EPIGENETIC REPROGRAMMING OF SOMATIC CELLS BY ZYGOTIC FACTORS

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Abstract

Cloning cattle using somatic cell nuclear transfer (SCNT) is an inefficient process, with approximately only 5% of transferred embryos developing to live offspring. SCNT produced cattle have a high mortality rate due to a number of developmental abnormalities caused mainly by the incorrect epigenetic reprogramming of the donor cell to a pluripotent state. Conventional bovine SCNT involves fusion of a somatic donor cell into an enucleated metaphase II (MII) arrested oocyte. The resulting embryo is cultured to the blastocyst stage before being transferred to a surrogate cow to produce live offspring. Zygotes were initially thought to be unsuitable as SCNT recipients in bovine, until it was revealed that using telophase II (TII) zygotes as opposed to MII oocytes results in improved *in vivo* development. Metaphase zygotes have also been used successfully as SCNT recipients in mouse to produce cloned blastocysts and it has been proposed that reprogramming factors sequester in the pronuclei of interphase zygotes. Little is known about the nature the nuclear reprogramming, however a few candidate reprogramming factors have emerged recently. TCTP is known to activate key pluripotency genes (*POU5F1* and *NANOG*) in somatic cell nuclei. It has also been identified as present in bovine oocytes with a high potential to reprogram somatic cells. Reprogramming of cell nuclei by *Xenopus* egg extract has found to require BRG1. Immunodepletion of BRG1 was shown to decrease the reprogramming ability of the egg extract, whist its over-expression increased reprogramming potential. HDAC1 has been found to initiate a transcriptionally repressive state in preimplantation mouse embryos possibly inhibiting transcription of reprogramming factors. Knockdown of HDAC1 using TSA is known to increase development of mouse NT embryos.

The aim of this study was to produce blastocysts using metaphase zygotes as recipients for SCNT in bovine. In addition, localisation and abundance of candidate reprogramming factors TCTP (*TPT1* gene), BRG1 and HDAC1 were examined in MII oocytes as well as TII, interphase and metaphase zygotes.

This study found metaphase zygotes are unsuitable as recipients for bovine SCNT using current methodologies, possibly due to the premature cleavage of the embryos. Control embryos produced using MII oocytes as recipients developed to blastocyst with an efficiency of ~ 11%. mRNA analysis of zygotes and oocytes did not reveal any significant differences in the relative concentrations of *TPT1* or *BRG1* between the samples. The TCTP and HDAC1 proteins showed a similar pattern of localisation in the MII oocytes and all stages of zygotes. Both proteins clearly localise to the maternal chromatin in the second polar body of TII zygotes. This finding has never previously been described and may in part explain why there is increased cloning efficiency observed when using TII zygotes as SCNT recipients.

This increased understanding of these reprogramming factors may increase our knowledge of the processes which occur during NT and lead to greater efficiency of bovine cloning for commercial and therapeutic purposes.

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List of Abbreviations

(v/v)	Volume per Volume
(w/v)	Weight per Volume
2D-PAGE	Two Dimensional Polyacrylamide Gel Electrophoresis
8c	8 Cell
B1-3	Blastocyst Grade 1-3
B199	Bicarbonate-buffered M199
BRG1	Brahma-Related Gene 1
BSA	Bovine Serum Albumin
CB	Cytochalasin B
cDNA	Complementary Deoxyribose Nucleic Acid
CHX	Cycloheximide
COC	Cumulus-Oocyte Complex
Cp	Copy Number
DEPC	Diethyl Pyrocarbonate
DMAP	Dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide Triphosphate

DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetra-acetic Acid
EGA	Embryonic Genome Activation
ESC	Embryonic Stem Cells
ESOF	Early Synthetic Oviduct Fluid
F12	Nutrient Mixture F-12
FCS	Fetal Calf Serum
g	Relative Gravitational Force
GCAP	Germ Cell Alkaline Phosphatase
GV	Germinal Vesicle
GVBD	Germinal Vesicle Break Down
h	Hours
H199	Hepes-buffered M199
H4K5	Histone H4 Lysine 5
HDAC1	Histone Deacetylase 1
hpf	Hours Post Fertilisation
HSOF	Hepes-buffered Synthetic Oviduct Fluid
ICC	Immunocytochemistry
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* Maturation
IVP	*In vitro* Production
KDa	Kilo Dalton

