Investigations of Methods for Non-Viral Generation of Bovine Cells to Pluripotency, With a View on Potential Use in Reproductive Technologies

Aimee Maree Grace

BRuSc (Hons I) University of New England 2011

A thesis submitted for the degree of Doctor of Philosophy of the University of New England

Submitted June 2014

Acknowledgements

I would like to acknowledge the financial support I have received from the University of New England and CSIRO Animal Food and Health Sciences (previously Livestock Sciences) as part of a joint UNE/CSIRO PhD Scholarship. I would also like to acknowledge funding received for the project though the CSIRO Food Futures National Research Flagship. Additionally, I would also like to recognise the generous financial support I received from the University of New England though the Keith & Dorothy Mackay Postgraduate Travelling scholarship that allowed me to attend an international conference that was an indispensable experience.

I would like to offer my most sincere thanks to my principle supervisor, Dr Sabine Schmoelzl. I could not have completed this thesis if it weren't for her continuous support, motivation, advice and understanding throughout the course of my PhD. I would also like to thank my associate supervisors, Dr Geoffrey Hinch and Dr Nicholas Andronicos, for your enthusiasm, insightful comments, and hard questions. I would also like to thank Dr Ian Cassady for all his input and assistance in the first year of my PhD, it was greatly appreciated.

To the staff and students at Chiswick, thank you for the friendships and the daily advice provided and interest you have taken in my project. A special mention must be made to Dr Peter Hunt, Jody McNally, Rhonda Davy and Dr Mary McMillan, and Grant Uphill, for the time you have all taken to help me with various aspects of my thesis, and for imparting your invaluable knowledge to me, I am so grateful, and will miss seeing you all regularly.

My most sincere thanks to Janet and my mother for helping me edit this thesis; I appreciate the time you both took out of your busy lives to help me more than you can imagine.
I would also like to thank all my friends and family for your encouragement, constant support, and for keeping me sane for the last three years; I promise I will now become social again! I wish to dedicate this thesis to my father, who gave me the ambition to follow my dreams and provided me the opportunity to do whatever I wanted to do. A special thankyou to my mother and sister, I could not have achieved any of this without your strength, love and encouragement. Finally, my most heartfelt thankyou to James, I cannot express how much I have valued your support, patience and that even though it has been stressful you have made me laugh several times a day during these last few months. I have no doubt that you have maintained my sanity; I could not have done this without you.

Abstract

Spermatogonial stem cells (SSCs) from livestock species have the potential to be used for reproductive technologies and for the production of transgenic animals. The isolation of a relatively pure population of SSCs from livestock species has proven difficult; however, the recent advances in the production of induced pluripotent stem (iPS) cells may provide an alternative source of SSCs through the differentiation of iPS cells toward the germline.

The general aim of this thesis was to produce bovine iPS cells that may be differentiated toward the germ line for potential use in reproductive technologies such as germ cell transplantation. Due to difficulties in isolating a pure population of bovine SSCs, methods to improve the enrichment of these cells are of interest in order to improve the success of bovine germ cell transplantation. A number of different methods to enrich bovine spermatogonia were trialled to determine which of the method for enrichment was most effective. The combination of enrichment by differential plating followed by separation of cells by discontinuous Percoll gradient centrifugation, was found to isolate the most enriched population of un-differentiated spermatogonia.

Additionally, the ability to better identify and characterise bovine testis cells, specifically SSCs, by the identification of new markers that identify SSCs or different sub-sets of spermatogonia in the bovine testis, would also contribute to knowledge of characterisation of the cells and may provide better methods for enrichment. Towards the first goal, the potential markers for bovine SSCs previously identified by a global proteomic screen and comparative analysis of gene expression levels in SSC enriched and depleted cell populations, DDX6, NAP1L4 and TKTL1, were investigated by gene expression analysis and immunohistochemistry. The three markers were shown to identify different stages of spermatogenesis.

