to their smaller size (Jia et al., 2010). Additionally they also show longer ectopic expression in the host cell due to lower activation of exogenous silencing methods (Jia et al., 2010). This gives transfected cells longer to switch on endogenous gene expression associated with reprogramming, thereby potentially improving the efficiency of producing fully reprogrammed iPS cells. However, long term expression of the transgenes can also slow the process of exogenous gene silencing in fully reprogrammed iPS cells, thereby potentially limiting their use (Chen, Z.-Y. Y. et al., 2003).

In the original study of iPS generation by minicircle vector, Jia et al. (2010) used a polycistronic minicircle with four reprogramming factors, *POU5F1* (OCT4), *NANOG*, *LIN28* and *SOX2* (OSNL), in combination with a green fluorescent protein (GFP) reporter gene, to produce human iPS cells. Adult adipose tissue stem cells were initially transfected with the minicircle by electroporation, followed by Lipofectamine™ transfections on days four and six. After selection for cells expressing the GFP reporter gene by flow cytometry, positive cells were cultured in iPS cell culture conditions, with iPS colonies observed 14-16 days post transfection. The study reported an average reprogramming efficiency of approximately 0.005%, making the minicircle iPS reprogramming more efficient than traditional episomal plasmids (0.001%) (Okita et al., 2008; Yu, J. et al., 2009) but still less efficient than virally derived cells (approximately 0.01-0.1%) (Okita et al., 2007; Wernig et al., 2007). Minicircle derived iPS cells showed ES cell-like morphology, stained for embryonic stem cell makers and exhibited all characteristics associated with pluripotency, in addition to a lack of integration into the host genome (Jia et al., 2010). This method of producing non-viral iPS cells that are free of exogenous DNA integration therefore has potential to be used in an applied setting, with the only drawback appearing to be the number of transfections needed to induce reprogramming.

Minicircle vectors have advanced the field of non-viral iPS cell generation by decreasing episome size and thereby increasing reprogramming efficiency, while limiting the potential for integration into the host genome. Additionally the derivation of iPS cells by minicircle transfection requires a relatively simple protocol. At this time minicircle derived iPS cells have only been reported in human cells, specifically by transfection of adipose tissue derived stem cells. The derivation of minicircle iPS cells in other species and cell types has not been well documented. In 2011, minicircle vectors became commercially available with both human and mouse derived factors in the so-called Yamanaka

(OSKM) and Thomson (OSNL) reprogramming factor combinations (STEMCELL Technologies 2011) (Applied Biological Materials Inc, 2014).

The use of iPS cells in reproductive technologies, including germ cell transplantation, offers enormous potential for the dissemination of genetics in livestock species. Pluripotent stem cells, including both embryonic stem (ES) cells and iPS cells, are capable of differentiating into cell types from all three germ lineages, including development into germ cells (Imamura et al., 2014). While iPS cells have previously been shown to contribute to the germline by the production of chimeras, it is also possible to differentiate ES and iPS cells into cells from the male and female germline *in vitro* (Imamura et al., 2010; Imamura et al., 2014; Kerkis et al., 2007; Yang, S. et al., 2012; Zhu et al., 2012). The differentiation of human iPS cells into both male and female germline cells has previously been achieved, although it is yet to be determined if the derivation of these cells can result in functional gametes (Easley et al., 2012; Medrano, Ramathal, Nguyen, Simon, & Reijo Pera, 2012; Park, T. et al., 2009).

The differentiation of germ cells *in vitro* is recognised as being difficult compared to differentiation of other cell types where detailed differentiation protocols have been described (Imamura et al., 2014). Preliminary experiments into the differentiation of pluripotent cells toward the germline were dependent on spontaneous differentiation and were not easily controlled. The increase in knowledge and understanding of germ cell differentiation has led to more consistent methods of producing germline cells using *in vitro* methods. However, there is still a lack of understanding of the complex mechanisms associated with germ cell differentiation, particularly in species other than humans and mice. Additionally, the mechanisms associated with the development and differentiation of germ cells are not easily controlled *in vitro* (Imamura et al., 2014).

Retinoic acid (RA) is most commonly used to differentiate pluripotent cells toward the germline and has been shown to induce the production of primordial germ cells (PGCs), spermatogonia and oocytes through the initiation of meiosis (Bowles & Koopman, 2007; Chen, W. et al., 2012; Hu, Y. et al., 2012; Kerkis et al., 2007; Lavagnolli et al., 2009; Silva, C. et al., 2009). Additionally, BMP4, testosterone or a combination of these growth factors, with or without retinoic acid, have also been shown to increase the expression of testis cell markers when supplemented during embryoid body formation or in adherent cultures (Nayernia et al., 2006; Panula et al., 2011; Silva, C. et al., 2009; Yang, S. et al., 2012; Zhu et al., 2012). Retinoic acid has been shown to initiate meiosis in PGCs. High

levels in the foetal female gonad and low levels in the foetal male gonad suggests that the up regulation of RA initiates oogenesis in the foetal female, while the down regulation in the male gonads suggests the role of RA suppression in halting meiosis until puberty (Bowles & Koopman, 2007; Koubova et al., 2006). Additionally, RA has been shown to induce male germ cells to enter meiosis *in vitro* (Eguizabal et al., 2011). However, RA only appears to be able to induce meiosis in PGCs at an early stage of development, and has no effect on meiosis after the cells have progressed further toward the male differentiation pathway (Ohta, Lin, Hogg, Yamamoto, & Yamazaki, 2010).

A recent study reported that lentivirally produced bovine iPS cells could be differentiated into female germ cells. In this instance embryoid bodies were cultured in 10% porcine follicular fluid and 0.5  $\mu$ M retinoic acid (RA), producing cells that expressed early and late female germ cell-specific genes *VASA*, *DAZL*, *Gdf9*, *Nobox*, *Zp2*, and *Zp3* (Cao et al., 2012). Additionally, porcine muscle derived stem cells have also been converted to female germ cells by culture with the small molecule reversine, and bovine follicular fluid at 10% or 20%. These cells expressed common female germ cell markers and displayed large round cellular morphology, comparable to oocytes (Lv et al., 2012).

While the differentiation of pluripotent cells into germ cells *in vitro* has been shown to be successful, to date, there has been no success in the production of a genuine line of spermatogonial stem cells derived *in vitro* from pluripotent stem cells (Imamura et al., 2014). Attempts to produce a line of spermatogonial stem cells from pluripotent stem cells have been made though the differentiation of mouse ES cells using treatment with retinoic acid (Nayernia et al., 2006). This treatment resulted in the production SSC like colonies. Further treatment of SSC like colonies with retinoic acid was reported to produce haploid cells that differentiated into sperm like cells. However, the putative sperm cells produced in this study resulted in abnormal pups that died within five months, possibly due to abnormalities in genetic imprinting (Nayernia et al., 2006).

A study conducted in 2011 demonstrated the use of a progressive approach of differentiation ES and iPS cells first to epiblast-like cells which were then differentiated into primordial germ cell-like cells that efficiently underwent spermatogenesis (Hayashi et al., 2011). These studies were further built on when it was shown that primordial germ cells could be produced from mouse iPS cells by a combination of *in vitro* differentiation followed by transplantation into male hosts. These cells were capable of producing functional gametes and healthy offspring (Hayashi & Saitou, 2013). These results indicate that the derivation of male germline cells from bovine iPS cells for use in transgenic

breeding and other reproductive technologies may be feasible in the future. Table 7-1 describes reports of *in vitro* differentiation of ES cells and iPS cells into germline cells from different species.

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Table 7-1. Reports of *in vitro* germ cell derivation from embryonic stem cells and induced pluripotent stem cells. (Table revised from Imamura et al. (2014)). ES= embryonic stem cells, iPS= induced pluripotent stem cells, PGCs= primordial germ cells, SSC= Spermatogonial stem cells, EGF=epidermal growth factor, GDNF= glial cell derived neurotropic factor, SCF= Stem cell factor.

Reference	Source Cells	Culture Methods	<i>In vitro</i> derived germ cells	Evaluation
Toyooka et al. (2003)	Mouse ES cells	EBs co-cultured with BMP4 expressing cells	PGCs	Expression of germ cell markers Transplantation into host testis
Mizuno et al. (2006)	Mouse ES cells	EBs co-cultured with BMP4 expressing cells	PGCs	Expression of germ cell markers
Nayernia et al. (2006)	Mouse ES cells	Adherent cultured with retinoic acid	SSCs and sperm	Expression germ cell markers Genomic imprinting Transplantation into host testis
Kerkis et al. (2007)	Mouse ES cells	EBs cultured with retinoic acid	Sperm	Expression of germ cell markers Haploid cells produced
Lavagnolli et al. (2009)	Mouse ES cells	EBs cultured with retinoic acid	PCGs, Sperm, Oocytes	Expression of germ cell markers Haploid cells produced
Imamura et al. (2010)	Mouse iPS cells	EBs co-culture with BMP4, EGF, GDNF, SCF expressing cells	PCGs, Oocytes	Expression of germ cell markers
Chen, W. et al. (2012)	Mouse ES cells	Adherent culture with retinoic acid (1 $\mu$ M)	PGCs	Expression of germ cell markers
Guo, Xin et al. (2012)	Mouse ES cells	EBs cultured with retinoic acid (1, 2 or 5 $\mu$ M)	PGCs	Expression of germ cell markers
Hu, Y. et al. (2012)	Mouse ES cells	EBs cultured with retinoic acid	Oocytes	Expression of germ cell markers Production of oestradiol

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Yang, S. et al. (2012)	Mouse iPS cells	EBs cultured with retinoic acid	Male germ cells	Expression of germ cell markers Transplantation into host testis
Zhu et al. (2012)	Mouse iPS cells	EBs cultured with retinoic acid	Male germ cells	Expression of germ cell markers Transplantation into host testis
Cai, H. et al. (2013)	Mouse ES and iPS cells	EBs cultured with retinoic acid (2 $\mu$ M)	PGCs, Male germ cells	Expression of germ cell markers Transplantation into host testis
Park, T. et al. (2009)	Human ES and iPS cells	Adherent culture with human foetal gonadal cells	PGCs	Expression of germ cell markers Genomic imprinting
Eguizabal et al. (2011)	Human ES and iPS cells	EBs cultured with retinoic acid (1 $\mu$ M)	PGCs and spermatids	Expression of germ cell markers Genomic imprinting Haploid cells produced
Panula et al. (2011)	Human ES and iPS cells	Adherent culture with BMP4	Spermatids	Expression of germ cell markers Haploid cells produced
Easley et al. (2012)	Human ES and iPS cells	Adherent culture in normal SSC growth media conditions	SSCs, spermatocytes and spermatids	Expression of germ cell markers Genomic imprinting Haploid cells produced
Medrano et al. (2012)	Human ES and iPS cells	Adherent culture with over expression of VASA/DAZL	Spermatids	Expression of germ cell markers Genomic imprinting Haploid cells produced
Cao et al. (2012)	Bovine iPS cells	EBs cultured with 10% porcine follicular fluid and retinoic acid (0.5 $\mu$ M).	Female germ cells	Expression of germ cell markers
Lv et al. (2012)	Porcine muscle derived stem cells	Reversine and 10% or 20% bovine follicular fluid	Female germ cells	Expression of germ cell markers Oocyte morphology

In Chapter 5 of this thesis (pg. 142), derivation of putative non-viral bovine iPS cells from bovine fibroblasts was described by using an episomal plasmid coding for human transcription factors *POU5F1* (OCT4), *SOX2*, *Nanog* and *LIN28* (OSNL). To date, no reports of bovine iPS cell generations have been made using the combination of these four transcription factors which are instead more commonly used in human iPS cell generation. The aims of this chapter were two-fold, first to determine if the commercially available Minicircle vector (STEMCELL Technologies, USA), also coding for the human transcription factor combination OSNL, could be used to produce bovine iPS cells, and, whether this method was more efficient than the use of episomal plasmids. This was achieved by transfecting bovine embryonic fibroblasts (BEFs) and bovine dermal fibroblast (BDFs) with the minicircle vector using a combination of electroporation and two rounds of Lipofectamine™ transfection. The efficiency of reprogramming and characteristics of putative iPS cells produced by this method were compared to putative iPS cells derived by transfection with an episomal plasmid carrying the same set of four transcription factors (OSNL – Addgene plasmid # 20922 (Yu, Junying et al., 2007)).

Previous chapters of this thesis (Chapter 5, pg. 142 and Chapter 6, pg. 178) have shown that the putative iPS cells, produced using non-viral methods, are capable of expressing markers from all three germ lineages, providing evidence for their pluripotent potential. Due to the pluripotent potential of these cells, the second aim of this chapter was to determine if these cells could be directed to differentiate toward the germline. This was tested by culturing embryoid bodies derived from both episomal plasmid and minicircle transfection with retinoic acid at 0.5mM or 1mM for two weeks. Gene expression of known (*DDX4*, *GATA4* and *UCHL1*) and putative testis cell makers (*DDX6*, *NAP1L4* and *TKTL1*; identified in Chapter 3, pg. 69) by embryoid bodies cultured in retinoic acid were analysed after two weeks of culture.