LCMM	LightCycler® Master Mix
LSOF	Late Synthetic Oviduct Fluid
M199	Medium 199
MBT	Midblastula Transition
M-Cdk	Mitosis Cyclin Dependent Kinase
MII Equi	MII Equivalent
MII	Metaphase II
mmHg	Millimetres Mercury
mRNA	Messenger Ribonucleic Acid
M-Zygote	Metaphase Zygote
NEBD	Nuclear Envelope Break Down
NT	Nuclear Transfer
NTC	No Template Control
osm	Osmolarity
P	Probability
p21	Cyclin Dependent Kinase Inhibitor
PAC	Pre-Activated Cytoplast
PB	Polar Body
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PPB	Pseudo Polar Body
PPHH	Pyruvate, Penicillamine, Hypotaurine, Heparin

PVA	Polyvinyl Alcohol
R.U	Relative Units
RNA	Ribonucleic Acid
RNAi	Interfering Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per Minute
RT	Reverse Transcriptase
SCNT	Somatic Cell Nuclear Transfer
SOF	Synthetic Oviduct Fluid
TCTP	Translationally Controlled Tumour Protein
TD	Total Development
TII	Telophase II
TM	Tight Morulae
TNT	Telophase II Nuclear Transfer
TPT1	Translationally Controlled Tumour Protein 1
U.V	Ultra Violet
V	Volts
w/o	Washout

LITERATURE REVIEW AND INTRODUCTION

1. Introduction

1.1 General introduction

Cloning cattle using Somatic Cell Nuclear Transfer (SCNT) is an inefficient process, where the surviving offspring only represent approximately 5% of the embryos transferred into the surrogate cow (Oback and Wells, 2003). Nuclear transfer (NT) involves adult donor cells being attached to enucleated MII oocytes (cytoplasts) then electrically fused at room temperature before placement of the fused couplets into *in vitro* culture drops (Schurmann *et al*., 2006). These clones have a high mortality rate due to suffering a number of developmental abnormalities such as higher birth weights, muscular-skeletal problems and incorrect placental formation (Wells *et al*., 2004). At the cellular level, a higher incidence of apoptosis (Park *et al*., 2004) and aberrant allocation of inner cell mass (ICM) (Koo *et al*., 2002) is evident. At the sub-cellular level, problems with DNA methylation patterns (Bourc'his *et al*., 2001) and the dysregulation of genes occurs (Humpherys *et al*., 2002). These abnormalities are referred to as the "Cloning Syndrome" and are thought to mainly be due to epigenetic defects (changes in chromatin structure, not involving a change in DNA base sequence) which occur during cell reprogramming, where the donor cell DNA is reprogrammed to a pluripotent embryonic state (Schurmann *et al*., 2006).

Zygotes were initially thought to be unsuitable for use in NT experiments as they could not support reprogramming of the donor nucleus (McGrath and Solter,

1984), whereas an unfertilised oocyte could (Kato *et al*., 1998). This suggests that factors essential for the correct reprogramming of the donor nucleus are lost to the oocyte cytoplasm upon fertilisation (McGrath and Solter, 1984). However, a study by Schurmann, Wells *et al*. (2006) revealed that early zygotes (in the telophase II stage) were suitable for bovine somatic cell NT and resulted in higher *in vivo* development of cloned offspring. At present, there is no evidence suggesting that MII or Telophase II (TII) zygote cytoplast recipients differ in their ability to reprogram a somatic cell (Oback, 2008). Egli, Rosains *et al*. (2007) then investigated the possibility that factors required for reprogramming or embryonic development located in the unfertilised oocyte become sequestered in the pronuclei of interphase zygotes. This could explain why zygotes enucleated in interphase cannot support development after NT, as removal of the pronuclei would also mean removal of the reprogramming factors. In these experiments, mouse zygotes were arrested in the metaphase stage of mitosis (where breakdown of the pronuclear envelope occurs, possibly releasing the reprogramming factors) using nocodazole. The metaphase nuclei were then removed and the resulting cytoplasts injected with donor cell chromosomes (Egli *et al*., 2007). It was found that these metaphase arrested zygotes injected with donor cells developed to the blastocyst stage, indicating they supported complete reprogramming of donor DNA. These results suggest that at least some of the reprogramming factors were not permanently lost from the oocyte following fertilisation, and may localise to the pronuclei at interphase (Egli *et al*., 2007).

