Chapter 1: Literature Review

1.1. General Introduction

The ability of spermatogonial stem cells (SSCs) to recolonise testis tissue following transplantation has generated interest in these cells for use in reproductive technologies (Brinster & Averbock, 1994; Brinster & Zimmerman, 1994). Spermatogonial stem cell transplantation has the potential to be applied in livestock industries such as beef cattle production as an alternative to artificial insemination (Hill & Dobrinski, 2006). A more in depth understanding of SSCs and their stem cell niche is required in order to enhance the success of SSC transplant experiments in the bovine. One current limitation of such experiments is the identification and purification of SSC populations from the bovine testis. Another consideration is the ability to generate the large numbers of SSCs required for successful transplantation experiments.

At present there are no definitive markers that can be used to distinguish SSCs from other cell types in the testis, limiting the ability to identify and purify SSC populations (Aponte, van Bragt, de Rooj & Pelt, 2005; Oatley & Brinster, 2006). Progress has been made in the identification of spermatogonia in the mouse and human testis, but to date there is still limited information available regarding spermatogonia in the bovine testis. The use of highly purified SSC populations is thought to greatly enhance the success of transplantation experiments (Shinohara et al., 1999). Better characterisation of bovine SSCs and their niche will aid in the ability to generate more highly purified populations of these cells. The identification of cell surface markers for SSCs is of particular interest to aid in identifying and selecting these cells from a mixed testis cell population.

The culture and expansion of spermatogonial stem cells has proven difficult. Some progress has been made in mice, but for livestock species such as cattle there has been little success in maintaining these cells in culture (Aponte et al., 2008; Izadyar et al., 2003). The inability to expand these cells to large numbers in culture limits their use in transplantation experiments. One alternative to the expansion of these cells in culture is the generation of pluripotent stem cells that can then be differentiated towards a germline lineage. Induced pluripotent stem (iPS) cells are generated by reprogramming somatic cells to an embryonic stem cell-like state (Takahashi et al., 2006). These cells have the ability to differentiate into any cell type, including germline cells, and thus they have the potential to be used in place of SSCs in transplantation experiments.

Induced pluripotent stem cells were first derived by the forced expression of four transcription factors in mouse fibroblasts in 2006 (Takahashi et al., 2006). Since then, there have been significant advances in reprogramming methods, resulting in iPS cells being produced from a number of different species. However, like embryonic stem cells, iPS cells from livestock species, including cattle, have proven difficult to establish and maintain (Huang et al, 2011; Sumer et al, 2011). There are a number of factors that determine the success of reprogramming experiments, including the choice of reprogramming factors, delivery of these factors, the target cell type, and the components of the cell culture medium (Maherali & Hochedlinger, 2008). Defining the optimal conditions for cell reprogramming will aid in the generation and maintenance of bovine iPS cells.

1.2. Spermatogonial Stem Cells

Spermatogonial stem cells (SSCs) are the adult stem cells found in the testis that are responsible for the continual production of spermatozoa throughout the lifetime of a male. SSCs are located on the basal membrane of the seminiferous tubules, and represent only a

very small percentage of the total testis cell population (Huckins & Clermont, 1968). SSCs, like other stem cells, are characterised by their ability to undergo both self-renewing and differentiating cell divisions. This allows for the production of millions of sperm daily, while still maintaining a base stem cell population. SSCs also have the ability to recolonise testis tissue and restore spermatogenesis following transplantation (Brinster & Zimerman, 1994). There is great interest in these cells based on their potential applications in regenerative medicine and reproductive technologies. Rodents represent the most well studied models of spermatogenesis. The information presented in the following sections refers almost exclusively to research carried out in the mouse and rat. It is important to note that there are differences in spermatogenesis between primates and non-primate mammals such as rodents.

1.2.1. Spermatogonial stem cells in mammals

SSCs are diploid cells located on the basal membrane of the seminiferous tubule (Huckins & Clermont, 1968). This cell population arises from a transient population of primordial germ cells (PGCs), formed from the epiblast and set aside in the early stages of embryo development (Gardner & Rossant, 1979). PGCs migrate via the hindgut to the genital ridges, where they colonise (Mintz & Russell, 1957). Throughout this migration phase the PGCs undergo proliferation (Tam & Snow, 1981). Once established in the genital ridges, PGCs become enclosed within cords formed by pre-cursor Sertoli cells and surrounded by peritubular cells, at which point they become known as gonocytes (Clermont & Perey, 1957; Huckins & Clermont, 1968). Gonocytes become arrested in the G0 phase of the cell cycle and remain mitotically quiescent until after birth (Clermont & Perey, 1957; Huckins & Clermont, 1968). During puberty gonocytes resume proliferation and establish the pool of SSCs that colonise the basement membrane and establish and maintain spermatogenesis following puberty (Huckins & Clermont, 1968).

Spermatogenesis in non-primate mammals is described using the A_s model, in which SSCs are described as A-single (A_s) spermatogonia (Huckins, 1971; Oakberg, 1971).Spermatogenesis begins when A_s spermatogonia divide and give rise to two daughter cells known as A paired (A_{pr}) spermatogonia. The two cells forming the A_{pr} spermatogonia do not complete cytokinesis and remain attached to each other by an intercellular bridge (Fawcett, Ito, & Slautterback, 1959; Weber & Russell, 1987). The A_s spermatogonia are considered to be the only spermatogonial subpopulation that has stem cell activity, although it has been hypothesized that cells in the A_{pr} population may act as potential germline stem cells and have the ability to break the cytoplasmic bridges and initiate self-renewal under certain conditions (Nakagawa, Nabeshima, & Yoshida, 2007). During spermatogenesis A_{pr} spermatogonia undergo further divisions in which cytokinesis remains incomplete, resulting in the formation of chains of cells attached via intracellular bridges (Huckins & Clermont, 1968). These chains of cells are known as A-aligned spermatogonia (A_{al}), and generally consist of 4, 8, or 16 cells (de Rooij & Russell, 2000). The A_{al} spermaotogonia are considered to be committed to the differentiation process and have lost the potential for stem cell activity. A_{al} spermatogonia undergo further differentiation without undergoing mitosis, to form A_1 spermatogonia (Chiarini-Garcia, Meistrich, Hou, & Singh, 2008). Following the formation of A_1 spermatogonia there are a number of rounds of cell division that result in the formation of A_2, A_3, A_4, Intermediate (In) and B spermatogonia successively (de Rooij & Russell, 2000). Type B spermatogonia undergo meiosis to produce haploid spermatids (Russell, Ettilin, Sinha, & Clegg, 1990). Figure 1.1 provides a schematic representation of the A_s model of spermatogenesis.

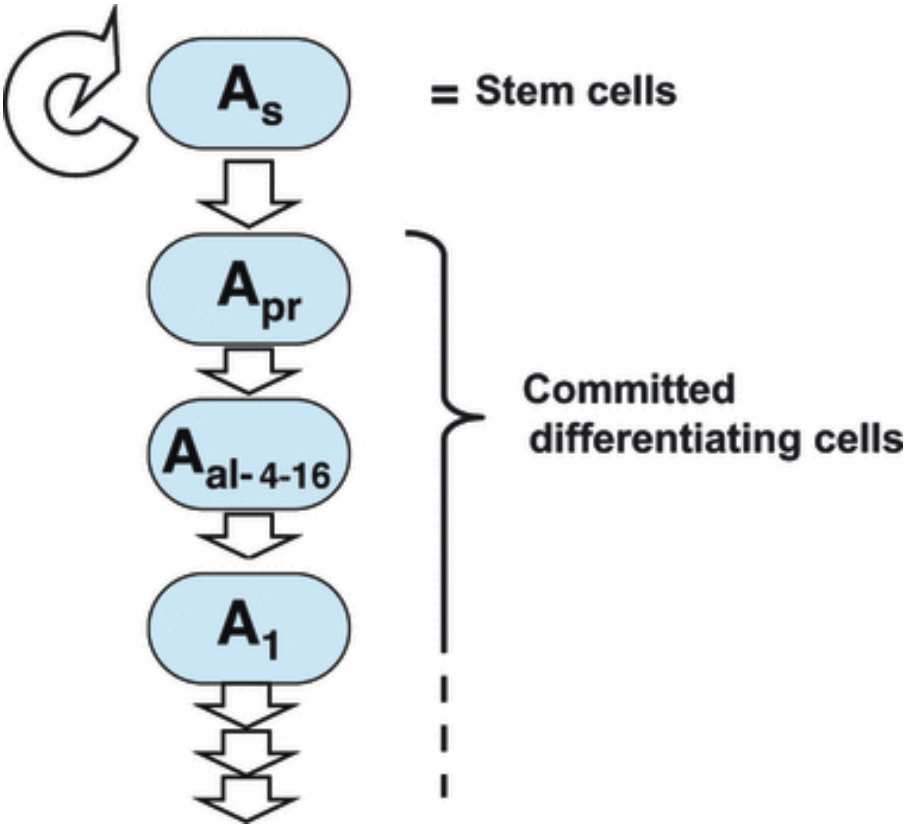

Figure 1-1 A schematic representation of the "A_s model" of spermatogenesis. (Yoshida, 2010)

The A_s, A_{pr} and A_{al} spermatogonia are collectively called the undifferentiated spermatogonia, while A_{1-4}, In and B spermatogonia onwards are known as differentiated spermatogonia (de Rooij & Russell, 2000). The undifferentiated spermatogonia share similar phenotypic characteristics and at present there are no specific characteristics that allow the three populations to be easily distinguished from each other (Oatley & Brinster, 2006). The undifferentiated and differentiated spermatogonia can be distinguished from each other by a number of distinct phenotypic characteristics, including the presence or absence of heterochromatin in the nucleus (de Rooij & Russell, 2000) and the expression of various molecular markers, which will be described in more detail later in this review.

1.2.2. The spermatogonial stem cell niche

The ability of SSCs to colonise and initiate spermatogenesis depends on the surrounding microenvironment, known as the stem cell niche. The stem cell niche is essential for mediating endocrine and paracrine signals that ensure SSC survival, maintain the SSCs in an undifferentiated state and also control cell differentiation. It is thought that the main cellular components of the SSC niche in the mammalian testis are Sertoli cells, Leydig cells and peritubular myoid cells, which secrete a number of factors involved in stem cell maintenance (Oatley, Racicot, & Oatley, 2010). Tight junctions between Sertoli cells form a protective blood-testis barrier (BTB) that plays an important role in regulating spermatogenesis by maintaining a selective substance flow across this barrier (Mok, Mruk, Lee, & Cheng, 2011).

Despite evidence that SSCs are located in specific niches, rather than being randomly distributed over the basal membrane (Chiarini-Garcia, Raymer, & Russell, 2003), no morphological differences have been detected between different areas of the basal membrane of the seminiferous tubules in any species. Sertoli cells always appear to display uniform morphology, and SSCs have not been reported to be accompanied by a specific type of Sertoli cell (De Rooij, 2009). Yoshida et al. (2007) observed that undifferentiated spermatogonia appear to be preferentially located in areas of the seminiferous tubules associated with the vascular network, close to the interstitial space. As cells differentiate they leave these vascularised areas and migrate through the seminiferous tubules. These findings imply that the SSC niches exist in areas of the basal membrane that border on the interstitial tissue, and that the transfer of supporting factors through the blood is critical for the maintenance of SSCs in the stem cell niche (Shetty & Meistrich, 2007; Yoshida et al., 2007).

Adhesion molecules are highly expressed by various adult and embryonic stem cells, although the function of these molecules in the stem cell niche is not clear (Marthiens, Kazanis, Moss, Long, & ffrench-Constant, 2010; Raymond, Deugnier, Faraldo, & Glukhova, 2009). Adhesion molecules are thought to play a vital role in retaining stem cells in the niche, as well as regulating which cells leave the niche (Ellis & Tanentzapf, 2010; Marthiens et al., 2010). Adhesion of stem cells to the supporting cells within the niche appears to be mediated by cadherins (Song, Zhu, Doan, & Xie, 2002). The expression of cadherins has also been implicated in determining which cells leave the niche, with cells competing for niche space based on levels of cadherin expression and down-regulation of cadherins in differentiating cells providing a mechanism by which they are displaced from the niche (Jin et al., 2008). There is also evidence to suggest that some adhesion molecules, such as integrins, may have a role in the regulation of cell division (Ellis & Tanentzapf, 2010).

SSCs and Sertoli cells have been shown to express a number of adhesion molecules including integrins and E-cadherin (de Rooij, Repping, & van Pelt, 2008). The expression of the adhesion molecule β1-integrin by both SSCs and Sertoli cells has been shown to be important for the correct homing of SSCs to the basal membrane following transplantation (Kanatsu-Shinohara et al., 2008). In addition, deficiency in β1-integrin results in SSCs that exhibit incomplete spermatogenesis, suggesting additional roles for β1-integrin beyond the migration of SSCs to the basal membrane (Kanatsu-Shinohara et al., 2008). At present little else is known regarding the role of adhesion molecules in the mammalian spermatogonial stem cell niche.

Some of the key regulatory factors involved in the regulation of SSC self-renewal secreted by cells in the stem cell niche include Glial-derived neurotrophic factor (GDNF), Colony stimulating factor 1 (CSF-1), Fibroblast growth factor 2 (FGF2), and the Ets variant

gene 5 (ETV5). GDNF, secreted by Sertoli cells, is thought to be a key regulator of SSC self-renewal, acting through the P13K/Akt pathway (Lee et al., 2007). GDNF appears to be essential for the maintenance of SSCs in an undifferentiated state in vivo (Hofmann, Braydich-Stolle, & Dym, 2005; Kubota, Avarbock, & Brinster, 2004). CSF-1 is expressed by both Leydig and myoid cells, and has been shown to enhance self-renewal of mouse SSCs (Kokkinaki et al., 2009; Oatley et al., 2009; Ryan et al., 2001). Increased expression of CSF-1 enhances the proliferation of undifferentiated spermatogonia in vitro (Kokkinaki et al., 2009; Oatley et al., 2009). Another growth factor secreted by Sertoli cells is FGF2. FGF2 has been shown to have a role in regulating self–renewal and proliferation of SSCs (De Rooij, 2009). FGF2 may also stimulate GDNF expression in Sertoli cells, and thus play a role in the maintenance of the SSC pool (Simon et al., 2007). The transcription factor Etv5 is also expressed by Sertoli cells, and plays a role in the maintenance of the undifferentiated SSC pool (Chen et al., 2005). Disruption of Etv5 impairs the ability of the Sertoli cells to support SSCs; however, it does not affect spermatogonial differentiation (Simon et al., 2007; X. Wu, Goodyear, Tobias, Avarbock, & Brinster, 2011).

In addition to factors that regulate self-renewal, cells in this stem cell niche also produce factors that regulate the differentiation of SSCs. Activin A and Bone morphogenetic protein 4 (BMP4) both promote differentiation of SSCs when added to *in vitro* cell cultures (Nagano, Ryu, Brinster, Avarbock, & Brinster, 2003). BMP4 has been shown to induce c-KIT expression in spermatogonia, which is an indication of spermatogonial differentiation (Pellegrini, Grimaldi, Rossi, Geremia, & Dolci, 2003). Stem Cell Factor (SCF) is also secreted by Sertoli cells, and can induce differentiation of undifferentiated spermatogonia by binding to the c-KIT receptor (De Rooij, 2009; Filipponi et al., 2007). By producing such factors the support cells of the spermatogonial stem cell niche help to maintain the balance between SSC self-renewal and differentiation that is required for continual spermatogenesis.

1.2.3. Molecular markers of spermatogonial stem cells

At present, the only available method to unequivocally confirm the presence of SSCs in a population is through a functional assay involving the transplantation of cells into a recipient animal and the determination of testis colonisation activity (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994). The ability to culture and manipulate SSCs directly could lead to advances in infertility treatment, as well as allowing for a method of germ-line transmission of modified genomes. In order to identify and isolate a pure population of SSCs, specific cell markers need to be identified. The identification of cell surface markers is of particular interest to enable physical selection of a SSC population using techniques such as magnetic or fluorescent activated cell sorting (MACS or FACS). A number of cellular markers have been identified, mostly through the study of murine SSCs, for spermatogonia in different stages of differentiation (Aponte, van Bragt, de Rooij, & Pelt, 2005; Hamra et al., 2004; Oatley & Brinster, 2006). However, there are currently no markers that have been shown to be specific for SSCs alone. Instead, the markers are specific for different subsets of spermatogonia, such as the undifferentiated spermatogonia, or for spermatogonia in general. These markers may be useful in identifying and/or creating cell populations that are enriched for a particular subset of spermatogonia; however, they cannot be used to isolate a pure population of SSCs. In addition, they cannot be used to identify only SSCs in a mixed cell population, although they can identify subsets of spermatogonia such as the undifferentiated spermatogonia.

1.2.3.1. Markers of spermatogonia in rodent and human models

The majority of studies carried out in order to characterise mammalian SSCs have used mouse or rat testis as a model. One of the first identifying characteristics of spermatogonia to be discovered was their ability to preferentially bind to laminin over other surfaces (Shinohara, Avarbock, & Brinster, 1999). Selection for the laminin binding receptors α6 and β1-integrin produced a cell population enriched for undifferentiated spermatogonia (Shinohara et al., 1999). Since then, numerous studies have identified markers for different subsets of spermatogonia in the rodent testis. Expression of KIT has been used to distinguish early differentiated (A_{1-4}) spermatogonia from the undifferentiated (A_s, A_{pr} and A_{al}) spermatogonia, as the undifferentiated spermatogonia are KIT negative (Schrans-Stassen, van de Kant, de Rooij, & van Pelt, 1999; von Schonfeldt, Wistuba, & Schlatt, 2004; Yoshinaga, Nishikawa, Ogawa, Hayashi et al., 1991). ZBTB16 (also known as PLZF) is expressed by undifferentiated spermatogonia, and has a role in the regulation of SSC self-renewal (Buaas et al., 2004; Costoya et al., 2004). Other markers expressed by undifferentiated spermatogonia and not by more differentiated spermatogonia in the rodent testis include THY1 (Kubota, Avarbock, & Brinster, 2003), GFRA1 (Buagew et al., 2005; Grasso et al., 2012; Hofmann et al., 2005; von Schonfeldt et al., 2004), DDX4 (also known as VASA) (Noce, Okamoto-Ito, & Tsunekawa, 2001; Raz, 2000; Toyooka et al., 2000), DAZL (Reijo et al., 2000), POU5F1 (also known as OCT4) (Ohbo, Yoshida, & Ohmura, 2003), SOX3 (Raverot, Weiss, Park, Hurley, & Jameson, 2005), NOTCH1 (von Schonfeldt et al., 2004), CD9 (Kanatsu-Shinohara, Toyokuni, & Shinohara, 2004) and STRA8 (Giuili, Tomljenovic & Labrecque, 2002; Oulad Abdelghani et al., 1996).

A comparison of spermatogonial markers in rodents and humans indicates that these cells share many, but not all, phenotypic characteristics (Dym, Kokkinaki, & He, 2009). A

number of markers of undifferentiated spermatogonia, including α6-integrin, THY1, DDX4, ZBTB16 and GFRα1, are conserved between species and can be used to identify undifferentiated spermatogonia in humans and rodents (Conrad et al., 2008; Dym, Kokkinaki et al., 2009). In contrast, other spermatogonial markers identified in rodents are not conserved in humans, or do not identify the same subset of spermatogonia. β1 integrin, a marker for undifferentiated spermatogonia in the rodent testis, is instead expressed by mature spermatocytes in humans (Schaller, Glander, & Dethloff, 1993). POU5F1 and KIT, markers of rodent undifferentiated and differentiating spermatogonia respectively, are not expressed in human spermatogonia (Looijenga et al., 2003; Rajpert-De Meyts et al., 2003). Additional markers of human spermatogonia have been identified that are not applicable in the rodent testis, such as TSPY (Schnieders et al., 1996). The expression of other markers of rodent spermatogonia, such as CD9 and STRA8, has not been examined in the human testis. Markers for human and rodent spermatogonia are summarised in Table 1.1.

Table 1-1: A comparison of markers for human and rodent spermatogonia. (Dym et al., 2009)

Marker	Human	Rodent	References
α6-integrin (CD49f)	+	+	(Shinohara et al., 1999; Conrad et al., 2008)
β1-integrin (CD29)	−	+	(Schaller et al., 1993; Shinohara et al., 1999)
CD9	?	+	(Kanatsu-Shinohara et al., 2004b)
CD133	+	?	(Conrad et al., 2008)
CDH1	?	+	(Tokuda et al., 2007)
CHEK2	+	?	(Bartkova et al., 2001)
GFRA1	+	+	(Meng et al., 2000; Conrad et al., 2008)
GPR125	+	+	(Seandel et al., 2007; Dym et al., 2009)
KIT	−	+	(Yoshinaga et al., 1991; Rajpert-De Meyts et al., 2003)
MAGE-A4	+	?	(Rajpert-De Meyts et al., 2003)
Neurogenin3	?	+	(Yoshida et al., 2004)
NSE	+	?	(Rajpert-De Meyts et al., 2003)
PLZF	+	+	(Buaas et al., 2004; Costoya et al., 2004)
POU5F1	−	+	(Looijenga et al., 2003; Ohbo et al., 2003)
RET	?	+	(Naughton et al., 2006)
STRA8	?	+	(Giuili et al., 2002)
Thy-1 (CD90)	+	+	(Kubota et al., 2003; Conrad et al., 2008)
TSPY	+	−	(Schnieders et al., 1996; Kido and Lau, 2006)

Markers known to be expressed (+) or absent (−) and unknown (?) in human and rodent spermatogonia are listed. Abbreviations: CDH1, cadherin 1, type 1, E-cadherin; CHEK2, CHK2 checkpoint homolog; GFRA1, GDNF family receptor alpha 1; GPR125, G protein-coupled receptor 125; MAGE-A4, melanoma antigen family A, 4; NSE, neurone-specific enolase; PLZF, promyelocytic leukemia zinc finger protein; POU5F1, also known as Oct-4; STRA8, stimulated by retinoic acid gene 8; TSPY, testis specific protein, Y-linked 1.

A number of the markers described above have been used to generate testis cell populations enriched for SSCs using MACS and/or FACS. A 30-fold enrichment of mouse SSCs has been achieved by selecting THY1 positive cells (Kubota et al., 2003), while selection of GFRA1 positive cells results in SSC enriched populations from both rodent and primate testes (Gassei, Ehmcke, Dhir, & Schlatt, 2010; Gassei, Ehmcke, & Schlatt, 2009). Using a combination of positive and negative selection for various markers it is now possible to produce highly enriched populations of SSCs. Selection of THY1 positive, MHC-1/KIT negative cells from the mouse testis has generated a population of cells enriched 25-fold for SSCs compared to unselected populations (Shinohara et al., 1999). However, despite these advances, there is still no marker or combination of markers able to produce pure populations of SSCs.

1.2.3.2. Markers of bovine spermatogonia

Markers of bovine spermatogonia are less well defined than those of human and rodent spermatogonia. Some markers identified in humans and/or rodents appear to be conserved in the bovine. DDX4 has been shown to be expressed by undifferentiated spermatogonia in both adult and foetal bovine testes (Bartholomew & Parks, 2007; Fujihara, Kim, Minami, Yamada, & Imai, 2011). ZBTB16 is expressed by a sub-population of bovine spermatogonia thought to include SSCs (Reding, Stepnoski, Cloninger, & Oatley, 2010). THY1 has also recently been demonstrated to be a marker for undifferentiated spermatogonia in the bovine testis (Reding et al., 2010). As in the rodent testis, KIT expression has also been used to identify more differentiated spermatogonia in the bovine testis (Izadyar et al., 2003; Izadyar, Spierenberg, Creemers, den Ouden, & de Rooij, 2002). In addition to the markers previously identified in humans and rodents, there have also been alternative markers identified for bovine spermatogonia. DBA (*Dolichos biflorus* agglutinin) binding and

UCHL1 (also known as PGP9.5) have both been identified as markers for bovine undifferentiated spermatogonia, although neither appear to be specific for the A_s spermatogonia (Ertl & Wrobel, 1992; Wrobel, Bickel, Kujat, & Schimmel, 1995). Expression of other markers identified in humans and/or rodents, such as GFRA1, STRA8, α6-integrin and β1-integrin, are yet to be described in the bovine testis.

As with markers of human and rodent spermatogonia, none of the markers identified for bovine spermatogonia are specific for SSCs alone, although they may be useful for generating SSC enriched cell populations. Few of the markers described above have been used to generate SSC enriched cell populations from the bovine testis. SSC enriched cell populations have been produced in the bovine using positive selection for DBA binding activity or THY1 expression, or have relied on alternative physical methods of enrichment such as density gradient separation or differential plating (Herrid, Davey, Hutton, Colditz, & Hill, 2009; Izadyar et al., 2002).

1.2.4. Spermatogonial stem cell transplantation

Germ cell transplantation involves the transfer of testis cells from a fertile donor into the seminiferous tubules of a recipient testis, allowing the recipient to produce donor-derived spermatozoa. Figure 1.2 shows a schematic representation of a typical testis cell transplantation experiment. Transplantation of SSCs and the subsequent colonisation and initiation of spermatogenesis was first described by Brinster et al. (1994). By definition, only stem cells can initiate spermatogenesis, therefore SSC transplantation provides a functional assay to characterise stem cell activity in testis cell populations. Since this landmark study, SSC transplantation has been successfully carried out in many species including rats (Jiang & Short, 1995), goats (Honaramooz, Behboodi, Blash, Megee, & Dobrinski, 2003), sheep (Herrid et al., 2009; Rodriguez-Sosa, Silvertown, Foster, Medin, & Hahnel, 2009), cattle

(Herrid, Vignarajan, Davey, Dobrinski, & Hill, 2006; Izadyar et al., 2003), pigs (Honaramooz et al., 2002) and dogs (Kim et al., 2008). This technology has the potential to be applied in a number of fields, including treatment of male infertility and the generation of transgenic animals. Another potential application of SSC transplantation in livestock is as an alternative to artificial insemination for dissemination of elite genetics in the beef cattle industry (Hill & Dobrinski, 2006). This review will focus on SSC transplantation and its application in cattle.

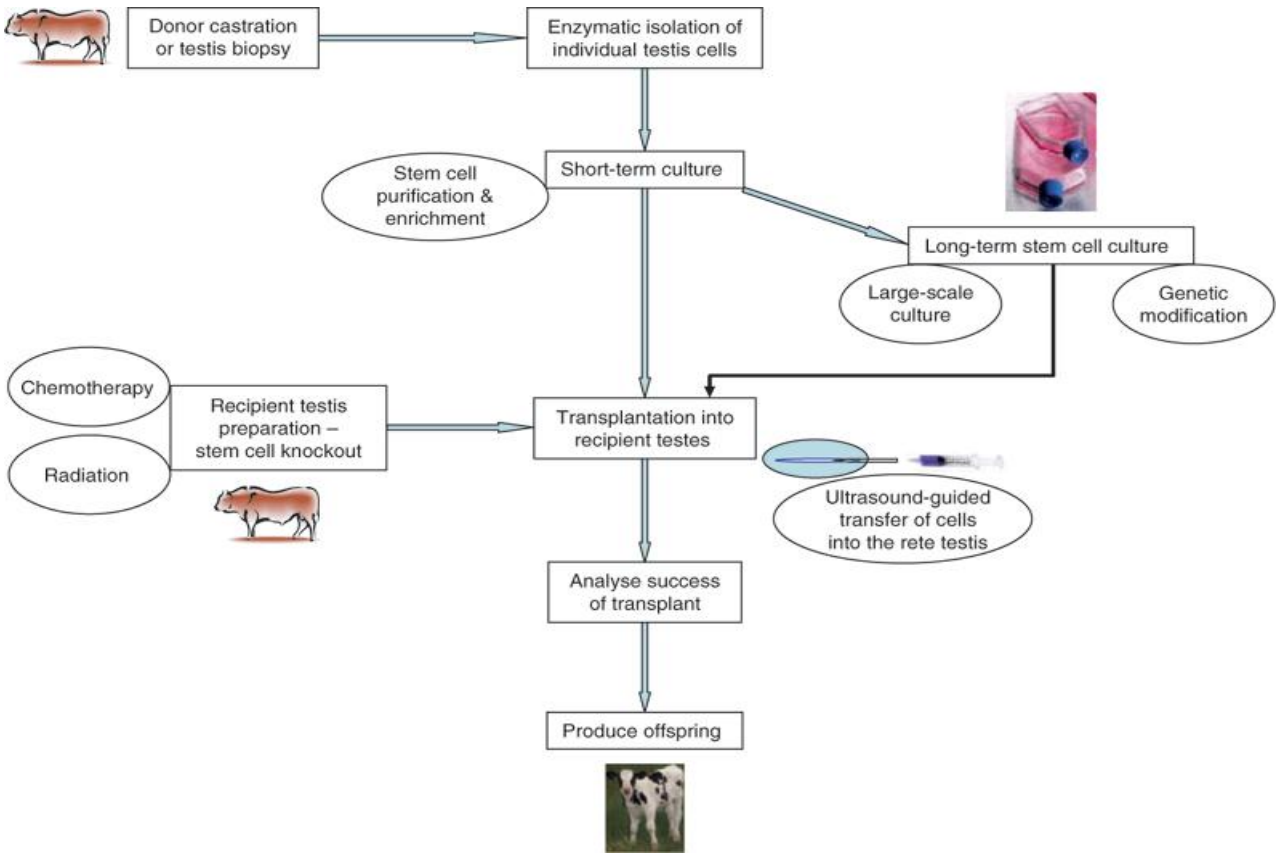

Figure 1-2: Schematic representation of the steps involved in a typical testis cell transplantation experiment in cattle (Hill & Dobrinski, 2006)

There are a number of factors that currently limit the success of SSC transplantation experiments. The first is preparation of the recipient testis. The endogenous stem cell population of the recipient testis needs to be ablated to create space on the basal membrane of the tubules for the transplanted cells to colonise (Brinster et al., 2003; Ogawa, Dobrinski, & Brinster, 1999). Across species this is generally achieved through the use of radiation

(Creemers et al., 2002; Herrid et al., 2009; Izadyar et al., 2003; Schlatt, Foppiani, Rolf, Weinbauer, & Nieschlag, 2002) or chemotherapeutic agents such as busulfan (Brinster et al., 2003; Honaramooz et al., 2005). However, there is a tendency for a small population of endogenous stem cells to survive such treatments, and these cells will re-initiate spermatogenesis, typically resulting in a recipient that produces spermatozoa from both endogenous and transplanted cells (Brinster et al., 2003).

At present, SSC transplantation is an inefficient process (Honaramooz & Yang, 2011). Studies in mice have shown that the most effective way to increase transplantation efficiency is to transplant a highly purified SSC population. A 10-fold enrichment of SSCs prior to transplantation resulted in a 10-fold increase in the number of colonies formed in the recipient mouse testis (Shinohara et al., 1999). However, as discussed previously, the lack of specific markers for bovine SSCs makes purification of these cells difficult, although enriched cell populations can be achieved (Herrid et al., 2009; Izadyar et al., 2002). Further characterisation and identification of markers for bovine SSCs will aid in the isolation of more highly purified SSC populations, which may in turn enhance the efficiency of SSC transplantation in this species.

A further limitation to SSC transplantation in the bovine is the large number of cells required. Although in theory only a small number of SSC are required to repopulate the recipient seminiferous tubules, the length of the tubules in the bovine means this would take a long period of time (Hill & Dobrinski, 2006). The efficiency of transplantation could therefore be increased by transplanting larger numbers of SSCs. It would be advantageous if large numbers of SSCs could be generated using an *in vitro* culture system. However, the conditions required for the long term culture and propagation of bovine SSCs are yet to be defined. Although some progress has been made, there still remain issues with long replication intervals and low survival rates of SSCs in long-term culture, and the tendency of

these cells to undergo differentiation in culture (Aponte et al., 2008; Izadyar et al., 2003; Nasiri et al., 2012; Oatley, Reeves, & McLean, 2004). An increased understanding of germ cells and their niche is of key importance to defining optimal culture conditions that will allow the *in vitro* expansion of SSCs for use in transplantation experiments.

1.3. Induced pluripotent stem cells

Until recently, cell fate was assumed to be fixed, and, once determined, unable to be altered or reversed. However, in recent years a number of experiments have shown that cell fate is less rigidly fixed than previously thought. Takahashi and Yamanaka (2006) demonstrated that the overexpression of key transcription factors could cause murine fibroblasts to be reprogrammed to an embryonic stem (ES) cell like state. Such cells are termed induced pluripotent stem (iPS) cells. Throughout this thesis pluripotency is defined as the ability to form all somatic and germ line cell types *in vivo*. Cells which do not meet these criteria, or that have not had their *in vivo* differentiation potential investigated are referred to as iPS-like. iPS cells share a number of characteristics of ES cells (Maherali et al., 2007; Okita, Ichisaka, & Yamanaka, 2007; Wernig, Meissner, & Foreman, 2007). iPS cells show similar morphology to ES cells, forming compact colonies with clear borders. iPS and ES cells express a number of markers of pluripotency including NANOG and REX1, and are positive for alkaline phosphatase activity (Takahashi & Yamanaka, 2006). Depending on species, they also express the cell surface markers SSEA1, SSEA3/4, Tra-1-60 and Tra-1-81 (Park, Zhao & West, 2008; Takahashi, Tanabe & Ohnuki; 2007; Takahashi & Yamanaka, 2006). ES and iPS cells also have the same potential for differentiation. Besides their ability to undergo what is considered to be unlimited self-renewal, these cells can also differentiate into cell types from all three embryonic germ lineages, and can contribute to the germline. A

further characteristic of fully reprogrammed iPS cells is the silencing of transgene expression as the cells own pluripotency machinery is activated.

iPS cells have the potential to be applied in a number of fields in place of ES cells. They are of particular interest to researchers in the field of regenerative medicine, as they can act as a source of patient-specific stem cells. iPS cells from livestock species are of interest for the generation of transgenic animals and in the development of novel reproductive technologies such as testis cell transplantation as they have the ability to differentiate into any cell type, including germline cells.

1.3.1. Factors affecting iPS generation

The direct reprogramming of differentiated somatic cells to pluripotent cells has been shown to be a highly inefficient process, with generally less than 0.1% of target cells undergoing complete reprogramming (Stadtfeld & Hochedlinger, 2010). There are a number of factors that have been shown to influence the success and efficiency of reprogramming experiments, including the choice of reprogramming factors, the method for delivery of reprogramming factors, the choice of target cell and the cell culture conditions. Figure 1.3 provides an overview of the steps involved in the generation of iPS cells, and the factors that influence the success of such experiments.