## 7.2 Materials and Methods

### 7.2.1 General

All experiments involving animals were approved and conducted under the F D McMaster Laboratory Animal Ethics Committee for CSIRO Animal, Health and Food Sciences (AEC 11/20). All cell culture reagents were purchased from Life Technologies Corporation (USA) unless stated

otherwise. Additional reagents were purchased from Sigma-Aldridge Chemical Industries (USA) or Stemgent (USA) and are indicated in the following. The Minicircle vectors 'STEMcircles' were purchased from STEMCELL Technologies (USA). Plasmids were purchased from Addgene plasmid repository (Plasmid # 20922 (Yu, Junying et al., 2007)). Primers used in this chapter were ordered through Biosearch Technologies (USA) and are shown in Table 7-2. This experiment was repeated three times with two technical replicates for each treatment per experiment.

### 7.2.2 Derivation of Bovine Fibroblasts

For bovine dermal fibroblasts (BDFs), skin samples from male Angus calves were taken during castration which was performed under general anaesthesia. For bovine embryonic fibroblasts (BEFs), a male *Bos taurus* bovine foetus was sourced from a local abattoir from a slaughtered cow. The foetus measured approximately 18 cm crown to rump, giving an estimated age of 60-70 days gestation. Primary cell cultures were prepared using standard methods described in Chapter 4 (pg. 114). Fibroblast cells were grown in fibroblast growth media (DMEM, 10% Foetal Bovine Serum (FBS), 2 mM GlutaMAX™, 100 µM non-essential amino acids (NNEA), with 1% anti-anti (10,000 U/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®) (all components from Life Technologies, USA)), and were passaged every two to three days.

### 7.2.3 Minicircle and Plasmid Transfection of Bovine Fibroblasts

Transfection of bovine embryonic and dermal fibroblasts (BEFs and BDFs respectively) with the minicircle vector (STEMCELL Technologies, USA) was conducted according to the protocol set out by Jia et al. (2010). On the day of transfection, approximately  $6 \times 10^6$  BEFs and BDFs were each transfected with 5 µg of minicircle vector (STEMCELL Technologies, USA) per  $1 \times 10^6$  cells using a Neon Transfector System (Life Technologies, USA) under the transfection protocol 1400 V, 20 Pulse width, 2 pulses. Transfected cells ( $1 \times 10^6$  cells) were plated into each well of a 6 well plate and grown in fibroblast growth media without antibiotics (DMEM, 10% Foetal Bovine Serum (FBS) (Life Technologies, USA), 2 mM GlutaMAX™, 100 µM non-essential amino acids (NNEA) (Life Technologies, USA)). After 48 hours, cells were examined for GFP expression using a Nikon Eclipse TE300 fluorescent inverted microscope (Nikon, Japan) equipped with a cooled PE light source (Cooled, USA); images were captured using ProgRes CapturePro 2.1 software (Jenoptik, Germany).

Three days after the initial transfection, cells were again transfected with 5 µg of minicircle vector per well using Lipofectamine™ 2000 (Life Technologies, USA) according to the manufactures instructions. In short, for each well of cultured cells, 5 µg of the minicircle vector were diluted in 250 µl Opti-MEM™ and mixed gently. For each well of cells 10 µl Lipofectamine™ 2000 reagent was diluted in 250 µl Opti-MEM™ and incubated at room temperature for 5 mins. Following the 5 min incubation the Lipofectamine™ and minicircle solutions were combined, mixed gently and incubated at room temperature for a further 20 minutes. After incubating the Lipofectamine™ /minicircle solution was added to each well and mixed gently. Media was changed to fresh fibroblast media without antibiotics after 6 hours to remove the Lipofectamine™. A further 48 hours after the second transfection, the cells were again transfected with 5 µg of minicircle vector/well with Lipofectamine™ 2000 as described above. The following day cells transfected with the minicircle vector were plated onto Mitomycin-C (Sigma-Aldridge Chemical Industries, USA) inactivated BEFs ( $2 \times 10^6$ / 10cm dish) at a density of  $2 \times 10^4$  cells per 10 cm dish. Inactivation of fibroblasts with Mitomycin-C and plating onto gelatine coated plates is described in Chapter 4 (pg. 117).

Plasmid transfection of BEFs and BDFs was conducted using the Neon Transfection System (Life Technologies, USA), according to the manufactures instructions as described in Chapter 5 (pg. 152). In brief, BEFs and BDFs at  $1 \times 10^7$  cells/ml were transfected with OSNL reprogramming plasmids (2 µg; Addgene Plasmid # 20922 (Yu, Junying et al., 2007)) using the electroporation protocol of 1400V, 20 Pulse width and 2 pulses. Cells were grown in culture for 24 hours in fibroblast media without antibiotics, after which 1% Anti-Anti (Life Technologies, USA) was added. When cells reached 80-90% confluence after 2-3 days, they were enzymatically dissociated using TrypLE™ Express (Life Technologies, USA) and plated at a rate of  $5 \times 10^4$  cells onto 10cm gelatine coated tissue culture dishes plated with Mitomycin-C inactivated BEF feeder layers ( $2 \times 10^6$  feeders cells/dish) as described in Chapter 4 (pg. 117).

Cells transfected with minicircle or plasmid vector were cultured in iPS media containing small molecule combination VPA-NaB-SB-PD (Minimum Essential Media Alpha (MEM-α), 20% FBS, 1% insulin-transferrin-selenium (ITS), 2 mM GlutaMAX™, 100 µM NEAA, 50U/mol penicillin, 50 mg/ml streptomycin, 0.1 mM β-mercaptoethanol (Sigma, USA), 4 ng/ml human leukemia inhibitory factor (LIF, Millipore, USA), and 10 ng/ml basic fibroblast growth factor (bFGF, Millipore, USA), supplement with small molecules 0.5 mM sodium butyrate (NaB) (Sigma, USA), 0.5 µM PD0325901 (PD) (Stemgent, USA) and 2 µM SB431542 (SB) (Stemgent, USA) and 1 mM valproic acid (VPA) (Sigma,

USA) for 21 days with media changed every two days. The cultures were incubated at 37°C in a 95%:5% air: CO<sub>2</sub> humidified atmosphere during the culture period. Control cultures of untransfected bovine fibroblasts were grown in parallel under the same conditions of the plasmid and minicircle transduced cultures for 21 days with media changed every two days.

#### **7.2.4 Analysis of Putative Bovine iPS Colonies**

Minicircle and plasmid derived iPS cultures were observed daily for colony formation, and the time to colony formation was recorded for each treatment. Reprogramming efficiency of cultures was calculated after 21 days of culture and was determined by the total number of colonies observed divided by the total number of transfected cells initially plated. Photographs of developing colonies were taken using a Nikon Eclipse TE300 inverted microscope (Nikon, Japan) equipped with a COOLED PE light source (COOLED, USA), and images were captured using ProgRes CapturePro 2.1 software (Jenoptik, Germany). Colonies were stained for alkaline phosphatase activity using the alkaline phosphatase live stain (Life Technologies, USA) according to manufacturer's instructions outlined in Chapter 4 (pg. 119). Immunofluorescent staining for pluripotency markers SSEA4 and Tra-1-60 was carried out using the ES Cell Characterisation kit (Millipore, USA) as described in Chapter 5 (pg. 154). Following culture of iPS colonies for 21 days, colonies were picked, washed in PBS, and used to derive embryoid bodies, or archived at -80°C until RNA isolation for qRT-PCR analysis.

#### **7.2.5 Embryoid Body (EB) Production and Differentiation of putative iPS Cells Toward the Germline**

Embryoid bodies were derived from putative iPS colonies from all treatments. In short colonies used to derive embryoid bodies were manually picked and transferred to ultra-low-adherent 6cm cell culture dishes, and grown in iPS media without growth factors or small molecules (Minimum Essential Media Alpha (MEM- $\alpha$ ), 20% FBS, 1% insulin transferrin-selenium (ITS), 2 mM GlutaMAX™, 100  $\mu$ M non-essential amino acids (NNEA), 50 U/mol penicillin, 50 mg/ml streptomycin, 0.1 mM  $\beta$ -mercaptoethanol (Sigma, USA)). Embryoid body cultures were supplemented with retinoic acid (Sigma-Aldrich Chemical Industries, USA) at 0 mM, 0.5 mM or 1 mM. Embryoid bodies were grown at 37°C in a 95%:5% air:CO<sub>2</sub> humidified atmosphere, with media changed every three days, for two

weeks, after which the EBs were harvested, frozen and stored at -80°C until qRT-PCR analysis was carried out for lineage markers, and known and putative testis cell markers.

### 7.2.6 mRNA Extraction and qRT-PCR Analysis

Messenger RNA was extracted from 5 pooled colonies or embryoid bodies for each treatment using Dynabeads® mRNA DIRECT™ Micro Kit according to the manufactures instructions (Life Technologies, USA) described in Chapter 4 (pg. 120). Complementary DNA synthesis was carried out using the SuperScript™ III First-Strand Synthesis System (Life Technologies, USA) described in Chapter 4 (pg. 120). qRT-PCR reactions for expression of pluripotency and lineage markers were carried out as described previously in Chapter 4 (pg. 120). Primers used for qRT-PCR reactions are shown in Table 7-2.

### 7.2.7 Statistical Analysis

For results of time to colony formation and number of colonies one-way ANOVAs with Tukey's multiple comparison tests were conducted using GraphPad Prism® software (GraphPad Software, USA) to determine if group means differed significantly from one another ( $P < 0.05$ ).

qRT-PCR reactions were analysed in Excel add-in Genex (Bio-Rad, USA). Ct values from the raw data were converted into gene expression data as a fold change relative to reference gene *RPS26*. Statistical analysis of data was performed using GraphPad Prism® software. For all results of qRT-PCR analysis two-way ANOVAs with Tukey's multiple comparison tests were used to determine if statistically significant differences existed between group means, ( $P < 0.05$ ). Results were presented as means  $\pm$  SEM for biological replications.

Table 7-2. Primers used in Chapter 7.

Gene Construct	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Size (bp)	Reaction Efficiency (%)	Melt Peak (°C)	Specificity
<b>Pluripotency Genes</b>						
POU5F1 (OCT4)	AAGCGGACGAGTATCGAGAA	ACACTCGGACCACGTCTTTC	133	113.8	86	Bovine mRNA
SOX2	ATGAAGGAACACCCGGATTA	CATGTGCGCGTAGCTGTC	186	81.2	89	Bovine
NANOG	GTCCTGGTCAAGAAACAAAA	TCTGGAACCAGGTCTTCACC	163	106.1	87	Bovine mRNA
c-MYC	GGAAGAAATTCGAGCTGCG	GTCGCAGATGAAGCTCTGGT	226	105.7	87	Bovine mRNA
<b>Lineage Markers</b>						
TUBB3	TGGAGCGCATCAGTGTCTAC	CAGTTGTTACCAGCCCCACT	172	99.3	87.5	Total
DESMIN	GGGACATCCGTGCTCAGTAT	GTGGCGGTACTCCATCATCT	155	120.8	86.5	Total
FoxA2	ACCACTACGCCTTCAACCAC	GGGGTAGTGCATCACCTGTT	134	85.8	86	Total
<b>Known Testis Cell Makers</b>						
DDX4 (VASA)	TACGCCAATTCGATGAAC	TTGCCACTTTTCTTTGTCAAG	225	89.4	83	Endogenous
GATA4	ACCAGCAGCAGTGAGGAGAT	TGGTGACTGGCTGACAGAAG	190	86.5	89	Endogenous
UCHL1 (PGP9.5)	CCCCTGAAGACAGAGCAAAG	CCGACATTGGCCTTCCTG	86	87.9	84.5	Endogenous
<b>Putative Testis Cell Markers (Chapter 3)</b>						
DDX6	TGCCATTCTCTTGCTTTGTG	CTCACTCCTTTTGCCTGGAG	148	100.7	82	Endogenous
NAP1L4	CGGAGTTCTGGTTCACCATT	AGTCATCGGGTTCAAAGTGG	165	93.8	83.5	Endogenous
TKTL1	TGAGCGCTTCATCGAGTGTT	AGCGAAGGTGCAAGCAAAAG	194	102.5	87.5	Endogenous
<b>Reference Gene</b>						
RPS26	TCATTCGGAACATCGTAGAGG	CCTGACTACCTTGCTGTGAAT				Endogenous/ Reference Gene

## 7.3 Results

To determine if a commercially available minicircle vector, 'STEMcircles' (STEMCELL Technologies, USA) was able to produce bovine iPS cells more efficiently than a polycistronic episomal plasmid carrying the same combination of factors (OSNL), BEFs and BDFs were transfected with either the minicircle vector or the episomal plasmid. Experiments were carried out with two technical replicated per treatment, and the experiment was repeated three times.

### 7.3.1 Minicircle Vectors are Expressed in Bovine Fibroblasts Following Transfection

The presence of GFP fluorescent cells indicated that the minicircle vector was expressed in bovine fibroblasts three days after the initial electroporation (Figure 7-1), and again 24 hours after each Lipofectamine™ transfection. The initial transfection efficiency for the plasmid derived eGFP control cultures was approximately, 85% this was representative of the transfection efficiency of the reprogramming constructs. Transfection efficiency for minicircle derived cultures was approximately 95%. GFP expression was lost from these cells approximately one week after plating transfected cells on feeders for iPS culture. This suggested that the expression of the minicircle vector had decreased below an observable level at this stage.