Egli *et al*. (2008) proposed that the developmental potential of a cell is determined by its transcriptional profile. Transcriptional regulators may dissociate from the

chromatin on entry into mitosis and reassociate with the chromatin following cell division, meaning new gene expression patterns are established (Egli *et al*., 2008). Transcriptional silencing in mitosis may be due to the condensed structure of the chromatin, and therefore provide an opportunity for changes in gene expression. Dissociation of transcriptional regulators from the chromatin during mitosis (and not interphase) may allow these factors to disperse into the cytoplasm. This would provide an opportunity to remove the genome of the mitotic zygote or oocyte which can then be used as a recipient for NT, leaving main factors that allow for the resetting of transcriptional profiles in the donor cell remaining in the recipient cytoplasm (Egli *et al*., 2008). Transcriptional profiles which regulate genes within a cell are flexible and reversible. When an adult cell is transferred into an unfertilised oocyte, the transcriptional machinery of the cell alters and becomes embryonic in nature (Beyhan *et al*., 2007; Brambrink *et al*., 2006). The molecular mechanisms underlying epigenetic reprogramming are still essentially a black box. No definitive reprogramming factors have so far been purified (Bortvin *et al*., 2003).

Candidate reprogramming factors analysed in this study are tumour translationally controlled protein 1 (TCTP); the gene of which is *TPT1* (Koziol *et al*., 2007), brahma-related gene 1 (BRG1) (Hansis *et al*., 2004) and Histone Deacetylase 1 (HDAC1) (Ma and Schultz, 2008).

TCTP has been found to activate transcription of *POU5F1* (also known as *OCT4*) and the *NANOG* (transcription factors which are both implicated in inducing

pluripotency of cells) (Koziol *et al*., 2007). It was found that a reduction in the oocyte content of TCTP reduced *POU5F1* transcription, suggesting TCTP is involved in the transcriptional activation of *POU5F1* (Koziol *et al*., 2007). *POU5F1* is normally expressed only in pluripotent cells (Byrne *et al*., 2003). It is activated during NT into oocytes and activation of *POU5F1* transcription is directly correlated to cloning efficiency (Bortvin *et al*., 2003). A reduction in *NANOG* also occurs after a down regulation of *TPT1* in oocytes, suggesting *TPT1* is also involved in the activation of *NANOG* transcription (Koziol *et al*., 2007). *NANOG* is thought to be an important pluripotency gene as its over-expression results in a significant increase of the nuclear reprogramming efficiency in cell fusion experiments (Silva *et al*., 2006). TCTP was found to bind to the *POU5F1* promoter, but not significantly to the *NANOG* promoter. This implies that TCTP regulates *NANOG* transcription indirectly, while it directly activates *POU5F1* transcription (Koziol *et al*., 2007). The correct activation of the *POU5F1* and *NANOG* genes is essential for correct nuclear reprogramming (Koziol *et al*., 2007).

Hansis, Barreto *et al*. (2004) looked to identify some genes and mechanisms involved with nuclear reprogramming in *Xenopus* egg extract. It was found that the pluripotency markers *OCT4* and Germ Cell Alkaline Phosphatase (*GCAP*) were up regulated in human 293T cells and human primary leukocytes, exposed to the egg extract. A screen was carried out to identify potential factors involved in the reprogramming process, and the chromatin remodelling ATPase BRG1 was discovered to have a major influence. Immunodepletion of BRG1 inhibited the oocytes ability to reprogram, whilst its overexpression increased the oocyte's ability to reprogram as the embryo progressed further through development

(Hansis *et al*., 2004). The chromatin remodelling factor BRG1 has been shown to dissociate from the chromatin during mitosis (Egli *et al*., 2008), meaning it is an important gene to consider for influencing donor cell reprogramming and development of metaphase zygote NT embryos.