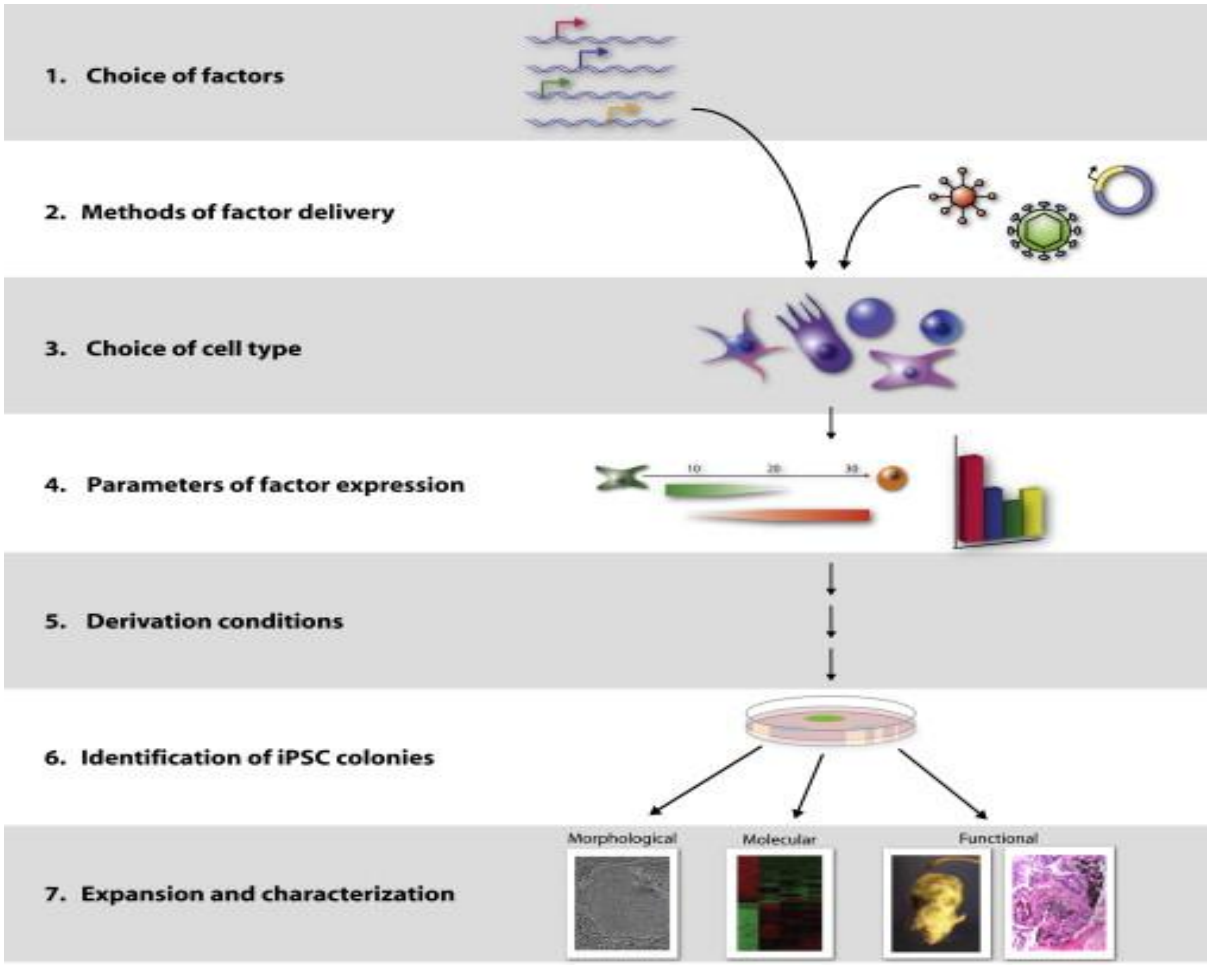

Figure 1-3: Overview of the iPSC derivation process and the parameters that affect reprogramming success (Maherali & Hochedlinger, 2008)

1.3.1.1. Choice of reprogramming factors

The first iPS cells, derived from murine fibroblasts, were generated by the over expression of four transcription factors: POU5F1, SOX2, KLF4, and c-MYC (Takahashi & Yamanaka, 2006). This core set of factors, referred to as the Yamanaka set of factors, have proved sufficient to reprogram various cell types in mice, as well as cells from a number of other species including humans (Takahashi, Tanabe, & Ohnuki, 2007), primates (Liu et al., 2008) and livestock species (Esteban et al., 2009; Huang et al., 2011; Li et al., 2011). Shortly afterwards an alternative set of four transcription factors, referred to as the Thomson set of factors, consisting of POU5F1, SOX2, LIN28 and NANOG, were used to successfully

reprogram human cells to pluripotency (Yu, Vodyanik, & Smuga-Otto, 2007). Various combinations of one or more of these six factors have been used to reprogram different cell types. Additional factors such as the SV40 large T antigen and hTERT have also been used to enhance reprogramming in different cell types (Mali et al., 2008; Park, Zhao, & West, 2008).

POU5F1 is considered to be the key factor required for all reprogramming experiments (Sterneckert, Höing, & Schöler, 2012). POU5F1 interacts with many other factors to establish pluripotency, binding to as many as 90 proteins (Pardo et al., 2010; Wang et al., 2006). POU5F1 has been shown to regulate the expression of other reprogramming factors including SOX2 and NANOG, as well as being involved in self-regulation (Masui et al., 2007). Although the introduction of exogenous NANOG is not required for the induction of pluripotency, there does appear to be an important role for NANOG in the reprogramming process. NANOG is thought to be instrumental in overcoming some barriers to the reprogramming process, and thus the inclusion of NANOG in the reprogramming cocktail may be useful in enhancing the reprogramming process in some cell types (Theunissen et al., 2011). Inclusion of NANOG in the reprogramming cocktail has also been shown to increase the efficiency of iPS cell derivation (Hanna et al., 2009).

The endogenous expression of some reprogramming factors by target cells can permit the exclusion of those factors from the reprogramming cocktail. Fibroblasts express both KLF4 and c-MYC, and it has been shown that these cells can be reprogrammed without the use of exogenous c-MYC (Nakagawa, 2008; Wernig, Meissner, Cassady, & Jaenisch, 2008). Neural progenitor cells that express endogenous SOX2 and c-MYC have been reprogrammed using only POU5F1 and KLF4 (Kim et al., 2008), and also by using POU5F1 alone (Kim et al., 2009). However, it has been noted that exclusion of reprogramming factors can result in lower reprogramming efficiencies and an increase in the time taken for cells to complete the reprogramming process (Kim et al., 2008; Nakagawa, 2008; Wernig et al., 2008).

The original set of Yamanaka factors, and to a lesser extent the Thomson factors, remain the standard for direct reprogramming experiments. However, a number of other small molecules and additional factors have been described that can functionally replace the role of some of these transcription factors. Substitutes for c-MYC are of particular interest as this oncogene is linked to tumourigenesis in iPS cell-derived chimeras (Nakagawa, 2008). The nuclear receptor NR5A2 can replace POU5F1 (Heng et al., 2010), while KLF4 can be replaced by estrogen-related receptor beta (ESRRB) (Feng et al., 2009). L-MYC and n-MYC have both been shown to act as substitutes for c-MYC in the reprogramming process (Blelloch, Venere, Yen, & Ramalho-Santos, 2007). Another transcription factor, GLIS1, has recently been identified that can replace c-MYC, and reprogram cells with less potential for tumour formation (Maekawa et al., 2011). The use of chemical compounds including histone deacetylase (HDAC) and DNA methyltransferase inhibitors and signalling pathway inhibitors can allow reprogramming in the absence of one or more reprogramming factors.

1.3.1.2. Delivery of reprogramming factors

The initial experiments that resulted in the creation of iPS cells used retro-viral mediated delivery of transcription factors to reprogram cells (Takahashi & Yamanaka, 2006). Retro-viral vectors are still commonly used for delivery of transgenes, as are lentiviral vectors. Viral vectors result in the permanent integration of transgenes into the target cell genome, resulting in stable expression of transgenes. Transgene integrations present a major hurdle to the use of these cells in clinical applications, as genomic insertions can alter gene function, and reactivation of transgenes following differentiation has been implicated in tumorgenesis (Kustikova et al., 2005; Nakagawa, 2008). Various approaches have been used to overcome this issue, including the use of non-integrating adenovirus (Stadtfeld, Nagaya,

Utikal, Weir, & Hochedlinger, 2008), and excisable vectors such as the *piggyBac* transposon (Kaji et al., 2009; Yusa, Rad, Takeda, & Bradley, 2009). Another approach aimed at limiting the number of pro-viral integrations in reprogrammed cells is the use of polycistronic viral vectors. These vectors allow delivery of all reprogramming factors within a single vector, and it has been shown that a single integration of one of these vectors can be sufficient to fully reprogram cells (Carey, Markoulaki, & Hanna, 2009; Shao et al., 2009). Polycistronic vectors do not result in integration-free reprogramming, but their use can result in fewer pro-viral integrations than the use of multiple viral vectors.

While viral vectors remain the most commonly used method to deliver reprogramming factors, recent research has increasingly explored the use of non-viral methods of transgene delivery. A number of approaches have been trialled, including the use of mRNA (Warren et al., 2010), protein (Kim et al., 2009; Zhou et al., 2009), and modified DNA (Jia et al., 2010; Narsinh et al., 2011). Although there has been some success in reprogramming using such methods, there is limited information available as to whether these methods can be used to successfully reprogram as wide a range of cells as viral methods have been shown to. In addition, the efficiency of reprogramming cells using non-viral methods is in general much lower than efficiency of viral reprogramming (Jia et al., 2010; Kim et al., 2009; Zhou et al., 2009).

1.3.1.3. Target cell type

The choice of target cell has been shown to have a large effect on the efficiency and success of reprogramming experiments. Initial reprogramming experiments were carried out using embryonic fibroblasts (Takahashi & Yamanaka, 2006). Since then a number of different cell types have been used, including embryonic and adult fibroblasts from different species (Takahashi et al., 2007; Wernig et al., 2007), keratinocytes (Aasen et al., 2008), gut

cells (Aoi, Yae, Nakagawa, & Ichisaka, 2008), neural progenitors (Kim et al., 2009; Kim et al., 2008), adipocytes (Sun et al., 2009) and mesenchymal stem cells (Niibe et al., 2011). Some cell types appear to be more amenable to the reprogramming process and reprogram with high efficiency, possibly due to high levels of endogenous expression of the transcription factors required for reprogramming.

In general, younger or less differentiated cells are considered to be easier to reprogram than older or more differentiated cells. It has been demonstrated that there are differences in reprogramming potential between adult and embryonic cells, with embryonic cells being shown to reprogram at a higher efficiency than their adult counterparts (Okada & Yoneda, 2011; Park et al., 2008). One possible explanation for this observed difference in reprogramming potential is that older cells, or those that have undergone several passages, may have acquired DNA damage which can be a major barrier to the reprogramming process (Hong et al., 2009; Marion et al., 2009).

The differentiation status of the target cells has been demonstrated to affect the reprogramming efficiency and also affect to some extent the differentiation potential of the reprogrammed cells. Less differentiated cells, such as adult stem cells, have been shown to reprogram at a higher efficiency than further differentiated cells (Eminli et al., 2009; Niibe et al., 2011). There is also evidence that fully differentiated cells retain an epigenetic memory, and more readily differentiate into their source lineage than into other lineages (Bar-Nur, Russ, Efrat, & Benvenisty, 2011; Kim et al., 2011; Polo et al., 2010).

In addition to cell age and differentiation status, there are other factors that need to be taken into account in selecting target cells for reprogramming experiments. These include the availability and ease of derivation of the target cell type, and the ease with which reprogramming factors can be introduced into the cells (Maherali & Hochedlinger, 2008). For

large scale reprogramming experiments the target cells need to be easily accessed and cultured. The delivery of all transcription factors is an essential step in the reprogramming process, and thus the target cells must be amenable to the chosen delivery method if reprogramming is to be successful.

1.3.1.4. Cell culture conditions

The basic cell culture conditions used for the derivation of iPS cells are generally based on the culture conditions used for the maintenance and expansion of ES cell lines. Culture conditions are well established for human and murine ES cells (Evans & Kaufman, 1981; Thomson et al., 1998). In other species, such as livestock animals, the conditions required for the derivation of ES cells are yet to be determined, and therefore cannot be used for the culture of iPS cells in these species (Gong et al., 2010; Zhao et al., 2011). In such cases attempts at generation of iPS cells are carried out using culture conditions adapted from human or mouse experiments, which may be sub-optimal for the maintenance of iPS cells from other species. There are a number of aspects of cell culture conditions that may be altered to enhance iPS generation, including the base media used, the use of serum or serum free media, the choice of feeder layers or feeder free conditions, and the temperature and oxygen levels used. In addition there are a number of small molecules and signalling pathway inhibitors that may be used to supplement the culture media, which will be reviewed separately.

The use of serum in cell culture medium is thought to have a significant effect on the derivation of ES and iPS cell lines. The components of serum are not well defined, and are subject to batch to batch variation. It has been speculated that some components of serum may drive differentiation of pluripotent cells (Horii, Nagao, Tokunaga, & Imai, 2003). The use of serum replacement has been shown to enhance the derivation of mouse ES cells

(Cheng, Dutra, Takesono, Garrett-Beal, & Schwartzberg, 2004; Horii et al., 2003; Lee et al., 2006), and has also been shown to enhance reprogramming of mouse fibroblasts (Blelloch et al., 2007). The use of serum replacement may aid in the reprogramming of cell types which for which standard serum conditions are unsuitable.

ES cell growth and survival relies on fibroblast-derived factors, and iPS cells are generally derived and maintained in co-culture with a fibroblast feeder layer. Mouse ES and iPS cells have been cultured in feeder-free conditions (Stadtfeld, Maherali, Breault, & Hochedlinger, 2008; Wernig, Lengner, & Hanna, 2008; Ying et al., 2008), but this has not yet proved possible for iPS cells from all species. Inactivated mouse embryonic fibroblasts (MEFs) are commonly used as feeder layers for iPS generation. There is some evidence to suggest that species specific, rather than mouse, fibroblasts may be more appropriate to use in reprogramming cells from other species (Nagy et al., 2011).

The *in vivo* environment in which ES cells exist is an hypoxic environment (Fischer & Bavister, 1993). The use of hypoxic conditions has been shown to prevent differentiation of ES cells in *in vitro* culture (Ezashi, Das, & Roberts, 2005), and also to enhance the reprogramming efficiency of both human and murine cells (Shimada, Hashimoto, Nakada, Shigeno, & Nakamura, 2012; Yoshida, Takahashi, Okita, Ichisaka, & Yamanaka, 2009). The reason for this is unclear, although research has shown that Hypoxia Induced Factors (HIFs) interact with one of the key reprogramming factors, POU5F1 (Covello et al., 2006). Hypoxic conditions may therefore enhance cell reprogramming by modulating the expression of POU5F1.

1.3.2. Induced pluripotent stem cells from livestock species

Attempts to generate ES cell lines from livestock species such as sheep and cattle have had limited success (Gong, Roach, Jiang, Yang, & Tian, 2010; Mitalipova, Beyhan, &

First, 2001; Saito et al., 2003; Wang et al., 2005; Zhao et al., 2011; Zhu, Sun, & Zhang, 2007). The derivation of iPS cell lines from mice and humans gives rise to the hope that these cells may be used as an alternative to ES cells in the future. However, despite advances in iPS cell technology it has proven difficult to derive iPS cells from some species using conventional protocols. In recent years there have been a number of attempts to reprogram cells from livestock and large animals, with varying degrees of success. Improvements in iPS technology will provide options for the derivation of pluripotent stem cells that can be used in the production of transgenic animals for biomedical or agricultural application.

The domestic pig represents an important model species for human disease, with potential applications for transplantation medicine. Due to the lack of success in obtaining porcine ES cell lines, attention has now turned to the generation of iPS cells in this species. In 2009 three independent groups reported the generation of iPS cells from porcine fibroblasts by the overexpression of the four transcription factors POU5F1, SOX2, KLF4 and c-MYC (Esteban et al., 2009; Ezashi et al., 2009; Wu et al., 2009). These studies employed either lentiviral or retroviral vectors carrying either the human or murine cDNAs for this set of reprogramming factors. One study found that the addition of two extra reprogramming factors, LIN28 and NANOG, resulted in a higher percentage of undifferentiated colonies during early reprogramming (Wu et al., 2009). All three of these studies generated cells that displayed characteristics of ES like-cells, including morphology, marker expression and differentiation potential. However, transgene silencing was not observed, and in one study that employed an inducible transgene vector differentiation occurred following the removal of Dox from the culture media (Wu et al., 2009). These results suggest that pluripotency was being maintained by reliance on exogenous rather than endogenous gene expression. Since these studies, further reports of porcine iPS cells have emerged, with similar results (Ezashi, Matsuyama, Telugu, & Roberts, 2011; Montserrat et al., 2011; Ruan et al., 2011). One group

reported that they were able to generate iPS cells without the use of POU5F1, instead using only three reprogramming factors; SOX2, KLF4 and c-MYC (Montserrat et al., 2011). To date none of the reported porcine iPS cells have been shown to undergo transgene silencing. This limits the potential use of such cells in a clinical setting as continued transgene expression can lead to instability and limit the differentiation potential of these cells.

Sheep are an important agricultural species in many parts of the world. There is interest in the production of genetically modified sheep to improve production traits and disease resistance, however the lack of authentic sheep ES cell lines limits research in this area. Ovine iPS cells, a possible substitute for ES cells, were first reported by Bao et al. (2011). These cells were generated using a set of six transcription factors (POU5F1, SOX2, KLF4, c-MYC, LIN28 and NANOG) delivered by an inducible lentiviral system. The authors found that although these factors were sufficient to generate ES cell-like colonies, these colonies could not be expanded. Addition of the SV40 large T antigen and hTERT to the reprogramming cocktail resulted in the generation of ovine iPS cells, displaying ES cell-like characteristics. In contrast, a second study showed that ovine iPS cells could be generated using only four reprogramming factors (POU5F1, SOX2, KLF4 and c-MYC) (Li, Cang, Lee, Zhang, & Liu, 2011). These cells were also generated using a drug- inducible lentiviral system, and in both cases transgene silencing remained incomplete and removal of doxycycline from the culture medium resulted in cell differentiation and loss of ES cell like characteristics. The most recent report of ovine iPS cells indicated that transgene silencing can occur with long term culture and passaging (Liu et al., 2012). This is the first report of iPS cells generated in a livestock species that do not rely on continual expression of exogenous genes to maintain pluripotency.

To date there have been only two reports of iPS cells generated from bovine cells. Sumer et al. (2011) reported the first generation of bovine iPS cells using retroviral-mediated

expression of human POU5F1, SOX2, KLF4 and c-MYC in adult dermal fibroblasts. Interestingly, this study found that the addition of NANOG to the reprogramming cocktail was necessary to generate pluripotent cells that could be maintained in long-term culture. These bovine iPS cells show all of the characteristics of fully reprogrammed cells except for transgene silencing. The second reported bovine iPS cells were generated using a non-viral polycistronic vector to deliver POU5F1, SOX2, KLF4 and c-MYC to embryonic fibroblasts (Huang et al., 2011). The putative iPS cells derived in this study showed characteristics of ES cells including morphology, expression of pluripotency markers, and the ability to differentiate into cell types indicative of all three embryonic germ layers, however, they were unable to be expanded in culture. These two reports provide conflicting evidence on the necessity of introducing exogenous NANOG for reprogramming bovine fibroblasts.

In addition to attempts to establish iPS cells from pigs, sheep and cattle, there have also been attempts to generate iPS cells from other livestock species such as the horse. The domestic horse is a valuable species in sport and recreation, and also has potential to act as a model species for a number of human medical conditions such as muscle, tendon and ligament injuries. Nagy et al. (2011) first reported the generation of iPS cells from equine fetal fibroblasts using a *piggyBac* transposon based method to deliver an inducible polycistronic vector carrying the four reprogramming factors POU5F1, SOX2, KLF4 and c-MYC. Using a cocktail of small molecules and signalling pathway inhibitors, the authors of this report were able to generate ES-like cells that expressed pluripotency markers, and had the ability to differentiate into cell types from all three germ layers both *in vitro* and *in vivo*. Transgene silencing was not complete in these cells, and they were shown to undergo rapid differentiation with the removal of doxycycline. A second group has since reported the generation of equine iPS cells (Khodadadi et al., 2012). This group found that they were able to generate these cells without the use of c-MYC (Khodadadi et al., 2012). These cells

showed similar characteristics to those generated using a four factor reprogramming method, and once again failed to silence exogenous transgenes.

Despite several attempts aimed at the generation of iPS cells from livestock species there has been to date no consensus reached on the best methods and culture conditions for the derivation of these cells. Table 1.2 summarises livestock reprogramming experiments to date. There has been wide variation in the reprogramming factors used, the method of delivery of these factors, and the culture conditions used including media composition. The lack of transgene silencing in livestock iPS cells acts as a barrier to the use of these cells in clinical applications, and also indicates that the optimal conditions for the reprogramming of these cells are yet to be defined. It remains unclear as to why standard methods used in reprogramming mouse and human cells are less effective in the reprogramming of livestock cells.

Table 1-2: Summary of livestock cell reprogramming experiments

Species	Reprogramming factors	Delivery method	Target cell type	Small molecules/ inhibitors	References
Pig	Human/murine POU5F1, SOX2, KLF4, c-MYC	Retrovirus	Embryonic fibroblasts	LIF, bFGF, 5-aza, PD0325901, CHIR99021	(Esteban et al., 2009)
Pig	Human POU5F1, SOX2, KLF4, c-MYC	Lentivirus	Foetal fibroblasts	FGF2	(Ezashi et al., 2009)
Pig	Human POU5F1, SOX2, KLF4, c-MYC, LIN28, NANOG	Inducible lentivirus	Adult dermal fibroblasts/ bone marrow cells	Nil	(Wu et al., 2009)
Pig	Murine SOX2, KLF4, c-MYC	Polycistronic retrovirus	Adult dermal fibroblasts	bFGF, LIF	(Montserrat et al., 2011)
Sheep	Murine POU5F1, SOX2, KLF4, c-MYC	Inducible lentivirus	Foetal fibroblasts	FGF2	(Li et al., 2011)
Sheep	Human POU5F1, SOX2, KLF4, c-MYC, LIN28, NANOG, SV40 Large T, hTERT	Inducible lentivirus	Adult dermal fibroblasts	Nil	(Bao et al., 2011)
Sheep	Human POU5F1, SOX2, KLF4, c-MYC	Retrovirus	Fibroblasts	bFGF, LIF	(Liu et al., 2012)
Cow	Human POU5F1, SOX2, KLF4, c-MYC, NANOG	Retrovirus	Adult dermal fibroblasts	LIF, bFGF	(Sumer et al., 2011)
Cow	Bovine POU5F1, SOX2, KLF4, c-MYC	Non-viral, poly-promoter plasmid	Embryonic fibroblasts	LIF, PD0325901, CHIR99021	(Huang et al., 2011)
Horse	Human POU5F1, SOX2, KLF4, c-MYC	*piggyBac* transposon	Foetal fibroblasts	LIF, bFGF, CHIR99021, PD0325901, A83-01, Thiazovivin, SB431542	(Nagy et al., 2011)
Horse	Human POU5F1, SOX2, KLF4	Retrovirus	Adult fibroblasts	LIF, bFGF	(Khodadadi et al., 2012)

1.3.3. Signalling pathways involved in reprogramming

Early reprogramming experiments identified a set of key transcription factors required to reprogram differentiated cells to an ES cell-like state, but very little was known about the mechanisms involved. Advances in iPS cell technology have allowed for the identification of a number of signalling pathways involved in cell reprogramming, providing insight into the mechanisms of cell reprogramming. These pathways include the JAK-STAT, BMP-SMAD, TGFβ, WNT/β-catenin and the MEK/ERK signalling pathways. Although most of these pathways appear to be conserved across species, there is some inter-species variation in the activation of these pathways during reprogramming and maintenance of pluripotency. Figure 1.4 summarises the signalling pathways involved in pluripotency.

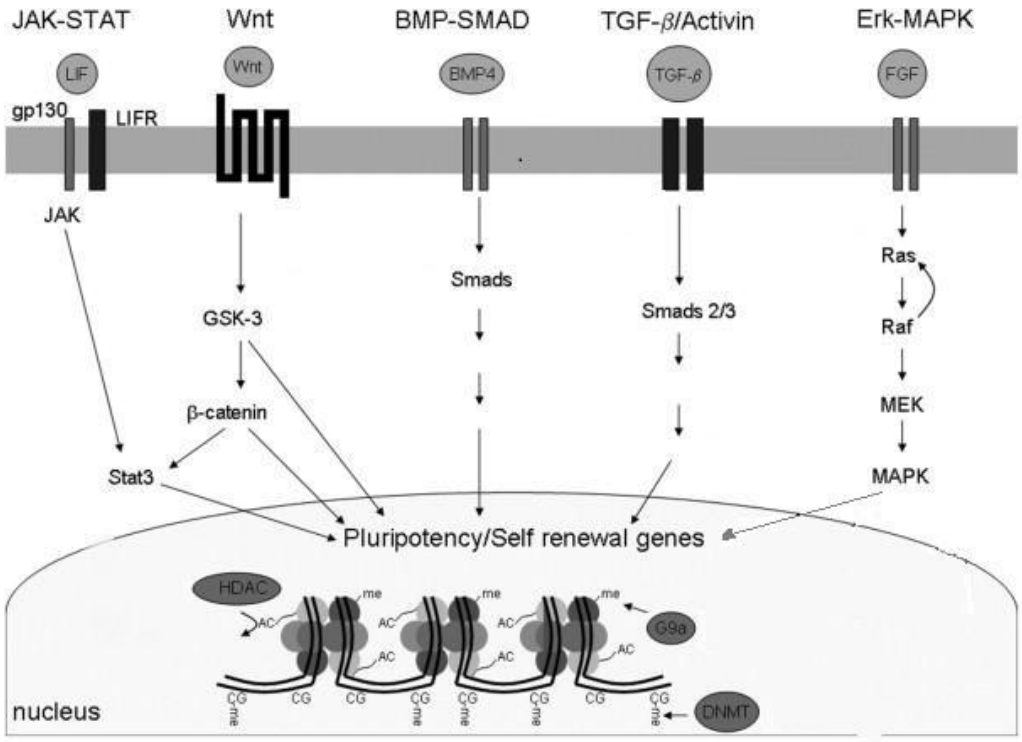

Figure 1-4: Signalling pathways involved in the maintenance of pluripotency. Modified from (Sumer, Liu, & Verma, 2010)

The JAK/STAT and BMP-SMAD pathways are both implicated in the maintenance and generation of murine ES and iPS cells. The JAK/STAT pathway is activated by binding of the cytokine leukaemia inhibitory factor (LIF) to its receptor, which in turn activates the Janus tyrosine kinase (JAK) (Davis et al., 1993). JAK in turn activates STAT3, which translocates to the nucleus where it acts as a transcription factor to regulate genes involved in self renewal and the maintenance of pluripotency (Burdon, Stracey, Chambers, Nichols, & Smith, 1999). In human and primate ES and iPS cells LIF cannot promote self-renewal via the JAK-STAT pathway, due to the low expression of the signalling components and the presence of inhibitors (Wei et al., 2005). The STAT3 pathway in human cells can be activated in response to LIF, but the level of activation is insufficient to maintain self-renewal (Sato, Meijer, Skaltsounis, Greengard, & Brivanlou, 2004). The murine BMP-SMAD pathway is stimulated by BMP4, which binds to its receptor and causes the release of SMAD proteins, which in turn regulates the expression of genes involved in the maintenance of pluripotency and self-renewal (Shi & Massagué, 2003). In contrast, activation of the BMP-SMAD pathway in human ES cells induces differentiation towards a trophectoderm lineage (Xu et al., 2002).

One of the early events in the reprogramming process is a mesenchymal-to-epithelial transition (MET), characterised by a morphological transformation to epithelial-like colonies (Li et al., 2010; Samavarchi-Tehrani et al., 2010). The TGFβ signalling pathway appears to play a central role in this transition. The TGFβ pathway is activated by the TGFβ ligand binding to its receptor and initiating a cascade of events that result in the activation of SMADs 2/3 which regulate the expression of a number of genes involved in self-renewal including NANOG (Shi & Massagué, 2003). Activation of the TGFβ pathway is thought to induce an epithelial-to-mesenchymal transition and blocks reprogramming in both murine and human cells (Maherali et al., 2007).

Activation of the WNT signalling pathway has been demonstrated to aid in sustaining ES and iPS cells in an undifferentiated state via the action of β-catenin (Marson et al., 2008; Miki, Yasuda, & Kahn, 2011; Sato et al., 2004). In the absence of WNT activation β-catenin is degraded by the action of a complex of molecules including the glycogen synthase kinase (GSK3b) (Hoppler & Kavanagh, 2007). When the WNT pathway is activated by WNT binding to the Frizzled family of receptors GSK3b is inhibited, and β-catenin is allowed to accumulate in the nucleus (Hoppler & Kavanagh, 2007; Sato et al., 2004). At present it is unclear exactly how WNT signalling stimulates reprogramming, as experiments in WNT-mediated reprogramming have shown that it does not result in the up-regulation of the common transcription factors involved in reprogramming, such as NANOG, POU5F1 or c-MYC (Lluis, Pedone, Pepe, & Cosma, 2008; Marson et al., 2008).

The MEK/ERK pathway is activated by growth factors including bFGF. The downstream effectors of this pathway, MAPK and ERK, regulate the activity of several transcription factors including c-MYC (Kunath et al., 2007). MEK/ERK signalling triggers differentiation of ES cells (Kunath et al., 2007), whereas inhibition of MEK/ERK signalling enhances the efficiency of ES and iPS cell production (Ying et al., 2008).

1.3.4. Barriers to the induction of pluripotency during reprogramming

Despite advances in iPS cell technology, and refinement of iPS protocols, the generation of reprogrammed cells remains a highly inefficient process. In addition, reprogramming experiments often result in the generation of cells that fail to complete the reprogramming process and become locked in a partially reprogrammed state. A number of barriers that prevent cells from either beginning or completing the reprogramming process have been hypothesized.

One major barrier encountered during cellular reprogramming is cell senescence or death. Pluripotent cells such as iPS cells are considered to be immortal, and can undergo unlimited cell division. In contrast, somatic cells such as fibroblasts have a restricted proliferation potential (Collado, Blasco, & Serrano, 2007), and must undergo a transition to cellular immortality during reprogramming. This is characterised by early events in the reprogramming process which include the up-regulation of genes involved in cell cycle progression and an increase in proliferation (Mikkelsen et al., 2008). The dramatic changes taking place in the cell during reprogramming appear to trigger the DNA damage response leading to stress-induced senescence or apoptosis (Banito et al., 2009; Hong et al., 2009). The DNA damage response is mediated primarily through activation of the tumour suppressing p53/p21 pathway, and numerous studies have shown that down-regulation of p53, p21 and related factors enhances both the efficiency and kinetics of the reprogramming process by preventing cells from entering the senescence or apoptosis pathways (Banito et al., 2009; Hong et al., 2009; Marion et al., 2009).

Studies in fibroblasts have demonstrated that key events in the reprogramming process include the down-regulation of the somatic cell program, activation of pluripotency markers, silencing of transgenes and morphological changes characteristic of a mesenchymal-to-epithelial transition (MET) (Li et al., 2010; Samavarchi-Tehrani et al., 2010; Stadtfeld et al., 2008; Wernig et al., 2008). The failure to complete any of these processes results in cells that are not considered to be fully reprogrammed and instead exist in a partially reprogrammed state. At present it is unclear why some cells are able to complete these processes while others fail to undergo one or more. There is some evidence to suggest some of these barriers can be overcome with continued passaging of cells. Complete down regulation of the cells endogenous somatic cell program, and effective silencing of viral

transgenes have both been shown to be achieved over time (Liu et al., 2012; Polo et al., 2010).

A further barrier to the reprogramming process is the level of transcription factor expression in target cells. It appears that there is an optimal level of expression of each reprogramming factor that is required for efficient iPS generation (Brambrink et al., 2008; Carey et al., 2011; Tiemann et al., 2011). The optimal expression levels and stoichiometry of reprogramming factors is yet to be well defined. Studies have suggested that high levels of SOX2 may be detrimental to the reprogramming process (Eminli et al., 2009), while conversely low levels of POU5F1 expression may be insufficient to induce reprogramming (Papapetrou et al., 2009; Tiemann et al., 2011). Further evidence of the importance of the correct stoichiometry of reprogramming factors is provided by studies employing polycistronic vectors for delivery of transgenes. Such vectors have been shown to support near equimolar expression of proteins, which may not be ideal for the induction of pluripotency, as evidenced by the significantly lower reprogramming efficiencies when compared to reprogramming using individual vectors (Carey, Markoulaki, & Hanna, 2009; Shao et al., 2009).

In addition to these molecular barriers, there also appear to be epigenetic barriers that a cell must overcome during the reprogramming process. Comparison of pluripotent ES or iPS cells with somatic cells indicates that pluripotent cells have what is considered to be a transcriptionally permissive chromatin state, characterised by histone acetylation and DNA demethylation (Azuara et al., 2006; Bernstein et al., 2006). Hypermethylation or deacetylation of chromatin can be barriers to the transition of cells to a fully reprogrammed state (Mikkelsen et al., 2008; Sridharan et al., 2009). This is likely due to the inability of the transcription factors involved in reprogramming to access and bind DNA in this state.

1.3.5. Small molecules and signalling pathway inhibitors in reprogramming

As knowledge of the signalling pathways involved in, and barriers to the reprogramming process increases, a number of small molecules have been identified that can enhance the reprogramming process. Such molecules can be used to activate or inhibit signalling pathways involved in pluripotency, or overcome specific barriers to the reprogramming process. These molecules can be divided into two general categories: molecules that are involved in DNA modification, and signalling pathway inhibitors. Table 1.3 provides a summary of small molecules and inhibitors used in enhancing the generation of pluripotent cells, and the signalling pathways they affect.

Table 1-3: Small molecules and signalling pathway inhibitors used to enhance iPS cell generation.

Molecule	Function	References
5-azacytidine	DNA methyltransferase inhibitor	(Huangfu, Maehr, & Guo, 2008; Mikkelsen et al., 2008)
RG108	DNA methyltransferase inhibitor	(Shi et al., 2008)
Valproic acid	Histone deacetylase inhibitor	(Huangfu et al., 2008)
Sodium butyrate	Histone deacetylase inhibitor	(Liang, Taranova, Xia, & Zhang, 2010; Mali et al., 2010)
Trichostatin A	Histone deacetylase inhibitor	(Huangfu et al., 2008)
6-Bromoindirubin-3-oxime	GSK3β inhibitor	(Sato et al., 2004)
CHIR99021	GSK3β inhibitor	(Ying et al., 2008)
A-83-01	TGFβ inhibitor	(Zhu et al., 2010)
SB431542	ALK5 inhibitor	(Lin et al., 2009)
PD0325901	MEK inhibitor	(Lin et al., 2009; Shi et al., 2008)
PD98059	MEK inhibitor	(Burdon et al., 1999)
PD184352	MEK inhibitor	(Ying et al., 2008)
PD173074	FGF receptor inhibitor	(Ying et al., 2008)
SU5402	FGF receptor inhibitor	(Ying et al., 2008)

DNA modification molecules that have been shown to enhance reprogramming include histone deacetylase (HDAC) inhibitors and DNA methyltransferases. These molecules appear to enhance the reprogramming process by promoting a transcriptionally permissive chromatin state. A number of HDAC inhibitors including valproic acid (VPA), trichostatin A and sodium butyrate (NaB) have all been shown to improve the efficiency and/or kinetics of the reprogramming process (Huangfu et al., 2008; Liang et al., 2010; Mali et al., 2010). The DNA methyltransferase inhibitors 5-azacytidine (5-aza) and RG108 have

been shown not only to increase the efficiency of the reprogramming process but also promote the conversion of partially reprogrammed cells into fully reprogrammed iPS cells (Huangfu et al., 2008; Mikkelsen et al., 2008; Shi et al., 2008).