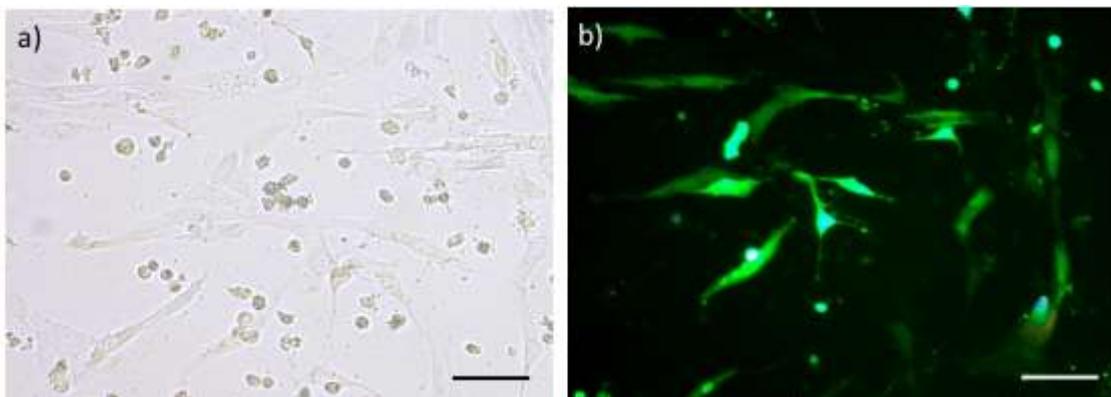


Figure 7-1. Expression of Green Fluorescent Protein (GFP) in bovine embryonic fibroblasts, transfected with minicircle vector, 3 days after initial transfection by electroporation) a) Brightfield image b) Fluorescent image. Scale bar represents 50  $\mu\text{m}$ .

### 7.3.2 Minicircle Vector Transfection Improves Reprogramming Efficiency

Following transfection of minicircle and episomal plasmid vectors into BDFs and BEFs, the cells were cultured in iPS media with the small molecule combination, VPA-SB-PD-NaB, for 21 days. There was no significant difference in time to colony formation observed between cultures transfected with minicircle or episomal plasmid, with colonies appearing on average after 10 days in culture (Figure 7-2a). However, for both BDF and BEF cells, the average number of colonies derived per 10cm dish after 21 days of culture, was significantly higher in cultures transfected with the minicircle vector compared to the OSNL episomal plasmid ( $P<0.05$ ). On average 31 colonies were produced in episomal plasmid cultures and 50 colonies in minicircle cultures (per 10 cm dish) (Figure 7-2b). This equated to a total reprogramming efficiency of 0.1% for minicircle vector derived colonies, and 0.062% for OSNL episomal plasmid derived colonies. Additionally, there was no significant difference in the number of colonies produced between BDF and BEFs transfected with the same reprogramming vector (Figure 7-2b). No colonies were observed in control cultures.

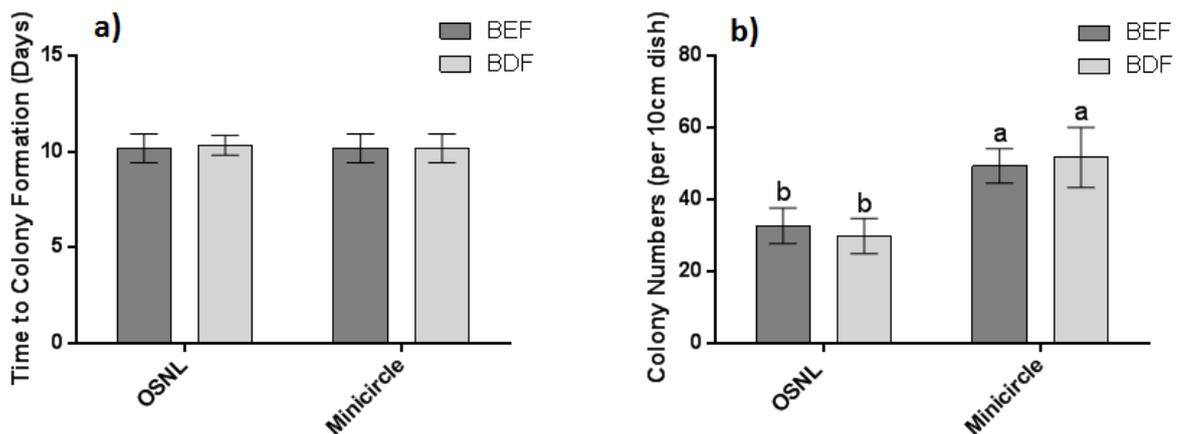
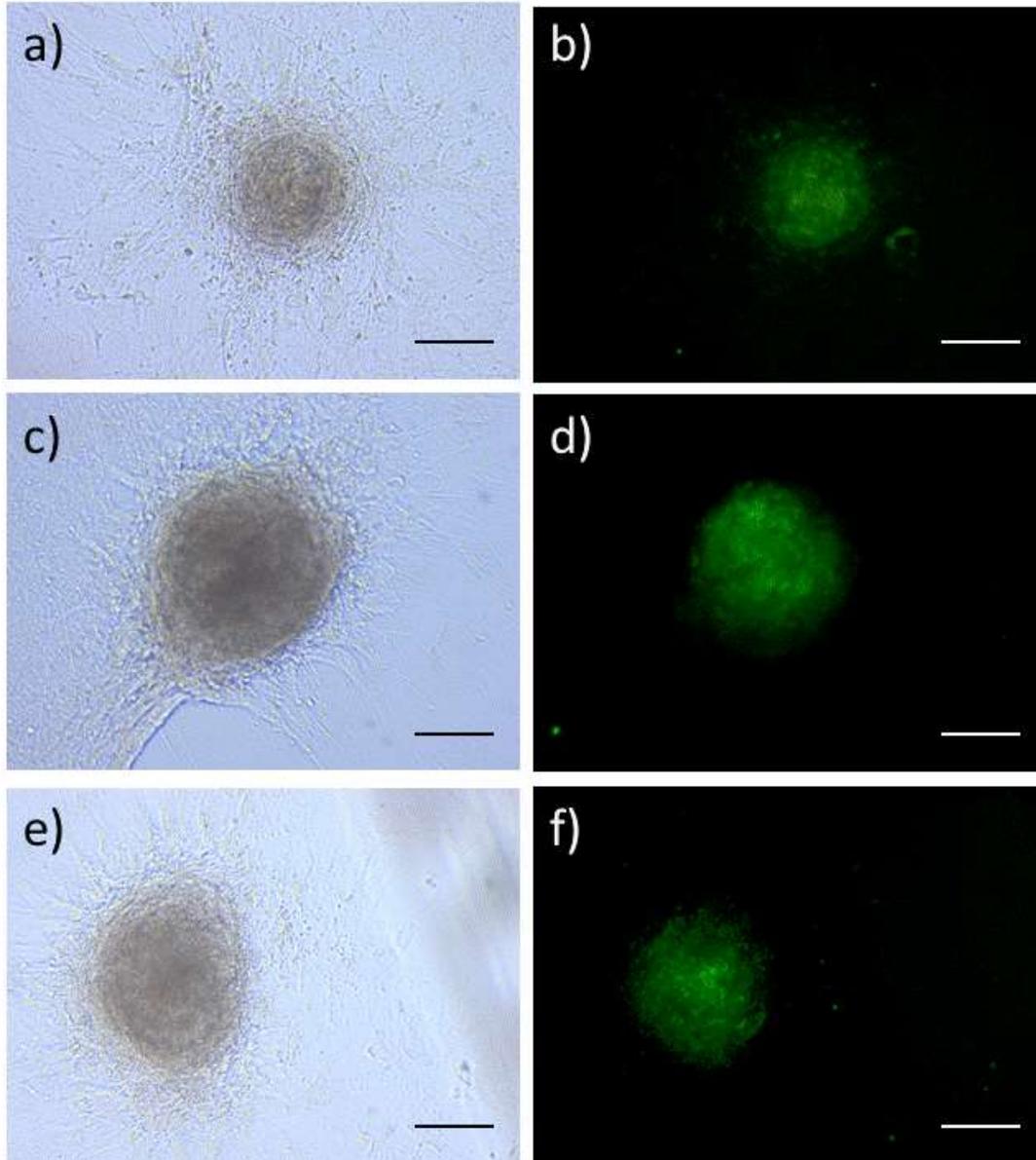


Figure 7-2. a) Time to Colony formation in days for putative bovine iPS colonies derived from BEF and BDF cells transfected with minicircle vector or OSNL plasmid (n=6,  $P>0.05$ ). b) Average numbers of putative bovine iPS colonies present per 10 cm dish after 21 days of culture for BEF or BDFs transfected with minicircle or OSNL plasmid. Different characters above error bars indicate a significant difference in treatment means (n=6,  $P<0.05$ ). Error bars represent SEM.

### **7.3.3 Minicircle and Episomal Plasmid Derived Putative Bovine iPS Colonies Express Alkaline Phosphatase**

Live alkaline phosphatase staining was carried out after 14 and 21 days of culture. After 14 days of culture, colonies derived from both minicircle (Figure 7-3a, b) and episomal plasmid (Figure 7-3c, d) cultures, showed strong fluorescent staining, indicating alkaline phosphatase activity. These colonies showed ES cell like morphology with small, round cells forming densely packed colonies with clearly defined borders (Figure 7-3a, c). After 21 days of culture, colonies appeared larger in both the minicircle and episomal plasmid cultures, and those that stained positively for alkaline phosphatase still displayed ES like morphology with clear borders and small tightly packed cells (Figure 7-3b,c). Some colonies became large, dome shaped structures and resembled embryoid bodies, these colonies did not stain positively for alkaline phosphatase (

Figure 7-8) and so acted as negative controls for alkaline phosphatase assays. None of the minicircle or OSNL plasmid derived colonies stained positively for stem cell markers SSEA-4 or Tra-1-60 (data not shown).



**Figure 7-3: Examples of alkaline phosphatase positive putative bovine iPS colonies. a) Small colony present in minicircle cultures after 14 days in culture staining positively for Alkaline Phosphatase, similar colonies also present in OSNL cultures. b, c) Larger colonies present in OSNL (b) and minicircle cultures (c) after 21 days in culture, also stain clearly positively for alkaline phosphatase activity in the centre of the colony and slightly weaker or absent at colony edges. Scale bars represent 100  $\mu\text{m}$ .**

### 7.3.4 Minicircle and OSNL Episomal Plasmid Derived Putative iPS Colonies Express Pluripotency Markers

Following 21 days of culture, putative iPS colonies from all treatments were analysed for pluripotency markers using qRT-PCR. All putative iPS cultures expressed high levels of *POU5F1* (OCT4), as did un-transfected BEFs, expressing 400 fold higher levels of *POU5F1* than un-transfected BDFs, which did not express *POU5F1*. However, the level of *POU5F1* expression by un-transfected BEFs was significantly lower than expression by all putative iPS colonies ( $P < 0.05$ ). *POU5F1* expression was highest in BEF minicircle-derived colonies, with approximately 71 fold higher expression than un-transfected BEFs. BDF OSNL-plasmid derived colonies expressed the next highest levels of *POU5F1* (7 000 fold higher than BDFs), BEF OSNL derived colonies (3 fold higher than BEFs) and BDF minicircle derived colonies (2 700 fold higher than BDFs) (Figure 7-4a).

*SOX2* was not expressed by un-transfected cell types, but was expressed by all putative iPS cultures, with the highest expression by BDFs transfected with the minicircle vector (25 000 fold compared to un-transfected cell type). BEF minicircle (11 500 fold higher than BEFs) and BDF OSNL transfected colonies (11 000 fold higher than BDFs) expressed moderate levels of *SOX2*. Low levels of *SOX2* were also expressed by BEF OSNL transfected colonies; however, these were still 700 fold higher than un-transfected BEF cells (Figure 7-4b).

*NANOG* was expressed by all iPS cultures, and also by un-transfected BEFs at a lower level than putative iPS colonies, however, the expression of *NANOG* by BEFs was still almost 200 fold higher than un-transfected BDFs, which did not express *NANOG*. The highest expression of *NANOG* was seen in BEF minicircle derived colonies (40 fold higher than BEFs), followed by BDF minicircle derived colonies (1400 fold higher than BDFs). OSNL episomal plasmid transfected colonies also expressed *NANOG*, with BDF OSNL plasmid derived colonies expressing higher levels of *NANOG* than un-transfected BDFs (1500 fold than BDFs), and BEF OSNL plasmid derived colonies (2.5 fold higher than BEFs) (Figure 7-4c).