Acetylation of histones plays a major role in regulating transcription in eukaryotic cells. Hyperacetylation of chromatin is associated with increased gene expression, possibly due to the unwinding of the nucleosome structure allowing better access of transcription factors. Histone Deacetylases (HDACs) are associated with the silencing of gene expression as they deacetylate histones by interacting with the gene promoter (Grozinger and Schreiber, 2002). HDAC1 is likely a major deacetylase in early mouse embryos, and is thought to be specifically involved in the deacetylation of histone H4K5 (Ma and Schultz, 2008). Ma and Schultz (2008) also discovered that HDAC1 is involved in initiating a transcriptionally repressive state, and is highly abundant in pre-implantation mouse embryos.

Exposure of activated NT reconstructs to HDAC inhibitors such as trichostatin A (TSA) can increase the efficiency of SCNT by preventing abnormal hypermethylation of histones in early mouse embryos (Kishigami *et al*., 2006). This provides an example of how inhibition of HDACs can lead to an improvement in epigenetic reprogramming of SCNT embryos.

Identification and quantification of these candidate reprogramming factors in the developing zygote will result in a better understanding of the epigenetic remodelling processes in cloned embryos. Better understanding of these processes may lead to increases in cloning efficiency and ultimately being able to use cloning for commercial and therapeutic purposes.

1.2 Metaphase zygote NT experiments

McGrath and Soulter (1984) transferred mouse embryonic nuclei into enucleated zygotes and concluded that development of these reconstructs was unable to be supported *in vitro* (McGrath and Solter, 1984). Several more attempts have been carried out in a variety of species using zygotes as recipients for NT; including rabbit (Modlinski and Smorag, 1991), pig (Prather and First, 1990) and rhesus (Meng *et al*., 1997). In all cases the vast majority of embryos produced unable to progress past the first two cleavage divisions (Greda *et al*., 2006). Wakayama, Tateno *et al.* (2000) concluded that when interphase mouse zygotes were used as recipients for SCNT, no development to blastocyst was observed (Wakayama *et al*., 2000).

Schurmann, Wells *et al.* (2006) found that by using early zygotic cytoplasts at the TII stage as NT recipients, *in vitro* development was similar to that of NT into metaphase II cytoplasts, however *in vivo* development was significantly increased. They concluded the reason for this increased development was that certain factors are delivered to the oocyte by the sperm during natural fertilisation (not artificial activation), leading to an increase in *in vivo* development. These factors may include phospholipase C, which in mouse spermatozoa induces long lasting Ca^{2+}

oscillations in the egg (Parrington *et al*., 2000) rather that the single large Ca^{2+} rise brought about by artificial activation . Also, the centrosome present in bovine oocytes may be removed during enucleation in standard NT experiments (Schurmann *et al*., 2006), and 3000 kinds of mRNA (Ostermeier *et al*., 2002) some that are delivered to the oocyte post fertilisation (Ostermeier *et al*., 2004). Micro RNAs delivered by the sperm (Ostermeier *et al*., 2005) may also play a role in the increased *in vivo* development as well as possible sperm derived transcripts which may arise during the fusion of the sperm and the oocyte (Schurmann *et al*., 2006).

Normal fertilisation (fig 1.1) involves sperm entering an MII oocyte to form a zygote, which then progresses through the anaphase II and telophase II cell cycle stages extruding a second polar body in the process. After formation of the second polar body the zygote progresses through the G_1 and S phases before again entering metaphase prior to cleavage (27 – 33 hpf). During NT of a somatic donor cell into an MII cytoplast (fig 1.2), the same series of events occur as in fig 1.1 but the reconstruct must be artificially activated. The embryo progresses to the metaphase stage before cleaving into a two cell approximately 25 hours post activation. When metaphase arrested zygotes are used as NT recipients (fig 1.3), the zygote has already progressed through the G_1 and S phases which occur during normal fertilisation. It can be assumed following NT the zygote will again progress through the anaphase and telophase stages (extruding a pseudo polar body) prior to cleavage (fig 1.3).