A number of signalling pathways that play a role in cellular reprogramming and maintenance of pluripotency have been identified, as described above. Inhibitors of these pathways have been used to enhance both the efficiency and kinetics of the reprogramming process. Activation of the WNT signalling pathway by the use of GSK3b inhibitors including 6-bromoindirubin-3-oxime (BIO) and CHIR99021 aids in maintaining ES cells in an undifferentiated state, and also allows the derivation of ES cells from cell types previously considered non-permissive (Lluis et al., 2008; Sato et al., 2004; Silva et al., 2008; Ying et al., 2008). Inhibition of the MEK/ERK pathway aids in the maintenance of pluripotency by stimulating self-renewal of ES and iPS cells. There are a number of inhibitors of the MEK/ERK pathway including PD0325901, PD98059, PD173074, PD184352 and SU5402 (Burdon et al., 1999; Silva et al., 2008; Sumer et al., 2010; Ying et al., 2008). Inhibitors of the TGFβ signalling pathway appear to enhance cellular reprogramming by promoting MET. Inhibitors of this pathway include the TGFβ inhibitor A-83-01 and the activin-like kinase 5 (ALK5) inhibitor SB431542 (Lin et al., 2009; Zhu et al., 2010).

Interestingly, it appears that these small molecules and signalling pathway inhibitors have synergistic roles, and are most effective when used in combination. Dual inhibition of the MEK and GSK3b pathways by PD0325901 and CHIR99021 (known as 2i) promotes the completion of reprogramming in murine cells and has also been used to promote reprogramming of porcine and bovine cells (Esteban et al., 2009; Huang et al., 2011; Ying et al., 2008). Inhibition of the GSK3b and MEK/ERK pathways with a combination of CHIR99021, PD184352 and SU5402 (known as 3i) has also been used to enhance the

propagation of pluripotent stem cells (Ying et al., 2008). The HDAC inhibitor NaB has been used in combination with MEK and TGFβ inhibitors to significantly enhance the efficiency and kinetics of human iPS generation (Zhang et al., 2011). Other similar combinations of inhibitors have also been used to enhance iPS generation and promote the completion of reprogramming in partially reprogrammed cells (Lin et al., 2009; Zhu et al., 2010). In each of these cases treatment with one or two small molecules or inhibitors was much less effective than using them in combination. This suggests that the use of DNA modifying compounds and the inhibition of multiple signalling pathways have a synergistic effect on enhancing the generation of iPS cells (Lin et al., 2009; Zhang et al., 2011; Zhu et al., 2010).

1.4. Germline differentiation

Pluripotent stem cells can be differentiated *in vitro* into cell types from all three embryonic germ layers when exposed to the appropriate conditions. Under the appropriate conditions they are also able to be differentiated into germline cells. Differentiation of germ cells from pluripotent stem cells has the potential to provide a source of germline cells for use in reproductive technologies. In order to successfully produce germline cells and potentially gametes *in vitro* a better understanding of *in vivo* germline specification is required.

1.4.1. Germline specification *in vivo*

In mammals, germ cells are derived from primordial germ cells (PGCs) arising from the epiblast early in development. Studies of *in vivo* germline specification have identified a number of key events and factors involved in the differentiation of pluripotent cells to a germline lineage. Signals from the extra-embryonic tissue appear to be critical for the establishment of PGCs in mammals, inducing epiblast cells to differentiate into PGC precursors (Lawson & Hage, 1994; Zhao & Garbers, 2002). Bone morphogenetic proteins (BMPs) including BMP4, BMP2 and BMP8b are expressed in the extra-embryonic ectoderm

and are thought to play a role in induction of PGC specification (Lawson et al., 1999; Ying, Liu, Marble, Lawson, & Zhao, 2000; Ying & Zhao, 2001). Inactivation of these genes in mice results in either reduced numbers of germ cells, or the complete failure to form PGCs (Wei et al., 2008a). WNT3 has also been shown to have a critical role in germline specification. WNT3 expression appears to control germline specification by allowing epiblast cells to respond to BMP4 signalling (Ohinata et al., 2009; Wei et al., 2008). WNT3 knockouts show impaired response to BMP signalling, which is essential for PGC formation. POU5F1 is also hypothesised to play a role in the formation of PGCs. Trophectoderm and epiblast cells quickly down-regulate POU5F1 expression as they differentiate, while POU5F1 expression persists in germline cells (Fuhrmann et al., 2001). At present the role of POU5F1 in germline specification is yet to be defined, and it remains to be determined whether POU5F1 expression is regulated by BMPs.

1.4.2. *In vitro* differentiation of pluripotent stem cells to germline cells

There have been a number of studies describing attempts to generate germ cells and/or mature gametes from pluripotent stem cells using *in vitro* culture systems. The two main methods employed for *in vitro* differentiation studies are differentiation in adherent culture, or differentiation through embryoid body (EB) formation (Marques-Mari, Lacham-Kaplan, Medrano, Pellicer, & Simon, 2009). These studies have identified a number of factors that promote the differentiation of cells towards the germline. The addition of retinoic acid (RA) has been shown to induce germ cell differentiation in mouse ES and iPS cells, including generating germ cells that can give rise to offspring (Geijsen et al., 2004; Nayernia et al., 2006; Silva et al., 2009; Yang et al., 2012; Zhu et al., 2012). Other studies have demonstrated that addition of BMP4 to cell culture media is sufficient to derive germ cells from pluripotent stem cells (Kee, Gonsalves, Clark, & Reijo Pera, 2006; Toyooka, Tsunekawa, Akasu, & Noce, 2003; Young, Dias, & Loveland, 2009). Germ cells have also

been derived *in vitro* using either testis or ovarian cell conditioned media (Lachman-Kaplan, Chy, & Trounson, 2006; Richards, Fong, & Bongso, 2008). Taken together, these studies indicate that both human and mouse ES and iPS cells have the potential to undergo germline differentiation in *in vitro* culture systems, however the efficiency of this process is yet to be optimised.

1.5. Conclusions

Spermatogonial stem cells (SSCs) have great potential for application in reproductive medicine and for reproductive technologies such as testis cell transplantation. However, at present the use of SSCs in such technologies is limited by a lack of knowledge of these cells and their niche environment. Numerous studies have identified a number of characteristics and molecular markers for different subsets of spermatogonia; however, to date no SSC specific markers have been identified. Maintaining and expanding these cells in culture has also proven difficult given the slow rate of cell division and tendency of cells to undergo spontaneous differentiation. A number of factors that are involved in maintaining these stem cells in their niche have also been determined, although the role of many of these factors is yet to be elucidated.

Compared to human and murine systems, information regarding bovine SSCs and their niche is extremely limited. Further research is required to characterise bovine SSCs and improve methods of identification and purification of these cells. Improvements in isolation and culture will not only allow these cells to be used in a wider range of applications, but will also provide insights that may assist in the generation of germline stem cells via *in vitro* differentiation from pluripotent stem cells.

Induced pluripotent stem (iPS) cells provide a potential alternative source of pluripotent stem cells that may be used in many applications including the generation of

germline cells for use in reproductive technologies. However, there are still a number of hurdles to be overcome before iPS cells can be considered for clinical application. At present the generation of iPS cells is a highly inefficient process. Investigation into the pathways involved in reprogramming has led to the identification of small molecules that can increase the efficiency of the iPS cell generation, however efficiency still remains low. In addition to the issue of efficiency there are also issues concerning the use of viral vectors in iPS cell generation. Transgene integrations associated with the use of viral vectors can alter gene function promote tumorgenesis. Although non-viral methods of iPS cell generation have proven successful the efficiency of such methods is in general lower than that of viral-mediated reprogramming. Continued research into the pathways involved in the reprogramming process could aid in increasing efficiency of both viral and non-viral cell reprogramming.

Despite rapid advances in iPS technology, generation of iPS cells from livestock species, including cattle, has proven difficult. At present it is unclear why reprogramming methods that are successful in other species, such as humans and mice, are less successful when applied to livestock cells. There are numerous factors that can influence the success of reprogramming experiments, and a number of barriers that must be overcome in order to generate fully reprogrammed cells. Further study is required to define the optimum conditions for the successful generation of bovine iPS cells.

In vitro differentiated germline cells and cells isolated directly from the testis represent two possible sources of SSCs for use in reproductive technologies such as testis cell transplantation. If the previously discussed limitations of iPS cell generation can be overcome, differentiating germline cells from iPS cells may hold an advantage over directly isolating cells from the testis. The material required for the generation of iPS cells can be obtained from an animal with little to no effect on the animal, and can be taken from any

animal, regardless of age. In addition, once established, iPS cells can quickly be expanded to large numbers. In order to achieve the long-term goal of generating bovine SSCs from iPS cells current understanding of both SSCs and iPS cells must be increased.

1.6. Aims

Therefore the general focus of this thesis is to contribute to the characterisation of bovine spermatogonial stem cells by describing molecular markers that could be used to identify and potentially purify SSC populations. These markers would also be of use to identify successful *in vitro* germline differentiation of bovine pluripotent stem cells generated using iPS cell technology.

Specifically, within the scope of this thesis the aim is to describe a screening platform that could be used to identify potential markers for bovine spermatogonia, and then use this platform to screen a number of candidate markers. A second aim of this thesis is to further examine any putative SSC markers identified using this platform to determine if they have potential for use in identification or purification of bovine spermatogonia. This thesis then aimed to explore the feasibility of deriving bovine iPS cells from fibroblasts using lentiviral-mediated expression of four transcription factors and finally explore the use of small molecules and signalling pathway inhibitors to enhance the reprogramming of bovine fibroblasts to pluripotency.

Chapter 2: Characterisation of bovine spermatogonial stem cells

2.1. Introduction

Spermatogonial stem cells (SSCs) in the adult testis allow for the continual production of spermatozoa throughout the post pubertal life of a male. The SSC population, also known as type A single (A_s) spermatogonia have the ability to self-renew, undergo differentiation, and have the ability to colonise testis tissue when transplanted. SSCs, located on the basal membrane of the seminiferous tubules, make up only a small percentage of the total testis cell population.

The ability of SSCs to recolonise testis tissue following transplantation has generated interest in these cells for use in reproductive technologies. The success of SSC transplantation experiments in mice (Brinster & Zimmermann, 1994) has led the way for application of this technique to other species, including livestock (Herrid, Davey, & Hill, 2007; Hill & Dobrinski, 2006; Honaramooz et al., 2003; Honaramooz et al., 2002). SSC transplantation has the potential to be applied in livestock industries such as beef cattle production as an alternative to artificial insemination (Hill & Dobrinski, 2006). An important step in enhancing the success of SSC transplantation experiments is the identification and purification of SSC populations (Johnston, Russell, & Griswold, 2000; Shinohara et al., 1999). At present there are no cellular markers that can be used to distinguish SSCs from all other cell types in the testis, although cell populations enriched for SSCs have been achieved in some species. The identification of a cell surface marker specific for SSCs is of particular interest to enable the identification and purification of these cells.

Bovine SSCs can be isolated directly from the testis (Herrid et al., 2006; Izadyar et al., 2002), or produced *in vitro* from the differentiation of pluripotent stem cells (Toyooka et

al., 2003). Regardless of the source, molecular markers are required to identify SSCs in culture. A number of markers have been identified for spermatogonia in different stages of differentiation, mostly through the use of murine models. Currently there are no cellular markers that have been shown to be specific for SSCs alone. Instead, such markers are specific for subsets of spermatogonia, such as the undifferentiated spermatogonia, or for spermatogonia in general (Aponte et al., 2005; Hamra et al., 2004; Oatley & Brinster, 2006). These markers have been useful in creating and identifying populations enriched for a particular subset of spermatogonia, but are not sufficiently specific to support selection of pure SSC populations (Aponte et al., 2005; Hamra et al., 2004; Oatley & Brinster, 2006).

A number of different cell surface proteins have been used for the isolation of stem cells from other tissues. Examination of these proteins for usefulness in enriching SSC populations has identified multiple proteins that could potentially be used as markers for SSCs, including CD9 (Kanatsu-Shinohara et al., 2004), KIT (Schrans-Stassen et al., 1999) and THY1 (Kubota et al., 2003). Immunocytochemical studies have also identified a number of genes that appear to be expressed specifically in undifferentiated spermatogonia, including *ZBTB16, GFRA1, POU5F1, SOX3, STRA8* and *NOTCH1* among others (as reviewed by Aponte et al., 2005). KIT is recognised as a marker of more differentiated spermatogonia in many species including humans (Izadyar et al., 2011) and mice (Schrans-Stassen et al., 1999; Yoshinaga, Nishikawa, Ogawa, & al., 1991). KIT expression is generally low or absent in early undifferentiated spermatogonia including SSCs, and enhanced as spermatogonia begin to differentiate (Schrans-Stassen et al., 1999). GFRA1 is the receptor for the growth factor GDNF, which regulates differentiation and self-renewal of SSCs. GFRA1 has been shown to be expressed by the single cell spermatogonia in the mouse testis (Grasso et al., 2012); however, co-expression of GFRA1 and KIT in some cells indicates that GFRA1 is not completely specific to SSCs (Hofmann et al., 2005). THY1 is thought to be a conserved

marker of undifferentiated spermatogonia in mammals. Selection for $THY1^+$ cells from primates (Hermann et al., 2009) and mice (Kubota et al., 2003) has resulted in highly enriched SSC populations. STRA8 has been shown to be expressed specifically in the developing male gonad during embryogenesis in the mouse (Oulad Abdelghani et al., 1996). In the adult mouse expression is restricted to pre-meiotic germ cells (Oulad Abdelghani et al., 1996). STRA8 is believed to have a role in the pre-meitotic phase of spermatogenesis (Miyamoto et al., 2002; Oulad Abdelghani et al., 1996).

In addition to the markers identified in mice, some general spermatogonial markers have been identified in other species. In the bovine, DBA (*Dolichos biflorus* agglutinin) binding activity (Ertl & Wrobel, 1992; Herrid et al., 2007; Izadyar et al., 2002) and UCHL1 (PGP9.5 - Protein Gene Product 9.5) (Wrobel et al., 1995) have both been identified as markers for early spermatogonia, although neither appears to be specific for SSCs. UCHL1 is thought to be expressed generally by undifferentiated spermatogonia, but is also expressed by a small proportion of later germ cell stages (Herrid et al., 2007). In addition, DDX4 (VASA) (Bartholomew & Parks, 2007; Fujihara et al., 2011) and ZBTB16 (PLZF) (Reding et al., 2010) have also been shown to be expressed in subsets of bovine spermatogonia. DDX4 is a conserved marker for the germ cell lineage across species, and is expressed in primordial germ cells and undifferentiated spermatogonia (Noce et al., 2001; Raz, 2000). DDX4 has been shown to be expressed by germ cells in both adult and foetal bovine testis (Bartholomew & Parks, 2007; Fujihara et al., 2011). ZBTB16 is expressed by a sub-population of UCHL1+ type A spermatogonia and is thought to be more restricted to SSCs (Kubota et al., 2003). During the preparation of this thesis it was demonstrated that THY1 is a marker for undifferentiated spermatogonia in the bovine testis (Reding et al., 2010). As in other species, all of these markers are general markers for subsets of spermatogonia, and are

not specific for SSCs. Many other cellular markers identified in other species are yet to be validated for the bovine.

A number of these different markers have been used to generate testis cell populations enriched for SSCs using MACS and/or FACS. Selection of $THY1^+$ cells in the mouse testis results in an approximate 30-fold enrichment for SSCs compared to unselected populations (Kubota et al., 2003). MACS selection of $THY1^+$ cells in the bovine testis has resulted in an enriched cell population consisting of 84% germ cells (Reding et al., 2010). $GFRA1^+$ selection by MACS has been used to generate enriched SSC populations in both rodents (Gassei et al., 2009; Grasso et al., 2012) and primates (Gassei et al., 2010). At present the most effective methods for enrichment of SSCs employ a combination of cellular markers to select for these cells. This strategy has been used to achieve highly enriched SSC populations in mice and humans. A 25-fold enrichment of SSCs has been achieved in mice by selecting cells that are $THY1^+$ and MHC-1/KIT^- (Shinohara et al., 1999). A similar strategy has yet to be employed in the enrichment of SSCs from the bovine testis.

Much of the work described above that has been carried out in an attempt to identify SSC markers has been undertaken using murine models. Some aspects of spermatogenesis differ between species (Dym, Kokkinaki et al., 2009). Some genes, such as those regulating mitosis, meiosis and germline development appear to be specific to developmental phases and may be conserved between different species (Hamra et al., 2004). However, it appears that many of the identified putative markers may still be species specific. Some spermatogonial markers identified in rodents are not expressed during spermatogenesis in the human, and conversely some human markers are not expressed during rodent spermatogenesis (Dym et al., 2009). Other markers are yet to be tested in more than one system. It is possible that there are some markers (those related to core regulatory genes involved in spermatogenesis) that are conserved between species, and other markers that are

species specific. Thus the use of any potential marker needs to be validated for any given target organism.

It is hypothesised that SSC markers identified in other species can be validated for use in bovine testis cell populations, and that previously unreported markers can be identified for bovine spermatogonia. These markers can then be used to identify and/or purify SSCs from testis cell populations or populations of pluripotent stem cells that have undergone germline differentiation *in vitro*. In order to test this hypothesis a quantitative reverse transcribed PCR (qRT-PCR) screening platform was developed to compare expression of candidate marker genes between SSC enriched and SSC depleted cell populations. Expression of established bovine spermatogonial markers *DDX4*, *UCHL1* and *ZBTB16* was used to confirm the effectiveness of this platform. This method allows for efficient screening of multiple candidates without the need to source specific antibodies. This screening platform was used to examine expression of the spermatogonial markers *KIT*, *THY1*, *STRA8* and *GFRA1* in the bovine testis. Finally, a number of putative SSC markers, identified by proteomic analysis of testis cell populations (Colgrave, Stockwell, McMillan, Lehnert, & Schmoelzl, submitted), were evaluated to determine whether they could identify bovine spermatogonia.

2.2. Materials and Methods

2.2.1. Collection of testis samples

Animal experiments were approved and conducted under guidelines of the F.D. McMaster Animal Ethics Committee, CSIRO Livestock Industries (Approval no. 10/14). Twelve pre-pubescent Angus bull calves aged between 4 and 8 months were castrated under general anaesthesia. Testes were kept on ice until dissection. The tunica albuginea and epididymis were removed.

2.2.2. Enzymatic isolation of testis germ cells

All cell culture reagents were purchased from Invitrogen, USA, unless otherwise stated. Cell suspensions of testis cells were obtained using the method described by Herrid et al. (2006). Briefly, approximately 10 g of tissue was dissected from the testis and placed in DMEM/F12 containing 100 U/ml penicillin and 100 mg/ml streptomycin. The tissue was chopped finely, then placed into a sterile tea strainer and ground using a syringe plunger. The resulting paste was transferred into DMEM/F12 with 1 mg/ml collagenase (Sigma, USA) and incubated at 37^{o}C in a shaking water bath until individual tubules could be seen under a microscope, approximately 15 minutes. The suspension was allowed to settle, the supernatant was removed, and the tissue was then washed 5 times in DPBS. The tissue was resuspended in DPBS containing 2.5 mg/ml trypsin, and incubated at 37^{o}C for up to 10 minutes. Once a single cell suspension was obtained, an equal volume of DMEM/F12 with 5% foetal bovine serum (FBS) was added to inactivate the trypsin. The resulting cell suspension was 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 10 ml DMEM/F12 + 5% FBS. Cell viability was assessed by trypan blue exclusion. Freshly isolated cells were used for subsequent SSC enrichment. Cell smears were prepared from freshly isolated cells. Cell samples were snap frozen in liquid nitrogen and stored at -80^{o}C until required for RNA isolation.

2.2.3. SSC enrichment

All cell culture reagents were purchased from Invitrogen, USA, unless otherwise stated. Cell populations were enriched for SSCs using either differential plating on tissue culture plastic or selection for *D. biflorus* agglutinin (DBA) positive cells using magnetic

activated cell sorting (MACS). For differential plating 5.5×10^5 cells/cm^2 were resuspended in DMEM/F12 + 5% FBS + Pen/Strep and incubated overnight at 37^{o}C, 5% CO_2. Loosely attached cells were dislodged by gently tapping the side of the culture flasks, and supernatant was collected (SSC enriched). The flask was then rinsed with DPBS, and attached cells dislodged with a cell scraper (SSC depleted).

For enrichment using MACS, 5×10^6 cells were collected, washed in DPBS then resuspended in 100 μl MACS buffer (DPBS containing 0.5% BSA +2 mM EDTA). DBA-FITC (10 μl, Vector Laboratories, USA) was added to each tube, mixed, and incubated on ice in the dark for 5 minutes. Cells were washed by adding 1 ml MACS buffer and centrifuging at 300 × g for 5 minutes. The wash step was repeated, and the resulting cell pellet resuspended in 90 μl MACS buffer. Anti-FITC microbeads (20 μl, Miltenyi Biotec, USA) were added, mixed well and incubated in the dark on ice for 15 minutes. Cells were washed as described previously, and resuspended in 500 μl buffer. MACS LS columns (Miltenyi Biotec, USA) were prepared by placing in a MACS Separator (Miltenyi Biotec, USA) and rinsing with 3 ml MACS buffer. The cell suspension was then loaded onto the column. The suspension was allowed to pass through the column, and the column was rinsed 3 times with 3 ml of buffer. The unlabelled (SSC depleted) cells passing through the column were collected as the MACS(-) fraction. The DBA labelled cells (SSC enriched) were removed from the column by adding 5 ml buffer and using the plunger supplied to flush the cells from the column. These cells were collected as the MACS(+) fraction.

Cell smears were prepared using cells from each treatment. Remaining cells were snap frozen in liquid nitrogen and stored at -80^{o}C until required for RNA isolation.

2.2.4. 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. Primary antibody against DBA-biotin (Vector Laboratories, USA; 1:10) was diluted in TBS containing 0.5% BSA, and incubated on smears for 30 minutes at room temperature. Smears were washed and incubated with Streptavadin Alexa-350 conjugate (Invitrogen, USA; 1:250) for 30 minutes at room temperature. Smears were washed in TBS-T and mounted with Prolong Gold (Invitrogen, USA). Smears were examined using a fluorescent microscope (Zeiss, Germany) and images captured using Axiovision software (Zeiss, Germany).

2.2.5. Quantitative Reverse Transcribed Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from frozen cell samples using an RNeasy midi kit (Qiagen, USA) including an on-column DNAse treatment step. cDNA was transcribed using a Superscript III first strand synthesis kit (Invitrogen, USA). Quantitative RT-PCR reactions were carried out in an 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 cDNA transcribed from 10 ng RNA. Reactions were performed in triplicate. Bovine specific primers for the genes of interest were designed using bovine sequences in the NCBI Genbank databases. The sequences of primers used in this Chapter are given in Table 2.1. Reaction efficiencies for each primer set were determined from a standard curve created using a serial dilution (100 ng, 20 ng, 4 ng, 0.8 ng and 0.16 ng) of cDNA transcribed from RNA extracted from isolated testis cells, with reactions carried out in triplicate. These efficiencies are given in Table 2.1. Appropriate reference genes, *ATP5G2* (ATP synthase*)* and *RPS26*, were determined using the sheep GeNorm kit (PrimerDesign, UK) to test

candidate reference gene expression in different testis cell fractions, and selecting the two most stably expressed candidates.

Table 2-1: Sequences of primer sets used in Chapter 2.

Gene	Forward Primer (5'-3')	Reverse Primer (5'–3')	Amplicon size (BP)	Reaction efficiency (%)	Melt peak (^{o}C)
ASB9	CTCTTCCCTAGGCGATACCC	AAGTGGAGACAGAGCCAGGA	162	86.5	84
ATIC	CAGTCGAACTCCGTGTGCTA	TACTGATCGATGGCATTGGA	197	94.2	85
BCL6B	TCAAGAGTCACGTTCGCATC	TGTTTCTGGCGCAGATGTAG	109	95.3	85.5
CLDN8	TTGCCAATTCCATCATCAGA	TTCGGCGTGATAGCTTTTCT	218	102.3	86.5
CSF1R	GTGGCTGTGAAGATGCTGAA	CCTTCCTTCGCAGAAAGTTG	199	126.9	87
DDX4	TACGCCCAATTCGATGAAC	TTGCCACTTTTCTTTGTCAAG	225	90.51	81
DDX6	TGCCATTCTCTTGCTTTGTG	CTCACTCCTTTTGCCTGGAG	148	100.7	82
GATA4	ACCAGCAGCAGTGAGGAGAT	TGGTGACTGGCTGACAGAAG	190	96.25	89
GFRA1	ATGTCCAATGACGTCTGCAA	GGAGTCCTGCAAGTTCAAGC	205	102.59	89.5
KIT	AGAGAATGTGGATCTGGTTG	AATGTCACGGAAGAATTGAC	231	97.06	80.5
MTHFD1	CCTGGCTCTCACCAGTTCTC	GAGTGCCCTCTAACGTCTGC	176	91.8	85.5
NAP1L4	CGGAGTTCTGGTTCACCATT	AGTCATCGGGTTCAAAGTGG	165	93.8	83.5
PARK7	CATCCTGGCTAAAGGAGCAG	CAGACTGGCATCAGGACAAA	151	105.7	84
PFN1	GACTGCCAAGACGCTAGTCC	ATGTGTGTGGGAAGGAGAGG	173	91.0	87
PHGDH	AGGAAAAGCTGCAGATTCCA	CTCCGTCTTGCAGGATCTTC	114	99.2	84
PRDX1	ATCTCATTCAGGGGCCTTTT	GTCTCAGCGTCTCATCCACA	103	91.5	82
SEPT7	ATTGCTCCTTCAGGACATGG	AACTGTTGGCATTCCTCTGG	128	96.6	80.5
STRA8	CTCGGAGGAGAGGAAGACCT	GTCCAGGAAACTTGCCACAT	202	111.2	85
THY1	CACCTCTGCCAATACCACCT	ATACCCCTCCATCCTTCCAC	196	96.3	85
TKTL1	TGTTGATGTGGCAACTGGAT	AGATGGCCACAAGATTGTCC	194	102.5	87.5
UCHL1	CCCCTGAAGACAGAGCAAAG	CCGACATTGGCCTTCCTG	86	102.62	84
ZBTB16	GGATGAAGACGTACGGGTGT	CCCACACAGCAGACAGAAGA	206	117.5	82.5

2.2.6. Statistical Analysis

C_t values from qRT-PCR were converted into expression data (fold change relative to the means of reference genes) using the Excel add-in Genex (Bio-Rad, USA). Statistical and correlation analyses of expression data were performed using GraphPad Prism software (Graphpad Software, USA). A one-way ANOVA with Tukey's multiple comparison test was used to determine if statistically significant differences existed between group means. Differences of $P < 0.05$ were considered to be significant. Results are presented as means ±SEM.

2.3. Results

2.3.1. Generation of SSC enriched and depleted testis cell populations

Enrichment for undifferentiated spermatogonia in samples prepared by differential plating and MACS was confirmed by staining cell smears to test for DBA binding activity. Cell counts of 400 cells from each cell smear showed a significant increase in DBA^+ cells in both the supernatant (41.05 ± 7.71%) and MACS(+) (66.49 ± 13.72%) fractions when compared to the initial isolate (Figure 2.1). The percentage of DBA^+ cells was significantly decreased in the attached and MACS(-) fractions compared to the supernatant and MACS(+) treatment groups.

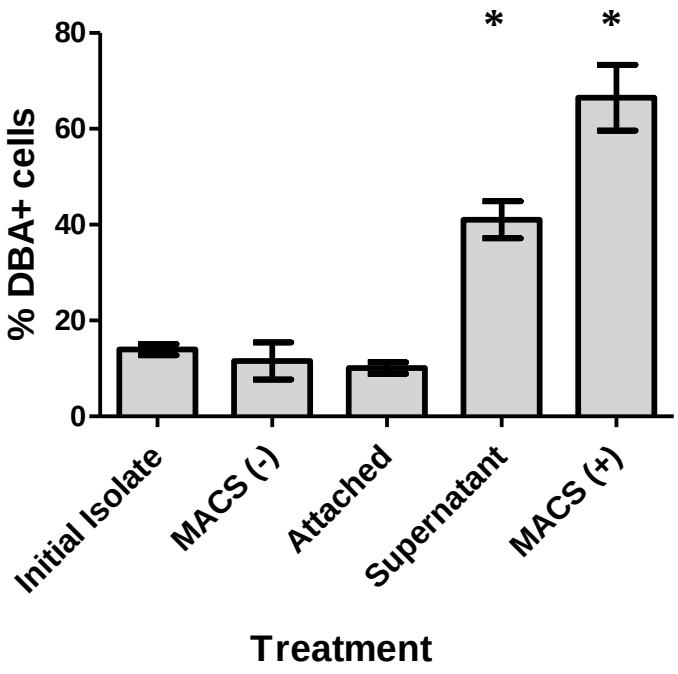

Figure 2-1: Percentage of DBA positive cells by treatment group. * indicates significant difference between group means when compared to the Initial Isolate, P < 0.01, n = 4 biological repeats, 400 cells scored per repeat.

2.3.2. Expression of established bovine spermatogonial markers *DDX4, ZBTB16* and *UCHL1*

Expression levels of the established spermatogonial markers *DDX4*, *ZBTB16* and *UCHL1* were compared across 5 different treatment groups: MACS(–) (SSC depleted), differential plating attached faction (SSC depleted), initial cell isolate (no enrichment/depletion), differential plating supernatant faction (SSC enriched) and MACS(+) (SSC enriched). Expression of the Sertoli cell marker *GATA4* was also compared across samples.

Expression of *DDX4* was significantly increased in both the supernatant and MACS(+) fractions when compared to the initial isolate (Figure 2.2 A). There was no

significant difference in expression between the MACS(-), attached and initial isolate fractions. *ZBTB16* showed a similar expression pattern, with significantly increased expression in the supernatant and MACS(+) fractions (Figure 2.2 B). *ZBTB16* expression was also significantly lower in the MACS(-) fraction, however there was no difference between the attached and initial isolate samples. The expression data for *UCHL1* followed a similar trend, with expression significantly increased in both the supernatant and MACS(+) fractions (Figure 2.2 C). *UCHL1* expression was also significantly decreased in the MACS(-) samples when compared to the initial isolate. No change in expression of *UCHL1* was detected between the initial isolate and attached fractions.

Expression of *GATA4* was significantly lower in the supernatant and MACS(+) fractions compared to the initial isolate (Figure 2.2 D). *GATA4* expression was significantly decreased in both SSC enriched populations (MACS(+) and Supernatant) when compared to the SSC depleted populations (MACS(-) and Attached).

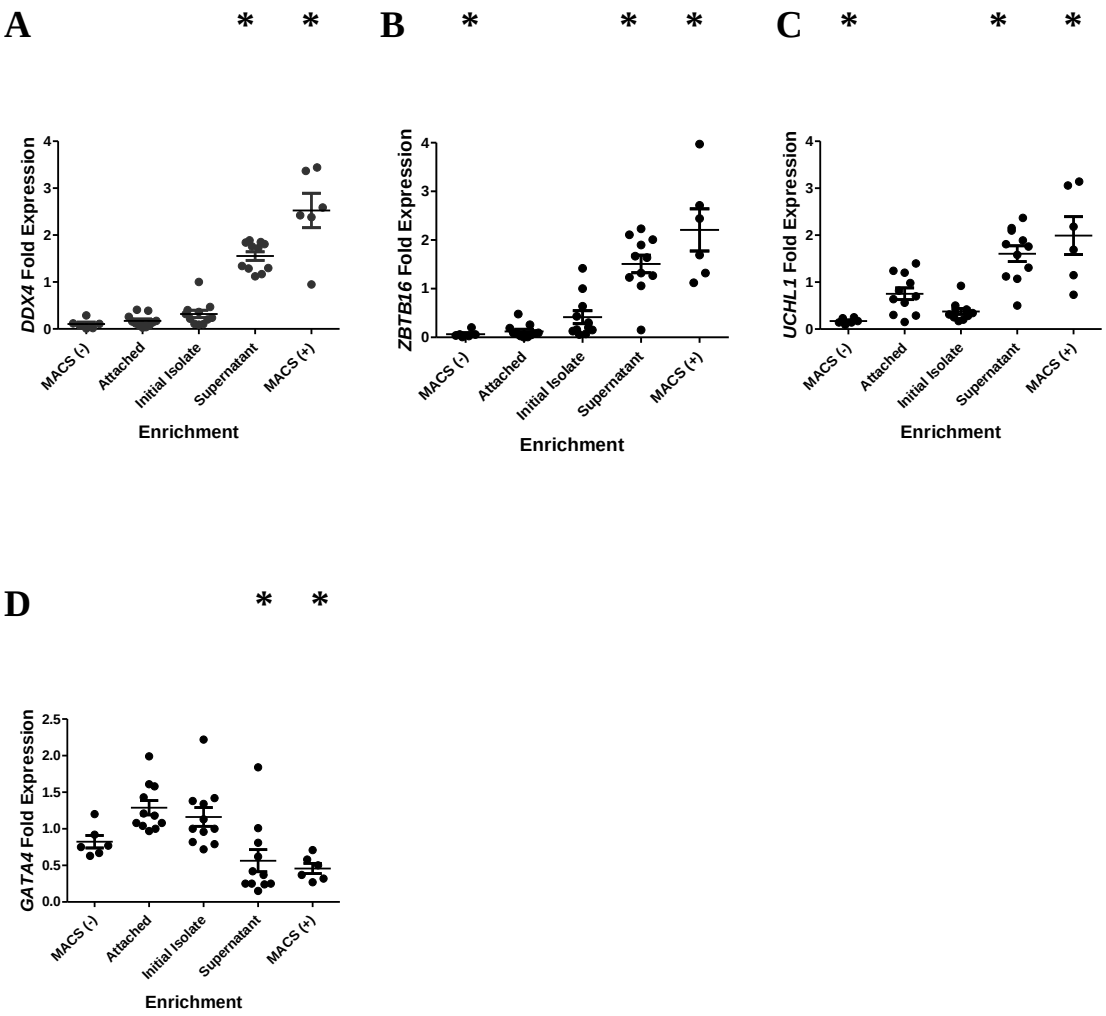

Figure 2-2: Expression of *DDX4* (A), *ZBTB16* (B), *UCHL1* (C) and *GATA4* (D) in SSC enriched and depleted testis cell samples. * indicates a significant difference in group means when compared to the Initial Isolate, P < 0.01. Fold expression indicates fold change over means of two reference genes. Each data point represents data from an individual animal n = 12 biological replicates.