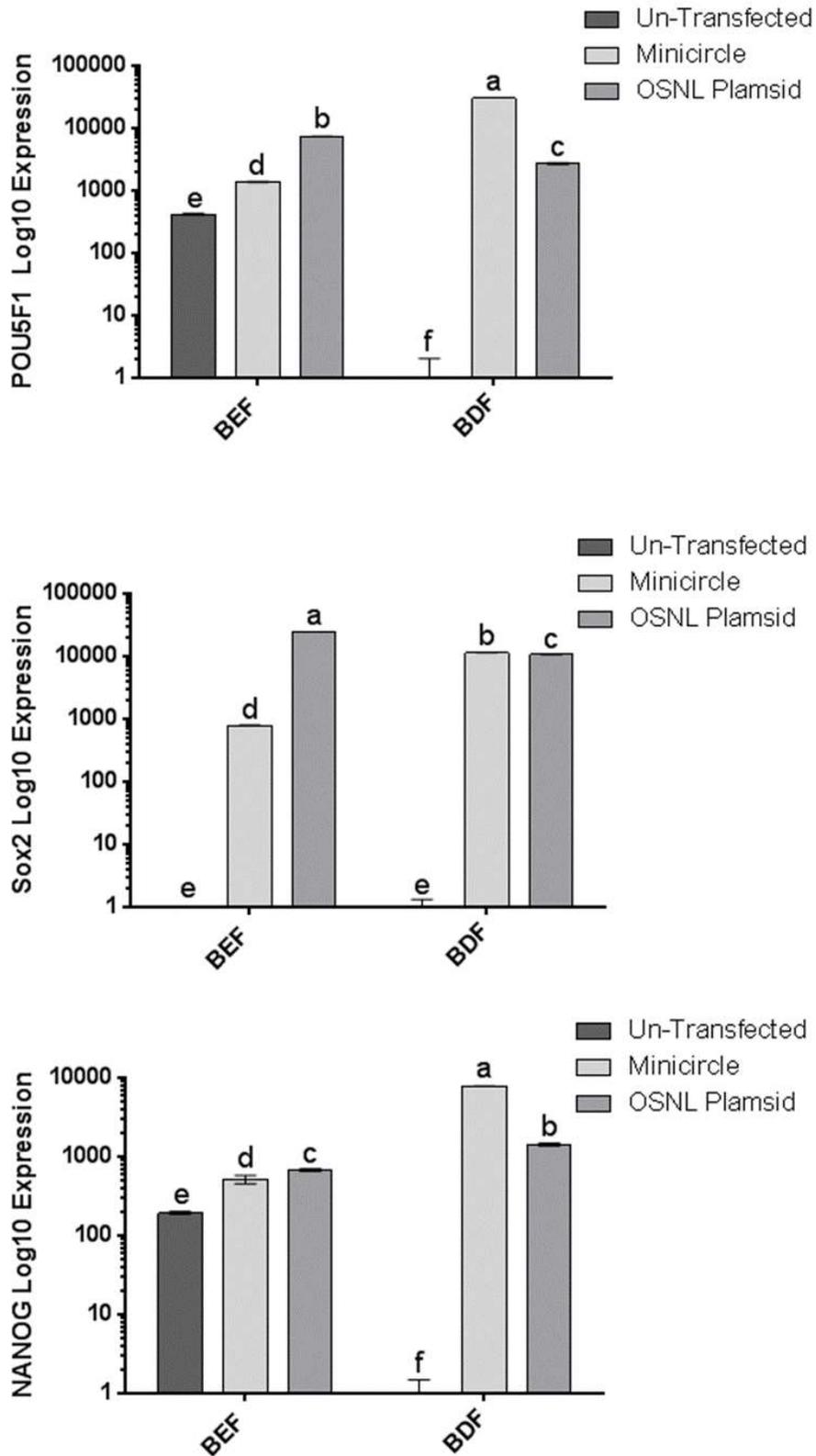


Figure 7-4: Log<sub>10</sub> Expression of pluripotency markers a) *POU5F1* (OCT4), b) *SOX2* and c) *NANOG*, by putative iPS colonies derived from BEF and BDFs transfected with minicircle vector or OSNL episomal plasmid, relative to housekeeper gene *RPS26*. Different characters above treatment means indicates a significant difference in expression (n=3, P<0.05). Error bars represent as ± SEM.

### 7.3.5 Minicircle and Episomal Plasmid Derived Embryoid Bodies Express Markers from all Three Germ Lineages

Following 21 days of culture, putative iPS colonies were manually picked from all treatments and used for embryoid body derivation. Embryoid body cultures were grown in the presence of retinoic acid (RA) at 0 mM, 0.5 mM or 1 mM for 2 weeks. After 7 days in culture embryoid bodies displayed characteristic morphology (Figure 7-5a) and continued to grow into very large structures after 14 days in culture (Figure 7-5b). The addition of retinoic acid to EB cultures did not alter the appearance of EBs compared to those not treated with RA.

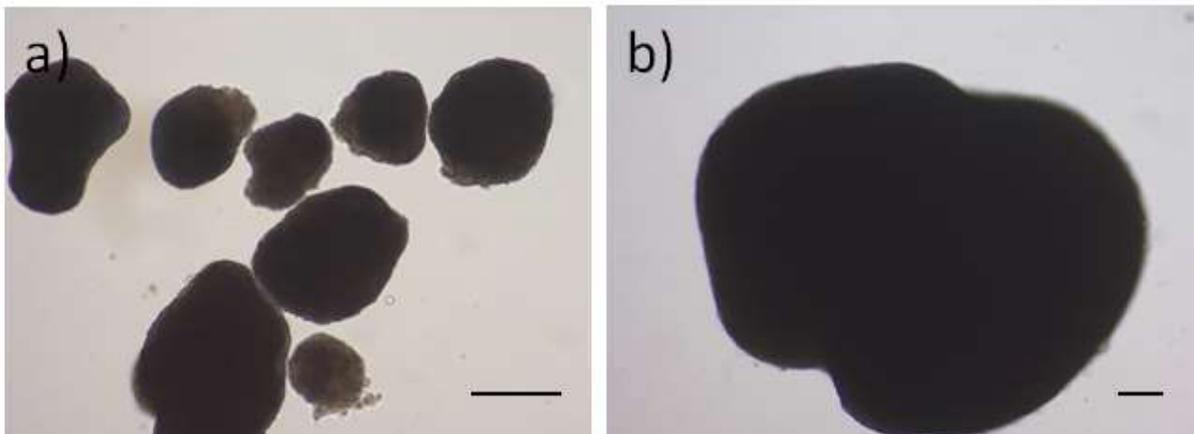


Figure 7-5. Typical morphology of embryoid bodies derived from putative bovine iPS cultures a) Embryoid bodies present after 7 days in culture. b) Large embryoid body present after 14 days of culture. Scale bars represent 100  $\mu\text{m}$ .

Following culture in the presence of retinoic acid for 14 days, embryoid bodies were harvested and analysed for lineage and testis cell markers by qRT-PCR analysis. Expression levels were compared to un-transfected BEF and BDF cell types which expressed moderate levels of the ectoderm and mesoderm markers *Tubb3* and *Desmin* (Figure 7-6).

In embryoid body cultures that were treated with 0 or 1 mM of RA, the ectoderm marker *Tubb3* was expressed by EBs derived from putative iPS colonies from BEF minicircle or episomal cultures. However, there was no difference in the levels of expression of these treatments to the expression profiles of un-transfected BEFs, which expressed equally high levels of *Tubb3*. BEF derived EBs that were treated with 0.5 mM RA, did not express levels of

*Tubb3* at an observable level (Figure 7-6a). Conversely, the same pattern of expression was not observed by EBs derived from BDF iPS cultures. The highest expression of *Tubb3* was observed in BDF minicircle derived EBs, treated with 0.5 mM RA, followed by BDF OSNL derived EBs treated with the same RA concentration, which expressed similar levels of *Tubb3* as un-transfected BDFs (Figure 7-6b). Moderate *Tubb3* levels were expressed by BDF minicircle derived colonies that were not treated with RA. While OSNL cultures treated with 0 mM or 1 mM expressed lower levels of *Tubb3*, and these low levels of *Tubb3* were no different to BDF minicircle derived EBs treated with 1 mM which did not express *Tubb3* at an observable level.

Mesoderm marker *Desmin* was expressed at the highest levels by BEF minicircle derived EBs that were treated with 0.5mM RA, and BEF OSNL derived EBs that were not treated with RA. Although other colonies expressed low levels of *Desmin*, these were not significantly different to the levels expressed by 0 mM RA control cultures, or un-transfected BEFs (Figure 7-6c). EBs derived from BDF minicircle cultures treated with 0.5 mM RA expressed the highest levels of *Desmin*, with all other cultures, including un-transfected BDFs, expressing lower levels, and minicircle colonies treated with 1 mM RA expressing negligible levels of *Desmin* (Figure 7-6d).

In BEF derived EBs, endoderm marker *FoxA2* was only expressed in cultures that were not treated with RA, with OSNL derived EBs expressing the highest levels followed by minicircle derived EBs (Figure 7-6e). Un-transfected BEFs and BDFs did not express *FoxA2* at an observable level. In EBs derived from BDF iPS cultures, the EBs derived from minicircle colonies treated with 0.5 mM RA expressed the highest levels of *FoxA2*. Minicircle and OSNL episomal plasmid cultures that were not treated with RA and OSNL cultures treated with 0.5 mM RA also expressed low levels of *FoxA2*. Cultures treated with 1mM RA did not express *FoxA2* levels different to 0 mM RA control cultures (Figure 7-6f).

Taken together, these results show that embryoid bodies formed from putative bovine iPS colonies, derived by transformation by minicircle or episomal plasmid, contained cells that expressed markers of all three germ lineages. This indicates that reprogramming events had taken place, which allows the change of cell lineage in differentiation to embryoid bodies.

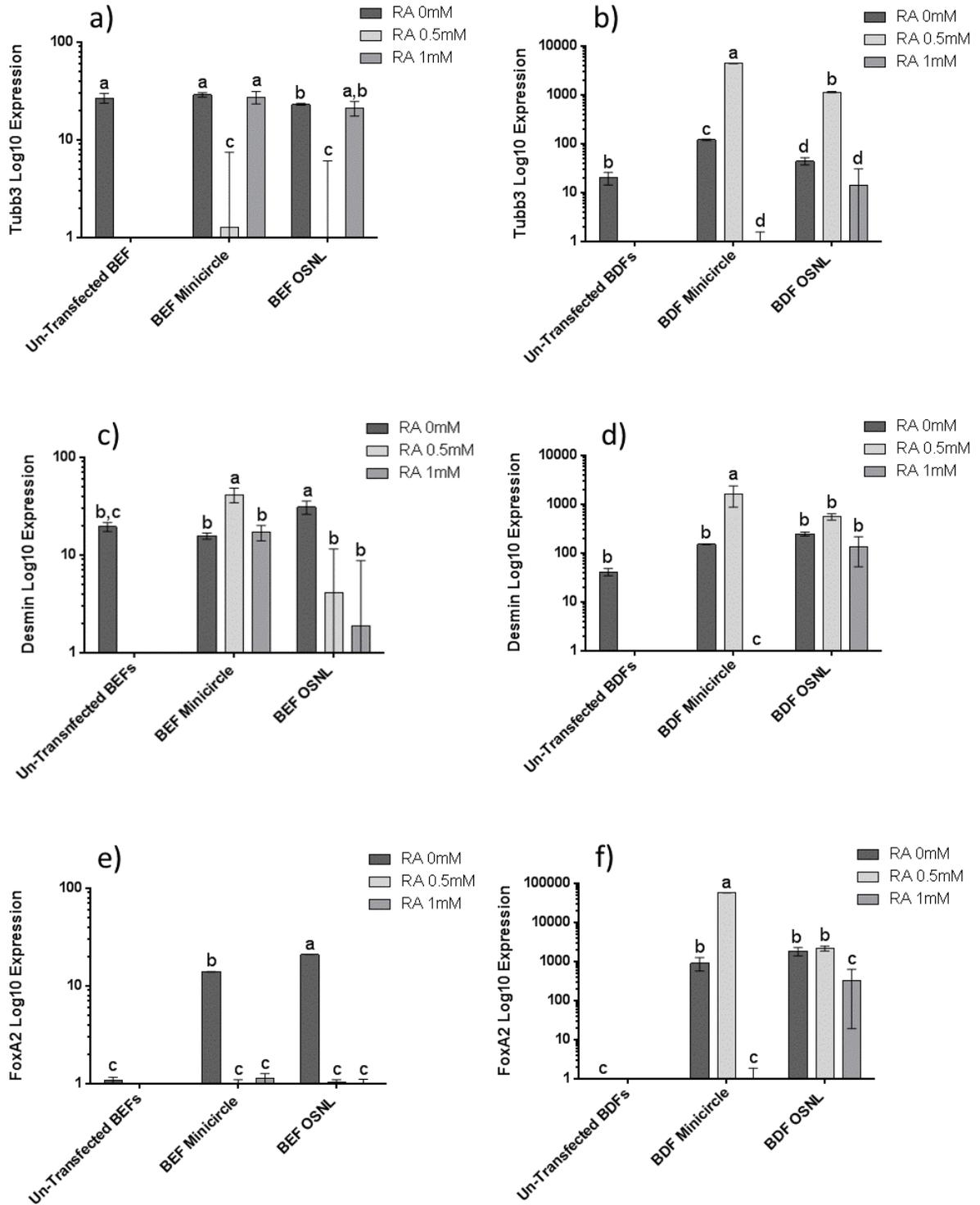


Figure 7-6: Expression of lineage markers by embryoid bodies derived from putative bovine iPS cultures, relative to housekeeper gene *RPS26*. a) Expression of *Tubb3* by BEF derived cultures b) Expression of *Tubb3* by BDF derived cultures. c) *Desmin* expression by BEF derived cultures. d) *Desmin* expression by BDF derived cultures. e) *FOXA2* expression by BEF derived cultures. f) *FOXA2* expression by BDF derived cultures. Different characters above treatment means indicates a significant difference in expression of lineage markers ( $n=3$ ,  $P<0.05$ ). Error bars represent as  $\pm$  SEM.