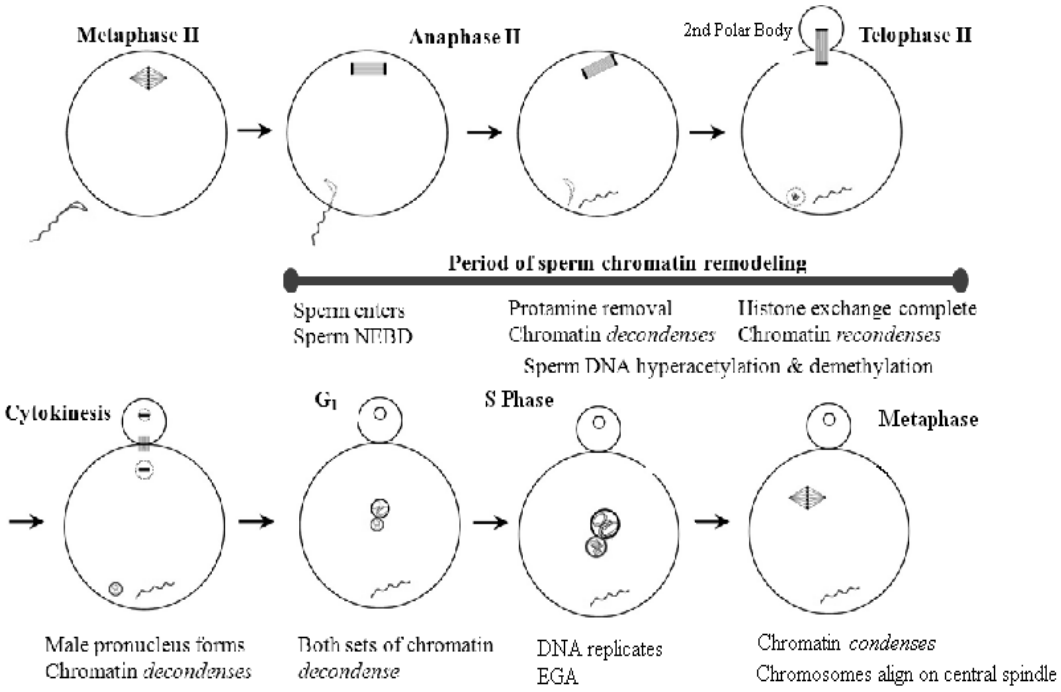


Fig 1.1 – Schematic diagram of normal fertilisation

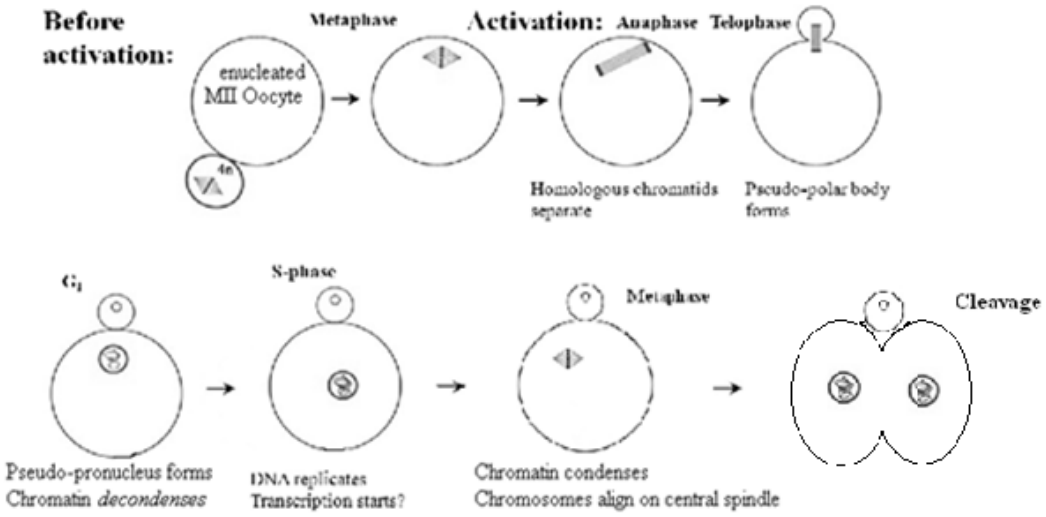


Fig 1.2 – Schematic of NT using metaphase donor cells with enucleated MII oocyte recipient