2.3.3. Expression of *STRA8, GFRA1, KIT* and *THY1* in the bovine testis

Expression levels of *STRA8*, *GFRA1*, *KIT* and *THY1* were compared across the different treatment groups using qRT-PCR. The expression data for *STRA8*, *GFRA1* and *KIT* followed a similar trend to that of *DDX4*, *ZBTB16* and *UCHL1*. For each gene, expression was significantly increased in both the supernatant and MACS(+) fractions compared to the initial isolate and the SSC depleted populations (Figure 2.3). *GFRA1* expression was also

significantly decreased in the MACS(–) samples when compared to the initial isolate. No change in gene expression was detected between the initial isolate and attached fractions for any of these three genes. Expression of *THY1* did not significantly differ across any of the treatment groups (Figure 2.3D).

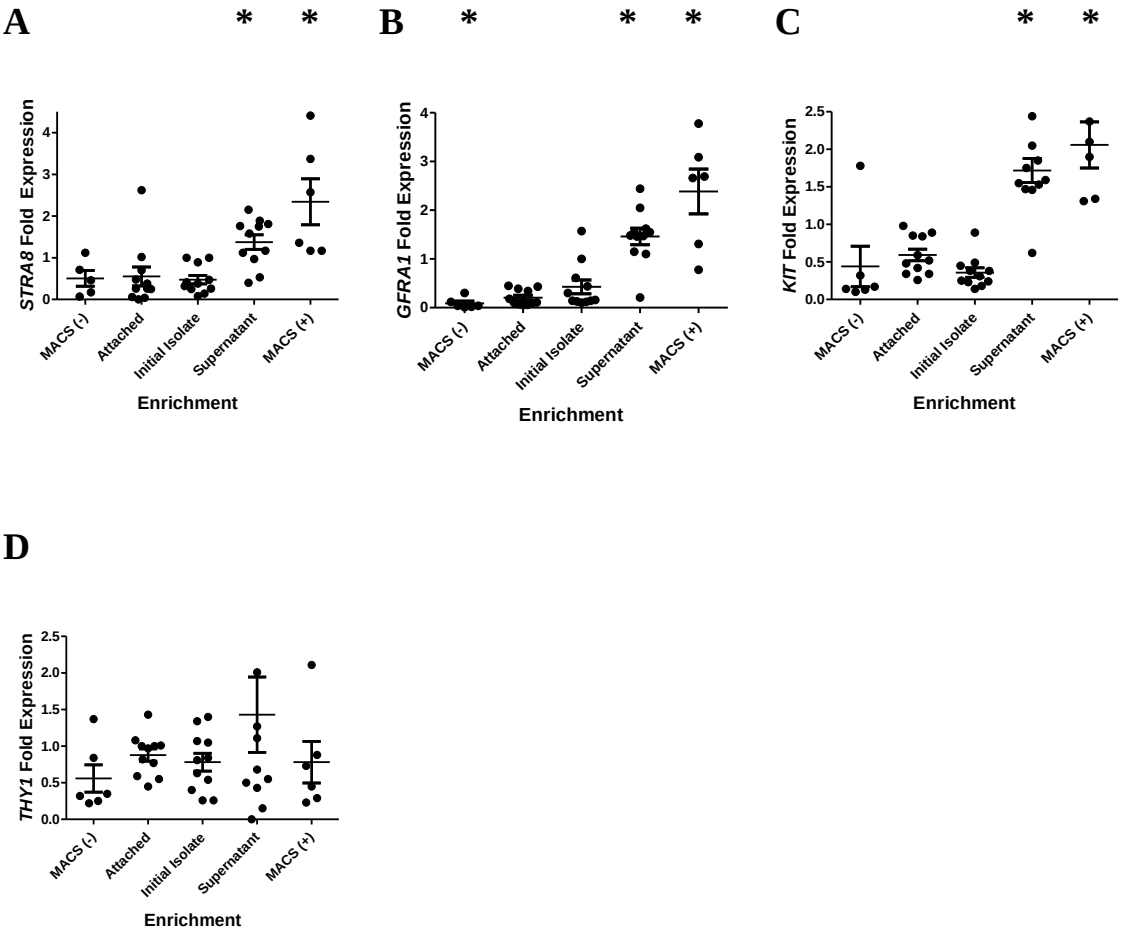

Figure 2-3: Expression of *STRA8* (A), *GFRA1* (B), *KIT* (C) and *THY1* (D) in SSC enriched and depleted testis cell samples. * indicates a significant difference in group means when compared to the Initial Isolate, P < 0.01. Fold expression indicates fold change over means of two reference genes. Each data point represents data from an individual animal, n = 12 biological replicates.

2.3.4. qRT-PCR analysis of candidate SSC marker genes

Expression levels of fourteen putative candidate marker genes were compared across the five different treatment groups. The genes examined were *ASB9, ATIC, BCL6B, CLDN8,*

CSF1R, DDX6, MTHFD1, NAP1L4, PARK7, PFN1, PHGDH, PRDX1, SEPT7 and *TKTL1* (Table 2.2).

CLDN8, BCL6B, PARK7, DDX6, NAP1L4 and *TKTL1* all showed a similar expression profile to that of established SSC markers. *CLDN8, BCL6B* and *PARK7* expression was significantly increased in both the supernatant and MACS(+) fractions compared to the initial isolate and SSC depleted fractions (Figure 2.4). No significant difference was seen between the MACS(-), attached and initial isolate fractions for any of these genes. Expression levels of *DDX6, NAP1L4* and *TKTL1* were all significantly increased in the supernatant fraction when compared to the initial isolate and attached fractions. Expression levels of these genes were not examined in the MACS(-) or MACS(+) populations.

No significant difference in gene expression levels was detected across treatment groups with regards to *ASB9, PFN1, PRDX1, MTHFD1, PHGDH* or *ATIC*. Expression of *CSF1R* was significantly increased in the supernatant fraction compared to the initial isolate; however, no other differences were detected across treatment groups. *SEPT7* expression was increased in the supernatant when compared to the initial isolate; however, there was no significant difference in expression between the supernatant and attached treatment groups.

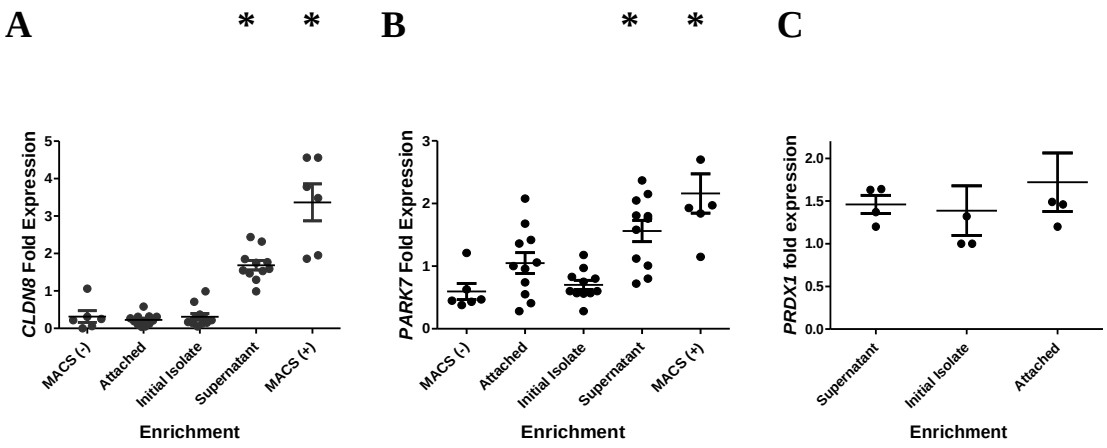

Figure 2-4: Expression patterns of candidate markers in SSC enriched and depleted testis cell samples. ***CLDN8*** **(A) and** ***PARK7*** **(B) are examples of candidates showing a similar expression pattern to that of established markers.** ***PRDX1*** **(C) is an example of a candidate that does not show any significant difference in expression across treatment groups. * indicates a significant difference in group means when compared to the Initial Isolate, P < 0.01 . Fold expression indicates fold change over means of two reference genes. Each data point represents data from an individual animal. n = 12 biological replicates for (A) and (B) and 4 biological replicates for (C).**

Table 2-2: Summary of variation in gene expression of candidate genes in SSC enriched and depleted testis cell samples. II: initial isolate; M(-): MACS(-); Att: attached; SN: supernatant; M(+): MACS(+). * indicates a significant difference in group means, ns indicates no significant difference in group means, - indicates no data is available.

GENE	II v M(-)	II v Att	II v SN	II v M(+)	M(-) v Att	M(-) v SN	M(-) v M(+)	Att v SN	Att v M(+)	SN v M(+)
ASB9	-	ns	ns	-	-	-	-	ns	-	-
ATIC	-	ns	ns	-	-	-	-	ns	-	-
BCL6B	ns	ns	*	*	ns	*	*	*	ns	ns
CLDN8	ns	ns	*	*	ns	*	*	*	*	*
CSF1R	ns	ns	*	ns	ns	ns	ns	*	ns	ns
DDX6	-	ns	*	-	-	-	-	*	-	-
MTHFD1	-	ns	ns	-	-	-	-	ns	-	-
NAP1L4	-	ns	*	-	-	-	-	*	-	-
PARK7	ns	ns	*	*	ns	*	*	ns	*	ns
PFN1	-	ns	ns	-	-	-	-	ns	-	-
PHGDH	-	ns	ns	-	-	-	-	ns	-	-
PRDX1	-	ns	ns	-	-	-	-	ns	-	-
SEPT7	-	ns	*	-	-	-	-	ns	-	-
TKTL1	-	ns	*	-	-	-	-	*	-	-

2.3.5. Correlation of novel candidate marker gene expression with established marker gene expression

Of the candidate spermatogonial markers tested, six (*CLDN8, BCL6B, PARK7, DDX6, NAP1L4* and *TKTL1*) showed expression patterns similar to that of established marker genes, with expression significantly increased in the SSC enriched treatment groups. Expression of these putative markers was correlated with expression of the established bovine spermatogonial markers *DDX4,UCHL1* and *ZBTB16,* and the Sertoli cell marker *GATA4* (Table 2.3). *CLDN8* expression showed a strong positive correlation with *DDX4* expression ($R^2 = 0.82$), and a positive correlation with both *UCHL1* and *ZBTB16* expression ($R^2 = 0.56$ and $R^2 = 0.44$ respectively; Figure 2.5). In contrast, *CLDN8* expression showed a weak negative correlation with expression of the Sertoli cell marker *GATA4* ($R^2 = 0.32$). *PARK7*

expression also showed a moderate positive correlation with *DDX4* ($R^2 = 0.62$), *UCHL1* ($R^2 = 0.56$) and *ZBTB16* ($R^2 = 0.48$); however, there was no significant correlation with *GATA4* expression. *BCL6B* showed only a weak correlation with *DDX4*, *UCHL1* and *ZBTB16* expression, and no significant correlation with *GATA4* expression. *DDX6* and *NAP1L4* both showed a moderate positive correlation with both *DDX4* and *ZBTB16* expression, and NAP1L4 also showed a moderate positive correlation with *UCHL1* expression. In addition, expression of these two genes also showed a moderate negative correlation with expression of the Sertoli cell marker *GATA4*. *TKTL1* showed only a weak correlation with *ZBTB16* expression, but no significant correlation with *DDX4*, *UCHL1* or *GATA4*. In summary, *CLDN8*, *DDX6* and *NAP1L4* showed the strongest correlation with expression of established markers of bovine spermatogonia.

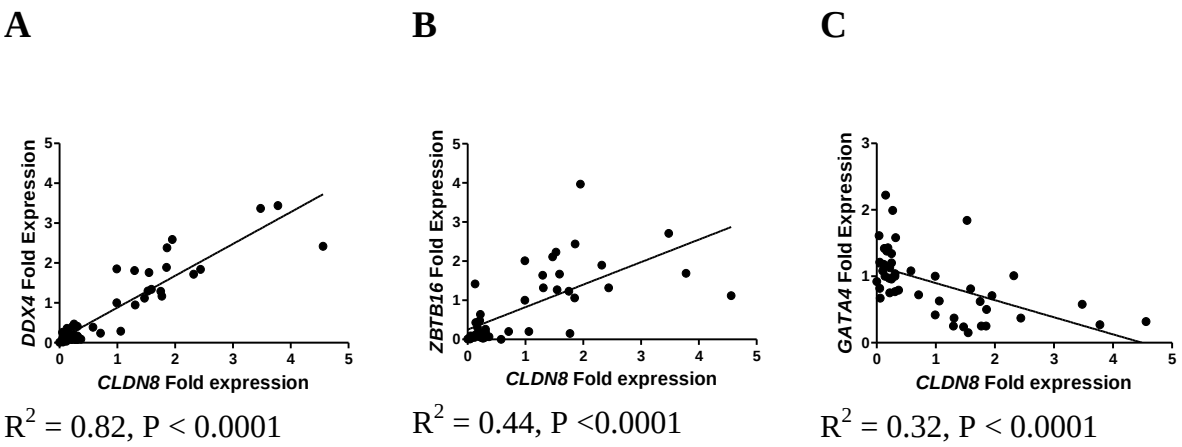

Figure 2-5: Correlation of expression of *CLDN8* with expression of *DDX4* (A), *ZBTB16* (B) and *GATA4* (C). Fold expression indicates fold change over means of two reference genes. Each data point represents data from an individual animal, n = 12 biological replicates.

Table 2-3: Summary of correlation data of 6 candidates (*CLDN8, PARK7, BCL6B, DDX6, NAP1L4* and *TKTL1*) when correlated with expression of *DDX4, ZBTB16* and *GATA4*

Candidate	Correlated with:	R^2 Value	P-value
BCL6B	*DDX4*	0.33	<0.0001
	UCHL1	0.24	0.001
	ZBTB16	0.35	<0.0001
	GATA4	-0.02	0.30
CLDN8	*DDX4*	0.44	<0.0001
	UCHL1	0.56	<0.0001
	ZBTB16	0.82	<0.0001
	GATA4	-0.32	<0.0001
DDX6	*DDX4*	0.58	0.003
	UCHL1	0.21	0.13
	ZBTB16	0.58	0.003
	GATA4	-0.45	0.02
NAP1L4	*DDX4*	0.46	0.01
	UCHL1	0.40	0.03
	ZBTB16	0.67	0.001
	GATA4	-0.59	0.003
PARK7	*DDX4*	0.48	<0.0001
	UCHL1	0.56	<0.0001
	ZBTB16	0.62	<0.0001
	GATA4	-0.04	0.17
TKTL1	*DDX4*	0.33	0.04
	UCHL1	0.06	0.45
	ZBTB16	0.29	0.07
	GATA4	-0.22	0.12

2.4. Discussion

The specific identification of SSCs within heterogeneous spermatogonial cell populations will aid in enhancing advanced reproductive technologies such as testis cell transplantation. Use of molecular markers, particularly cell surface markers, to identify and purify SSC populations is critical to the success of such technologies. Previous studies have identified a number of markers for various subsets of spermatogonia in different species. In

this chapter it was investigated whether such markers could be used to identify undifferentiated spermatogonia in bulls. In addition, a number of putative SSC marker candidates were evaluated to determine whether they may be used as markers to identify and potentially purify spermatogonia from the bovine testis.

DBA binding activity has been demonstrated to be a general marker for undifferentiated spermatogonia in the bovine, and DBA binding has previously been used to enrich SSCs from testis cell populations (Herrid et al., 2009; Izadyar et al., 2003). The results presented confirmed that selection of testis cells by differential plating and MACS yielded cell populations enriched for DBA^+ cells. DDX4, ZBTB16 and UCHL1 have been identified as markers of type A spermatogonia in a number of species including the bovine. The results presented here show that increased expression of *DDX4*, *ZBTB16* and *UCHL1* was positively correlated with enrichment of DBA^+ SSCs, and negatively correlated with DBA^- testis cell populations. In addition, there was a decrease in expression of the Sertoli cell marker *GATA4* (Imai et al., 2004) in the SSC enriched samples, indicating a depletion of Sertoli cell numbers in these cell populations. The increase of DBA^+ cells, together with the increased expression of early spermatogonial markers and decrease in Sertoli cell markers, indicate that the methods described in this chapter can be used to effectively generate cell populations enriched for undifferentiated spermatogonia, including SSCs, from the bovine testis. The ability to prepare these enriched cell populations provided a platform for screening novel spermatogonial marker candidates by comparing gene expression levels between SSC enriched and depleted samples. A putative SSC marker would be expected to show a similar gene expression pattern to that shown by established SSC markers, and would also be expected to show a strong correlation with expression of established marker genes. The methods described provide an initial screening platform for testing candidates to determine whether they are more highly expressed in SSC enriched cell populations when compared to

SSC depleted or unenriched cell populations. This screening method is based upon mRNA levels, which may not be a true representation of protein levels, thus any putative markers identified using these methods are required to undergo further analysis. Techniques such as immunocytochemical analysis, and *in-situ* hybridisation studies, could provide a better understanding of the type of cells expressing these putative markers, and aid in determining whether such markers are specific for SSCs or a particular sub-population of bovine spermatogonia. Finally, functional analysis of the cells expressing these candidate markers is required before these markers can be used to isolate, identify and manipulate SSCs *in vitro*.

GFRA1, STRA8, KIT and THY1 have been shown to be molecular markers of different subsets of spermatogonia in rodents and humans. Expression of these molecules has yet to be fully explored in the bovine testis. Based on the evidence gathered from mice, KIT has been used to identify more differentiated spermatogonia in the bovine testis (Izadyar et al., 2003; Izadyar et al., 2002). THY1 has recently been demonstrated to be a conserved marker for a subset of undifferentiated spermatogonia in the bovine testis (Reding et al., 2010). The role and expression patterns of GFRA1 and STRA8 in the bovine testis have yet to be determined. The results of this chapter demonstrated that *GFRA1, STRA8* and *KIT* were expressed by cells in the bovine testis, and expression of all three increased with increasing enrichment for undifferentiated spermatogonia. Further investigation is required to more accurately define the cell population expressing each of these markers. However, taken together with information gathered from other species, the results presented here strongly suggest that GFRA1, STRA8 and KIT are conserved markers for undifferentiated spermatogonia in the bovine and other species. These genes may be used as effective markers for studying undifferentiated spermatogonia in the bovine testis.

Interestingly, THY1, recently demonstrated to be a conserved marker of undifferentiated spermatogonia in the bovine testis (Reding et al., 2010), did not show an

expression pattern consistent with other established molecular markers. Selection of $THY1^+$ cells in rodents and non-human primates has been shown to result in highly enriched SSC populations. Reding et al. (2010) showed that THY1 is expressed by a rare sub-population of bovine spermatogonia, and selection for $THY1^+$ cells resulted in a cell population that was also enriched for $ZBTB16^+$ cells. In contrast, the data presented here demonstrated that, although THY1 is expressed in the bovine testis, there was no significant difference in *THY1* expression levels between SSC enriched and depleted populations. These contrasting results may be explained by the methods used in each study to select and analyse testis cell populations. Reding et al. (2010) selected for $THY1^+$ cells, and analysed this cell population to determine whether the $THY1^+$ cells also expressed other spermatogonial markers. They found that the $THY1^+$ cell population accounted for only approximately 0.05% of the total testis cell population. In the present study SSC enriched and depleted testis cell populations were prepared by differential plating or MACS, and the expression of a number of genes was compared across these different populations. Thus the SSC enriched populations used here were heterogenous rather than pure populations, containing higher numbers of spermatogonia than unenriched testis cell populations. The proportion of undifferentiated spermatogonia in the most highly enriched samples, as indicated by DBA binding activity, was 66.49 ± 13.72%. It is possible that this level of enrichment was insufficient to detect differences in expression levels of a gene that is expressed by such a small population of cells in the testis. It would be interesting to be able to examine THY1 expression in a highly SSC enriched and less heterogeneous cell population. However, at this time methods for generating more highly purified populations of bovine spermatogonia have not been established.

The expression of a number of potential markers of bovine spermatogonia was examined by comparing the expression patterns of these candidates with those of established marker genes. These candidates were identified from proteomics screening which identified

proteins that were more abundant in SSC enriched testis cell populations when compared to SSC depleted populations (Colgrave et al., submitted). Of the genes tested, six candidates, *CLDN8, PARK7, BCL6B, DDX6, NAP1L4* and *TKTL1,* showed expression patterns consistent with those of established markers of undifferentiated bovine spermatogonia. Of these candidates *CLDN8, DDX6* and *NAP1L4* also showed a significant positive correlation with expression of *DDX4* and *ZBTB16*. These three candidates also showed a significant negative correlation with expression of the Sertoli cell marker *GATA4*. The expression patterns, taken together with the strong correlations with established spermatogonial markers, indicate that these three genes are strong candidates for further analysis. CLDN8 has been reported to be expressed in both human (Dube, Chan, Hermo, & Cyr, 2007) and rat (Gregory & Cyr, 2006) epididymis, although its role is better understood in renal tissue where it acts as a tight junction protein regulating paracellular ion permeability (Amasheh et al., 2009; Angelow, Schneeberger, & Yu, 2007). Expression of a number of claudin genes has been described in testis tissue where they have been shown to be important in the formation of the blood-testis barrier (Gye, 2003; Morrow, Mruk, Cheng, & Hess, 2010). At present CLDN8 expression has not been reported in the bovine testis. DDX6 has been shown to be expressed in both the testis and ovary in mice, where it appears to play an essential role in gametogenesis (Matsumoto, Kwon, Kim, & Akao, 2005). The DDX6 ortholog, Ste13, is essential for sexual reproduction in yeast (Maekawa, Nakagawa, Uno, Kitamura, & Shimoda, 1994), and the *C. elegans* ortholog, CGH-1, is required for gametogenesis (Matsumoto et al., 2005). A role for DDX6 in the bovine testis is yet to be determined. NAP1L4, a nucleosome assembly protein, has been shown to be expressed ubiquitously in both humans (Hu, Lee, Johnson, & Feinberg, 1996) and pigs (Li et al., 2012), although in humans it is expressed at higher levels in the testis than in other tissues (Hu et al., 1996). NAP1L4 is involved in the incorporation of H3t, a testis-specific H3 variant, into nucleosomes in the human testis (Tachiwana, Osakabe,

Kimura, & Kurumizaka, 2008). It has been proposed that NAP1L4 may have a specific function in chromatin modification during meiosis in human male germ cells (Tachiwana et al., 2008). It is possible that NAP1L4 would have a similar function in bovine germ cells. Of the putative marker genes identified in this chapter, CLDN8 is of particular interest for further analysis as the CLDN8 protein is expressed on the cell surface. The identification of a cell surface marker for spermatogonia is of particular interest to enable live cell selection using techniques such as MACS and FACS.

At present no cellular markers have been identified that are expressed specifically by SSCs and not by other subsets of spermatogonia. Until such a marker is identified, the generation and identification of pure SSC populations will remain difficult if not impossible. The most effective methods to generate SSC enriched cell populations use a combination of positive and negative selection for a number of spermatogonial markers (Shinohara et al., 1999). It appears that such a strategy may also pose the best chance of generating a more highly purified population of bovine spermatogonia. At present the only SSC enriched cell populations reported in the bovine have been generated using only a single cellular marker, or alternative methods of purification such as percoll gradiants (Herrid et al., 2009; Izadyar et al., 2002). This study has identified a number of spermatogonial markers that are conserved in the bovine testis. Although none of these markers are specific for SSCs, when used in conjunction they may be able to separate a more highly enriched SSC population than when used individually. Selection of THY1/GFRA1^{+}, KIT^{-} cells, for example, would possibly generate a cell population highly enriched for early undifferentiated spermatogonia. Additional markers, such as the putative markers identified in this study, may be used to enhance the purification process by providing additional selection and/or identification tools.

2.5. Conclusion

The identification and purification of bovine spermatogonial stem cells will aid in the development of reproductive technologies such as testis cell transplantation. This chapter has described a platform for screening of candidate spermatogonial markers by analysing gene expression levels between SSC enriched and depleted cell populations. A number of spermatogonial markers identified in other species, such as STRA8, KIT and GRFA1 appear to be conserved markers for bovine spermatogonia. CLDN8, DDX6 and NAP1L4 have been identified as putative markers of spermatogonia in the bovine testis. Further analysis of these putative markers is required to determine whether they may be used to enhance the purification of spermatogonia cell populations, either alone or in combination with other cellular markers. Further analysis is also required to determine whether these markers can be used to identify germline cells derived from pluripotent stem cells *in vitro*. CLDN8 is of particular interest for further study given that it is expressed on the cell surface, and may enable physical selection of SSCs from heterogeneous populations.

Chapter 3: CLDN8 expression in the bovine testis

3.1. Introduction

Spermatogonial stem cell (SSC) transplantation has been successful in a variety of species, including mice, goats, pigs and cattle (Brinster & Zimmermann, 1994; Honaramooz et al., 2003; Honaramooz et al., 2002; Izadyar et al., 2003), and could be applied in livestock industries, such as beef cattle production, as an alternative to artificial insemination (Herrid et al., 2006; Hill & Dobrinski, 2006). The efficiency and success of SSC transplantation experiments in cattle could be improved by increasing our knowledge of bovine SSCs and their stem cell niche.

Chapter 2 described a qRT-PCR screening platform designed to aid in the identification of molecular markers of bovine SSCs. Such markers could be used for the identification of SSCs isolated from the testis, or SSCs generated *in vitro* by the differentiation of pluripotent stem cells. Surface markers for bovine SSCs may also be used to purify SSCs from heterogenous cell populations. The methods described in chapter 2 were used to identify a number of putative markers of bovine SSCs. The identification of a cell surface marker is of particular interest for enabling physical selection of cells via MACS or FACS. Of the putative markers identified in chapter 2, only CLDN8 is expressed on the cell surface. CLDN8 was therefore selected as the best candidate for further analysis, with no further analysis of other putative markers being undertaken. The methods described in chapter 2 were sufficient to identify CLDN8 as a potential SSC marker; however, further investigation is required in order to define the cell type/s expressing CLDN8 in the bovine testis.

Adhesion molecules are highly expressed on both adult and embryonic stem cells, although the function of these molecules in the stem cell niche is not clearly understood (Marthiens et al., 2010; Raymond et al., 2009). They are thought to be vital to retaining stem cells in the niche, as well as regulating which cells leave the niche (e.g. differentiating cells) (Ellis & Tanentzapf, 2010; Marthiens et al., 2010) and there is evidence to suggest that adhesion molecules, such as integrins, may play a role in regulating cell division (Ellis & Tanentzapf, 2010; Marthiens et al., 2010).

The ability of SSCs to maintain spermatogenesis is dependent on the surrounding microenvironment known as the stem cell niche. Sertoli cells have been demonstrated to be one of the main components of the spermatogonial stem cell niche (Oatley et al., 2010). Sertoli cells and SSCs have been shown to express a variety of adhesion molecules including integrins and E-cadherin (de Rooij et al., 2008). The expression of adhesion molecules by SSCs and Sertoli cells has been shown to be important for the correct homing of SSCs to the basal membrane following transplantation (Kanatsu-Shinohara et al., 2008). A deficiency of β1-integrin results in SSCs that fail to complete spermatogenesis, suggesting that β1-integrin has a role in the regulation of spermatogenesis in addition to its role in the migration of SSCs to the basal membrane (Kanatsu-Shinohara et al., 2008). At this time little else is known about the role adhesion molecules play in the spermatogonial stem cell niche; however, these molecules may have essential roles in regulating other aspects of stem cell–niche interactions,

The claudin family of proteins are involved in the formation of cell to cell tight junctions and the regulation of paracellular transport (Koval, 2006). CLDN8 has been reported to be expressed in both human (Dube et al., 2007) and rat (Gregory & Cyr, 2006) epididymis, although its role is better understood in renal tissue where it acts as a tight junction protein regulating paracellular ion permeability (Amasheh et al., 2009; Angelow et

al., 2007). Expression of a number of claudin genes has been described in testis tissue where they have been shown to be important in the formation of the blood-testis barrier (Gow et al., 1999; Gye, 2003; Morrow et al., 2010; Pelletier, 2011). Further studies have produced evidence that Claudins are expressed outside of tight junctions (Gregory, Dufresne, Hermo, & Cyr, 2001; Kiuchi-Saishin et al., 2002; Morrow et al., 2009; Rahner, Mitic, & Anderson, 2001) and it has been suggested that claudins may have other functions, including the ability to act as cell adhesion molecules (Gregory et al., 2001; Morrow et al., 2009).

It is hypothesised that CLDN8 is expressed by cells in the bovine testis and may be a potential surface marker for type A spermatogonia. In order to test this hypothesis, levels of *CLDN8* expression were compared in SSC enriched and depleted cell populations. Immunocytochemical and flow cytometric analyses were then used to further characterise the sub-population of testis cells expressing the CLDN8 protein.

3.2. Materials and Methods

3.2.1. Collection of testis samples

Animal experiments were approved and conducted under the guidelines of the F.D. McMaster Animal Ethics Committee, CSIRO Livestock Industries (Approval no. 10/14). Pre-pubescent Angus bull calves aged between 4 and 8 months were castrated under general anaesthesia. Testes were collected and kept on ice until dissection. The tunica albuginea and epididymis were removed prior to enzymatic digestion of testis tissue.

3.2.2. Enzymatic isolation of testis cells and SSC enrichment

Two-step enzymatic digestion of testis tissue was carried out as described previously (chapter 2, pg 46). Testis cell populations were enriched for SSCs by differential plating or MACS as described previously (chapter 2, pg 46-47). Cell smears were prepared from all cell

samples. The remaining cells were either snap frozen in liquid nitrogen for RNA isolation, or processed immediately for intracellular flow cytometric analysis.

3.2.3. Quantitative Reverse Transcribed Polymerase Chain Reaction (qRT-PCR)

qRT-PCR reactions were carried out as described previously (chapter 2, page 50). Bovine specific primers for the genes of interest were designed with Primer3 (http://frodo.wi.mit.edu/) using bovine sequences in the NCBI Genbank databases. The sequences of primers used in this chapter are given in Table 3.1. Appropriate reference genes (*ATP5G2* (*ATPsynth)* and *RPS26*) were determined using the sheep GeNorm kit (PrimerDesign, UK). Total RNA was extracted from frozen cell samples using an RNeasy Midi kit (Qiagen, USA). cDNA was synthesised using a Superscript III first strand synthesis kit (Invitrogen, USA). qRT-PCR reactions were carried out in triplicate in an 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 cDNA transcribed from 10 ng RNA.

Table 3-1: Sequences of primer sets used in Chapter 3.

Gene	Forward Primer (5'-3')	Reverse Primer (5'–3')	Amplicon size (BP)	Reaction efficiency (%)	Melt peak (^{o}C)
CLDN8	TTGCCAATTCCATCATCAGA	TTCGGCGTGATAGCTTTTCT	218	102.3	86.5
DDX4	TACGCCCAATTCGATGAAC	TTGCCACTTTTCTTTGTCAAG	225	90.51	81
GATA4	ACCAGCAGCAGTGAGGAGAT	TGGTGACTGGCTGACAGAAG	190	96.25	89
GFRA1	ATGTCCAATGACGTCTGCAA	GGAGTCCTGCAAGTTCAAGC	205	102.59	89.5
UCHL1	CCCCTGAAGACAGAGCAAAG	CCGACATTGGCCTTCCTG	86	102.62	84
ZBTB16	GGATGAAGACGTACGGGTGT	CCCACACAGCAGACAGAAGA	206	117.5	82.5

3.2.4. Immunocytochemistry

Cell smears were fixed in Modified Davidsons Fixative (MDF: 14% Ethyl Alcohol, 37.5% Formalin 37-39%, 6.25% Glacial Acetic Acid) for 2 minutes at room temperature and rinsed with Tris-buffered saline with 0.1% Tween 20 (TBS-T). Primary antibodies against CLDN8 (Santa Cruz, USA; 1:50), ZBTB16 (previously known as PLZF) (Santa Cruz, USA; 1:50) and GATA4 (Santa Cruz, USA; 1:100), as well as DBA-biotin (Vector Laboratories, USA; 1:10) were diluted in TBS containing 0.5% BSA, and incubated on smears for 30 minutes at room temperature. Mouse and rabbit IgG were included as controls. Smears were washed and incubated with Alexa-594 conjugated anti-mouse IgG (Invitrogen, USA; 1:250), Alexa-468 anti-rabbit IgG (Invitrogen, USA; 1:250) and Streptavadin Alexa-350 conjugate (Invitrogen, USA; 1:250) for 30 minutes at room temperature. Smears were washed in TBS-T and mounted with Prolong Gold (Invitrogen, USA). Smears were examined using a fluorescent microscope (Zeiss, Germany) and images captured using Axiovision software (Zeiss, Germany).

3.2.5. Flow Cytometric Assays

Freshly isolated cells were processed immediately for FACS analysis to minimise cell aggregation. Cells were fixed and permeabilized using a BD cytofix/cytoperm kit (BD Biosciences, USA), then incubated with primary antibodies against CLDN8 (Santa Cruz, USA; 1:100), ZBTB16 (Santa Cruz, USA; 1:100) and GATA4 (Santa Cruz, USA; 1:100) for 30 mins on ice. Cells were washed twice before incubation with secondary antibodies Dylight-649 conjugated anti-mouse IgG (Abcam, USA, 1:500) and R-PE conjugated anti-rabbit IgG (Invitrogen, USA; 1:1000) for 30 mins on ice. Cells were washed three times, then analysed on a BD LSR II flow cytometer (BD Biosciences, USA). Data was analysed using FCS Express software (De Novo Software, USA). Multicolour flow cytometry compensation

was performed using three colour beads (BD Biosciences, USA) and PeakFlow claret flow cytometer reference beads (Invitrogen, USA)

3.2.6. Statistical Analysis

C_t values from qRT-PCR were converted into relative expression data using the Excel add-in Genex (Bio-Rad, USA). Statistical and correlation analyses of expression and flow cytometric data were performed using GraphPad Prism software (GraphPad Software, USA). An unpaired t-test was applied to determine statistically significant differences between group means. Unpaired t-test with Welch's correction was performed to provide a valid t-test when there was unequal variance between group means. Differences of $P < 0.05$ were considered to be significant. Results are presented as means ±SEM.

3.3. Results

3.3.1. Analysis of *CLDN8* expression by qRT-PCR

Expression levels of *CLDN8* were compared across 5 different treatment groups ranked by level of SSC enrichment/depletion: MACS(–) (SSC depleted); Differential plating attached fraction (SSC depleted); Initial Isolate; Differential plating supernatant fraction (SSC enriched); MACS(+) (SSC enriched). The expression profile of *CLDN8* was compared with a panel of established molecular markers for undifferentiated spermatogonia (*ZBTB16, DDX4, GFRA1*, and *UCHL1*), and with the Sertoli cell marker *GATA4*.