Gene expression by embryoid bodies, of known and putative testis cell markers, *DDX4* (*VASA*), *GATA4*, *UCHL1* and *DDX6*, *NAP1L4*, *TKTL1*, were analysed by qRT-PCR. The expression of *DDX4* was detected in EBs treated with 0.5 or 1mM RA that were derived from BDF OSNL putative iPS colonies. *DDX4* levels in this treatment significantly increased ( $P<0.05$ ) compared to 0 mM RA control cultures, with an increase of between 10 to 33 fold for 0.5 mM and 1 mM RA supplemented cultures respectively (Figure 7-7a). *DDX4* was not detected in any other cultures.

Very low levels of *GATA4* were detected in all EB cultures; however, there was no statistical difference in the *GATA4* expression between treatments, and there was no difference in expression of *GATA4* in any treatments compared to the expression of 0 mM RA control cultures (Figure 7-7b).

High levels of *UCHL1* expression were detected in EBs treated with 0.5 or 1mM RA that were derived from BDF minicircle putative iPS colonies, with an increase of 900 and 1300 respectively compared to control 0 mM cultures (Figure 7-7c). Although *UCHL1* expression was variable between replicates, there was strong expression in all samples transformed with minicircles and treated with RA, while there was no detectable *UCHL1* expression in any other sample.

*DDX6* expression was seen only in EBs that were supplemented with 0.5 mM RA, and derived from BEF cultures transformed with plasmid vector, and in EBs derived from BDFs transformed with minicircles. BEF plasmid derived EBs expressed the highest levels of *DDX6*, with 3.5 fold higher expression than BDF minicircle derived EBs ( $P<0.05$ ). In all other treatments, *DDX6* gene expression was low and no statistically significant differences were detected between the other culture or vector conditions examined (Figure 7-7d).

*NAP1L4* gene expression was seen in EBs treated with 0.5 and 1 mM RA that were derived from BDF minicircle cultures, and in EBs , treated with 1mM RA that were derived from BDF OSNL episomal plasmid iPS cells. These levels of expression were statistically higher than in

0 mM RA control cultures ( $P < 0.05$ ), with fold increase of between 100 and 200 compared to control cultures (Figure 7-7e). No other cultures expressed *NAP1L4* levels significantly different to 0 mM RA control cultures (Figure 7-7e).

*TKTL1* expression was observed in BDF minicircle derived EBs, that were not treated with RA. The expression levels of *TKTL1* in these EBs were approximately 450 fold higher than the other culture or vector conditions analysed. No other cultures expressed significant levels of *TKTL1* (Figure 7-7f).

These results indicate that embryoid bodies derived from putative bovine iPS colonies produced by minicircle or episomal plasmid transformation, are able to differentiate into cells that express known and putative markers of bovine germ cells.

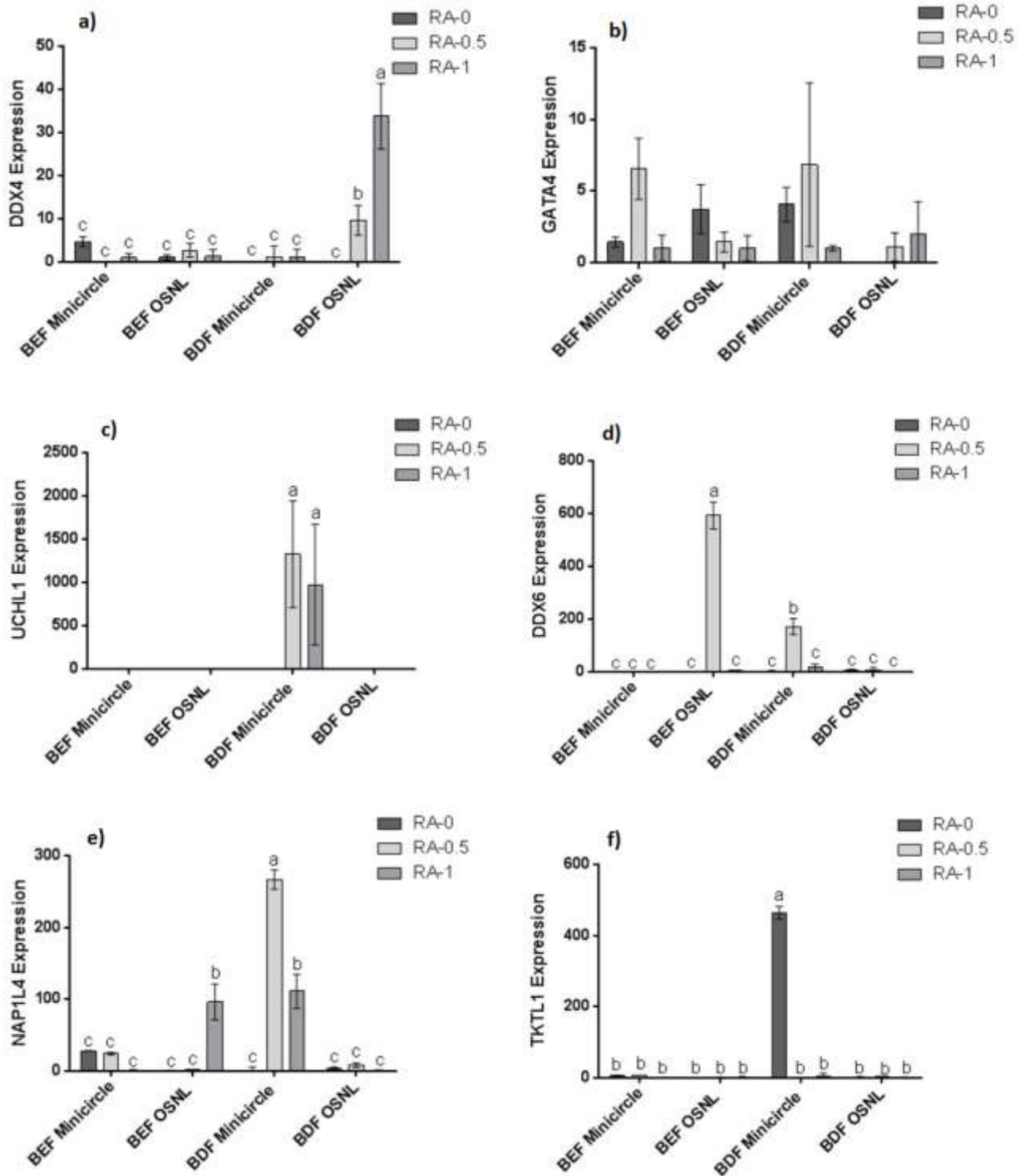


Figure 7-7. Expression of testis cell markers, relative to housekeeper gene RPS26, by embryoid bodies derived from BDF and BEF iPS colonies transfected with minicircle or OSNL episomal plasmid vector, treated with 0mM, 0.5mM or 1mM retinoic acid (RA) for 14 days a) *DDX4* (VASA), b) *GATA4*, c) *UCHL1*, d) *DDX6*, e) *NAP1L4*, f) *TKTL1*. Different characters above treatment means indicate a significant difference in expression of testis markers ( $P < 0.05$ ,  $n = 3$ ). Error bars represent  $\pm$  SEM.

### 7.3.6 Spontaneously Differentiating iPS Colonies Express Markers from all Three Germ Lineages

Some iPS colonies formed embryoid body like structures under iPS culture conditions. These colonies were larger than iPS like colonies that stained for AP activity, forming large spherical bodies that also appeared denser than iPS like colonies with good morphology. Such spontaneous differentiation was commonly observed in both minicircle and OSNL episomal plasmid derived cultures, and in cultures from previous episomal iPS experiments outlined in previous chapters of this thesis. These colonies showed similar morphology to examples shown in Figure 7-8. These colonies did not stain for alkaline phosphatase activity or other stem cell markers SSEA-4 or TRA-1-60.

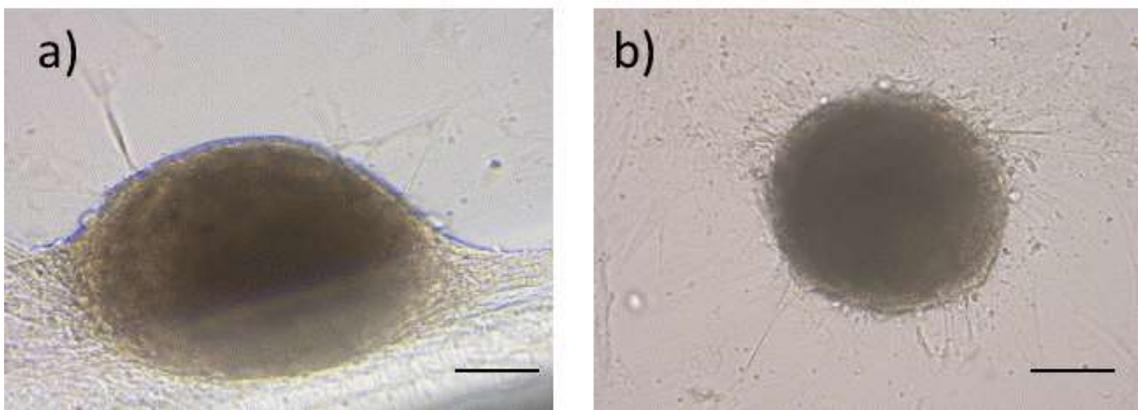


Figure 7-8. Large embryoid body like colonies present following 21 days in culture, these do not stain positively for alkaline phosphatase. Scale bars represent 200  $\mu\text{m}$ .

The pluripotency status of these colonies was of interest. Colonies from putative iPS cultures derived from BDF cells that displayed EB like morphology were analysed using qRT-PCR for pluripotency markers *POU5F1* (OCT4), *SOX2* and *NANOG*, and lineage markers *Tubb3*, *Desmin* and *FoxA2*. qRT-PCR analysis of expression of pluripotency markers showed that the EB-like colonies did not express *POU5F1* or *SOX2* levels any different to the un-transfected BDF. However, EB-like colonies did express high levels of *NANOG*, an indicator of pluripotent cells (Figure 7-9a). qRT-PCR analysis of expression of lineage markers showed that EB-like colonies expressed makers for all three germ lineages, while un-transfected BDFs expressed low levels of *Tubb3* and *Desmin*, and expression levels in un-transfected BDFs were significantly lower than the expression by EB like colonies (Figure 7-9b). This indicated that these putative iPS colonies may have undergone partial reprogramming, but had not

remained pluripotent in cultures, and instead may have spontaneously differentiated in to cell types representative of the three germ lineages.

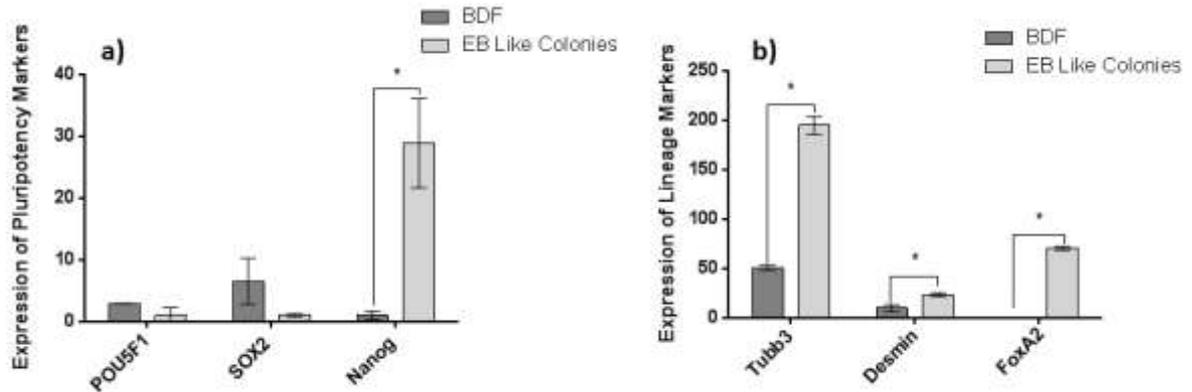


Figure 7-9. Expression of pluripotency and lineage markers, by embryoid body- like putative bovine iPS colonies, relative to housekeeper gene *RPS26*. a) Expression of pluripotency markers *POU5F1*, *SOX2* and *NANOG*, by embryoid body like colonies observed in BDF iPS cultures compared to un-transfected BDFs. b) Expression of lineage markers *Tubb3*, *Desmin* and *FoxA2* by embryoid body like colonies observed in BDF iPS cultures compared to un-transfected BDFs. \* indicates a significant difference in mean expression by BDFs and EB like iPS colonies ( $P<0.05$ ,  $n=3$ ). Error bars represent as  $\pm$  SEM.

## 7.4 Discussion

The generation of induced pluripotent stem cells (iPS) cells by non-viral or non-integrative methods, offer vast possibilities in clinical applications, with their potential to differentiate into any cell type in the body, including germ cells. The direct delivery of episomal plasmids coding for transcription factors has provided a relatively simple and effective method for producing iPS cells without transgene integration. However, the reprogramming efficiency of plasmid derived iPS cells is generally far lower than efficiencies achieved using lentiviral methods (0.001% compared to 0.1-1% respectively) (Stadtfield & Hochedlinger, 2010). Minicircle vectors have provided an interesting alternative to episomal plasmid delivery. Minicircle vectors boast more efficient reprogramming rates than episomal plasmids in the production of human iPS cells by their smaller size and therefore improved transfection efficiency, and by their longer ectopic expression (Jia et al., 2010). Minicircle vectors were first used to derive human iPS cells from adipose derived stem cells using a combination of OCT4 (*POU5F1*), *SOX2*, *NANOG* and *LIN28* (OSNL), in conjunction with a GFP reporter, resulting in a reprogramming efficiency of approximately 0.005% (Jia et al., 2010).