Examining methods to produce bovine iPS cells, different combinations of reprogramming factors resulted in differences in reprogramming efficiency as expected. Encouragingly, bovine dermal fibroblasts showed comparable reprogramming potential to bovine embryonic fibroblasts. Non-viral methods of transformation proved to be an effective alternative to viral transduction, with a commercially available non-integrating minicircle system providing particularly promising results. A systematic investigation of the effects of small molecules on reprogramming events identified combinations with significant effects on reprogramming efficiency and pluripotency gene activation. Though the cells described in this thesis are not considered to be fully reprogrammed, they are described as being putative iPS cells, referring to their characteristics being iPS cell ‘like’.

In vitro differentiation of the generated putative iPS cells lead to expression of some markers expressed by the germline which is a promising result for future research.

Candidate's Certification

I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

I certify that any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

Aimee Maree Grace

June 2014

Table of Contents

List of Figures

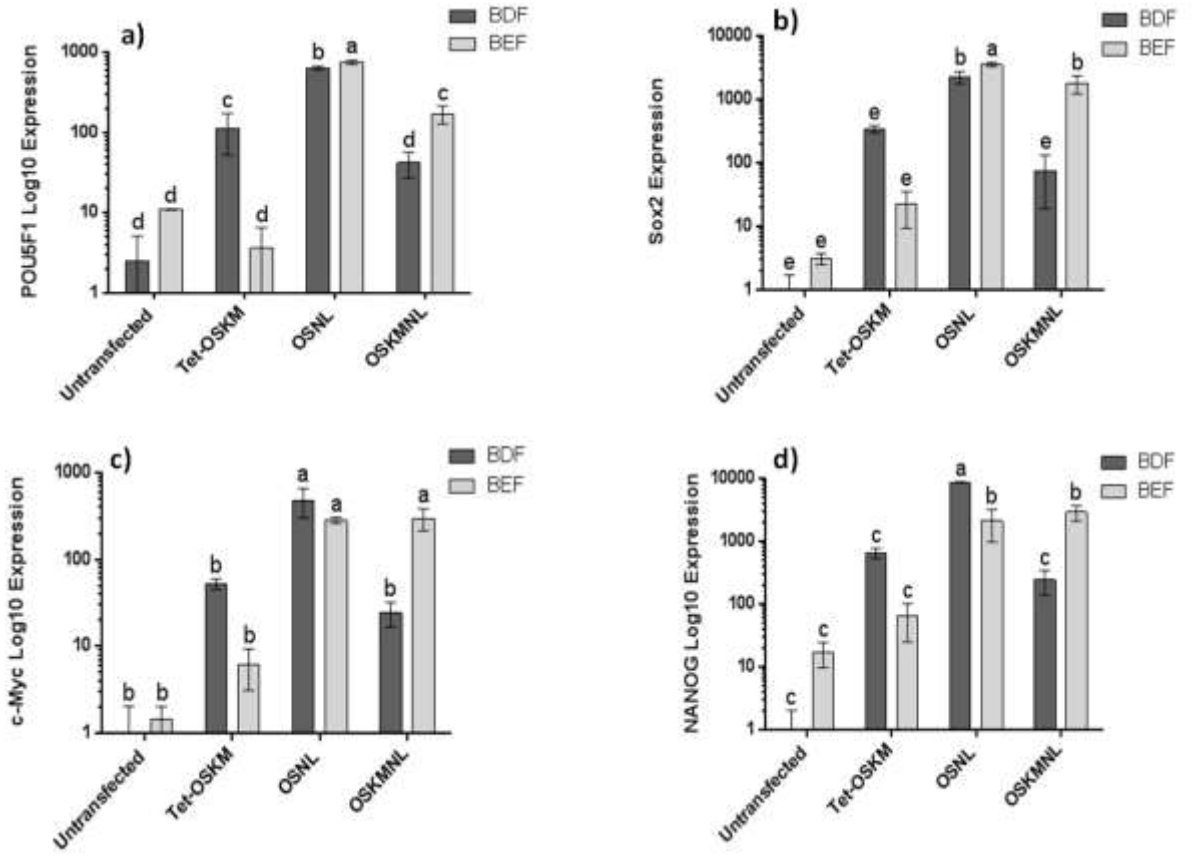

Figure 4-8. Log10 Expression of pluripotency markers relative to housekeeper gene *RPS26*, in putative bovine iPS colonies, produced by lentiviral transduction of reprogramming constructs coding for Tet-OSKM, OSNL or OSKMNL. (a) *POU5F1* (OCT4), (b) *SOX2*, (c) *c-MYC* and (d) *NANOG*. Different characters above treatment means indicate a significant difference between expression of pluripotency markers (P<0.05 n=3).Error bars represent as ± SEM. 131

List of Tables

List of Abbreviations

BDF: Bovine dermal fibroblast

BEF: Bovine embryonic fibroblast

bFGF: basic fibroblast growth factor

BMP: Bone morphogenetic protein

BTB: Blood-testis barrier

DBA: *Dolichos biflorus* agglutinin