Expression of the established spermatogonial markers *DDX4*, *GFRA1, UCHL1* and *ZBTB16* was significantly increased with SSC enrichment (Figure 3.1 B-E). Conversely, expression of the Sertoli cell marker *GATA4* decreased as enrichment for SSCs increased (Figure 3.1F). Expression of *CLDN8* followed a similar pattern to that of the established spermatogonial markers (Figure 3.1G). *CLDN8* expression was significantly increased in the

SSC enriched supernatant and MACS(+) cell populations. *CLDN8* expression was on average approximately 3-fold higher in the MACS(+) samples when compared to the initial isolate. There was no significant decrease in expression of *CLDN8* in the SSC depleted samples when compared to the initial isolate.

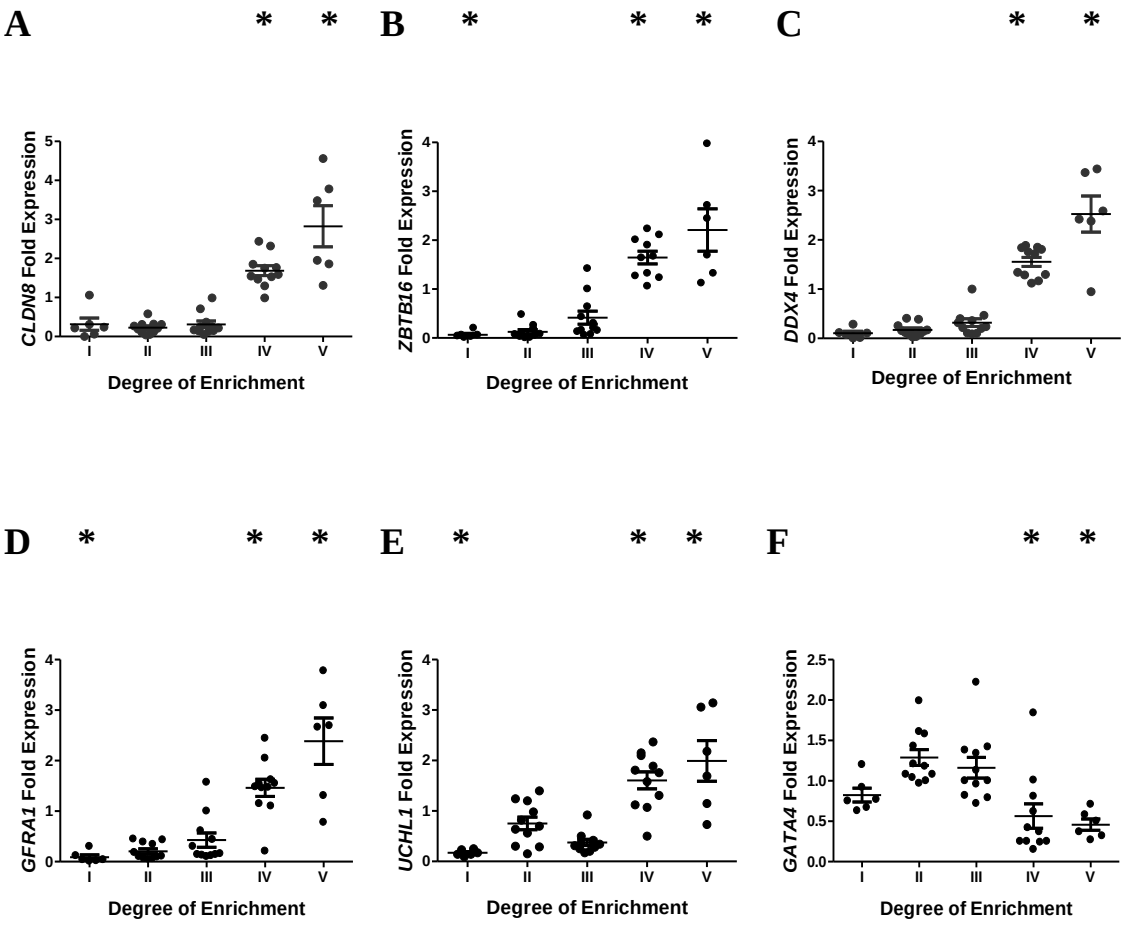

Figure 3-1: Changes in expression of *CLDN8* (A), *ZBTB16* (B), *DDX4* (C), *GFRA1* (D), *UCHL1* (E) and *GATA4* (F) in SSC depleted and enriched testis cell samples. Degree of enrichment: I = MACS(-), II = Attached, III = Initial Isolate, IV = Supernatant, V = MACS(+). * indicates a significant difference in group mean when compared to the mean of the initial isolate (P < 0.05). Fold expression indicates fold change over means of two reference genes. Each data point represents data from an individual animal,, n = 12 biological replicates

CLDN8 expression was correlated with expression of *ZBTB16, DDX4, GFRA1*, and *UCHL1*, and the Sertoli cell marker *GATA4* (Figure 3.2). *CLDN8* expression showed a strong positive correlation with *DDX4* expression (R^2 = 0.82, P <0.0001). There was also a positive correlation between *CLDN8* expression and that of other established spermatogonial markers. In contrast, *CLDN8* expression showed a weak negative correlation with expression of the Sertoli cell marker *GATA4* (R^2 = 0.3, p<0.0001).

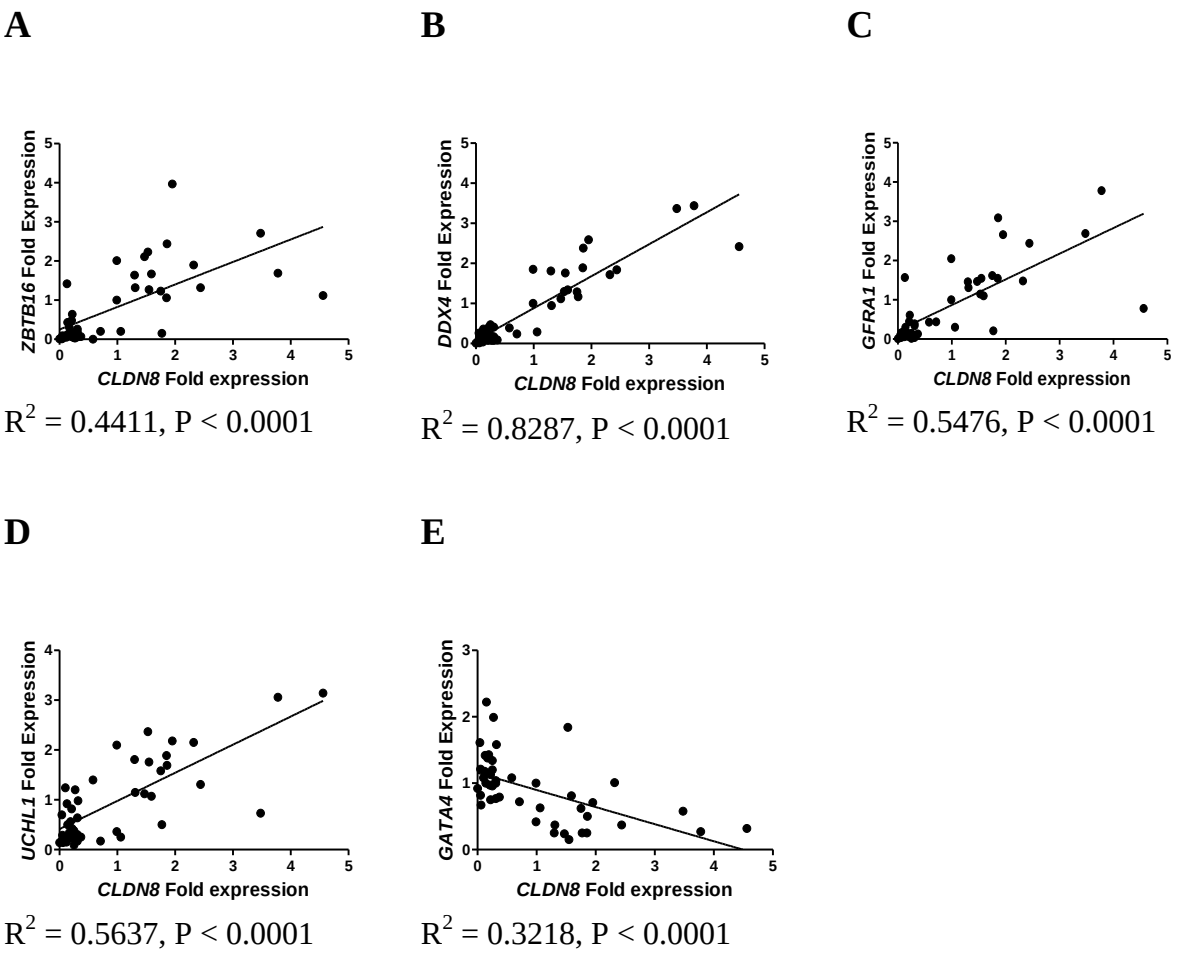

Figure 3-2: Correlation of *CLDN8* expression with expression of *ZBTB16* (A), *DDX4* (B), *GFRA1* (C), *UCHL1* (D) and *GATA4* (E) in the bovine testis. Fold expression indicates fold change over means of two reference genes. Each data point represents data from an individual animal, n = 12 biological replicates.

3.3.2. Analysis of CLDN8 expression by immunohistochemistry and flow cytometry

Cell smears from the five treatment groups were stained using DBA-FITC and antibodies against CLDN8, ZBTB16 and GATA4. Cells were identified that stained positive for DBA and ZBTB16 which were also CLDN8 positive (Figure 3.3).

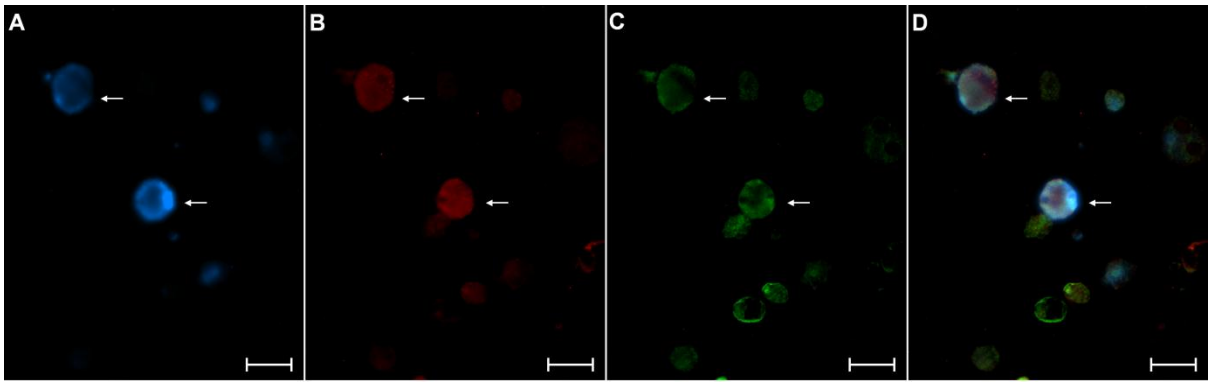

Figure 3-3: Examination of testis cells from initial isolate from pre-pubertal bull testis, stained for DBA binding activity (A), ZBTB16 (B), CLDN8 (C) and Merged image (D). Arrows indicate cells positive for DBA, ZBTB16 and CLDN8 expression. Scale bars = 50 μm.

There was also a population of cells that stained positive for CLDN8 expression but were negative for DBA and/or ZBTB16. In the MACS(+) samples, the population of CLDN8 positive cells that were DBA and ZBTB16 negative stained positive for GATA4 (Figure 3.4B). Very few $CLDN8^{+}/GATA4^{+}$ cells were observed in SSC depleted samples (Figure 3.4A). Attempts at immunostaining tissue sections with the CLDN8 antibody to determine the localisation of CLDN8 within the seminiferous tubule were unsuccessful.

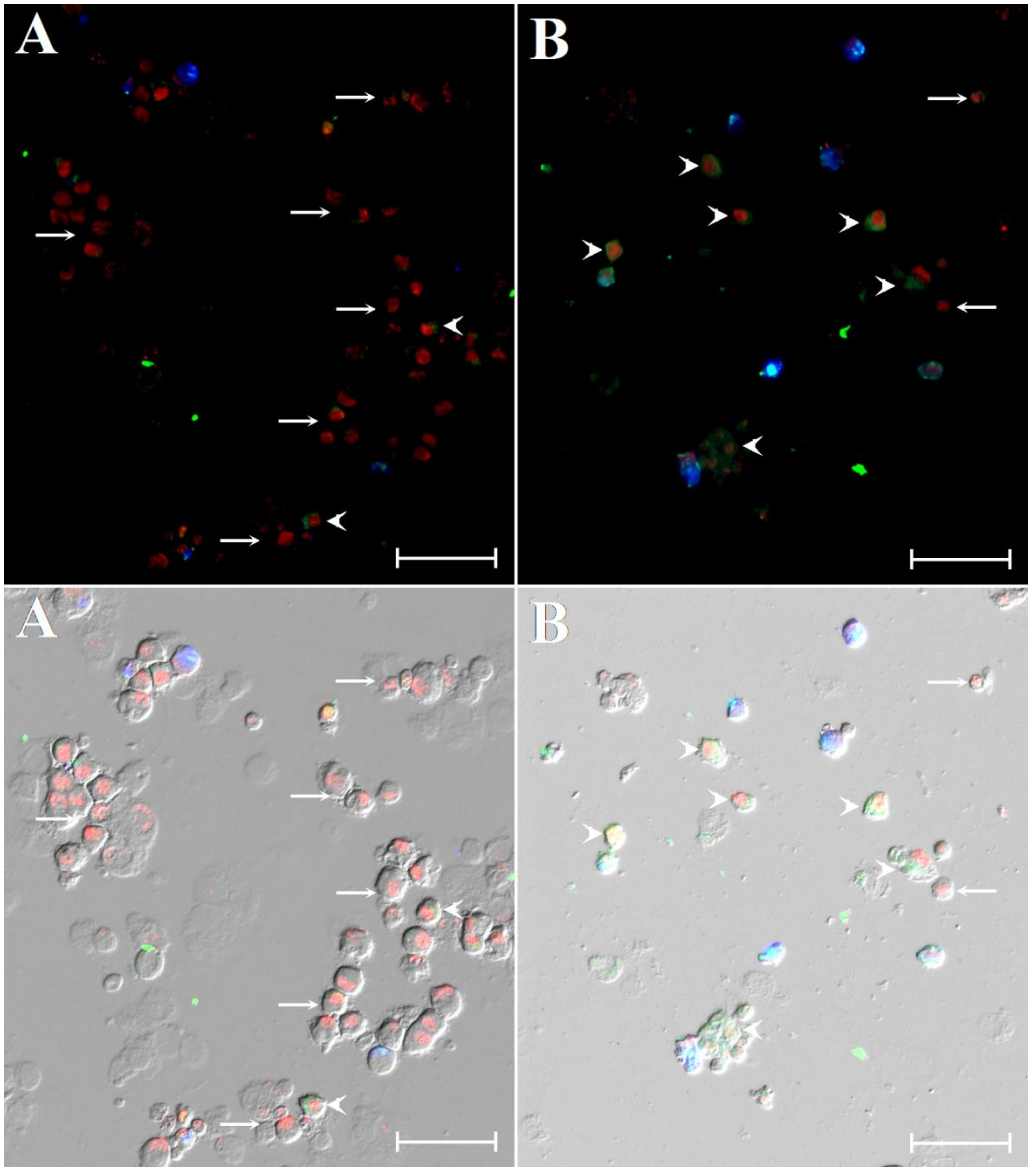

Figure 3-4: Comparison of MACS(-) (A) and MACS(+) (B) cell fractions stained for DBA (blue), CLDN8 (green) and GATA4 (red). Arrows indicate areas of GATA4+/CLDN8- cells. Arrowheads indicate GATA4+/CLDN8+ cells. Upper panel fluorescence micrographs, lower panel brightfield micrographs of corresponding fields. Scale bars = 50 μm.

FACS analysis of MACS(+) and MACS(–) cell populations confirmed that MACS selection resulted in a significant enrichment of DBA^+ cells (56.74%, ±5.36; Figure 3.5A). FACS analysis also confirmed that there was a significantly higher percentage of $CLDN8^+$ cells in the MACS(+) population compared to the initial isolate or MACS(-) population (Figure 3.5B). In agreement with observations from cell smears, a significantly higher

percentage of CLDN8$^+$ Sertoli cells were observed in MACS(+) samples compared to MACS(–) and initial isolate samples (Figure 3.5C), indicating that the majority of CLDN8$^+$ Sertoli cells co-purify with DBA$^+$ spermatogonia.

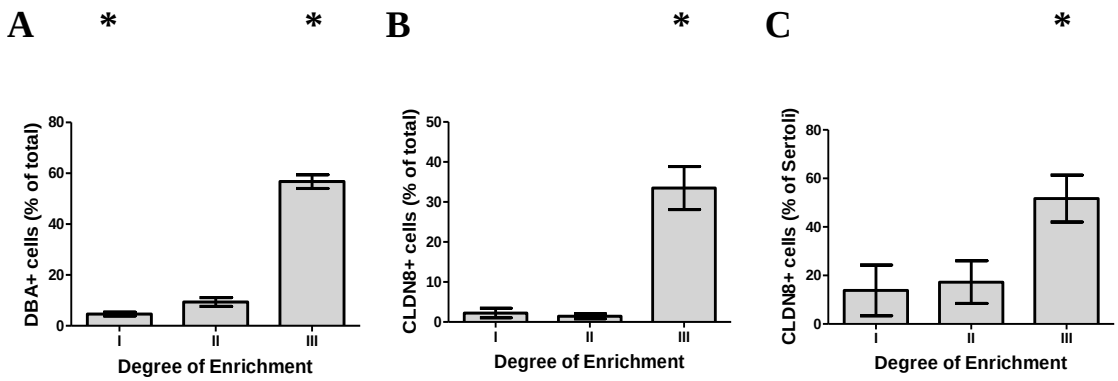

Figure 3-5: FACS analysis of MACS(-), Initial Isolate and MACS(+) cell fractions. Percentage of total cell population positive for DBA binding activity (A); Percentage of total cell population positive for CLDN8 (B); and Percentage of Sertoli cells (GATA4$^+$ cells) positive for CLDN8 (C). Degree of enrichment: I = MACS(-), II = Initial Isolate, III = MACS(+). * indicates a significant difference in group mean when compared to the initial isolate ($P < 0.05$, n = 4 biological replicates).

3.4. Discussion

CLDN8 was previously identified as a putative marker for bovine SSCs (chapter 2). CLDN8 is expressed on the cell surface, and is therefore of particular interest for enabling physical selection of SSCs via MACS or FACS. However, further analysis was required to confirm whether CLDN8 could be useful for the identification and/or purification of bovine SSCs. The experiments described in this chapter were designed to determine the cell type/s expressing CLDN8 in the bovine testis, and whether it could be used as a surface marker for undifferentiated spermatogonia.

Expression of *CLDN8* was compared in SSC enriched and depleted cell samples, using the expression of established spermatogonial markers as controls for the degree of

enrichment. The results of this chapter confirmed that *CLDN8* was expressed by cells in the bovine testis, following a similar pattern as established spermatogonial markers, with significantly higher expression in cell samples enriched for SSCs. There was a positive correlation between *CLDN8* expression and that of established spermatogonial markers, with the highest correlation between *CLDN8* and *DDX4*. *DDX4* is a highly conserved molecular marker for the germ cell lineage across species, expressed in primordial germ cells and undifferentiated spermatogonia (Noce et al., 2001; Raz, 2000). In order to further identify the cell types expressing CLDN8 in the testis, cell smears were stained with a CLDN8 specific antibody. Both DBA (Ertl & Wrobel, 1992; Herrid et al., 2007; Izadyar et al., 2002) and ZBTB16 (Reding et al., 2010) have been used previously to identify type A spermatogonia in the bovine testis. ZBTB16 has been reported to be expressed by a sub-population of bovine spermatogonia, and may be restricted to SSCs and very early spermatogonia (Reding et al., 2010). The identification of cells positive for DBA and ZBTB16 in addition to CLDN8, demonstrated that CLDN8 was expressed by at least a subset of type A bovine spermatogonia. A population of cells expressing both CLDN8 and GATA4 was also identified, indicating that CLDN8 expression is not limited to spermatogonia, and that it is also expressed by Sertoli cells.

CLDN8 was not expressed uniformly across all Sertoli cells, but instead was expressed by only a subpopulation of Sertoli cells. Interestingly, it was observed that the population of CLDN8 expressing Sertoli cells was found almost entirely within the SSC enriched MACS(+) cell fraction. SSC depleted MACS(-) samples were also CLDN8 depleted. Flow cytometric analysis of SSC enriched and depleted cell samples supported this observation. It appears that selecting for DBA positive cells using MACS also selects for $CLDN8^{+}$ /DBA^{-} Sertoli cells. A plausible explanation for this finding is the presence of claudin-mediated cell adhesion complexes between $CLDN8^{+}$ cells, allowing DBA^{-}/$CLDN8^{+}$

cells to be co-purified with $DBA^+/CLDN8^+$ cells during MACS selection. In the experiments described in this chapter it was observed that the enriched cell populations obtained using MACS tended to re-aggregate quickly after dissociation, an indication of cellular adhesiveness (Kubota et al., 1999). This may also explain the previously observed limitation of enrichment of DBA positive cells by MACS and other enrichment methods (Herrid et al., 2009). In this chapter approximately 50% of the MACS(+) population consisted of $CLDN8^+$ Sertoli cells. An interesting follow-on study would be to disrupt claudin bonds in testis cell preparations to determine whether this would facilitate selection of a more highly purified population of DBA^+ spermatogonial stem cells. A fragment of the *Clostridium perfringens* enterotoxin has been identified which specifically inhibits the formation of claudin bonds (Sonoda et al., 1999). If such a peptide could be used to disrupt CLDN8 bonds in testis cell cultures it may allow the selection of $DBA^+/CLDN8^+$ cells by MACS without contamination by $DBA^-/CLDN8^+$ cells.

The expression of CLDN8 in Sertoli cells in the bovine testis was not unexpected, as a number of members of the claudin protein family have been shown to play a role in the formation of tight junctions, including in the blood-testis barrier (Morrow et al., 2010; Pelletier, 2011). In addition, CLDN5 has been reported to be expressed by both Sertoli and germ cells in the mouse testis (Morrow et al., 2009). The role that CLDN5 plays in germ cells, which do not form tight junctions, is currently unknown. Other members of the claudin family have also been shown to be expressed outside of tight cell junctions in various tissues (Gregory et al., 2001; Rahner et al., 2001). It has been suggested that claudins may play a role in cellular adhesion in these circumstances (Gregory et al., 2001; Morrow et al., 2009).

SSCs express a number of adhesion molecules such as integrins and cadherins, which appear to be essential for normal spermatogenesis to occur (Marthiens et al., 2010; Raymond et al., 2009). Kanatsu-Shinohara et al. (2008) showed that β1-integrin is essential in the

localisation of SSCs to their niche using a murine model, suggesting that adhesion molecules are not only important for the migration of SSCs to the basal membrane, but also for maintenance of SSC health. SSCs are not randomly distributed on the basal membrane of seminiferous tubules, but rather are located in specific areas referred to as stem cell niches (Yoshida et al., 2007). It has been speculated, therefore, that there could be functional differences between the stem cell niches and other areas (De Rooij, 2009). Sertoli cells are thought to be an important component of the stem cell niche (Oatley et al., 2010). However, so far there has been no evidence for a subpopulation of SSC-supportive Sertoli cells. The evidence presented in this study supports the hypothesis that a $CLDN8^{+}$ subpopulation of Sertoli cells may be linked to spermatogonial stem cell niches, possibly playing a role in cellular adhesion within the niche. The localisation of CLDN8 in the seminiferous tubule could not be determined by immunocytochemical analysis of testis tissue sections during the course of this study. Therefore there is a lack of in situ evidence of any direct interaction between CLDN8 positive spermatogonia and Sertoli cells. Functional experiments would be needed in order to confirm and/or define a role for CLDN8 in the SSC niche.

Increased understanding of the spermatogonial stem cell niche has implications both for the success of testis cell transplantation and the long term culture of SSCs. At present the conditions required for the long term culture and propagation of bovine SSCs are unknown. SSCs have low survival rates and a tendency to differentiate in culture (Aponte et al., 2008; Izadyar et al., 2003; Nasiri et al., 2011; Oatley et al., 2004). A better understanding of the SSC niche may aid in defining the optimal conditions required for the maintenance of SSCs in culture. The success of testis cell transplantation experiments requires SSCs to correctly home to and colonise the stem cell niches of the recipient testis (Brinster & Zimmermann, 1994). The cells of the SSC niche secrete factors essential for the survival of SSCs (Oatley et al., 2010). Further characterisation of the SSC niche may aid in the identification of factors

essential for SSC survival, which may be used to enhance survival of SSCs throughout the transplantation process and increase the efficiency of transplantation experiments.

3.5. Conclusion

This chapter has shown that CLDN8 is expressed in the bovine testis, by type A spermatogonia and also by a subset of Sertoli cells. This subpopulation of CLDN8 expressing Sertoli cells appears to interact with spermatogonia. Collectively, these results suggest a role for CLDN8 in the bovine testis beyond the formation of tight junctions within the blood-testis barrier. It is possible that CLDN8, expressed by spermatogonia and Sertoli cells, may play a role in maintaining SSCs in the stem cell niche in the bovine testis. Although not specific for SSCs alone, CLDN8 expression may be used in conjunction with other markers to aid in the identification of germline cells derived from pluripotent stem cells *in vitro*.

Chapter 4: Reprogramming of bovine fibroblasts to pluripotency.

4.1. Introduction

Until recently, cell fate was thought to be determined early in development, and once determined, unable to be altered or reversed. However, in recent years a number of experiments have shown that cell fate is less rigidly fixed than previously thought. Takahashi and Yamanaka (2006) demonstrated that retroviral mediated over-expression of a set of four key transcription factors was sufficient to reprogram murine fibroblasts to an embryonic stem (ES) cell-like state. These cells were named induced pluripotent stem (iPS) cells, and like ES cells, have the ability to undergo self-renewal, and also differentiate into cell types from all three germ lineages. iPS cells share a number of characteristics of ES cells, including morphology, expression of pluripotency and cell surface markers, and developmental potential. iPS cells have now been produced from several species including humans, rats and primates (Liao et al., 2009; Liu et al., 2008; Takahashi et al., 2007).

The first iPS cells, derived from murine fibroblasts, were generated by the over expression of four transcription factors: POU5F1 (OCT4), SOX2, KLF4 and c-MYC (Takahashi & Yamanaka, 2006). An alternative set of four transcription factors: POU5F1, SOX2, NANOG and LIN28 have also been used to successfully reprogram human cells to pluripotency (Yu et al., 2007). Various combinations of these six factors have been used to reprogram different cell types from other species (Kim et al., 2009; Kim et al., 2008; Maherali & Hochedlinger, 2008; Nakagawa, 2008). The number of factors required for successful iPS cell generation appears to vary depending on the culture conditions and target cell type. The endogenous expression of reprogramming factors by target cells can permit the exclusion of these factors from the reprogramming cocktail. Neural progenitors that express

endogenous SOX2 and c-MYC can be reprogrammed by exogenous POU5F1 and KLF4, or POU5F1 alone (Kim et al., 2009; Kim et al., 2008). Fibroblasts expressing KLF4 and c-MYC can be reprogrammed without the expression of exogenous c-MYC (Nakagawa, 2008; Wernig et al., 2008). POU5F1 is widely considered to be the key reprogramming factor that is required for all cell reprogramming (Sterneckert et al., 2012). However, NANOG also has an important role in reprogramming, and has been shown to help overcome some barriers to the reprogramming process (Theunissen et al., 2011). Inclusion of NANOG in the cocktail of reprogramming factors has also been demonstrated to increase the efficiency of iPS cell generation from some cell types (Hanna et al., 2009).

The transcription factors required for iPS cell generation were originally introduced to murine fibroblasts using retroviral vectors (Takahashi & Yamanaka, 2006). Other viral vectors including lenti- and adenoviral, have been used to deliver transgenes in successful reprogramming experiments (Stadtfeld et al., 2008; Yu, Vodyanik, & Smuga-Otto, 2007). Successful reprogramming has since been reported using non–viral methods of transgene delivery including mRNA (Warren et al., 2010), protein (Kim et al., 2009; Zhou et al., 2009) and modified DNA (Jia et al., 2010; Narsinh et al., 2011); however, to date, viral-mediated expression of transgenes remains the most common and reliable method for the induction of cellular reprogramming. Viral mediated reprogramming using separate vectors to deliver reprogramming factors is limited by the inefficiency of the process, and requires identification of cells that carry all of the necessary transgenes following infection with a cocktail of viral vectors. One improvement to this strategy is the use of polycistronic vectors to deliver all of the reprogramming factors using a single virion. Polycistronic vectors carrying four reprogramming factors have been used to successfully reprogram both human and murine cells (Carey, Markoulaki, & Hanna, 2009; Shao et al., 2009). It has been demonstrated that a single copy of a polycistronic transgene is sufficient to fully reprogram

cells (Carey, Markoulaki, & Hanna, 2009). However, reprogramming with polycistronic vectors often results in significantly reduced reprogramming efficiency when compared to the use of multiple vectors each carrying a single transgene. This decrease in efficiency may be a result of the stoichiometry of factor expression from polycistronic vectors, which may not be ideal for the induction of pluripotency (Carey et al., 2011; Tiemann et al., 2011).

Since the establishment of mouse embryonic stem cell lines there have been concentrated efforts to establish equivalent lines from other species. The derivation of ES cell lines from livestock species such as sheep and cattle has so far been unsuccessful (Gong et al., 2010; Mitalipova et al., 2001; Saito et al., 2003; Wang et al., 2005). The successful derivation of iPS cell lines from mouse and human cells gave rise to hopes that these may be used as alternatives to ES cells in the future, but despite advances in iPS cell technology it has proven difficult to derive iPS cells from some species using conventional protocols, particularly livestock species.

To date there have been only two reports of the generation of iPS-like cells from bovine somatic cells, both published during the preparation of this thesis. The first employed retroviral vectors to transduce bovine adult fibroblasts with human POU5F1, SOX2, KLF4 and c-MYC, which proved insufficient to produce stable bovine iPS lines (Sumer et al., 2011). The addition of NANOG to the reprogramming cocktail resulted in the generation of iPS-like cells which could be maintained in a pluripotent state in long term culture (Sumer et al., 2011). The second reported bovine iPS-like cells were generated using a non-viral polycistronic vector encoding the bovine cDNAs for POU5F1, SOX2, KLF4 and c-MYC, which was used to transduce bovine embryonic fibroblasts (Huang et al., 2011).

Studies aimed at generating iPS cells from livestock species other than cattle have employed a variety of methods. Ovine foetal fibroblasts have been reprogrammed to

pluripotency by lentiviral-mediated delivery of murine POU5F1, SOX2, KLF4 and c-MYC under a doxycycline inducible system (Li et al., 2011). However, an alternative study found that the human homologs of these four factors were insufficient to reprogram adult ovine fibroblasts (Bao et al., 2011). The addition of NANOG and LIN28 along with the SV40 large T antigen and the catalytic subunit of human telomerase, hTERT, was required in order to generate stable iPS-like clones (Bao et al., 2011). There have been four reports of the generation of porcine iPS-like cells, each employing either the human or murine homologs of POU5F1, SOX2, KLF4 and c-MYC, and using either lentiviral or retroviral vectors (Esteban et al., 2009; Ezashi et al., 2009; Montserrat et al., 2011; Wu et al., 2009). Two of these studies used embryonic porcine fibroblasts (Esteban et al., 2009; Ezashi et al., 2009), while the other two studies reported porcine iPS-like cells generated from adult fibroblasts (Montserrat et al., 2011; Wu et al., 2009). The most recent of these studies reported induction of reprogramming without the use of POU5F1 (Montserrat et al., 2011).

In addition to the variation in reprogramming factor choice and delivery method, protocols for generating iPS cells from livestock species have also varied in the cell culture conditions used, including the base media, use of serum or serum-free media, and the use of small molecules or inhibitors. There has been no consistent protocol established for the generation of iPS cells from livestock species, and to date there is no evidence to show that any of the methods described above can be replicated and consistently produce livestock iPS-like cells in different laboratories.

It is hypothesised that bovine somatic cells can be reprogrammed to pluripotency by the over-expression of the four transcription factors POU5F1, SOX2, KLF4 and c-MYC. In order to test this hypothesis a polycistronic, Dox-inducible lentiviral vector (Carey, Markoulaki, & Hanna, 2009) was selected to deliver these transcription factors into adult bovine dermal fibroblasts. This vector carried the murine cDNAs for POU5F1, SOX2, KLF4

and c-MYC, separated by 2A peptides and under the control of an inducible Tet promotor. It is also hypothesised that NANOG plays an important role in the generation of bovine iPS cells, therefore the effect of adding NANOG to the reprogramming cocktail was also examined.

4.2. Materials and Methods

All animal experiments were approved and conducted under the guidelines of the F. D. McMaster Laboratory Animal Ethics Committee, CSIRO Livestock Industries (approval No. 10-14). All cell culture reagents were purchased from Invitrogen, USA, unless otherwise stated.

4.2.1. Derivation of bovine fibroblasts

Skin samples were obtained from pre-pubertal Angus breed male calves under general anaesthesia. Primary cell cultures of dermal fibroblasts were prepared using standard methods. Briefly, the epidermis was dissected into small pieces and placed into TrypLE Express (Invitrogen, USA), and incubated at 37^{o}C for approximately 1 hour with agitation. The resulting suspension was filtered through a 30μm filter, and the cells collected by centrifugation at 400 x g for 5 mins. Cells were resuspended in fibroblast growth media (DMEM, 5% FBS, 2 mM Glutamax, 100μM NEAA, 100 U/ml penicillin and 100 mg/ml streptomycin), cultured at 37^{o}C, 5% CO_2 and passaged approximately every 4 days.

4.2.2. Lentiviral production

All plasmids used for the production of lentivirus were obtained through the plasmid repository Addgene (www.addgene.org). All plasmids used for lentiviral production are listed in Table 4.1. Figure 4.1 shows a schematic representation of the Tet-OSKM construct.