The first aim of this chapter was to determine if the use of a commercially available minicircle vector, with the same features first described by Jia et al. (2010), was able to produce bovine iPS cells more efficiently than, or with similar success as episomal plasmid transfection described in chapters 5 and 6 of this thesis.

To date, no reprogramming experiments for bovine iPS cell derivation have reportedly used a combination of OCT4, SOX2, NANOG and LIN28 (OSNL) reprogramming factors. However previous experiments outlined in this thesis have shown the combination of these factors is sufficient to produce putative bovine iPS colonies similar to those produced using the more common combination of OCT4, SOX2, KLF4 and c-MYC (OSKM). Additionally, it has been reported that NANOG may enhance reprogramming of bovine cells (Sumer, Liu, Malaver-Ortega, et al., 2011), and this thesis has provided some evidence to support this (Chapter 4, pg. 105). For this reason, it was rational to assume minicircle vector carrying the OSNL combination may be used to reprogram putative bovine iPS cells.

In this chapter, the derivation of putative bovine iPS cells was shown to be possible using minicircle vectors or episomal plasmids carrying a combination of the four transcription factors *POU5F1* (OCT4), SOX2, NANOG and LIN28. There were no differences observed between time to colony formation of putative iPS cultures transfected with the minicircles or episomal plasmids, indicating the minicircle vector did not accelerate the kinetics of reprogramming. However, as observed in previous experiments of minicircle iPS derivation, the reprogramming efficiency of total putative iPS colonies was significantly higher in minicircle derived cultures than iPS cultures derived using an episomal plasmid, 0.1% and 0.062% respectively. As the same combination of human transcription factors were used for reprogramming, the increase in reprogramming efficiency is not likely to be as a result of factor combination and is instead believed to be due to the reduced size of the minicircle vector resulting in higher transfection efficiencies of the minicircle vector compared to the episomal vector, as seen in the experiments described in this chapter. Additionally, the minicircle vectors are expressed in the cell for a longer period of time, thereby improving reprogramming efficiency compared to episomal vectors (Jia et al., 2010). Due to removal of the bacterial backbone during the production of the minicircle vector, the size of the episome to be delivered is at least 3kb smaller than similar plasmid vectors (González et al.,

2011) leading to an increased transfection efficiency. Additionally, minicircle vectors are also expressed more persistently and more strongly than episomal plasmids, adding to the increased reprogramming efficiency (Jia et al., 2010). Additionally, although the longer ectopic expression timeframe shown by minicircle vectors compared to episomal plasmids can improve initial reprogramming efficiencies, transgene silencing in reprogrammed cells can also be delayed, impairing the use of these cells for clinical applications (Jia et al., 2010). Minicircle expression lasts for approximately 14 days in dividing cells, but in non-dividing cells, while minicircle DNA expression drops after the first week of culture, transient expression may be present for months (Chen, Z.-Y. Y. et al., 2003). In this experiment the expression of GFP was lost following one week of culture of transfected cells on feeders for iPS derivation. This is in agreement with the statement that the minicircle vectors decrease expression after one week. However, it is unlikely that in this case, all minicircle vectors were lost from putative iPS cells as these cells were not rapidly dividing and as such the minicircle vectors would not have been lost by dilution. PCR for the expression of minicircle transgenes would confirm their continued presence in the cell.

While the reprogramming efficiency of minicircle and plasmid derived bovine iPS cells was very high compared to previous reports by Jia et al. (2010), the reprogramming efficiencies described in this chapter are calculated from total colonies observed from the original number of transfected fibroblasts, not the number of fully reprogrammed colonies, as is the general convention. As no colonies produced in this study could be described as being fully reprogrammed due to their lack of expression of stem cell markers SSEA-4 and Tra-1-60, and their inability to be expanded further in culture, the reprogramming efficiencies described in this chapter should only be compared to each other. Even so, the results clearly showed that more putative iPS colonies were produced in cultures transfected with the minicircle vector than the episomal plasmid, adding evidence to the hypothesis that the minicircle vectors may be more efficient for iPS cell reprogramming than plasmids.

However there are limitations to the minicircle protocol for deriving iPS cells. Reports of successful generation of fully reprogrammed iPS cells using the minicircle vectors have only been described in human cells, with iPS cell derivation only reported from reprogramming of adipose tissue derived stem cells (Jia et al., 2010). To date, the protocol described by Jia et al. (2010) for human iPS cells generation has not been reported to successfully derive iPS

cells from adult cell types including fibroblasts. Embryonic and dermal fibroblasts were used in the experiment outlined in this chapter but did not result in fully reprogrammed bovine iPS cells. There is a potential to use bovine adipose derived stem cells for future reprogramming experiments, however, due to time and logistical restraints this was not feasible for this project.

Again, in this chapter the putative minicircle iPS cells described were not fully reprogrammed and so cannot be claimed to be true minicircle derived iPS cells. Differences in the extent of reprogramming between minicircle and plasmid derived bovine iPS cells were difficult to determine with no one treatment showing an expression pattern of pluripotency markers that was clearly better than others. The ideal stoichiometry of pluripotency markers expression for bovine is not yet known, making it difficult to decide if one treatment of either BEF or BDFs transfected with episomal plasmid or minicircle vector expressed markers at a more ideal rate than others. Suggestions have been made that high expression of OCT4 and NANOG in addition to moderate SOX2 expression is ideal for human and mice iPS cells (Papapetrou et al., 2009; Tiemann et al., 2011). However, this has not been determined for bovine cells. BEF minicircle iPS colonies did show an expression pattern of high *POU5F1* and *NANOG* and moderate *SOX2*, suggesting optimal expression of these genes. However, all treatments were able to produce embryoid bodies that expressed markers of all three germ lineages, regardless of cell type or reprogramming vector used, indicating their pluripotential state. For this reason, it is rational to assert that minicircle vectors may be more efficient at delivering reprogramming factors to bovine cells for reprogramming than episomal plasmid vectors, on the basis of increased reprogramming efficiency as evidenced by higher colony numbers.

Regularly in the experiments outlined in this chapter, and other iPS experiments outlined in chapters 5 and 6 of this thesis, large colonies were observed which had an embryoid body like appearance. These colonies did not stain positively for alkaline phosphatase as other small colonies of the same culture did. It was of interest to determine if these colonies expressed pluripotent markers, or if they had instead begun to differentiate spontaneously in culture. Quantitative RT-PCR analysis confirmed that these colonies did not express pluripotency markers *POU5F1* or *SOX2* but did express *NANOG*. *NANOG* expression has a well-defined role in pluripotency with overexpression in human ESCs enabling self-renewal

and maintenance of pluripotency for multiple passages (Darr, Mayshar, & Benvenisty, 2006). In contrast the down-regulation of NANOG is well documented in differentiating cells (Hyslop et al., 2005; Langroudi, Forouzandeh, Soleimani, Atashi, & Golestaneh, 2013). This may suggest that these colonies were once pluripotent but have begun to lose their pluripotency. Alternatively it is possible that some cells in these EB like colonies are still pluripotent and so still express high levels of NANOG. If this hypothesis were to be tested protein expression analysis of the colonies could be conducted to determine if NANOG is still being expressed and by which cells. Additionally, these EB-like colonies did express markers from all three germ lineages *Tubb3* (ectoderm), *Desmin* (mesoderm) and *FoxA2* (endoderm). This provides evidence that the embryoid body like colonies may have been transiently pluripotent, but then began to spontaneously differentiate in culture. This provides further evidence that the putative iPS colonies observed in this study were not stably reprogrammed. Reprogramming cells to a putative, though most like transient pluripotent state has been achieved in the experiments outline in this chapter. However, it is likely that these cells were not stably reprogrammed as a consequence of being unable to sufficiently define culture conditions needed to maintain pluripotent bovine cells. A better understating of culture conditions required for the culture of bovine pluripotent cells may allow for their expansion in culture and to encourage them to remain in a pluripotent state.

As the production of iPS cells moves towards clinical applications, the need for efficient production of pluripotent cells free from transgene integrations will become paramount. Advances have been made in the culture of bovine iPS cells and hopefully in the future, the development of a robust protocol for deriving bovine iPS cells may allow minicircle bovine iPS cells to be utilised further due to their improved transfection and reprogramming efficiency.

The second aim of this chapter was to determine if putative iPS colonies produced in these experiments were able to be differentiated *in vitro* toward the germline. As stated previously, the *in vitro* generation of functional gametes from mouse ES and iPS cells has recently been achieved (Hayashi et al., 2011). The derivation of these gametes was complex and involved the initial differentiation of ES and iPS cells into epiblast-like cells (EpiLCs), which are highly similar to pregastrulating epiblasts but different from epiblast stem cells. This initial differentiation step was included as the production of primordial germ cell like

cells (PGCLCs) was reported to be more easily achieved from the epiblast-like cellular state. These cells were then cultured in a floating culture, similar to EB culture, and were supplemented with 15% knockout serum replacement (KSR) and cytokines including BMP4 to induce differentiation toward the germline. This method was complex but effective, but it remains unknown whether similar derivation of EpiLCs from bovine iPS cells is feasible. The putative iPS colonies derived in the experiments outlined in this chapter were not fully reprogrammed, and hence, did not provide suitable material to attempt to replicate the procedure outlined by Hayashi et al. (2011) for the derivation of murine gametes. If genuine bovine iPS cells are obtained in the future, there is a possibility for this method of directed differentiation to be employed to artificially produce bovine germ cells.

In this chapter rudimentary attempts were made to differentiate putative bovine iPS cells toward the germline, through supplementation of EBs with retinoic acid (RA). Initial analysis of EBs showed that all cultures were able to produce embryoid bodies (EBs) that expressed markers from all three germ lineages; suggesting a state of pluripotency was achieved, and may have the potential to be differentiated toward the germline. To test the latter, embryoid bodies produced from putative iPS cultures derived from transfection of BEF or BDFs with episomal plasmids or minicircles, were cultured for two weeks in the presence of RA at 0 mM, 0.5 mM or 1 mM. The expression of known and putative testis cell markers (characterised in this thesis in Chapter 3) by embryoid bodies was analysed.

In the absence of RA, of the known and putative markers analysed only *TKTL1* was expressed, and the expression of *TKTL1* only occurred in the BDF minicircle derived EBs. As *TKTL1* is expressed by other bovine tissue including brain, kidneys and liver, as well as a subset of differentiating spermatogonia, it is possible that these EBs underwent spontaneous differentiation toward these tissue types rather than the germline.

BEF OSNL derived EBs expressed putative markers, *DDX6* and *NAP1L4*, believed to be associated with both early stage and more differentiated spermatogonia in the bovine testis. However, *DDX6* and *NAP1L4* have also been shown to be expressed by other bovine tissues including the brain, kidneys and lungs. Hence, *DDX6* and *NAP1L4* expression does not confirm the presence of testis cells in EBs, but also does not exclude their presence. BDF OSNL derived EBs were the only treatment to express later stage spermatogonia marker

*DDX4* (previously known as *VASA*). However, *DDX4* is also expressed in bovine liver tissue, and as such may not represent the development of testis cell but instead may suggest the differentiation of liver cells. Conversely, BEF minicircle derived EBs did not express any testis cell markers at observable levels, indicating that they did not differentiate into germ cell-like cells. Further analysis did suggest that these cells differentiated into cells of the mesoderm as indicated by *Desmin* expression.

When cultured with 0.5 or 1mM RA, BDF minicircle derived EBs expressed known spermatogonial marker *UCHL1* (previously known as PGP 9.5). *UCHL1* is also expressed by numerous bovine tissues including adipose tissue, brain, intestines, lungs and kidneys. Additionally these EBs also expressed putative testis cell markers *DDX6* and *NAP1L4*, which are also expressed by other tissues expressing *UCHL1*. However, *Desmin*, a marker of the mesoderm, is expressed in tissues including the adipose tissue, brain, intestines, lungs and kidneys, but is not expressed by testis cells. The lack of *Desmin* expression by BDF minicircle derived EBs treated with 1mM RA, in conjunction with their expression of known and putative testis cell markers *UCHL1* and *NAP1L4*, provides evidence that it is possible these EBs did differentiate towards the germline.

While differentiation of EBs is generally spontaneous, the supplementation of EB cultures with factors that can direct differentiation has previously been shown to enhance differentiation toward the germline. However, the differentiation of pluripotent cells toward the germline is more difficult than directed differentiation of other cell types. Additionally, the differentiation of haploid germ cells is even more difficult due to the necessary induction of meiosis required to produce germ cells (Imamura et al., 2014). Although haploid cells were not the desired cell type this thesis aimed to produce, and instead the production of diploid spermatogonial stem cells would be preferred, the ability to differentiate pluripotent stem cells to spermatogonial stem cells that do not differentiate into germ cells is also difficult to achieve, and has yet to be reported. The process of differentiation toward the germline is not easy to control, and it is even more difficult to achieve this process in livestock species where testis cells and mechanisms of differentiation are less completely understood than in humans and mice. In the experiments outlined in this chapter, the supplementation of EBs with retinoic acid led to the expression of known and putative bovine testis cell markers. However, this study has not confirmed if the cells expressing

these markers are part of the germline. To date, no markers exist that specifically and uniquely identify bovine testis stem cells. Therefore it remains difficult to determine if cells present in the EB colonies are germ cells or other cells expressing similar genes including cell types of the brain or kidneys.