DMEM: Dulbecco's Modified Eagle Medium

Dox: Doxycycline

DPBS: Dulbecco's phosphate buffered saline

EB: Embryoid body

ES cell: Embryonic stem cell

FACS: Fluorescent activated cell sorting

FBS: Foetal bovine serum

GFP: Green fluorescent protein

HDAC: Histone deacetylase

ICM: Inner cell mass

iPS cell: Induced pluripotent stem cell

LIF: Leukaemia inhibitory factor

MACS: Magnetic activated cell sorting

MDF: Modified Davidsons Fixative

MET: Mesenchymal-to-epithelial transition

MEM: Minimum Essential Medium

Mit-C: Mitomycin C

NaB: Sodium Butyrate

NEAA: Non-essential amino acids

OSKM: POU5F1 (OCT4); SOX2; KLF4; c-MYC

OSKMNL: POU5F1 (OCT4); SOX2; KLF4; c-MYC; NANOG; LIN28

OSNL: POU5F1 (OCT4); SOX2; NANOG; LIN28

PD: PD0325901 (Small Molecule)

PCR: Polymerase chain reaction

PGC: Primordial germ cell

qRT-PCR: Quantitative Reverse transcribed polymerase chain reaction

RA: Retinoic acid

SB: SB431542 (Small Molecule)

SCNT: Somatic cell nuclear transfer

SEM: Standard error margin

SCF: Stem cell factor

SSC: Spermatogonial stem cell

TBS: Tris buffered saline

Tet: Tetracycline

VPA: Valproic acid

Gene Synonyms

Gene Symbol	Synonym	Gene Name
c-MYC		v-Myc avian myelocytomatosis viral oncogene homolog
DDX4	*VASA*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4
DDX6	*RCK/p54*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6
DES		Desmin
FOXA2		Forkhead box A2
FSCN1	*FAN1, HSN, SNL*	Fascin 1, 55 kDa actin-bundling protein, Singed-like protein, p55
GATA4		GATA binding protein 4
IQGAP1	*KIAA0051*	Ras GTPase-activating-like protein IQGAP1
KLF4		Kruppel-like factor 4
NANOG		Nanog homeobox
NAP1L4		Nucleosome assembly protein 1-like-4
NES		Nestin
LIN28	CSDD1, LIN28A, ZCCHC1	Zinc finger CCHC domain-containing protein 1
POU5F1	*OCT4*	POU class 5 homeobox 1
SOX2		SRY (Sex determining region Y) box 2
TKTL1		Transketolase-like 1
TLN1		Talin 1
TUBB3		Tubulin, beta 3 class III
UCHL1	*PGP9.5*	Ubiquitin carboxyl-terminal esterase L1
VIM		Vimentin
ZBTB16	*PLZF*	Zinc finger and BTB domain containing 16