Table 4-1: Plasmids used in the production of lentivirus

Plasmid	Description	Addgene Plasmid No.	Reference
TetO-FUW-OSKM	Polycistronic inducible vector encoding murine *Pou5f1*, *Sox2*, *Klf4* and *c-MYC*	20321	(Carey, Markoulaki, & Hanna, 2009)
FUW-OSKM	Constitutive polycistronic vector encoding murine *Pou5f1*, *Sox2*, *Klf4* and *c-MYC*	20328	(Carey, Markoulaki, & Hanna, 2009)
FUW-M2rtTA	Reverse tetracycline transactivator	20342	(Hockemeyer et al., 2008)
HUW-TetO-hNanog	Inducible vector encoding human *NANOG*	25700	(Hanna et al., 2009)
psPAX2	Lentiviral packaging plasmid	12260	
pMD2.G	Lentiviral envelope plasmid	12259	
PGK-H2BeGFP	Constitutive vector encoding green fluorescent protein	21210	(Kita-Matsuo et al., 2009)

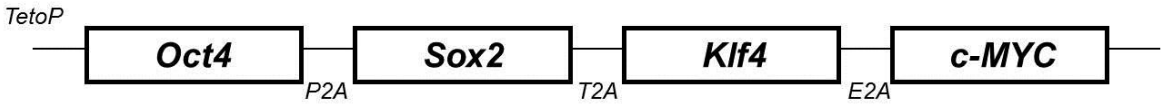

Figure 4-1: Schematic representation of the Tet-OSKM construct used for lentiviral production. Construct consists of cDNAs for murine *Pou5f1* (*Oct4*), *Sox2*, *Klf4* and *c-MYC* separated by self-cleaving 2A peptides (Carey, Markoulaki, & Hanna, 2009)

Lentivirus was produced using the Lipofectamine LTX system (Invitrogen, USA). Packaging plasmid (2 μg), envelope plasmid (1 μg) and vector plasmid (1 μg) were resuspended in 500 μl MEM. PLUS reagent (4 μl) was added and incubated at room

temperature for 5 mins. Lipofectamine LTX reagent (8 μl) was added and incubated for 30 mins at room temperature. The resulting mix was added directly onto a 70% confluent culture of 293 LTV cells in fibroblast growth media without antibiotics in a T25 tissue culture flask. Cells were cultured at 37^{o}C, 5% CO_2 for 16 hours, before medium was changed to fresh lentiviral collection medium (DMEM, 2mM Glutamax, 100μM NEAA). After a further 24 hours culture the supernatant was collected, filtered through a 45μm filter and snap frozen in liquid nitrogen.

4.2.3. Lentiviral transfection and cell culture

BDFs were infected simultaneously with a lentiviral vector carrying the reverse tetracycline transactivator M2rtTA and the lentiviral Tet-OSKM construct. Further BDF cultures were infected additionally with a Tet-NANOG lentiviral construct in conjunction with the Tet-OSKM and M2rtTA lentiviruses. BDFs (5×10^4) were plated per well of a 6 well tissue culture dish. Cells were infected with lentivirus containing supernatant, diluted 1:2 with fibroblast growth media and supplemented with 8 μg/ml polybrene (Sigma, USA). This infection was repeated after 24 hours. Twenty-four hours following the second infection the cells were changed to fresh fibroblast growth media. After 24 hours of recovery, the medium was changed to iPS medium (Minimum Essential Medium Alpha, 20% FBS, insulin-transferrin-selenium (ITS), 2mM Glutamax, 100μM NEAA, 50 U/mol penicillin, 50 mg/ml streptomycin, 0.1mM β-mercaptoethanol (Sigma, USA), 4 ng/ml human leukaemia inhibitory factor (LIF, Millipore, USA), and 10 ng/ml basic fibroblast growth factor (bFGF, Millipore, USA). Transduced cells were enzymatically dissociated and 5×10^4 cells plated onto 10 cm tissue culture dishes containing mitomycin-C inactivated MEF feeder layers. Transgene expression was induced by the addition of 2 μg/ml doxycycline (Sigma, USA). Cells were cultured for up to 3 weeks, with media changed every two days. After the first seven days of

culture MEF conditioned iPS medium was used in place of iPS medium. Media were supplemented daily with 2μg/ml doxycycline.

4.2.4. Alkaline phosphatase and immunofluorescent staining

Cells were fixed with 4% paraformaldehyde in PBS for 1-2 minutes at room temperature. The cells were rinsed with TBS-T (20 mM Tris-HCl, 0.15M NaCl, 0.05% Tween-20) and then stained for alkaline phosphatase activity using reagents and protocol from the ES Cell Characterisation Kit (Millipore, USA). Alkaline phosphatase stain was left in place for 15 minutes for each experiment.

Immunofluorescent staining was carried out on live cell cultures. Cell cultures were incubated at 37^{o}C, 5% CO_2 with mouse anti-Tra-1-60 IgM (Millipore, USA; 4μg/ml) or mouse anti-SSEA4 IgG (Millipore, USA; 4μg/ml) in iPS media without LIF and bFGF for 1 hour. Cell cultures were washed twice with pre-warmed PBS, then incubated for 1 hour with Alexa Fluor 488 goat anti-mouse IgM (Invitrogen, USA; 2μg/ml) or Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, USA; 2μg/ml) in iPS media without LIF and bFGF. Cultures were washed twice with PBS then replaced in complete iPS media before visualisation on a fluorescence microscope (Nikon, Japan). Images were captured using ProgRes CapturePro 2.1 software (Jenoptik, Germany).

4.2.5. Polymerase chain reaction (PCR) and Quantitative Reverse Transcribed Polymerase Chain Reaction (qRT-PCR)

For PCR, genomic DNA was extracted from pooled cell colonies using an Allprep RNA/DNA micro kit (Qiagen, USA). Each reaction contained 0.5μg DNA, 45μl PCR Supermix (Invitrogen, USA) and 0.2μM forward and reverse primer. Sequences of all primers used are given in Table 4.2. PCR reactions included an initial denaturation step at

95^{o}C for 5 minutes, followed by 30 cycles of denaturation at 95^{o}C for 1 minute, annealing at 60^{o}C for 30 seconds, and extension at 72^{o}C for 1 minute, with a final extension step at 72^{o}C for 5 minutes. PCR products were separated on a 1% agarose gel at 100V.

For qRT-PCR total RNA was extracted from pooled cell colonies using an Allprep RNA/DNA micro kit (Qiagen, USA). For each experiment between 8 and 12 colonies were manually picked from feeder layers. cDNA was synthesised using a Superscript III first strand synthesis kit (Invitrogen, USA). qRT-PCR reactions were carried out in triplicate as described previously (chapter 2, page 50). Primer efficiencies for all primer sets were determined as previously described (chapter 2, page 50). These are given in Table 4.2. Appropriate reference genes (RPL19 and RPS26) were determined by using the sheep GeNorm kit (PrimerDesign, UK), as described previously (chapter 2, page 50).

Table 4-2 Sequences of primer sets used in Chapter 4.

Gene/construct	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon size (bp)	Reaction efficiency (%)	Melt peak (^{o}C)	Specificity*
Tet-OSKM	TGAGGCTACAGGGACACCTT	TCAACATCACCTGCTTGCTT	240	89.8	87.5	Ectopic
NANOG	GTCCCGGTCAAGAAACAAAA	TCTGGAACCAGGTCTTCACC	163	106.1	87	Endogenous
POU5F1	AAGCGGACGAGTATCGAGAA	ACACTCGGACCACGTCTTTC	133	113.8	86	Total
SOX2	ATGAAGGAACACCCGGATTA	CATGTGCGCGTAGCTGTC	186	81.2	89	Total

* Indicates whether primers are specific for ectopic or endogenous transcripts, or both (total)

4.3. Results

4.3.1. Integration and expression of the Tet-OSKM transgene

Suitability of the lentiviral constructs to infect BDFs was tested by the infection of BDFs with a control GFP lentivirus. FACS analysis of BDFs after 48 hours in culture indicated that the GFP transgene had been introduced into the target cells, and high levels of GFP expression were observed. The infection efficiency of the GFP control virus was approximately 90% as determined by FACS analysis (Figure 4.2).

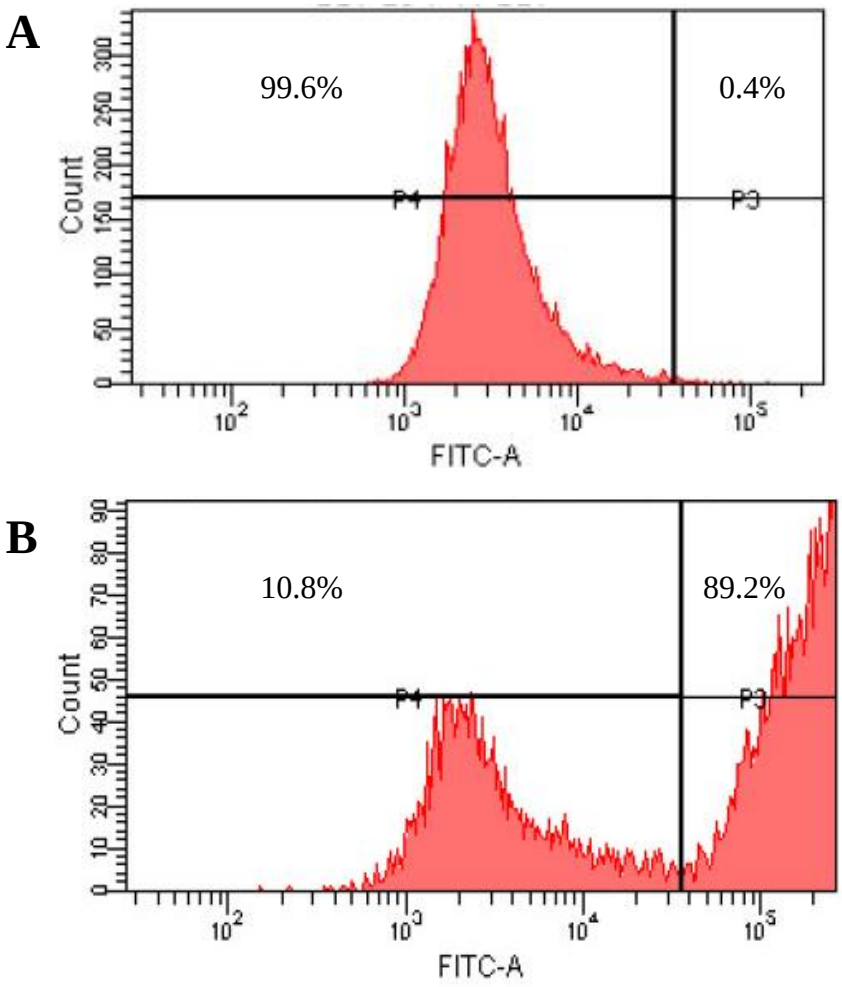

Figure 4-2: FACS analysis of BDFs (A) and GFP transduced BDFs (B). Infection efficiency of control GFP lentivirus is approximately 90%.

PCR analysis with transgene-specific primers demonstrated that the transgene was successfully integrated into the genomic DNA of BDFs following infection with the Tet-OSKM lentivirus (Figure 4.3 A). RT-PCR analysis of mRNA from infected BDFs using transgene specific primers confirmed expression of the transgene construct 4 days post-infection (Figure 4.3 B).

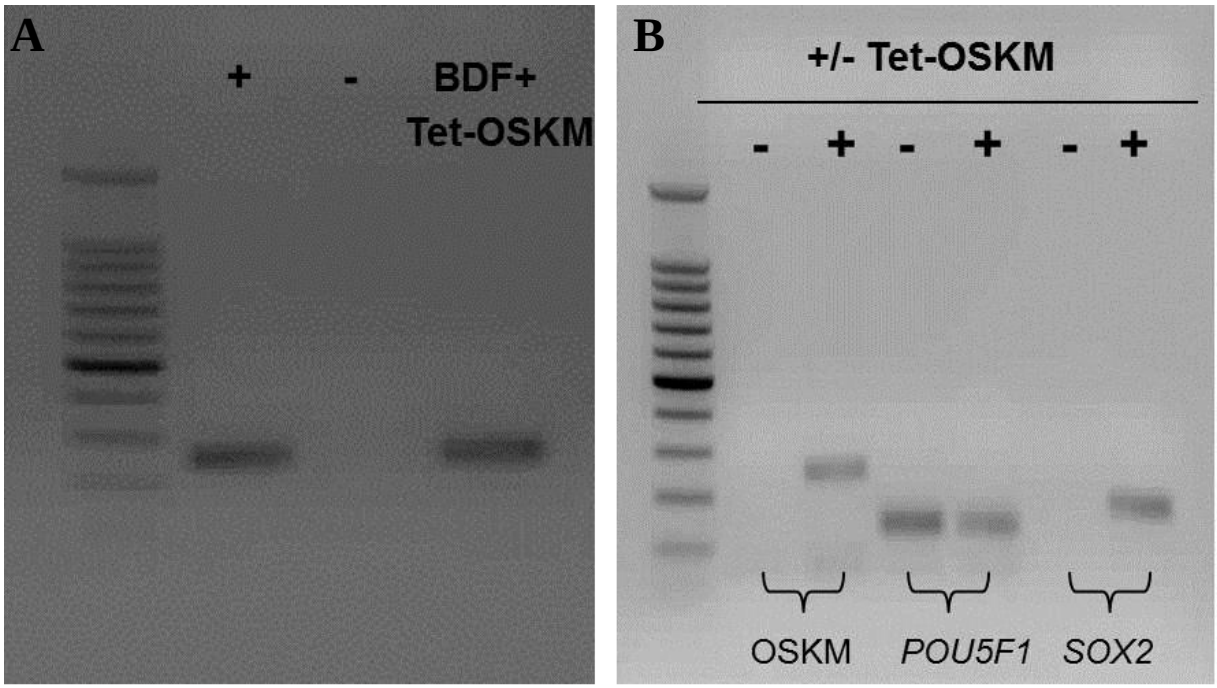

Figure 4-3: (A) PCR verification of integration of Tet-OSKM transgene into target cell genomic DNA using transgene-specific primers. + indicates positive control, - indicates uninfected BDF. (B) RT-PCR indicating expression of Tet-OSKM transgene (OSKM), *POU5F1* and *SOX2*. – indicates uninfected BDF, + indicates BDF + Tet-OSKM.

4.3.2. Transgene expression from inducible and constitutive OSKM vectors

A time course experiment comparing transgene expression levels in BDFs transfected with the inducible Tet-OSKM lentivirus and a constitutively expressed OSKM lentivirus over 11 days indicated that the Dox inducible Tet-OSKM transgene was expressed at much higher levels than the constitutively expressed transgene at all time points (Figure 4.4). The Tet-OSKM construct was therefore used in all further experiments.

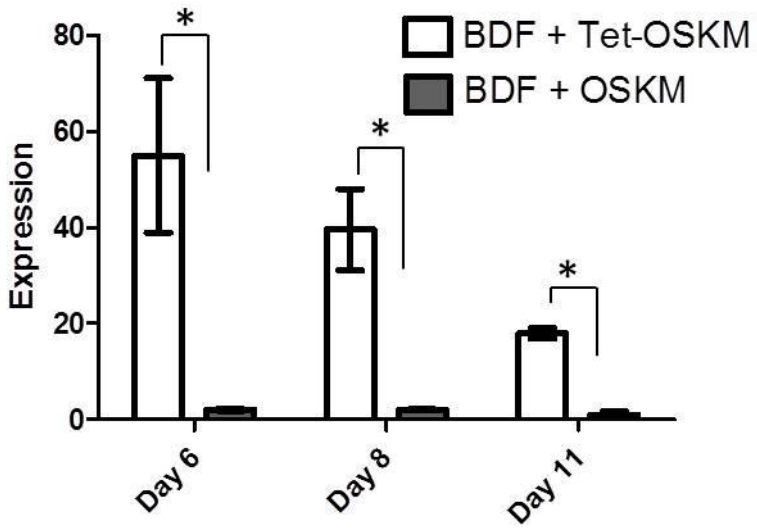

Figure 4-4: Expression of the inducible Tet-OSKM and constitutive OSKM transgene by BDFs over 11 days. * indicates significant difference between group means, P < 0.05, n = 2 biological replicates. Expression indicates fold change compared to the means of two reference genes.

4.3.3. Characterisation of putative bovine iPS cell colonies

BDF cultures transfected with the Tet-OSKM construct began to develop colonies after approximately 21 days in culture. These colonies did not show consistent morphology across plates. Some colonies showed ESC-like morphology, being flat, tightly compact, and having clear borders. Other colonies were less compact, showed heterogeneity of cells, or did not display clear borders. Figure 4.5 shows the various colony types observed at day 21 post-induction. No colony formation was observed in non-transfected BDF cultures.

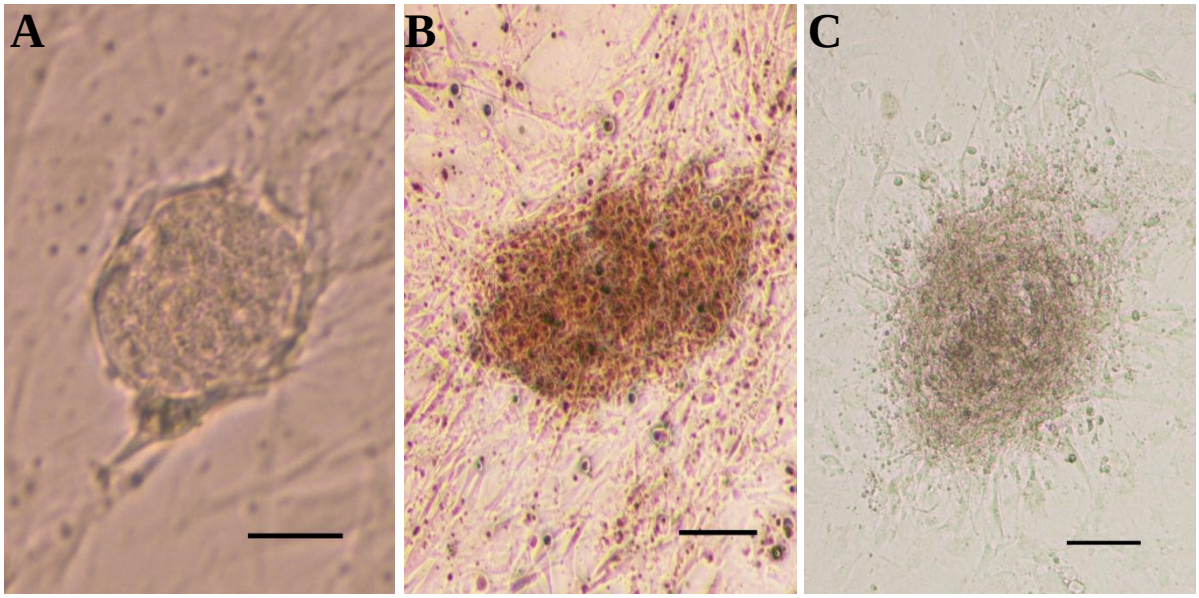

Figure 4-5: Cell colonies obtained from Tet-OSKM transduced BDFs after 21 days culture. (A) Colony showing ES cell-like morphology. (B) and (C) colonies showing non-ES cell-like morphology. Scale bars = 50 μm.

Cell colonies obtained from Tet-OSKM transfected BDFs were analysed for alkaline phosphatase activity, and expression of pluripotency surface markers. Colonies stained for alkaline phosphatase activity showed no or only low background levels of activity. Live staining for expression of the cell surface markers SSEA4 and Tra-1-60 failed to identify any colonies expressing these surface markers. RT-PCR analysis revealed that these cells did not express *NANOG*, a marker of pluripotency. Due to the limited amount of material available no immunocytochemical analysis of transcription factors or NANOG was carried out.

A number of colonies were mechanically picked and transferred onto fresh feeder layers. These colonies failed to expand after 7 days. Some colonies appeared to undergo slow growth for 1 or 2 days following passaging, before becoming quiescent, while other colonies did not show any growth after passaging and appeared to become immediately quiescent.

4.3.4. Effect of NANOG on the reprogramming of bovine fibroblasts

Colony development in BDF cultures transduced with the Tet-OSKM construct were compared with that in BDF cultures transduced with the Tet-OSKM transgene in combination with a Tet-NANOG vector (Tet-OSKM+N). No difference was observed in the time taken for BDFs infected with either Tet-OSKM or Tet-OSKM+N to form cell colonies, or in the number of colonies formed per 10 cm dish (Figure 4.6). No colony formation was observed in non-transfected BDF cell cultures.

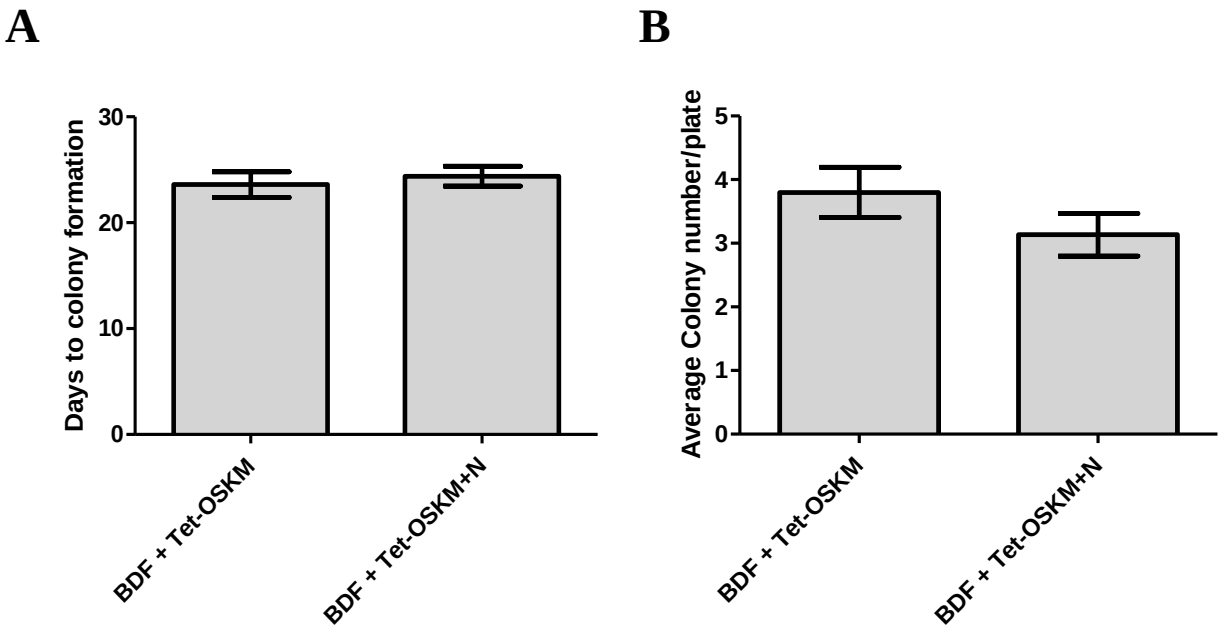

Figure 4-6: Comparison of time taken to colony formation (A) and colonies formed per plate (B) from BDF cultures transduced with Tet-OSKM or Tet-OSKM+N. No significant difference was detected between the two groups ($P > 0.5$, $n = 5$)

There were no morphological differences between colonies from Tet-OSKM and Tet-OSKM+N treated BDF cultures. Colonies from Tet-OSKM+N transduced BDF cultures were negative for alkaline phosphatase activity, and did not express cell surface markers SSEA4 or Tra-1-60. These colonies also failed to expand when mechanically picked and passaged.

4.4. Discussion

Since the first reported reprogramming of somatic cells to pluripotency via overexpression of a set of four transcription factors (Takahashi & Yamanaka, 2006), iPS cells have been generated from a number of species (Liao et al., 2009; Liu et al., 2008; Takahashi et al., 2007). To date there has been limited success in generating fully reprogrammed iPS cells from livestock species such as cattle (Huang et al., 2011; Sumer et al., 2011), and as yet no reproducible protocol for reprogramming these cells has been described. This chapter describes attempts to generate iPS cells from bovine adult fibroblasts using a drug- inducible polycistronic vector.

The results presented in this chapter demonstrate that a polycistronic lentiviral vector was suitable for the delivery of multiple reprogramming factors, resulting in the stable integration of the transgene construct into the genomic DNA of bovine dermal fibroblasts (BDFs). BDFs expressing the transgene construct were able to form cell colonies exhibiting embryonic stem (ES) cell-like morphology. However, these putative iPS cell colonies failed to express the pluripotency associated surface markers Tra-1-60 or SSEA4. Examination of alkaline phosphatase activity, a general indicator of pluripotency, indicated that these cell colonies showed no, or only low levels of activity. The putative iPS cell colonies were unable to be expanded in culture, and did not express NANOG, an additional marker of pluripotency. Although the putative iPS cells described in this chapter did not exhibit these characteristics, they did appear to be morphologically distinct from the initial fibroblast population. It is therefore hypothesised that the cell colonies generated by the expression of the Tet-OSKM transgene in adult bovine fibroblasts represent a population of partially reprogrammed cells.

Partially reprogrammed cells have been reported in a number of different studies, and at present it is difficult to say why these cells fail to undergo full reprogramming. There are a

number of aspects of the reprogramming protocol used in these experiments that may be responsible for the failure of cells to complete the reprogramming process. The differentiation status and source of the target cell type are known to have a significant impact on the success of reprogramming attempts. Different target cell types have been shown to have a greater potential for reprogramming than others, as indicated by variations in reprogramming efficiencies between cell types (Aasen et al., 2008; Byrne, Nguyen, & Reijo Pera, 2009; Niibe et al., 2011). Some cell types are considered to be unreprogrammable, while less differentiated cell types are considered to be easier to reprogram than fully differentiated lines. Thus multipotent cells such as adult stem cells have been shown to be easier to reprogram than unipotent somatic cells (Eminli et al., 2009; Niibe et al., 2011). The experiments described here were carried out using adult bovine dermal fibroblasts as the targets of reprogramming. Such cells have previously been shown to be amenable to the reprogramming process (Sumer et al., 2011), although adult cells are generally considered to be more difficult to reprogram than embryonic cells (Okada & Yoneda, 2011; Park et al., 2008). An interesting follow on study would be to compare the reprogramming potential of embryonic fibroblasts to adult fibroblasts under the conditions described here. It may also be possible to generate fully reprogrammed cells under the conditions described if the chosen target cell type is more amenable to the reprogramming process.

The reprogramming experiments described in this chapter were carried out using a set of four transcription factors: POU5F1, SOX2, KLF4 and c-MYC. NANOG has previously been shown to be important in the generation of bovine iPS-like cells (Sumer et al., 2011), and may be necessary for the successful generation of iPS cells (Theunissen et al., 2011). BDF cultures were therefore co-infected with a lentiviral vector carrying a Tet-NANOG construct in combination with the Tet-OSKM vector. No difference in colony formation was detected when fibroblasts were transduced with the Tet-OSKM vector as compared to the

Tet-OSKM+N combination. Whether NANOG is required for the generation of bovine iPS cells is under debate. Of the two reports of bovine iPS-like cells, one found that NANOG was required for the generation of stable iPS-like cells (Sumer et al., 2011), while the other was able to generate such cells without the use of NANOG (Huang et al., 2011). There were significant differences in the reprogramming conditions used in these two studies, leading to the conclusion that NANOG is required for reprogramming under certain conditions, while it can be dispensed with under alternative conditions. Various combinations of the six transcription factors that make up the Yamanaka and Thomson sets of reprogramming factors have been used to generate iPS cells in different species (Bao et al., 2011; Sumer et al., 2011; Takahashi & Yamanaka, 2006; Yu, Vodyanik, & Smuga-Otto, 2007). It appears that under the optimal culture conditions the number of factors required for successful reprogramming can be minimised (Kim et al., 2009; Kim et al., 2008; Nakagawa, 2008; Tsai et al., 2011; Wernig et al., 2008). It is possible that both the OSKM and OSKM+N sets of factors are equally effective in generating the partially reprogrammed cells described here. Under the conditions described in this chapter neither set of factors was sufficient to drive the complete reprogramming of bovine somatic cells to iPS cells. It remains to be tested whether either set of factors would be capable of generating bovine iPS cells under alternative culture conditions than those described in this chapter.

The species of origin of the transcription factors used in reprogramming may also have an impact on the ability of cells to reprogram completely. The transcription factors used in this study were murine cDNAs of POU5F1, SOX2, KLF4 and c-MYC. Although these transcription factors are highly conserved between species, it is possible that bovine cells are unable to completely process or recognise one or more of these factors, resulting in cells that fail to undergo complete reprogramming. Murine transcription factors have been used successfully in reprogramming cells from other livestock species (Pig: Esteban et al., 2009;

Sheep: Y. Li et al., 2011), however they have not yet been successfully used in cattle. Human transcription factors have been used to successfully reprogram bovine cells (Sumer et al., 2011). A comparative study of human and murine factors in reprogramming bovine cells could potentially help to identify whether it is the reprogramming factors themselves, or other aspects of the reprogramming protocol that are responsible for the failure of cells to undergo complete reprogramming.

Generation of iPS cells is a highly inefficient process. One factor that can influence the efficiency of iPS cell generation is the choice of vector for delivery of reprogramming factors. In this chapter a polycistronic vector was used to deliver all four reprogramming factors using a single virion. Polycistronic vectors have been used to successfully reprogram both human and murine cells (Carey, Markoulaki, & Hanna, 2009; Shao et al., 2009), although they are not without intrinsic problems. Reprogramming using polycistronic vectors results in a significantly lower reprogramming efficiency than reprogramming using single vectors, possibly as a result of the stoichiometry of factor expression from polycistronic vectors (Carey, Markoulaki, & Hanna, 2009; Okita, Nakagawa, Hyenjong, Ichisaka, & Yamanaka, 2008). Polycistronic vectors utilising self-cleaving 2A peptides, such as the Tet-OSKM vector used in this study, have been shown to support near equimolar expression of proteins (Szymczak, Workman, & Wang, 2004). The use of separate vectors allows for variation in the number of proviral integrations of each individual factor, thereby allowing for reprogramming of cells that express the optimal ratio of factors. However, the optimal expression levels and stoichiometry of reprogramming factors is not well defined (Brambrink et al., 2008; Carey et al., 2011; Papapetrou et al., 2009; Tiemann et al., 2011). A previous study has reported successful generation of bovine iPS cells using a polycistronic vector (Huang et al., 2011), although the vector used in that case was constructed with each of the reprogramming factors under the control of an independent promoter, rather than all four

under the control of a single promoter as in the Tet-OSKM vector used here. Other attempts at reprogramming livestock cells have used separate vectors in order to induce pluripotency (Esteban et al., 2009; Ezashi et al., 2009; Li et al., 2011; Sumer et al., 2011; Wu et al., 2009). Due to the limited amount of material available no analysis of protein levels of reprogramming factors was carried out in this study. It is possible that the partial reprogramming of cells described in this chapter is a result of sub-optimal levels of expression of the four reprogramming factors used. This may be overcome by the use of individual vectors carrying each of the reprogramming factors. A comparison of cellular reprogramming using separate vectors versus the polycistronic vector was not conducted as part of this study, but such a comparison is warranted in future research.

In addition to these aspects of the reprogramming protocol, there are also a number of barriers to the reprogramming process that may result in partially reprogrammed cells. One early event in the reprogramming process is the down-regulation of the cells somatic markers (Stadtfeld et al., 2008). It appears that only a subset of cells expressing reprogramming factors can undergo this down-regulation of somatic cell markers (Wernig et al., 2008), while other cells are refractory to this process (Stadtfeld & Hochedlinger, 2010). Another early event in the reprogramming process involves cells undergoing a mesenchymal-to-epithelial (MET) morphological transition (Li et al., 2010; Samavarchi-Tehrani et al., 2010; Stadtfeld et al., 2008). During development the opposite process is fundamental for the correct differentiation of cell types (Chaffer, Thompson, & Williams, 2007; Zeisberg & Kalluri, 2004). It is therefore required that the intrinsic nature of the cells be overcome in order to undergo this crucial step in the reprogramming process. Another barrier encountered in the reprogramming process is the onset of cellular senescence (Banito et al., 2009; Hong et al., 2009). In addition to these molecular barriers there are also epigenetic barriers to the reprogramming process. DNA hypermethylation can present a barrier to the reprogramming

process, as the promoters of pluripotency genes are stably silenced by DNA methylation in somatic cells (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2008). This methylation pattern can interfere with transcription factor binding during the reprogramming process, which requires demethylation of promoters (Sridharan et al., 2009). Histone H3 and H4 acetylation of chromatin is also considered to be important for the generation of iPS cells (Azuara et al., 2006; Bernstein et al., 2006). High levels of histone deacetylase activity can inhibit the reprogramming process, while use of histone deacetylase inhibitors has been shown to improve the kinetics and efficiency of iPS cell generation (Huangfu et al., 2008; Liang et al., 2010; Mali et al., 2010). The partially reprogrammed cells described here may be a result of a failure to overcome any one or more of these barriers, although further investigation is required in order to pinpoint the cause. The failure of these cells to expand in culture once passaged may indicate the onset of cellular senescence, but this was not investigated further in this study.

Despite being unable to generate fully reprogrammed bovine iPS cells, this chapter has laid a solid foundation for future work in this area and has raised a number of questions for future research. Two major avenues for further research are the reprogramming protocol, and the culture conditions used for the generation of reprogrammed bovine cells. The reprogramming protocol implemented in this study was insufficient for generating fully reprogrammed bovine cells. There were a number of aspects of this protocol that may be revised in order to improve the reprogramming process, including the choice of reprogramming factors, the method of delivery of these factors, and the target cell type. Culture conditions for mouse and human iPS cells have been derived from the conditions used to culture embryonic stem cells in these species. Such culture conditions are unknown for bovine ES cells as these cells have proven problematic to establish and maintain (Gong et al., 2010; Mitalipova et al., 2001; Saito et al., 2003; Wang et al., 2005). Thus the culture

conditions used in this and other studies involving iPS cell generation from livestock species have involved the adaptation of methods used in reprogramming human and murine cells. It is possible that these conditions were sub-optimal for the generation of iPS cells in the bovine, and this may be one of the barriers preventing the derivation of fully reprogrammed bovine iPS cells. Variations in reprogramming media composition are likely to have a significant effect on the reprogramming of bovine cells. There are a number of signalling pathway inhibitors and small molecules that have been used to enhance the reprogramming process in a number of species including the bovine (Huang et al., 2011). Investigation into the effect of different small molecules and signalling pathway inhibitors should be a starting point for further research attempting to overcome the barriers currently preventing the generation of completely reprogrammed bovine iPS cells.

4.5. Conclusion

To date the generation of iPS cells from livestock species such as cattle has proven difficult. A number of attempts have been made, however as yet no reproducible protocol has been established for the reprogramming of these cells. In this chapter it has been demonstrated that delivery of the murine transcription factors POU5F1, SOX2, KLF4 and c-MYC using a polycistronic, drug inducible lentiviral vector was sufficient to induce the formation of stem cell-like colonies, but was insufficient to fully reprogram adult bovine fibroblasts to pluripotency. The addition of a fifth transcription factor, NANOG, did not help to overcome the barrier to reprogramming. Therefore, to generate fully reprogrammed bovine cells, culture parameters other than transcription factors warrant further investigation. It is hypothesised that optimisation of the reprogramming protocol by modifying the culture conditions may enable further progress towards the reprogramming of bovine somatic cells to

pluripotency. Several small molecules and signalling pathway inhibitors have been identified which aid in the generation of fully reprogrammed iPS cells in different species. These molecules may be of use in enhancing the reprogramming of bovine somatic cells to pluripotency.