It is possible that combinations of other growth factors or the forced over-expression of certain genes could be used as an alternative to enhance differentiation toward the germline. Genetic factors that enhance germ cell differentiation have been determined through knock-down screens of genes expressed by PGCs, these have determined that the over expression of *LIN28* promotes differentiation of EB cultures into PGCs (West, J. et al., 2009). Additionally, RNA-binding protein *DAZL* has also been identified as a meiosis inducing factor in mice and humans. Low expression of *DAZL* by ES cells has been shown to result in fewer PGCs when EBs were differentiated (Haston, Tung, & Reijo Pera, 2009). Conversely the over expression of *DAZL* induces the production of sperm and oocyte like cells (Yu, J. et al., 2009). However, though some of the sperm like cells produced in the study by Yu, J. et al. (2009) were motile, they were unable to produce embryos. The over expression of *DAZL* and related genes *DAZ* and *BOULE* is also able to facilitate germ cell differentiation in humans. Additionally, the forced expression of all three genes, *DAZL*, *DAZ* and *BOULE*, has been shown to produce haploid spermatid-like cells (Kee, Angeles, Flores, Nguyen, & Reijo Pera, 2009). While the over expression of *VASA* has also been shown to promote germ cell differentiation and meiosis of human ES and iPS derived cells (Medrano et al., 2012). Another study has identified *STELLA*, in combination with retinoic acid assists germ cell differentiation of human ES derived cells (Wongtrakoongate, Jones, Gokhale, & Andrews, 2013). It should be noted that the approaches outlined in these studies required stable pluripotent cell lines, or at least fully reprogrammed iPS cells, for their differentiation protocols. The aim of this chapter was to test if the putative iPS cells derived in these experiments could, in principle, be directed to differentiate toward the germline. Hence differentiation of EBs in the presence of retinoic acid was appropriate. However, if this line of experiments were to be investigated further, there is a potential to use the differentiation techniques outlined above to differentiate bovine iPS cells to germ cells.

Although successful attempts have been made to derive functional mouse germ cells by *in vitro* differentiation of pluripotent cells, a robust protocol for doing so in other species has

yet to be established. Once protocols for the derivation of germ cells *in vitro* have been refined the technology may be used for novel reproductive and transgenic breeding applications in many species including livestock. The ability to use iPS cells for the derivation of germ cells offers further applications, with the potential to produce unlimited supplies of genetic material for use in genetic dissemination of superior animals, and to create transgenic livestock in an efficient manner. The use of iPS cells to produce germ cells also offers potential application in artificial breeding technologies to conserve rare, endangered or potentially extinct species (Selvaraj, Wildt, & Pukazhenth, 2011).

## **7.5 Conclusion**

The efficient production of induced pluripotent stem cells, free of transgene integration, is essential for the application of these cells in clinical technologies. The production of non-viral bovine iPS cells that may be differentiated toward the germline provides potential applications of these cells to be used in artificial breeding technologies such as germ cell transplantation, or in transgenic breeding applications.

Here the reprogramming efficiency of producing putative bovine iPS cells by episomal plasmid or minicircle vector transfection was compared. The reprogramming efficiency of minicircle derived putative iPS colonies was higher than plasmid derived iPS colonies. The improvements in reprogramming efficiency by using minicircle vectors, was most likely due to their smaller size and therefore ease of transfection, in addition to their extended ectopic expression in the host cell compared to episomal plasmid DNA. However, no putative iPS colonies derived from either minicircle or episomal plasmid transfection could be described as being fully or stably reprogrammed. In addition to their inability to be expanded in culture, these cells also lacked expression of stem cell markers SSEA-4 and Tra-1-60, However, these transient, putative iPS-like colonies did express markers from all three germ lineages, suggesting their pluripotential. Additionally, derivation of embryoid bodies from these putative iPS colonies in the presence of retinoic acid resulted in the expression of some known and putative testis cell markers. It is therefore possible that the cells expressing bovine testis cell markers may have begun to differentiate toward the germline.

## Chapter 8 : General Discussion and Conclusions

The primary aim of this thesis was to investigate the feasibility of producing bovine induced pluripotent stem (iPS) cells that could be used for differentiation toward the germ line *in vitro*. Successful and robust production of germline competent bovine iPS cells would provide numerous applications in the livestock industries, both in the production of transgenic livestock, and for use in artificial breeding technologies such as germ cell transplantation.

Germ cell transplantation, as a novel artificial breeding technology for livestock, offers enormous potential for the dissemination of highly valuable genetics in extensive livestock production, and may be used to improve the productivity of isolated cattle herds in the north of Australia. However, the success of this technology requires the ability to isolate a large and relatively pure population of spermatogonial stem cells (SSCs) (Hill & Dobrinski, 2006). The lack of knowledge about bovine germ cells and their development, the inability to effectively culture these cells *in vitro* and the absence of markers that specifically identify bovine SSCs, has limited the success of this technology (Aponte, P. & de rooij, 2008; Izadyar et al., 2003). The recent generation of induced pluripotent stem (iPS) cells has provided an alternative to the traditional method of castrating a donor animal to isolate SSCs for transplantation, with the possibility to instead differentiate iPS cells toward the germ line. It is hoped that iPS generation and germline differentiation may be used in the future for breeding technologies including germ cell transplantation, to improve the genetics and production traits of livestock species.

Unfortunately the efficiency of donor cell colonisation in germ cell transplantation is low, but has shown be improved by the transplantation of a more enriched population of SSCs (Bugeaw et al., 2005; Shinohara et al., 1999; Shinohara, Orwig, Avarbock, & Brinster, 2000). Additionally, the ability to effectively differentiate pluripotent stem cells toward the germ line requires a better understanding of the development of bovine germ cells. Obtaining a pure population of spermatogonial stem cells by enhanced enrichment methods, would also allow the improved study of development, characterisation and identification of markers of SSCs and different sub-sets of spermatogonia. Enrichment of bovine spermatogonia has

been previously reported using numerous methods including differential plating, magnetic activated cell sorting (MACS), fluorescent activated cell sorting (FACS), velocity sedimentation (STA-PUT) and discontinuous gradient centrifugation (Bryant et al., 2013; Herrid et al., 2009; Izadyar et al., 2002). For the identification of specific markers, an even higher enrichment of bovine undifferentiated spermatogonia would be more desirable than what is currently achievable.

Chapter 2 of this thesis compared enrichment protocols currently used for isolating undifferentiated populations of bovine spermatogonia. It was shown that a combination of differential plating followed by Percoll discontinuous density centrifugation, resulted in a more enriched population of undifferentiated spermatogonia than differential plating or Percoll treatment alone, with a 4-10 fold increase in DBA positive cells.

In an attempt to further improve the enrichment of spermatogonia, a peptide capable of interrupting bonds, potentially formed by the adhesion molecule Claudin-8, between a subset of sertoli cells and spermatogonia was tested. Testis cell isolations were treated with the C-CPE<sub>290-319</sub> peptide prior to MACS enrichment. However, there was no evidence observed that treatment with this peptide resulted in enhanced enrichment of DBA positive cells when using MACS enrichment. This may be due to the numerous other cell adhesion molecules that may be associated with interactions between spermatogonia and Sertoli cells. Further studies into the use of peptides to disrupt tight junction bonds formed as a result of Claudin-8 interactions could employ the use of labelled peptides to identify successful binding, and the substrate to which the peptide binds. This information would be very useful in determining more efficient methods of enrichment of bovine SSCs.

The enrichment of bovine SSCs is in part limited by the lack of markers that uniquely identify bovine SSCs. The identification of markers, in particular cell surface markers, would aid in developing improved enrichment strategies. Additionally, the identification of markers for SSCs could contribute to knowledge of cellular mechanisms and allow for the better characterisation of bovine germ cells, including the potential to identify germline differentiation *in vitro*. The characterisation of SSCs and developing spermatogonia is important for the *in vitro* culture of these cells. To date, no robust protocol for the culture of bovine SSCs *in vitro* has been established, with a propensity of SSCs to spontaneously

differentiate or undergo apoptosis when in culture (Aponte, P. & de Rooij, 2008; Izadyar et al., 2003). To successfully establish culture conditions to more efficiently grow and study these cells, improved characterisation is essential. This may allow the development of culture systems that more closely mimic the natural stem cell niche of the bovine testis and encourage self-renewal, and may identify conditions supporting normal differentiation of SSCs *in vitro*. Further research is needed to improve the characterisation of bovine SSCs. This information will also be important for the success of germ cell transplantation, as improved understanding of the mechanisms involved in the colonisation process of transplanted donor cells into the recipient testis, may allow for improvements to the efficiency of transplantation.

Studies have identified that some spermatogonial markers are conserved between species; however, the expression of only few markers have been confirmed in the bovine. Screening methods, such as proteomics and transcriptomics analysis of testis populations can be used to identify potential candidate markers for spermatogonia and SSCs. Recently, a short list of cellular markers that may be associated with bovine spermatogonia was identified by proteomics profiling (Colgrave et al., 2013). In Chapter 3 of this thesis potential candidates from this short list were initially evaluated for gene expression associated with spermatogonia enriched populations of bovine testis cells by qRT-PCR analysis. Initial analysis of candidate markers identified three putative markers associated with bovine spermatogonia enriched populations, *DDX6*, *NAP1L4* and *TKTL1*. Further characterisation by immunohistochemical staining of antibodies against these markers was conducted on sections of bovine testis and cell smears from pre-pubertal, pubertal and post-pubertal (sections only for the latter) *Bos taurus* bulls. Although none of the tested putative markers were shown to identify bovine SSCs exclusively, identification of different sub-sets of the bovine spermatogonia population was demonstrated. These markers may therefore be useful for further elucidating developmental processes in the bovine testis. As the markers characterised in Chapter 3 were associated with the cytoplasm, possibly as membrane proteins, or in the nucleus, but not the cell surface, they have limited potential to be used to improve the enrichment of bovine spermatogonia. The further analysis of the proteins shortlisted as part of the proteomics profiling study could identify more markers that are associated with bovine spermatogonia, thereby improving our knowledge of these cells.

Despite enormous advances in iPS cell generation, difficulties in establishing iPS cells from some species including bovine and other livestock species have been encountered. There have been a small number of reports of successful bovine iPS cell generation over recent years; however, there has yet to be a robust or reproducible protocol for their derivation described (Cao et al., 2012; Huang, B. et al., 2011; Sumer, Liu, Malaver-Ortega, et al., 2011). Additionally, no reports have shown transgene silencing of bovine iPS cells, indicating that these cells do not meet the stricter criteria for iPS cell generation. The lack of transgene silencing has been a common problem for the production of all livestock iPS cells, with transgene silencing reported in only one report of ovine iPS cells (Liu, J. et al., 2012). Despite advances in the production of bovine iPS cells, it is evident that optimal conditions for the production of fully reprogrammed cells are yet to be described. The identification of a reproducible and robust protocol for bovine iPS cell generation will lead to the use of these cells in a wide range of applications, including their use in transgenic breeding and artificial reproductive technologies.

The first report describing the generation of fully reprogrammed bovine iPS cells claimed that NANOG was essential for the reprogramming of cells to a pluripotent state (Sumer, Liu, Malaver-Ortega, et al., 2011). This claim has not been verified by other studies, with bovine iPS generation reported without exogenous NANOG expression (Cao et al., 2012). However, NANOG has been shown to improve the reprogramming efficiency of some other cell types that have previously been difficult to reprogram (Hanna et al., 2009).

Chapter 4 of this thesis compares the reprogramming efficiency of using different combinations of transcription factors, including the NANOG for reprogramming bovine iPS cells. Three reprogramming cocktails, OCT4 (POU5F1), SOX2, KI4 and c-MYC (OSKM) (Takahashi & Yamanaka, 2006), OCT4, Sox3, NANOG and LIN28 (OSNL) (Yu, Junying et al., 2007), and a combination of all six factors (OSKMNL) (Yu, Junying et al., 2007), were compared for their ability to reprogram bovine somatic cells to pluripotency. Although no colonies produced in this experiment could be described as being fully reprogrammed, cultures that were transduced with the combination of reprogramming factors containing NANOG (OSNL and OSKMNL) expressed higher levels of reprogramming factors *POU5F1*, *SOX2*, *c-MYC* and *NANOG* than cultures reprogrammed using the OSKM combination. The results from chapter 4 of this thesis suggest that NANOG may improve reprogramming of

bovine somatic cells to pluripotency which is in keeping with the previous findings of Sumer, Liu, Malaver-Ortega, et al. (2011). However, it is evident from the results presented here that while the addition of NANOG may enhance reprogramming, it is not sufficient for successful production of fully reprogrammed bovine iPS cells under the tested culture conditions. If the production of bovine iPS cells is to be successful in the future the effect of different culture conditions and media should be analysed in an attempt to find optimal conditions for the derivation of bovine iPS cells.

The first reports of iPS cell generation used retroviral and lentiviral vectors to deliver transcription factors to target cells for reprogramming (Takahashi & Yamanaka, 2006; Yu, Junying et al., 2007). Transduction with these vectors resulted in the stable integration of exogenous transgenes in the host genome which, due to the potential for insertional mutations and tumour formation, limits the use of these cells for clinical applications (Kane et al., 2008). Production of iPS cells using non-viral or non-integrative vectors has resulted in the production of pluripotent cells without transgene integration, and are therefore safe for potential use in clinical applications (Kane et al., 2008; Narsinh et al., 2011; O'Doherty et al., 2013; Okita et al., 2008). However, the production of non-viral iPS cells inherently results in lower reprogramming efficiencies than viral methods; this has limited the adoption of non-viral reprogramming in livestock species, where iPS generation is already difficult. Only one report of the generation of non-viral bovine iPS cells has been made previously (Huang, B. et al., 2011); however, the production of non-viral bovine iPS cells is essential for the potential use of these cells in a clinical setting.

Chapter 5 of this thesis describes experiments on the derivation of non-viral bovine iPS cells using electroporation with episomal plasmids. This experiment used a 2x2x3 factorial design where two cell types, bovine embryonic or dermal fibroblasts (BEFs and BDFs respectively), were reprogrammed by the lentiviral or non-viral transfection of one of three different combinations of transcription factors OSKM, OSNL or OSKMNL. Here it was shown that the use of non-viral reprogramming enhances reprogramming kinetics, with non-viral colonies appearing after 10 days of culture as opposed to 12 days by lentiviral cultures. It is possible the slower lentiviral kinetics was due to suboptimal lentiviral titres, decreasing the transduction efficiency (Mátrai et al., 2010). In contrast, the number of colonies produced, and therefore reprogramming efficiency, was not shown to be effected by lentiviral or non-

viral reprogramming, but was affected by cell type and transcription factor combination. It was shown that BDF non-virally derived iPS cultures produced significantly more colonies than lentiviral or non-viral BEF derived iPS cultures. Furthermore, the use of the OSKMNL construct resulted in fewer colonies than Tet-OSKM and OSNL derived cultures, possibly due to the large size of the reprogramming construct limiting transfection efficiency (Ribeiro et al., 2012). Transfection of the OSKMNL transcription factor combination as single transcription factors, rather than as a large polycistronic plasmid, could be tested to determine if reprogramming efficiency improved. However, the use of single transcription factors would result in the variability of multiple plasmid transfection events. The use of protein analysis for pluripotency markers, either by staining or western blots, would be beneficial if this line of experiments was to be followed in the future. This information would give further evidence to confirm the expression of pluripotency markers by these putative iPS colonies and therefore giving further evidence that a reprogramming event occurred.

It is interesting that none of the colonies described in Chapters 4 or 5 displayed alkaline phosphatase activity. As these colonies displayed other traits of pluripotent cells including expression of pluripotency markers, it is possible that these colonies did express AP activity at some stage during reprogramming. If the pluripotency status of these colonies was to be investigated further, a time course of alkaline phosphatase activity could be conducted where colonies were stained daily with live staining to identify at what stage they expressed AP.

Embryoid bodies derived from BEF OSKMNL iPS colonies were one of only two treatments that produced EBs that expressed markers from all three germ lineages, indicating their pluripotency. This may suggest that although reprogramming efficiency is low, cells that are transfected with OSKMNL may advance further towards a state of full reprogramming than other treatments. Additionally, BDF OSNL derived EBs also expressed markers from all three germ lineages. If these experiments were to be repeated in the future, teratoma assays would provide further evidence for the pluripotent nature of these cells. Teratoma assays were not conducted in these experiments as the cells produced were not considered fully pluripotent, and logistically the low cell numbers produced prevented the possibility of conducting an assay. The ability of these two cultures to produce cells with pluripotent characteristics, may be a result of these reprogramming constructs containing NANOG, in

agreement with the report by Sumer, Liu, Malaver-Ortega, et al. (2011) that claims NANOG is essential for bovine iPS cell reprogramming.

Small molecules have been found to enhance reprogramming efficiency and are able to help convert partially reprogrammed cells to a fully reprogrammed state by their ability to overcome barriers to reprogramming (Zhang, Y. et al., 2012). Previous iPS cell experiments outlined in chapters 4 and 5 employed the use of a combination of three small molecules, HDAC inhibitor, sodium butyrate (NaB), MEK inhibitor, PD0325901 and TGF- $\beta$  inhibitor SB431542 (NaB-SB-PD), which was previously found to accelerate reprogramming kinetics and improve the characteristics of putative bovine iPS colonies (McMillan, 2012). Chapter 6 describes experiments to determine the effect on reprogramming of different combinations of small molecules, NaB, PD, SB and another small molecule, the HDAC inhibitor valproic acid (VPA). The results showed that the addition of valproic acid to small molecule combination, NaB-SB-PD (VPA-NaB-SB-PD), improved reprogramming efficiency further and enhancing reprogramming to produce putative bovine iPS cells that displayed more characteristics of pluripotent cells than cells previously derived using the three factor combination. The small molecules used in this experiment only represent a small number of the total small molecules available to assist in reprogramming. Further research is needed to determine if there is a more effective small molecule combination to enhance reprogramming of bovine cells to pluripotency. Analysis of different combinations of small molecules could be tested in not only the culture conditions outlined in this thesis, but also in other commonly used or novel conditions to identify optimal conditions of bovine iPS cell production.

The use of different reprogramming methods has also been shown to affect reprogramming efficiency. Chapter 7 describes experiments to determine if the use of a commercially available non-viral, non-integrative minicircle vector could be used to reprogram bovine cells more efficiently. Minicircle vectors have higher transfection efficiency due to their small size, and in addition are expressed for a longer period of time in the target cell than episomal vectors, thereby improving reprogramming efficiency (Jia et al., 2010). Results of this experiment supported the reports by Jia et al. (2010), that minicircle transfection resulted in higher reprogramming efficiencies in bovine iPS cultures than episomal vectors carrying the same combination of transcription factors. However, like iPS cells previously described in this thesis, cells produced by minicircle transfection were also not fully reprogrammed.

However, minicircle vectors may provide an efficient and safe method for the derivation of bovine iPS cells once a robust protocol for the production of bovine iPS cell culture has been established, dependent on available reprogramming factor combinations. The number of minicircle vectors available commercially has grown since the time these experiments were conducted. Minicircle vectors that contain human or mouse factors in the OSNL or OSKM combinations are now commercially available. The use of different minicircle vectors for the production of bovine iPS cells could be used in future experiments to determine if reprogramming factors from different species, or different reprogramming factor combinations influences the reprogramming of bovine cells to pluripotency. There is potential to produce a minicircle vector containing bovine factors, this may improve the production of bovine iPS cells further, particularly if a reproducible culture protocol is established for bovine iPS cells.

Although the cells described in Chapter 7 were not fully reprogrammed, embryoid bodies derived from these cultures were able to express markers from all three germ lineages, indicating their pluripotent state and therefore potential to be differentiated into cells of the germline. Recently, the *in vitro* derivation of functional gametes from murine iPS cells resulting in healthy and fertile offspring has been demonstrated (Hayashi & Saitou, 2013), while numerous other studies have shown the derivation of putative male and female germ cells is possible *in vitro* (Bowles & Koopman, 2007; Ohta et al., 2010; Silva, C. et al., 2009). The possibility to use this technology in other species, including livestock, may result in the generation of functional gametes that could be used to efficiently disseminate livestock genetics of a wide area (Kues, W., Nowak-Imialek, Haridoss, & Niemann, 2010).

Chapter 7 describes preliminary attempts to differentiate embryoid bodies derived from putative bovine iPS cells toward the germ line by exposure to the differentiating, and meiosis inducing agent, retinoic acid (RA). Resulting EBs from some treatments expressed markers associated with bovine testis cells. These preliminary findings do provide hope that it may be possible to differentiate bovine iPS cells toward the germ line for use in reproductive technologies in the future. Future experiments could be conducted where bovine iPS cells were cultured *in vitro* or *in vivo* in the presence of Sertoli cells and other somatic testis cells to induce differentiation toward the germ line. However, this would only be feasible when bovine iPS cells that could be passaged extensively were derived.

It is evident from the results presented here that the putative bovine iPS cells described throughout this thesis underwent only partial reprogramming. There are a number of aspects of the reprogramming protocol used throughout this thesis that could have contributed to partial reprogramming of these cells. These factors include the use of human and murine transcription factors for reprogramming as opposed to the use of bovine factors. However, reprogramming factors do not seem to be species specific in function, murine cells have been reprogrammed with human factors, and cells from other species have been reprogrammed with murine or human factors (Liu, J. et al., 2012; Park, K.-M. et al., 2013; Sartori et al., 2012; Song et al., 2013). Additionally, the use of polycistronic vectors, preventing control of optimal stoichiometric expression of transcription factors may have also prevented full reprogramming. Furthermore, the choice of target cell type may have also influenced reprogramming, with embryonic and adult dermal fibroblast used due to ease of acquisition, rather than adult stem cells or cells that have been shown to be more amenable to reprogramming (Jia et al., 2010; Niibe et al., 2011). There are almost endless possible experiments for future research into optimising the production of bovine iPS cells. This thesis has provided a good background for the use and analysis of small molecules in bovine iPS culture, but this work should be expanded on, as the resulting bovine iPS cells described in this thesis being only partially reprogrammed.

Of perhaps most interest is the specific cell culture conditions used in this thesis, as cell culture components including base media composition, the use of serum or serum replacement and even the specific type of serum used, can all impact on the success of reprogramming. iPS cell culture conditions have generally been derived from the conditions needed for embryonic stem cell growth, these conditions vary between species but have been defined for humans, mice and some other species. However, to date, conditions for successful and replicable bovine ES culture have not been reported (Gong et al., 2010; Mitalipova et al., 2001; Wang, L. et al., 2005). Instead, variations of mouse and human iPS culture conditions have been used to generate bovine iPS cells. In reports on production of bovine iPS cells, the culture conditions used vary widely, and for all protocols published to date, no replication of the results has been published by other groups, providing evidence the cell culture conditions reported previously are likely suboptimal, and possibly vulnerable to small differences in laboratory environments. This notion is in keeping with meta-analysis

on iPS and ES cell lines from different labs which found strong correlation between gene expression signatures and specific labs, supporting the hypothesis that micro-environmental context is highly relevant for pluripotent stem cell function (Newman & Cooper, 2010). Further research into the optimal cell culture conditions for bovine iPS and ES cells may provide insights into the conditions needed for successful bovine iPS cell generation and the potential culture of ES cells.

Results from this thesis have shown that alterations of different aspects of reprogramming, including reprogramming method, and cell culture conditions can have a significant effect on the reprogramming process. Important progress has been made in this thesis in enhancing reprogramming efficiency of putative bovine iPS, through the systematic investigation of small molecules to aid the reprogramming process. However, as the putative bovine iPS cells derived as a result of this thesis were considered only partially reprogrammed, further research addressing different aspects of the reprogramming process, is important to determine if further alterations could result in deriving a robust and reproducible protocol for the derivation of bovine iPS cells.

## **8.2 Conclusions**

This thesis has contributed to the better characterisation of bovine testis cells, with the potential to better understand the mechanisms associated with the development of bovine spermatogonia, through the identification of three novel bovine spermatogonial markers, which have been shown to identify spermatogonia of different stages of development.

This thesis has also established that the derivation of at least partially reprogrammed bovine iPS cells that display characteristics of pluripotent stem cells is possible. Furthermore, the experiments outlined in this thesis identified small molecule combinations which support reprogramming efficiency, and further reprogramming success. The systematic comparison of different combinations of small molecules, with different combinations of reprogramming factors, has provided a powerful insight into the complexities of reprogramming outcomes. Further research is needed to generate a robust and reproducible protocol for the derivation of fully reprogrammed bovine iPS cells.

In this thesis it was also established that in the bovine, dermal fibroblasts have at least similar reprogramming potential to embryonic fibroblasts. This hypothesis is supported by data from several different experiments conducted for this thesis, and this result is highly encouraging for the further development of protocols for bovine iPS cells for use in reproductive technologies, as dermal fibroblasts are easily and ethically available.

The ability to differentiate bovine iPS cells toward the germline *in vitro* is essential for the successful use of these cells in transgenic and artificial breeding applications, this thesis has provided evidence that differentiation towards the germline from a state of induced pluripotency is possible in principle. For the application of iPS cells in germ cell transplantation, *in vitro* meiosis is not required or even desirable, and hence evidence that pluripotent stem cells might be able to be committed towards diploid germ cell precursors is highly encouraging for future work.

There are still difficulties encountered in the production of livestock, and in particular bovine iPS cells. Furthermore, even in murine models the mechanisms of differentiating pluripotent stem cells toward the germline are poorly understood and the process of directed differentiation has proven difficult. Given these complications, it is necessary for further improvements be made for both iPS cell generation and differentiation protocols of bovine cells before these cells are of use in reproductive technology applications. If robust protocols for the derivation of fully pluripotent bovine iPS cells are established, there would be a potential for practically unlimited amount of bovine pluripotent cells to be cultured. Of these cells, a smaller percentage may be successfully differentiated toward the germ line, and may be potentially used in reproductive technologies. Hence, livestock iPS cells do offer immense possibilities if robust protocols for their derivation and differentiation are produced.

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