Chapter 5: Use of small molecules to enhance the reprogramming of bovine fibroblasts to pluripotency

5.1. Introduction

Induced pluripotent stem (iPS) cells have been produced from a number of species including mice, humans and primates. Despite advances in iPS cell technology, there has been limited success in generating iPS cells from livestock species, including cattle. Chapter 4 described attempts to generate bovine iPS cells using a drug inducible, polycistronic lentiviral vector encoding murine cDNAs for *Pou5f1, Sox2, Klf4* and *c-MYC*. The methods described were sufficient to induce the formation of stem cell-like colonies, but were insufficient to reprogram adult bovine fibroblasts to a fully pluripotent state. This chapter further explores the reprogramming of bovine fibroblasts using modified culture conditions in an attempt to enable further progress towards the reprogramming of bovine cells to pluripotency.

The first iPS cells were derived from murine embryonic fibroblasts (Takahashi & Yamanaka, 2006). Since this time, iPS cells have been produced from numerous different cell types. The source of the target cells is thought to have an impact on the success of reprogramming attempts. Some cell types appear to have a greater reprogramming potential than others, generating iPS cells at a much higher efficiency than other cell types (Aasen et al., 2008; Byrne et al., 2009; Niibe et al., 2011). Younger and/or less differentiated cells are generally considered to be more amenable to the reprogramming process than older or more differentiated cells, and often reprogram at higher efficiencies (Park et al., 2008). Park et al. (2008) investigated the reprogramming potential of a variety of human cell types including ESC derived fibroblasts, foetal cells and adult cell lines and found that transduction of POU5F1, SOX2, KLF4 and c-MYC into the more developmentally mature cell lines such as

adult dermal fibroblasts resulted in a decrease in proliferation and an increase in cellular senescence. In contrast to ESC-derived and fetal cell lines, the adult lines failed to give rise to colonies with obvious ESC-like morphology under the same conditions, indicating that there is a difference in reprogramming potential between human embryonic and adult cell types. A similar difference in reprogramming potential has been observed between mouse embryonic fibroblasts and adult tail-tip fibroblasts (Okada & Yoneda, 2011). One possible explanation for the difference in reprogramming potential between embryonic and adult cells is that older cells, or those that have undergone several passages, may have acquired DNA damage, which is a major barrier in the reprogramming process (Hong et al., 2009; Marion et al., 2009).

There is limited information regarding any difference in reprogramming potential between embryonic and adult cells in livestock species. Several studies attempting to derive iPS cells from livestock species have been carried out using embryonic cells as the targets for reprogramming (Esteban et al., 2009; Ezashi et al., 2009; Li et al., 2011), although adult cells from a number of species have also been used to generate iPS-like cells (Bao et al., 2011; Nagy et al., 2011; Wu et al., 2009). The first reported bovine iPS cells were generated from adult dermal fibroblasts obtained from ear skin punches (Sumer et al., 2011). In preliminary experiments described by Huang et al. (2011) a line of bovine embryonic fibroblasts were found to yield the highest number of iPS-like colonies when compared to various foetal and adult cell lines, however this study did not further investigate any differences in reprogramming potential between adult and embryonic lines. The results of these studies indicate that both adult and embryonic bovine fibroblasts are amenable to the reprogramming process; however, it remains to be confirmed whether one cell type is easier or more efficient to reprogram.

The process of reprogramming cells to pluripotency is highly inefficient, and can result in the generation of partially reprogrammed cells. As understanding of the signalling

pathways involved in the reprogramming process increases, various small molecules and inhibitors have been identified that can enhance this process. These include molecules that alter the DNA methylation or histone acetylation state of target cells, as well as inhibitors of the MEK/ERK, TGFβ and WNT signalling pathways. Pluripotent stem cells are considered to have a transcriptionally permissive chromatin state, characterised by DNA de-methylation and histone acetylation (Azuara et al., 2006; Bernstein et al., 2006). The DNA methyltransferase inhibitor 5-azacytidine (5-aza) and histone deacetylase (HDAC) inhibitors such as valproic acid and sodium butyrate (NaB) have been used to enhance reprogramming by altering the chromatin state of target cells until they more closely resemble that of ES cells (Huangfu et al., 2008; Mikkelsen et al., 2008). The MEK/ERK signalling pathway regulates the activity of a number of transcription factors, including c-MYC, and triggers differentiation from ESCs to lineage commitment (Kunath et al., 2007). Inhibition of the MEK/ERK pathway through the use of the MEK inhibitors PD0325901 or PD98059 or the ERK inhibitor PD184352 promotes cell reprogramming by blocking lineage commitment signalling and promoting self-renewal (Ying et al., 2008). During early development, cells undergo an epithelial to mesenchymal transition (EMT), which is essential for the correct differentiation into different lineages (Chaffer et al., 2007). The opposite process, a mesenchymal to epithelial transition (MET), is one of the early hallmarks of the reprogramming process (Li et al., 2010; Samavarchi-Tehrani et al., 2010). The TGFβ signalling pathway is involved in promoting the EMT during development, and can be inhibited by the TGFβ inhibitor A-83-01 or by the activin-like kinase 5 (ALK5) inhibitor SB431542. Inhibition of TGFβ signalling appears to enhance cell reprogramming by promoting MET (Zeisberg & Kalluri, 2004) and also regulating expression of NANOG (Ichida et al., 2009), an important transcription factor in reprogramming. Activation of the WNT signalling pathway has been shown to play an important role in the maintenance of

ESCs in an undifferentiated state, and also appears to have a role in the promotion of cell reprogramming (Marson et al., 2008; Miki et al., 2011). The WNT signalling pathway can be affected by inhibitors of glycogen synthase kinase-3 (GSK3-β) including BIO and CHIR99021. At present it is unclear how activation of the WNT signalling pathway facilitates cell reprogramming, although it is suggested that downstream components of the pathway regulate the expression of core transcription factors involved in maintaining pluripotency.

Many signalling pathway inhibitors and other small molecules have been used in combination to aid in or enhance the reprogramming of different cell types to pluripotency. Dual inhibition by the MEK inhibitor PD0325901 and the GSK3-b inhibitor CHIR99021 (known as 2i) promotes the complete reprogramming of both murine fibroblasts and neural stem cell derived cells (Silva et al., 2008). The 2i combination has also been used to promote or enhance reprogramming of bovine (Huang et al., 2011) and porcine (Esteban et al., 2009) cells. A combination of PD0325901 with the HDAC inhibitor NaB and the ALK5 receptor inhibitor SB431542 significantly enhances the efficiency and kinetics of reprogramming human cells, and also promotes partially reprogrammed cells to undergo complete reprogramming (Zhang et al., 2011). Other similar combinations (NaB/PD0325901/A-83-01 and PD0325901/SB431542/Thiazovivin) have also been shown to enhance and promote the reprogramming of human cells to pluripotency (Lin et al., 2009; Zhu et al., 2010). In all of these cases treatment with one or two of the molecules was far less effective than when the molecules were used in combination, suggesting that the activation or inhibition of multiple signalling pathways has a synergistic effect on enhancing iPS cell generation.

To date, the use of small molecules in reprogramming cells from livestock or large animal species has not been fully explored. The majority of iPS cells reported in livestock

species such as sheep and pigs were obtained without the use of small molecules (Bao et al., 2011; Ezashi et al., 2009; Li et al., 2011; Wu et al., 2009). Nagy et al. (2011) employed a cocktail of five small molecules and signalling pathway inhibitors in reprogramming equine fibroblasts. As this was the first report of equine iPS cells it is unclear whether these molecules are required for the reprogramming of equine fibroblasts, or whether these molecules simply enhance the reprogramming process. Esteban et al. (2009) demonstrated that 2i supplemented media promoted the complete reprogramming of porcine cells by enhancing cell compaction and increasing proliferation. The use of 2i media in non-viral reprogramming of bovine embryonic fibroblasts was investigated by Huang et al. (2011), who reported that 2i media promoted the reprogramming of bovine cells to pluripotency, with induction of molecular markers of pluripotency only occurring when cells were cultured in the presence of 2i/LIF. In contrast, the first reported bovine iPS cells were generated without the use of any inhibitors (Sumer et al., 2011). The few studies that have employed signalling pathway inhibitors in reprogramming livestock cells suggest that these molecules can have a significant impact on the reprogramming process, and suggest that the signalling pathways involved in reprogramming cells to pluripotency are conserved among species. Other small molecule combinations that have proved effective in enhancing iPS generation in human and mouse models are yet to be tested to determine if they have similar effects on the reprogramming of bovine cells.

Previous attempts at reprogramming bovine fibroblasts to pluripotency, described in chapter 4 of this thesis, resulted in only partially reprogrammed cells. The first aim of this chapter was to determine whether the differentiation state of the target cell affects the capacity of the cell to reprogram. This was determined by investigating the reprogramming potential of adult versus embryonic bovine fibroblasts. In addition, this chapter investigated whether use of a cocktail of small molecules and signalling pathway inhibitors can enhance

the reprogramming process and enable the generation of fully reprogrammed bovine iPS cells. The small molecule cocktail selected was a combination of the HDAC inhibitor NaB, the MEK inhibitor PD0325901 and the ALK5 receptor inhibitor SB431542.

5.2. Materials and Methods

5.2.1. Primary cell cultures

Primary cell cultures of bovine dermal fibroblasts (BDFs) were established as previously described (chapter 4, Pg 82). Primary cell cultures of bovine embryonic fibroblasts (BEFs) were established using similar methods. Briefly, skin samples were obtained from an approximately 65 day old foetus (abattoir material, age estimated from crown-rump length), and dissected into small pieces. Tissue was digested in TrypLE Express (Invitrogen, USA) for 1 hour at 37^{o}C with agitation. The resulting suspension was filtered through a 30 μM cell strainer, and the cells plated onto tissue culture flasks in fibroblast growth media (DMEM, 5% FBS, 2mM Glutamax, 100μM NEAA, 100U/ml penicillin and 100mg/ml streptomycin). Cells were passaged twice before cryopreservation for later use.

5.2.2. Lentiviral production, transfection and cell culture

Production of lentivirus and transfection of BDF/BEFs were carried out as described previously (chapter 4, Pg 83-85), with the following modifications: Following the second lentiviral infection cells were plated onto tissue culture flasks in either iPS media, or iPS media supplemented with 0.5mM sodium butyrate (Sigma, USA), 0.5μM PD0325901 (Stemgent, USA) and 2μM SB431542 (Stemgent, USA) (designated as NaB-PD-SB) and cultured for 3 to 5 days. Cells were then enzymatically dissociated and transferred onto 10 cm tissue culture dishes containing mitomycin-C inactivated BEF feeder layers. Cells were cultured in either iPS or iPS + NaB-PD-SB media until colonies formed.

5.2.3. Embryoid body formation

Cell colonies were manually harvested at day 12, and placed into low attachment culture dishes, in iPS media without bFGF and LIF, and cultured for 7 days at 37^{o}C, 5% CO_2. Embryoid bodies (EBs) were harvested on day 7 and pooled for RNA extraction prior to qRT-PCR analysis

5.2.4. Characterisation of putative iPS cells

Alkaline phosphatase staining of cell colonies was carried out as described in chapter 4 (pg 85). Cell cultures were examined under an inverted microscope (Nikon, Japan) and images captured using ProgRes CapturePro 2.1 software (Jenoptik, Germany).

Quantitative Reverse Transcribed Polymerase Chain Reaction (qRT-PCR) was carried out as previously described (chapter 2, pg 50). Sequences of all primer sets used in this chapter are given in Table 5.1.

Table 5-1: Sequences of primer sets used in Chapter 5.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon size (bp)	Reaction efficiency (%)	Melt peak (^{o}C)
Tet-OSKM construct	TGAGGCTACAGGGACACCTT	TCAACATCACCTGCTTGCTT	240	89.8	87.5
NANOG	GTCCCGGTCAAGAAACAAAA	TCTGGAACCAGGTCTTCACC	163	106.1	87
REX1	TACTCAGCCCTGGAGAAGGA	CTCTTCTGCCCAGGAAACAG	188	127.3	87
ALPL	CCGAAACACAAGCACTCTCA	AAGGGTCAGTCGCATTGTTC	142	101.0	86.5
THY1	CACCTCTGCCAATACCACCT	ATACCCCTCCATCCTTCCAC	196	96.3	85
FOXA2	ACCACTACGCCTTCAACCAC	GGGGTAGTGCATCACCTGTT	134	85.8	86
AFP	GAAATTTGCCGTGAAAAGGA	CAGAACTGGAAAGGGTGGAA	138	104.8	83.5
NESTIN	AAAGAAGGCTGGGATCCTGT	CCTCAAACTCTTCCGACAGC	130	108.9	88
TUBB3	TGGAGCGCATCAGTGTCTAC	CAGTTGTTACCAGCCCCACT	172	99.3	87.5
DESMIN	GGGACATCCGTGCTCAGTAT	GTGGCGGTACTCCATCATCT	155	120.8	86.5
BMP4	TGAGCCTTTCCAGCAAGTTT	CTTCCCCGTCTCAGGTATCA	180	99.2	82.5

5.2.5. Statistical analysis

Ct values from qRT-PCR were converted into expression data using the Excel add-in Genex (Bio-Rad, USA). Statistical and correlation analyses of expression data were performed using GraphPad Prism software (Graphpad Software, USA). An unpaired t-test was applied to determine if significant differences existed between group means. Differences of $P < 0.05$ were considered to be significant. Results are presented as means ±SEM.

5.3. Results

5.3.1. Reprogramming potential of embryonic and adult fibroblasts

To investigate whether adult and embryonic cells showed a difference in reprogramming potential bovine dermal and embryonic fibroblasts were transduced with both the Tet-OSKM and the M2rtTA vector. There was no significant difference in time to colony formation between Tet-OSKM transduced BDFs and BEFs (Figure 5.1). In iPS media colony formation occurred after approximately 21 days regardless of cell type. In NaB-PD-SB supplemented media colony formation occurred after approximately 12 days, regardless of cell type. No colony formation was observed in non-transduced BDFs or BEFs in either iPS or NaB-PD-SB supplemented media.

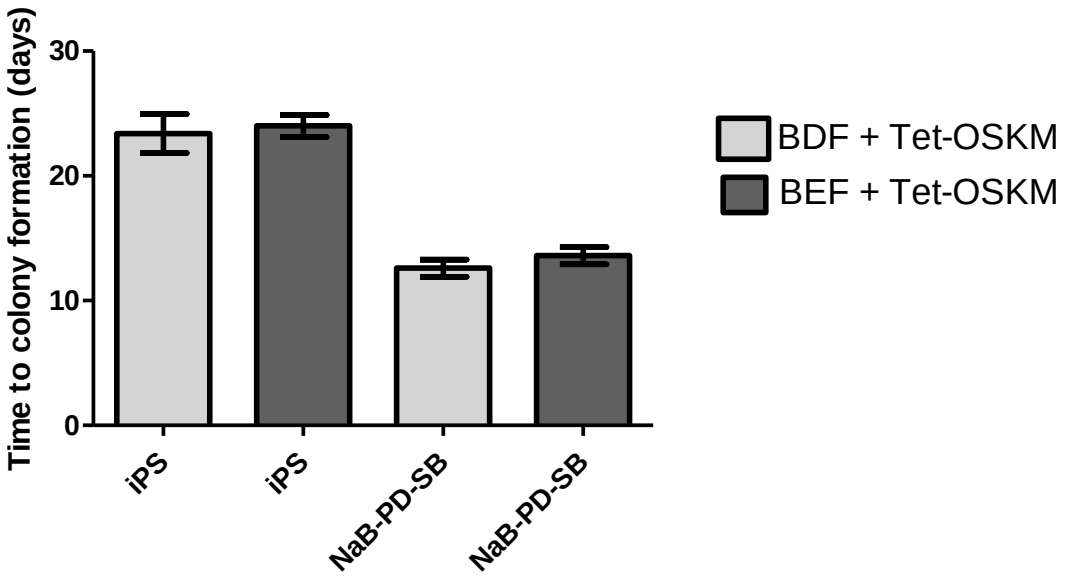

Figure 5-1: Comparison of reprogramming potential of adult and embryonic cells. No difference was observed in time to colony formation between BDF and BEF cell types, with colony formation occurring after an average of 21 days in iPS media and 12 days in NaB-PD-SB supplemented media (P > 0.15, n = 5 biological replicates).

There was no significant difference in the number of colonies formed per plate between Tet-OSKM transduced BDFs and BEFs (Figure 5.2). An average of 3 colonies per 10 cm dish had formed after 25 days in iPS media, and 15 days in NaB-PD-SB supplemented media, regardless of cell type.

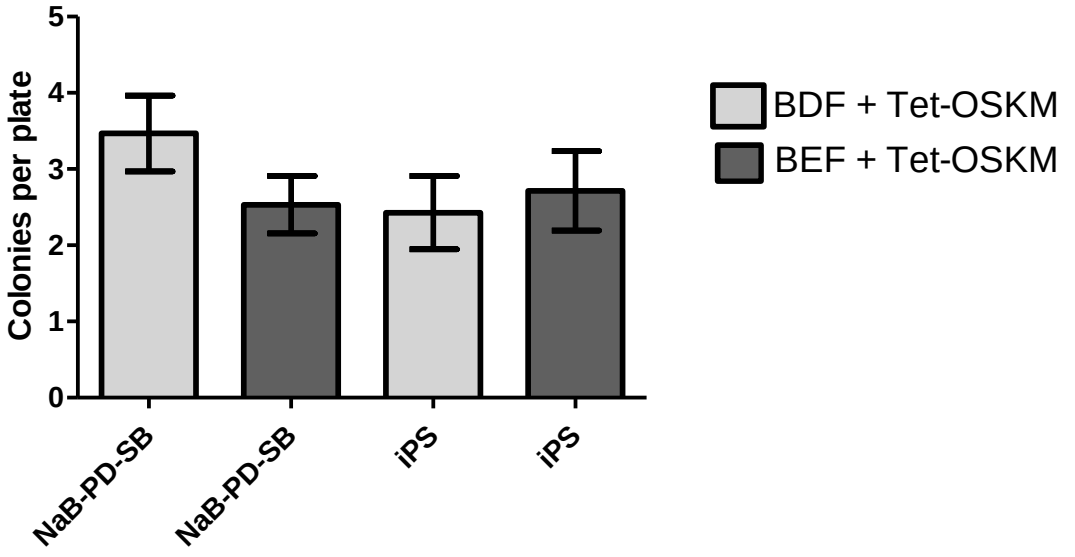

Figure 5-2: Comparison of reprogramming potential of adult and embryonic bovine fibroblasts. No significant difference was observed in the number of colonies formed per plate in cells cultured in iPS media for 25 days, or NaB-PD-SB supplemented media for 15 days (P > 0.3, n = 5 biological replicates).

5.3.2. Effect of a small molecule cocktail on reprogramming of bovine cells

To investigate whether a cocktail of small molecules would enhance the reprogramming process in bovine cells, Tet-OSKM transduced BDFs and BEFs were cultured in iPS media alone or iPS media supplemented with the small molecule cocktail designated NaB-PD-SB. Cells cultured in NaB-PD-SB supplemented media formed colonies after a significantly shorter time period (approximately 12 days) compared to cells cultured in iPS media alone (approximately 21 days: Figure 5.3).

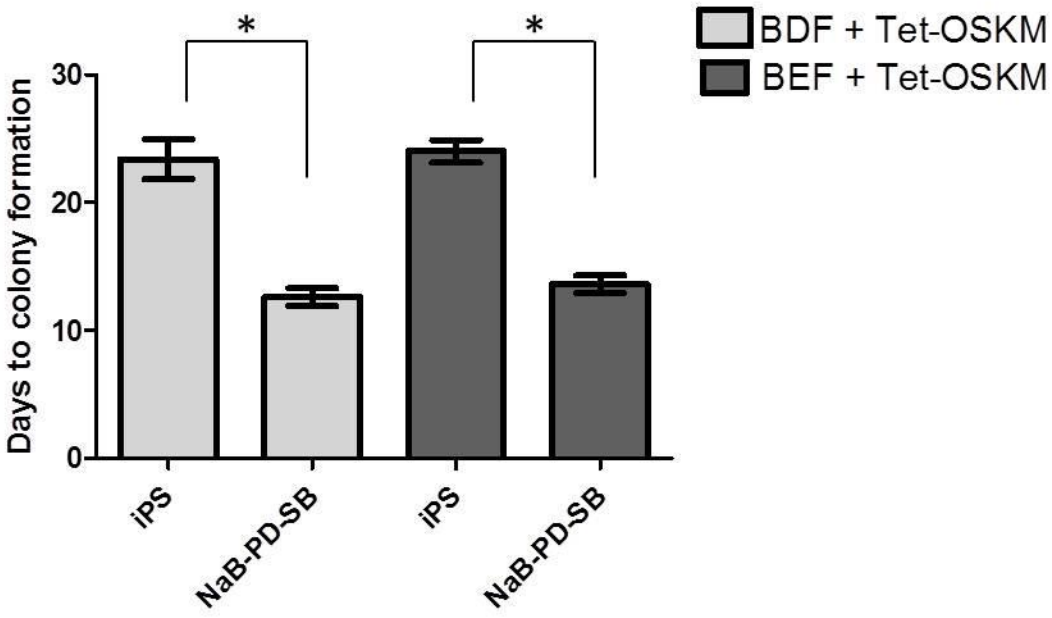

Figure 5-3: Time (days) to colony formation in Tet-OSKM transduced BDFs and BEFs when cultured in iPS media alone, or in iPS media supplemented with NaB-PD-SB. * indicates a significant difference between group means, P < 0.01, n = 5 biological replicates.

Colonies formed from cells cultured in NaB-PD-SB supplemented media showed more consistent cell morphology than those cultured in iPS media alone (Figure 5.4). Some colonies generated in the iPS media alone showed embryonic stem cell-like morphology, while others were irregularly shaped, heterogeneous or lacked clear borders (Figure 5.4A). These colonies were slow growing, and failed to expand following passaging. Colonies generated in NaB-PD-SB media showed consistent morphology, being composed of tightly compact small cells, with well-defined colony borders (Figure 5.4B). These colonies grew quickly, forming large, dome-shaped masses similar in appearance to embryoid bodies (Figure 5.4C).

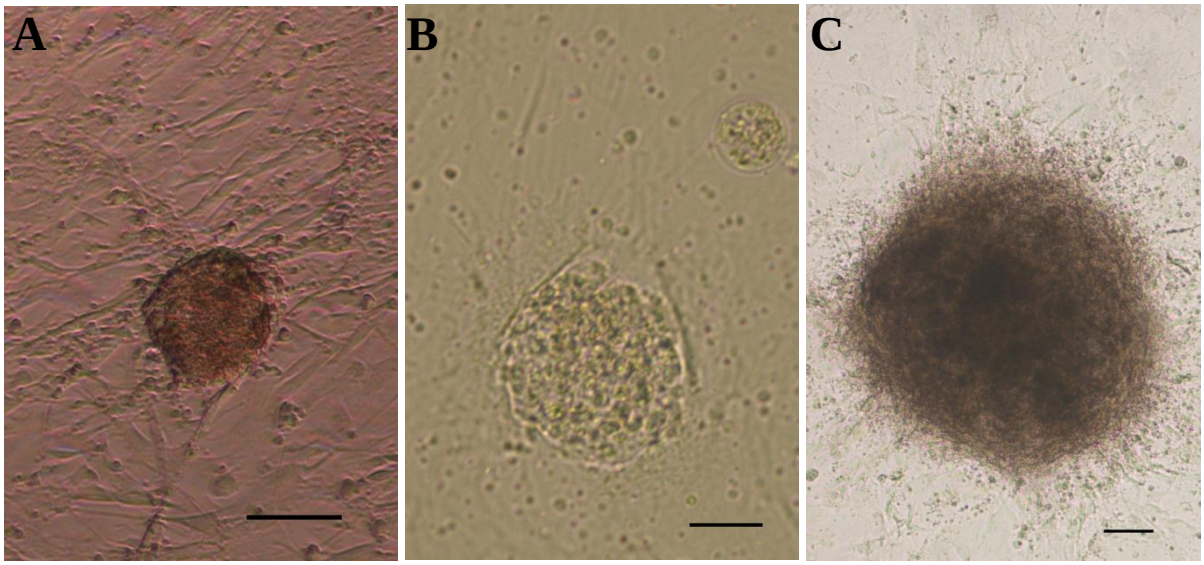

Figure 5-4: Colony morphology of Tet-OSKM transduced BDF in iPS media alone (A); NaB-PD-SB supplemented media at day 12 post induction (B); and in NaB-PD-SB supplemented media at day 21 post induction (C). Scale bars = 50 μm.

Alkaline phosphatase staining indicated that colonies formed in iPS media alone were negative for alkaline phosphatase activity. However, colonies generated in NaB-PD-SB supplemented media showed alkaline phosphatase activity at day 12 (Figure 5.5A), whereas alkaline phosphatase activity was absent in colonies 20 days after induction (Figure 5.5B).

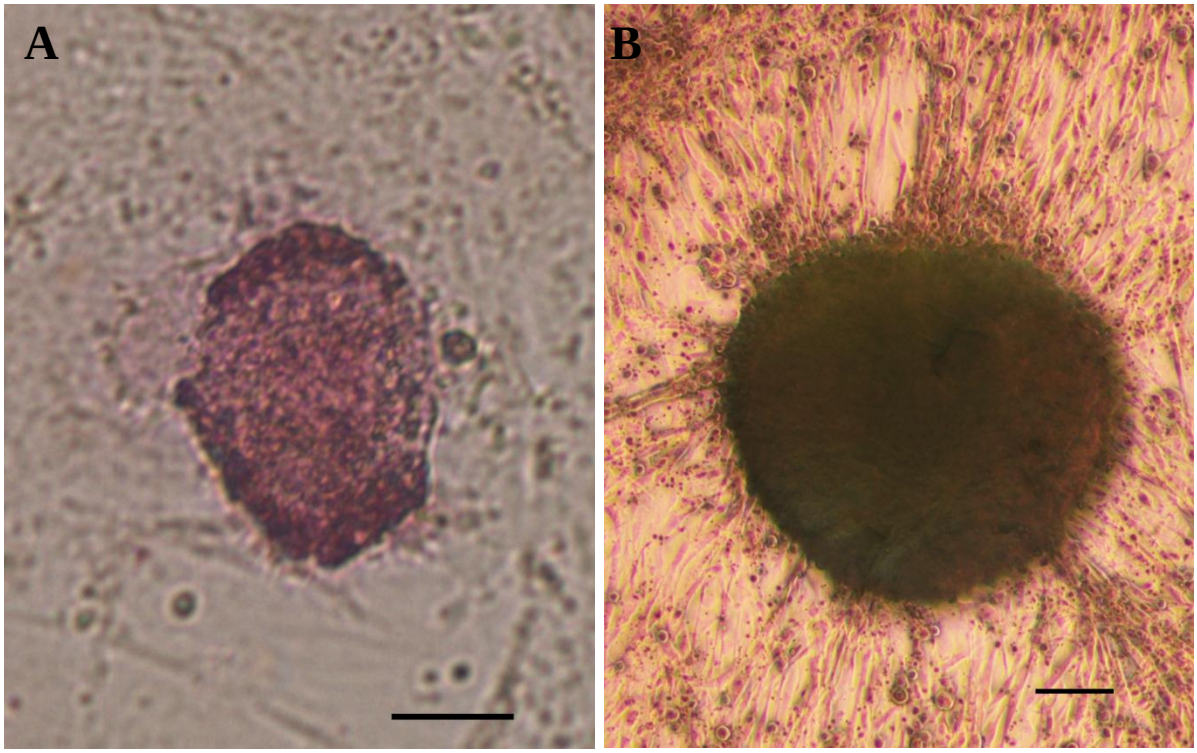

Figure 5-5: Alkaline phosphatase activity in Tet-OSKM transduced BDF in NaB-PD-SB supplemented media at day 12 (A: AP activity present) and day 20 (B: AP activity absent). Scale bars = 50 μm.

5.3.3. Characterisation of cell colonies obtained in NaB-PD-SB supplemented media

Colonies obtained from Tet-OSKM transduced BDFs and BEFs grown in NaB-PD-SB supplemented media were analysed by qRT-PCR to investigate expression of the pluripotency markers *NANOG*, *REX1* and *ALPL* (Figure 5.6). Colonies generated from Tet-OSKM transduced BDFs (Figure 5.6A) and BEFs (Figure 5.6B) both showed significantly increased gene expression of the pluripotency markers *NANOG* and *ALPL* compared to uninfected cells of the same type. No significant differences in *REX1* expression were observed between uninfected cells and cell colonies for either BDFs or BEFs.

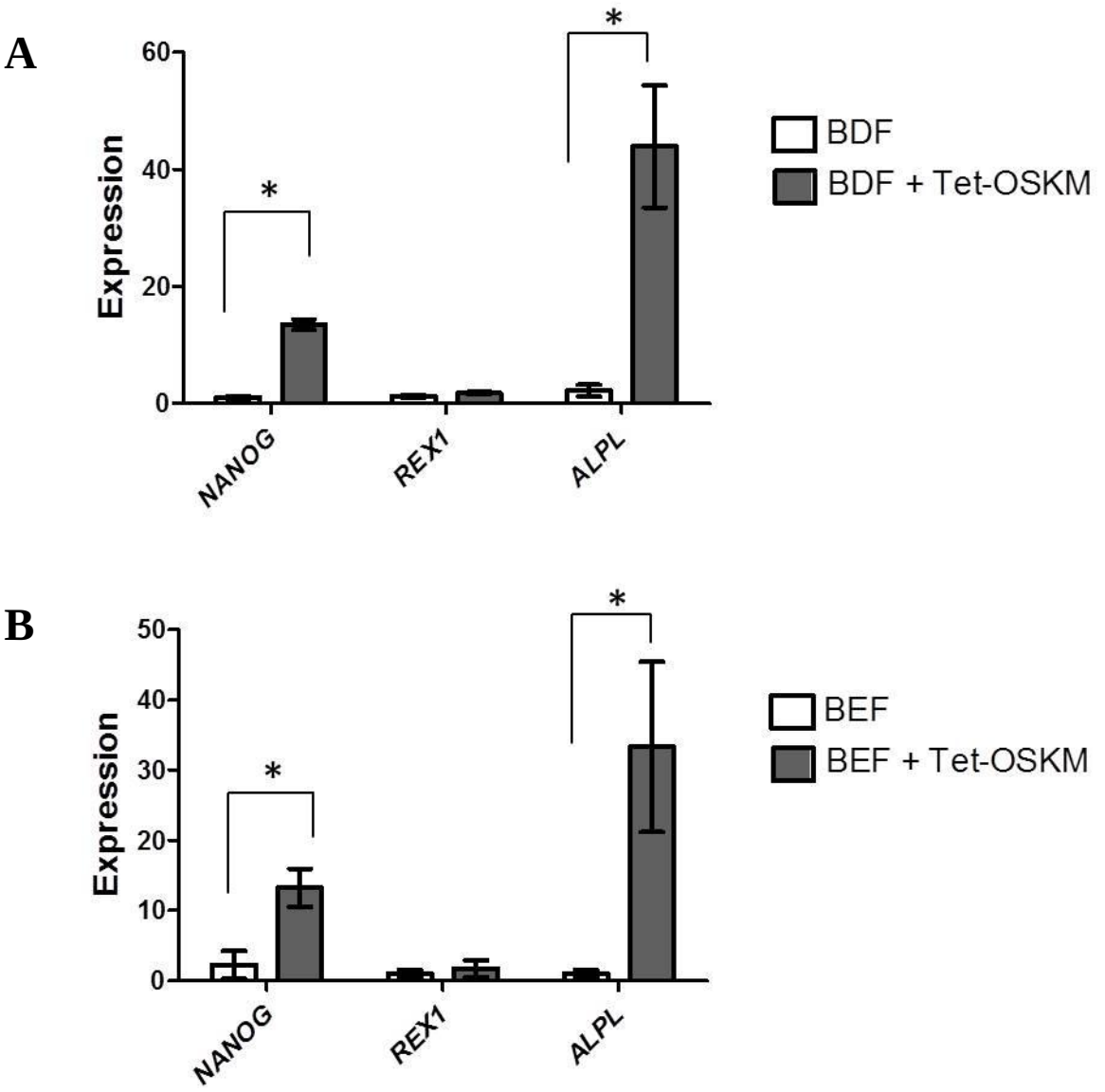

Figure 5-6: Expression of pluripotency markers *NANOG, REX1* and *ALPL* in Tet-OSKM transduced BDF (A) and Tet-OSKM transduced BEF (B) colonies. * indicates a significant difference in group means, P < 0.01, n = 2 biological replicates. Expression indicates fold change compared to the means of two reference genes.

Given the embryoid body-like appearance of colonies, qRT-PCR was used to investigate the expression of markers for the three embryonic germ layers (Figure 5.7). Both Tet-OSKM transduced BDFs (Figure 5.7A) and BEFs (Figure 5.7B) showed a significant increase in expression of the ectoderm marker *TUBB3* compared to non-transduced cells, indicating differentiation towards a neural lineage. There were no significant changes in

expression of the endoderm marker *AFP*, the mesoderm marker *DESMIN* or the fibroblast marker *THY1* in Tet-OSKM transduced BDFs or BEFs compared to non-transduced cells.

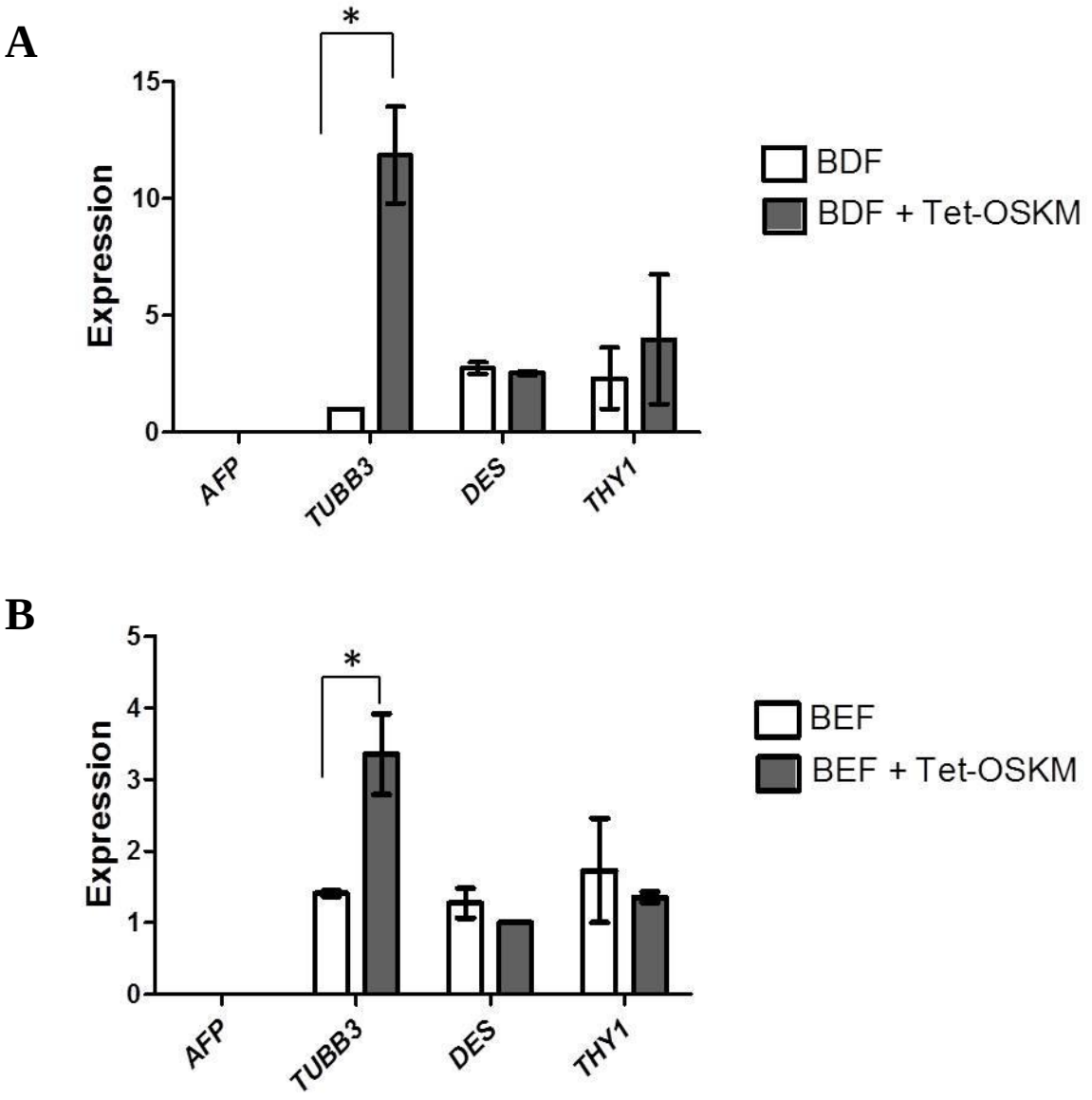

Figure 5-7: Expression of Endoderm (*AFP*), Ectoderm (*TUBB3*), Mesoderm (*DES*) and fibroblast (*THY1*) markers in Tet-OSKM transduced BDF (A) and Tet-OSKM transduced BEF (B) colonies. * indicates a significant difference in group means, P < 0.01, n = 2 biological replicates. Expression indicates fold change compared to the means of two reference genes..

5.3.4. Differentiation potential of reprogrammed bovine fibroblasts

To investigate the differentiation potential of Tet-OSKM transduced BDFs and BEFs, single colonies were mechanically picked and plated in non-adhesive dishes. The cells were allowed to differentiate for 7 days, by which time they had formed simple embryoid bodies (EBs; Figure 5.8).

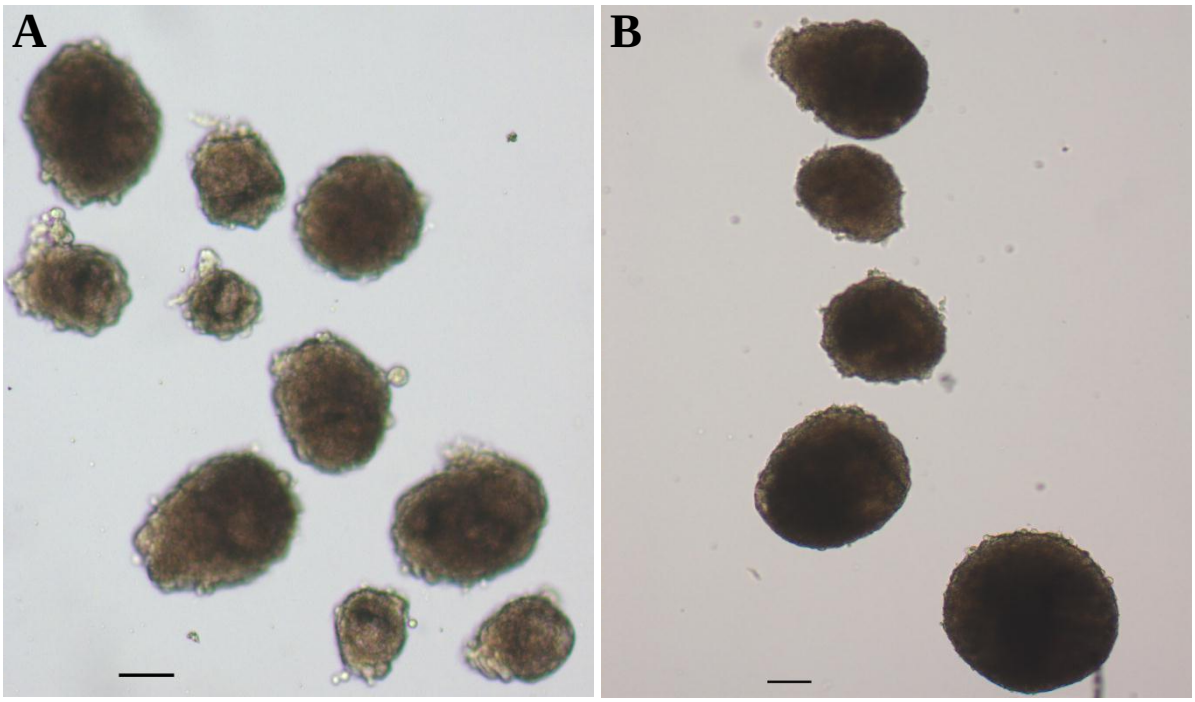

Figure 5-8: Simple embryoid bodies formed from Tet-OSKM induced BDFs (A) and BEFs (B), after 7 days. Scale bars = 100 μm.

Quantitative RT-PCR analysis of the EBs revealed that putative iPS cells from both BDFs and BEFs could differentiate into cell types indicative of the three embryonic germ lineages. EBs derived from BDF + Tet-OSKM showed increased expression of the endoderm marker *FOXA2*, the ectoderm markers *NESTIN* and *TUBB3*, and the mesoderm marker *DESMIN* (Figure 5.9A). EBs derived from BEF + Tet-OSKM showed increased expression of the ectoderm marker *TUBB3* and the mesoderm marker *DESMIN*, but no expression of the endoderm markers *FOXA2* or *AFP* (Figure 5.9B). Despite removal of doxycycline from

culture media used for EB formation, EBs formed from Tet-OSKM transduced BDF and BEF colonies showed significant ($P < 0.05$) expression of the Tet-OSKM transgene 7 days following doxycycline withdrawal.

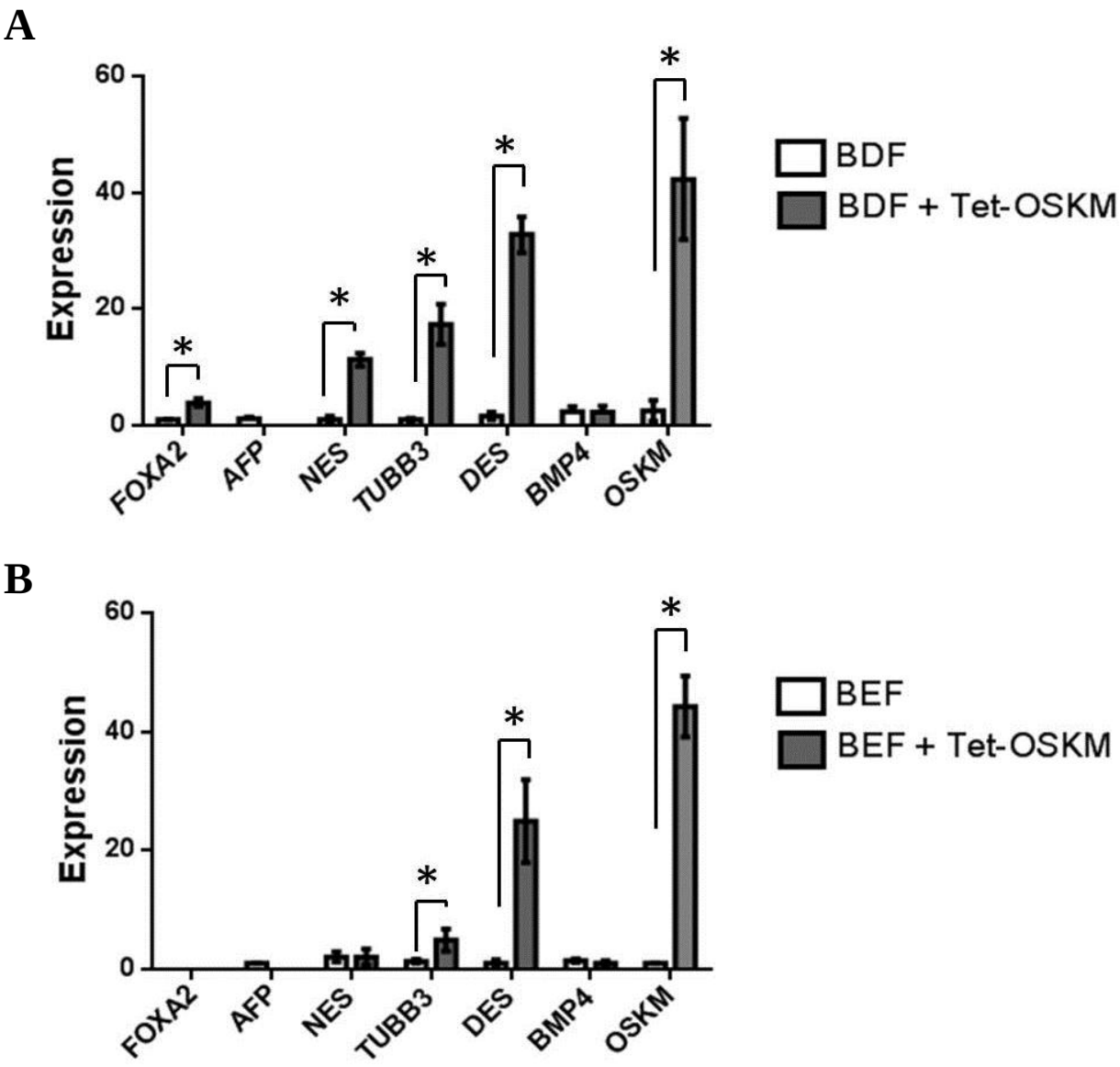

Figure 5-9: Differentiation potential of putative bovine iPS cells. Expression of Endoderm (*FOXA2, AFP*), Ectoderm (*NES, TUBB3*) and Mesoderm (*DES, BMP4*) markers by (A) BDF + Tet-OSKM and (B) BEF + Tet-OSKM EBs, compared to uninfected fibroblasts. * indicates a significant difference in group means, $P < 0.01$, n = 2 biological replicates. Expression indicates fold change compared to the means of two reference genes..

5.4. Discussion

To date, generation of fully reprogrammed iPS cells from livestock species has proven difficult. Previous efforts to reprogram bovine fibroblasts using a polycistronic drug-inducible vector resulted in cell colonies that exhibited some stem-cell like properties but did not display all of the characteristics of fully reprogrammed iPS cells (chapter 4). This chapter investigated whether the differentiation state of the target cells presented a barrier to reprogramming, by comparing the reprogramming potential of bovine adult and embryonic cells. This chapter also describes attempts to enhance the reprogramming process by modifying culture conditions with the inclusion of a cocktail of signalling pathway inhibitors.

The results of this chapter did not provide any evidence for a difference in reprogramming potential of adult versus embryonic bovine fibroblasts. Under the culture conditions examined both adult and embryonic fibroblasts exhibited the same potential for colony formation, with no difference detected in either colony number or time taken to form colonies. The colonies formed from both cell types expressed pluripotency markers, and appeared to undergo spontaneous differentiation towards a neural cell type, as indicated by the increased expression of the neural marker TUBB3. In general, younger or less differentiated cells are thought to be more amenable to the reprogramming process than older or more differentiated cells. Previous studies have shown that both bovine embryonic (Huang et al., 2011) and adult (Sumer et al., 2011) fibroblasts are able to be reprogrammed to a pluripotent state, but to date there has not been any comparison between the reprogramming efficiency of the two cell types. The results described in this chapter suggest that cell age may not be a factor that influences the reprogramming potential of bovine dermal fibroblasts. In contrast, human adult dermal fibroblasts have been shown to reprogram at a much lower efficiency than human ESC derived fibroblasts (Park et al., 2008), and mouse embryonic fibroblasts are more amenable to the reprogramming process than adult tail-tip fibroblasts

(Okada & Yoneda, 2011). It is possible that the two cell types used in this study were not far apart enough in age to show a difference in reprogramming potential. The embryonic fibroblasts used in this study are more developmentally mature than ESC derived cells, which may account for the inability to detect a difference in reprogramming potential when compared to adult fibroblasts. A difference in reprogramming efficiency similar to that seen in human cells may only be able to be detected when comparing ESC derived cells with adult cells. It would be interesting to compare bovine ESC derived fibroblasts with the embryonic and adult cells used in this study to determine whether the same difference in reprogramming efficiency exists.

The only detectable difference between the two cell types used in this study was in the differentiation potential of cell colonies. Embryoid bodies (EBs) formed from dermal fibroblast colonies were able to differentiate into cell types indicative of all three embryonic germ layers. EBs formed from embryonic fibroblast colonies showed expression of markers indicative of ecto- and mesodermal cell types, but did not express any markers indicative of an endodermal cell type. EBs formed from embryonic cell colonies also expressed only one ectodermal marker in contrast to the two expressed by adult cell EBs, and all of the lineage markers expressed by the embryonic cell EBs were expressed at lower levels than in the EBs derived from adult cell colonies. Collectively, these results suggest that cell colonies formed from embryonic fibroblasts have a slightly lower potential for differentiation than the cell colonies formed from adult fibroblasts. The reason for this is unclear, although there are a number of factors that can affect the ability of cells to differentiate into different lineages, including a failure to undergo transgene silencing, and the retention of epigenetic memory. It is possible that one of these factors could account for the difference in differentiation potential observed between BEF and BDF derived EBs. It has been shown that incomplete silencing of transgenes can limit the differentiation potential of reprogrammed cells

(Brambrink et al., 2008; Griscelli et al., 2012; Okada & Yoneda, 2011). Continued high levels of ectopic expression of reprogramming factors can completely eliminate the capacity of reprogrammed cells to differentiate (Brambrink et al., 2008). The experiments described in this chapter sought to overcome this potential difficulty by using an inducible transgene vector (Carey et al., 2009), effectively allowing the transgenes to be silenced by removal of doxycycline from the culture medium. However, the detection of transgene expression in EBs after removal of doxycycline indicated that the Tet-inducible system could be leaky. Therefore the lack of transgene silencing cannot be excluded as a factor that limits the differentiation potential of reprogrammed bovine cells. Studies have shown reprogrammed cells that retain residual DNA methylation signatures characteristic of their tissue of origin may favour differentiation towards cell types related to the tissue of origin, and be restricted in their potential to differentiate into cell types characteristic of other lineages (Bar-Nur et al., 2011; Kim et al., 2010; Kim et al., 2011; Polo et al., 2010). The results described here indicated that the reprogrammed cells demonstrated a propensity to differentiate into ectodermal cell lines in culture, and EBs formed from these cells appear to more readily differentiate towards ecto- and mesodermal cell types than to endodermal cell types. This may be a reflection of the epigenetic state of the cells. Both cell types used in this study are derived from the ectodermal lineage, therefore if they retain an epigenetic memory it would not be surprising that they may more readily differentiate into other cell types of this lineage rather than into cell types indicative of other lineages. The use of DNA altering molecules has been shown to be able to overcome the epigenetic memory of reprogrammed cells by promoting an ESC like DNA-methylation pattern (Huangfu et al., 2008; Mikkelsen et al., 2008). A follow on to this study could investigate the use of DNA methylation modulators to determine whether they may enhance the differentiation potential of reprogrammed bovine cells by overcoming the limitations imposed by cell epigenetic memory.

The use of the small molecule cocktail NaB-PD-SB as described in this chapter significantly increased the kinetics of the reprogramming process, with colony formation from Tet-OSKM transduced embryonic and adult fibroblasts occurring approximately 9 days earlier in supplemented iPS media than in iPS media alone. This is in agreement with the results of a study using the same cocktail of molecules in reprogramming of human cells (Zhang et al., 2011). An increase in the kinetics of the reprogramming process has also been observed when using various other combinations of molecules and also when using sodium butyrate alone (Liang et al., 2010; Lin et al., 2009; Mali et al., 2010; Shimada et al., 2012; Zhu et al., 2010). The acceleration of the reprogramming process when using these small molecules is most likely due to the effect of histone modification and/or inhibition of the TGFβ pathway, although further analysis would be required in order to confirm this hypothesis. HDAC inhibitors such as sodium butyrate may accelerate the reprogramming process by promoting a more relaxed chromatin state which facilitates binding of the transcription factors involved in the reprogramming process (Huangfu et al., 2008). TGFβ pathway inhibitors may accelerate the reprogramming process by promoting the MET that occurs early in the reprogramming process (Li et al., 2010). This study is the first time that this combination of molecules has been used in reprogramming bovine fibroblasts. The observation of increased kinetics of reprogramming, as seen when these molecules are used in other species, suggests that the key pathways involved in reprogramming are conserved between species. It remains to be determined whether the small molecules used in this study are sufficient to affect a change in kinetics when used alone, or whether this effect is only detected when these molecules are used in combination. Previous studies suggest that the largest effect is observed when small molecules are used in combination rather than alone (Zhang et al., 2011), but this is yet to be tested in reprogramming of bovine cells.

In addition to the decreased interval between transduction and colony formation, the NaB-PD-SB cocktail also appears to have had a significant effect on the completeness of the reprogramming process. Previous experiments using the same target cells, lentiviral vectors and reprogramming protocol in iPS media alone were insufficient to generate fully reprogrammed bovine cells. In those experiments, cell colonies that exhibited some stem cell-like characteristics were obtained, but those colonies failed to exhibit more stringent pluripotency characteristics such as alkaline phosphatase activity, expression of cell surface markers or expression of pluripotency markers such as NANOG. In this study, the addition of the small molecule cocktail NaB-PD-SB has allowed for the generation of cell colonies which exhibited alkaline phosphatase activity, expression of pluripotency marker NANOG, and differentiation into embryoid bodies expressing markers characteristic of all three embryonic germ layers. These results suggest that the components of the NaB-PD-SB cocktail have a significant effect on the reprogramming process, and may promote cells to undergo complete rather than partial reprogramming. This effect has also been observed in human cells, where use of the 3 molecule cocktail appeared to suppress the growth of partially reprogrammed cells while selectively promoting the generation of fully reprogrammed iPS cells (Zhang et al., 2011). Similar results have also been described when treating human cells with PD0325901 and SB431542 alone (Lin et al., 2009). This effect has also been observed in studies using sodium butyrate in the reprogramming of murine cells (Liang et al., 2010). It appears that the use of small molecules and signalling pathway inhibitors assist partially reprogrammed cells to overcome barriers that would otherwise inhibit them from achieving a fully reprogrammed state. Cells encountering one or more of these barriers will fail to undergo complete reprogramming, but can be rescued by the use of an appropriate inhibitor or inhibitors. This also explains why the most striking results have been achieved by using cocktails of small molecules and inhibitors rather than single factors

alone, as this allows multiple barriers to be overcome. Not all cells will necessarily encounter the same barriers, therefore a "shotgun" approach using multiple inhibitors and molecules appears to be a viable method to improve the efficiency of iPS cell generation across species by promoting reprogramming of cells that would otherwise fail to complete the reprogramming process.

Interestingly, although the NaB-PD-SB cocktail accelerated the kinetics and completeness of the reprogramming process, it did not appear to have an impact on the efficiency of reprogramming. There was no significant difference observed in efficiency between the supplemented and non-supplemented media, with both treatments yielding the same average number of colonies per plate. This is in contrast to results of studies carried out using human cells, where it has been shown that the addition of the NaB-PD-SB or other similar cocktails of small molecules significantly increased the reprogramming efficiency of these cells (Lin et al., 2009; Shimada et al., 2012; Zhang et al., 2011; Zhu et al., 2010). These molecules are thought to increase the efficiency of the reprogramming process in much the same way as they increase the kinetics: by facilitating transcription factor binding and aiding cells to progress through MET. The increase in efficiency observed in these studies is an increase in the percentage of colonies exhibiting ESC like characteristics such as alkaline phosphatase activity or expression of surface markers such as TRA-1-60, rather than an increase in the total number of colonies formed (Zhang et al., 2011). Due to the tendency of the reprogrammed bovine cells generated in this study to rapidly differentiate in culture, not all colonies were screened for alkaline phosphatase or surface marker activity, and instead total colony number was recorded. This variation in methodology may account for the lack of an increase in efficiency observed in this chapter.

Although the bovine iPS-like cells obtained in this study exhibited some of the characteristics of fully reprogrammed cells, there are still a number of issues that require

further examination. The vector, reprogramming protocol and culture conditions used in this chapter were sufficient to generate reprogrammed bovine cells; however, these cells could not be maintained in a pluripotent state under the stated culture conditions. Colony morphology, and the expression of neural markers indicated that these cells underwent spontaneous differentiation in culture shortly after reprogramming. The propensity of cells within colonies to spontaneously differentiate appeared to occur within seven days of the appearance of iPS-like colonies and suggested that long term culture and proliferation of these cells in a pluripotent state is not possible. Further investigation is required in order to define the culture conditions for the maintenance of pluripotent bovine cells. The results of this chapter have demonstrated the significant impact that alterations in culture media composition can have on the reprogramming process, with the addition of three compounds to the culture media resulting in faster and more complete cell reprogramming. There are a number of aspects of media composition that may be altered in order to help maintain cells in a pluripotent state for a longer period of time, including the base media, use of serum or serum replacement, and the addition of small molecules and/or signalling pathway inhibitors. This chapter has defined conditions sufficient to induce reprogramming in bovine somatic cells. The analyses carried out in this chapter were limited by the availability of material for analysis, given the low reprogramming efficiencies followed by spontaneous differentiation of cells. The next challenge is to define the optimal culture conditions for the expansion and maintenance of these cells in a pluripotent state. This would then allow for *in vivo* functional assays, such as teratoma formation, to be carried out, increasing the stringency of pluripotency testing and allowing for more thorough characterisation of these cells.

5.5. Conclusion

Bovine iPS cells have proven difficult to obtain using standard iPS protocols. This chapter has demonstrated that the use of embryonic rather than adult cells as the targets of reprogramming does not overcome reprogramming barriers. However, the use of a cocktail of small molecules and signalling pathway inhibitors does have a significant effect on the reprogramming of bovine fibroblasts. The use of the NaB-PD-SB cocktail not only accelerated the kinetics of the reprogramming process, but also promoted cells to undergo more complete reprogramming than when cultured without this cocktail. In contrast to the results of previous studies in human cells, this small molecule cocktail did not have an effect on the reprogramming efficiency of bovine cells. The cell colonies generated in this study show many of the characteristics of fully reprogrammed cells; however, further investigation is required to define the optimal culture conditions required to maintain these cells in a pluripotent state for long-term culture.

Chapter 6: General Discussion and Conclusion

6.1. General Discussion

The central aim of this thesis was to produce bovine induced pluripotent stem (iPS) cells with the potential to be differentiated towards a germline lineage *in vitro*. The ability to produce such cells could have a profound impact on livestock production as they could be used in, and improve the efficacy of, advanced reproductive technologies including testis stem cell transplantation in cattle. In order to be able to identify germline cells derived from *in vitro* culture it was first necessary to characterise bovine spermatogonial stem cells (SSCs) and identify molecular markers for these cells. To this end, this thesis examined the expression of established spermatogonial markers in the bovine testis, and developed a platform that was used for screening candidate spermatogonial markers. A number of potential markers for bovine spermatogonia were identified, which can then be examined more closely to determine which type/s of spermatogonia they identify. The use of standard iPS methods for reprogramming bovine somatic cells to pluripotency was then explored, but did not result in fully reprogrammed cells. However, this paved the way for further reprogramming experiments employing alternative culture conditions. Subsequent experiments using a cocktail of small molecules and signalling pathway inhibitors greatly enhanced the reprogramming of bovine somatic cells.

A limitation to the use of SSCs in reproductive technologies such as testis cell transplantation is the lack of specific markers that can be used for the identification and/or purification of these cells. Studies in rodents and humans have identified a number of spermatogonial markers that can be used to create enriched, but not pure, SSC populations (Aponte et al., 2005; Hamra et al., 2004; Oatley & Brinster, 2006). However, much less is known about SSCs and their niche in the bovine testis. Some molecular markers appear to be

conserved between species, while other markers appear to be species specific, thus any potential markers must be tested in the species of interest. The experiments described in chapter 2 enabled the development of a method for screening potential markers of bovine SSCs by comparative analysis of gene expression levels in SSC enriched and depleted cell populations. Previously, potential molecular markers would generally be assessed by use of immunocytochemical analysis. The methods described in chapter 2 can be used to conduct an initial screen of multiple candidate markers, and identify candidates that need to undergo further analysis. These methods can also be used to test putative markers for which no antibody exists, a problem that arises when working in species other than human and mouse, as epitopes can vary between species.

The screening platform described in this thesis was used to confirm that a number of spermatogonial markers identified in other species are conserved markers of spermatogonia in the bovine testis. These findings contribute to current knowledge of bovine spermatogonia, and should aid in future manipulation of these cells. Studies in mice have shown that highly purified SSC populations can be generated by using a combination of positive and negative selection against a number of spermatogonial markers (Shinohara et al., 1999). The identification of an increased number of bovine spermatogonial markers improves the chances that such a strategy could also be used to generate highly purified SSC populations from the bovine testis. It has been demonstrated that one of the most effective ways to increase the efficiency of testis cell transplantation experiments is to transplant a cell population enriched for SSCs (Shinohara et al., 1999). To date some methods have been described for the enrichment of bovine SSCs (Herrid et al., 2009; Izadyar et al., 2002), however these methods in general do not produce highly enriched cell populations, and could be improved by the identification of additional SSC markers. In addition to allowing for purification or enrichment of SSCs, increased knowledge of bovine spermatogonial markers

will allow for more effective identification of SSCs in cell cultures systems. This includes being able to identify pluripotent cells that have undergone germline differentiation in cultures. The *in vitro* differentiation of pluripotent cells towards the germline has potential to provide an alternative source of SSCs. Without a comprehensive knowledge of SSC markers, determining the success of *in vitro* differentiation experiments would be problematic.

A number of previously unidentified putative markers of bovine spermatogonia were identified in chapter 2. The identification of cell surface markers is of particular interest to enable physical selection of spermatogonia, therefore one of these putative markers, the membrane protein CLDN8, was selected for further analysis. The results presented in chapter 3 demonstrated that CLDN8 is expressed in the bovine testis. CLDN8 is expressed by a subset of bovine spermatogonia, and also by a subset of Sertoli cells, and it appears that there is an interaction between these two cell populations. CLDN8 can therefore not be used as a sole marker for the identification and/or purification of SSC populations from mixed cell populations, as selection for CLDN8 expressing cells will select for Sertoli cells in addition to spermatogonia. Other members of the claudin protein family have been described in the testis, where they play a role in the formation of tight junctions in the blood-testis barrier (BTB) (Morrow et al., 2010; Pelletier, 2011). These results suggest that CLDN8 plays a role in the bovine testis outside of the BTB, and led to the hypothesis that CLDN8 may have a role in the maintenance of bovine SSCs in the stem cell niche. SSC self-renewal and division is regulated by the stem cell niche microenvironment, and Sertoli cells have been demonstrated to be the main support cells of the SSC niche (Oatley et al., 2010). Adhesion molecules, expressed by both SSCs and Sertoli cells, are among the factors that have been shown to play a role in the maintenance of SSCs in the stem cell niche (de Rooij et al., 2008; Kanatsu-Shinohara et al., 2008; Marthiens et al., 2010). Characterisation of the SSC niche and the factors involved in stem cell maintenance is important for the development of *in vitro*

culture protocols for these cells. Once removed from the stem cell niche and placed into artificial culture systems SSCs tend to either differentiate or undergo apoptosis (Aponte et al., 2008; Izadyar et al., 2003; Nasiri et al., 2011). In order to maintain a normal balance of self-renewal and differentiation in *in vitro* culture it is important to mimic as closely as possible the *in vivo* stem cell environment. This knowledge is also important for the success of testis cell transplantation experiments. The success of such experiments relies on the transplanted stem cells correctly homing to and colonising available niches once in the recipient testis. Increased understanding of the SSCs and their niche environment may aid in identifying factors that may enhance the survival of these cells during the homing and colonisation processes.

Reproductive technologies such as testis cell transplantation in livestock require significant numbers of SSCs to be effective (Hill & Dobrinski, 2006). However, the long term maintenance and expansion of bovine SSCs in *in vitro* culture systems has proven problematic (Aponte et al., 2008; Izadyar et al., 2003; Nasiri et al., 2011). An alternative approach to generating the required cell numbers is to produce SSCs *in vitro* from pluripotent stem cells. Induced pluripotent stem cells can be produced by reprogramming somatic cells to an embryonic stem (ES) cell-like state by the overexpression of a set of transcription factors (Takahashi & Yamanaka, 2006). The advantage of iPS cells over ES cells is that iPS cells are patient specific, and can be generated from somatic cells that can be harvested with little or no harm to the individual. Despite advances in iPS cell technology, iPS cells have proven difficult to establish in some species, including livestock species. During the preparation of this thesis two reports of the generation of iPS cells from bovine cells were published (Huang et al., 2011; Sumer et al., 2011). These two reports employed significantly different strategies, and although both generated cells that appear to show characteristics of fully reprogrammed iPS cells, in both cases these cells failed to undergo transgene silencing,

indicating that pluripotency was likely reliant on endogenous rather than exogenous gene expression. This has been a recurring problem in reprogramming livestock cells, with only one recent report describing iPS cells from a livestock species that have undergone transgene silencing (Liu et al., 2012). At present it appears that, although there has been some success in producing bovine iPS, the optimal methods and conditions for generating these cells have yet to be defined. Standard protocols exist for reprogramming human and murine cells (Okita, Hong, Takahashi, & Yamanaka, 2010; Park, Lerou, Zhao, Huo, & Daley, 2008), while no such protocols exist for reprogramming livestock cells. Defining a standard set of conditions necessary for reprogramming these cells will pave the way for iPS cells from these species to be used in a wide range of applications. Until these cells can be routinely and consistently produced, their use in applications such as the production of transgenic livestock will remain theoretical rather than applied.

Chapter 4 describes attempts to produce bovine iPS cells using standard iPS methods. Although these methods were sufficient to produce cell colonies exhibiting some stem cell-like characteristics, they did not result in the generation of fully reprogrammed bovine iPS cells. There are a number of factors that influence the success of reprogramming experiments, including the target cell type, the reprogramming factors used, the method of delivery of reprogramming factors, and the basic cell culture conditions (Maherali & Hochedlinger, 2008). In addition, a number of barriers to the reprogramming process have been described, and the failure of cells to overcome one or more of these barriers will result in cells that either do not begin, or are unable to complete, the reprogramming process. There are a number of aspects of the reprogramming protocol employed in this thesis that could have contributed to the generation of only partially reprogrammed cells. These include the use of murine transcription factors in a bovine system, use of a polycistronic vector, use of adult dermal fibroblasts as target cells, and the basic cell culture conditions used. Further investigation into

each of these individual components is required in order to establish which aspect/s of this protocol are preventing the generation of fully reprogrammed cells. The basic cell culture conditions, including media composition, are of particular interest. Culture conditions for iPS cell generation are usually based on the culture conditions required for ES cell maintenance in the species of interest. However, at present, the conditions required for the long term maintenance of bovine ES cells are not known (Gong et al., 2010; Mitalipova et al., 2001; Saito et al., 2003; Wang et al., 2005). Instead, mouse or human ES cell culture conditions are used in attempts to generate bovine iPS cells. It is possible that these conditions are sub-optimal for the generation iPS cells from livestock species. Further research into cell culture conditions and media composition may provide insights into the optimal conditions for the generation and maintenance of bovine iPS cells. Such research also has implications in enhancing the derivation of ES cell lines from such species.

Chapter 5 describes experiments designed to test whether alterations to the reprogramming protocol used could enhance the success of reprogramming experiments. There are a number of small molecules and signalling pathway inhibitors that have been shown to increase the efficiency of iPS cell generation. In addition to increasing efficiency, a number of these molecules appear to be able to overcome reprogramming barriers and aid in the conversion of partially reprogrammed cells into completely reprogrammed iPS cells. The results showed that a combination of three small molecules/inhibitors could significantly enhance the success of reprogramming experiments involving bovine somatic cells, resulting in the generation of cell colonies that showed most of the characteristics of pluripotent stem cells. The small molecules used in this study represent only a subset of molecules that have been shown to impact on cell reprogramming. There are numerous other molecules, and combinations of molecules, that may also increase the success of iPS generation in the bovine. Further research is required to determine if more effective combinations exist.

However, it is clear that alterations to a single aspect of the reprogramming protocol can have a significant effect on the success of reprogramming experiments. There are a number of other aspects of the reprogramming protocol that have not been examined in this thesis, including the use of individual vectors versus a polycistronic vector to deliver transgenes, the use of a variety of target cell types, employing bovine or human transcription factors in place of murine factors, and use of alternate methods of transgene delivery. Modifications to one or more of these aspects may also have a dramatic impact on the success of reprogramming experiments. Further research is required to determine if further alterations to the reprogramming protocol and/or the cell conditions can further enhance the success of bovine iPS cell generation.

The results described here highlight opportunities for future research in a number of areas. Firstly, the methods described in chapter 2 provide an opportunity to efficiently screen multiple candidate markers for bovine SSCs and identify candidates that should undergo further analysis. These methods have already been used to identify a number of putative markers that should undergo further characterisation. One of these candidates, CLDN8, was chosen for further examination in chapter 3. The other putative markers identified have yet to undergo further analysis. Further analysis of CLDN8 as a potential marker for bovine SSC has raised questions as to a possible role for this protein in the SSC niche. Functional experiments are needed to confirm and/or define such a role. This thesis also raises a number of issues requiring further investigation with regards to the generation of bovine iPS cells. This thesis has described conditions sufficient to generate iPS-like cells that display most of the characteristics of pluripotent stem cells. However, these cells are produced at low efficiency, and are unable to be maintained in a pluripotent state for long term culture. Further research is required to better define the conditions required for both the generation and maintenance of bovine iPS cells.

6.2. Conclusions

This thesis has demonstrated that bovine postnatal somatic cells can be reprogrammed to a state of pluripotency. Further research is required to determine the conditions allowing the long-term maintenance of these cells in culture. To support the ultimate goal of germline differentiation of bovine pluripotent cells this thesis has contributed to the characterisation of spermatogonial stem cells and the spermatogonial stem cell niche in the bovine. The methods described herein can be used to identify further spermatogonial stem cell markers and thus aid in improving the identification and purification of bovine spermatogonia.

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