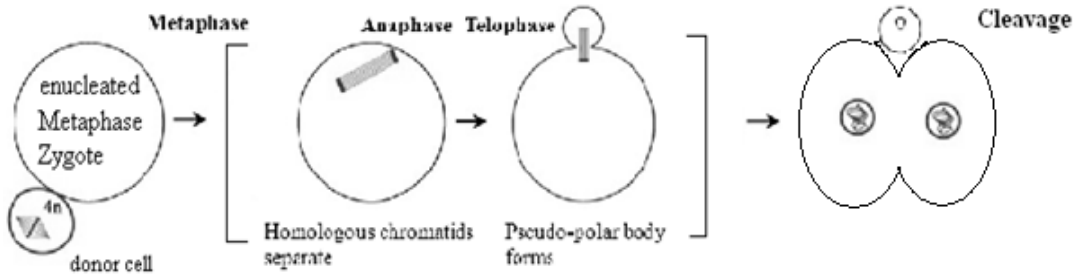


Fig 1.3 – Hypothetical schematic of NT using metaphase donor cells with enucleated metaphase zygote recipients

Greda, Karasiewicz *et al*. (2006) used mouse interphase zygotes as recipients for NT using blastomeres of various genetic profiles (strains) from 8 cell embryos as donors. They employed an alternative enucleation technique when removing the pronucleus of the interphase zygotes. This technique allows them to remove the nuclear envelope with the attached chromatin, whilst leaving the liquid pronuclear contents and nucleoli to remain in the cytoplasm ("selective enucleation"). Development of the embryos was then compared between groups where the selective enucleation technique was used and those which underwent complete enucleation (removal of the entire pronucleus and pronuclear contents). When complete enucleation was used no development was observed beyond the four-cell stage. Using selective enucleation however, development to blastocyst on day 5 was observed for two strains of blastomeres with an average efficiency of 39%. They concluded that cytoplasmic components which are needed for successful reprogramming of donor nuclei are incorporated into growing pronuclei, therefore removal of these pronuclei would mean removal of the reprogramming components which would not allow the embryo to develop correctly (Greda *et al*., 2006).

Egli, Rosains *et al*. (2007) considered the theory that factors required for either reprogramming or embryonic development in the cytoplasm of oocytes become sequestered in the pronuclei of zygotes. They consequently showed that the cytoplasm of metaphase-arrested mouse zygotes could support the nuclear reprogramming of somatic cells and embryonic stem cells (ESCs) in the mouse. It is suggested that the ability of the cytoplasm to reprogram somatic nuclei fluctuates with cell cycle, and that one or more factors required for this reprogramming localise to the pronuclei during interphase. The fact that embryos have now been produced from NT of somatic cells into zygotes of various stages shows that reprogramming factors are not lost from the cytoplasm of the oocytes following fertilisation (Egli *et al*., 2007).

The effects on *in vitro* development using donor cells of various cell cycle stages for bovine NT has been previously examined (Tani *et al*., 2001). Fusing metaphase donor cells into MII oocytes resulted in development to blastocyst. NT embryos which extruded a second polar body developed to blastocyst at an efficiency of 25% and were found to be diploid. Those embryos which did not extrude a second polar body were found to be tetraploid and developed to blastocyst at an efficiency of 30%. There was no significant difference in development to blastocyst between those embryos which did extrude a second polar body, and those which did not. Only those embryos which were diploid (had extruded a second polar body) were transferred to recipient cows. Of the five embryos transferred, one live calf was produced. It was therefore concluded that non-activated oocytes receiving metaphase donor cells have the potential to develop into normal calves (Tani *et al*., 2001).

1.3 Candidate reprogramming factors

1.3.1 TCTP

Translationally Controlled Tumour Protein (TCTP), the gene of which is *TPT1*, has been shown to bind to the microtubules of all mammalian cells during most stages of the cell cycle but detaches from the spindle after anaphase and binds again at the G_1 phase (Gachet *et al*., 1999). It was discovered that TCTP is phosphorylated by the protein kinase Plk, which is the likely cause of TCTP detachment from the mitotic spindle (Yarm, 2002).

Koziol, Garrett *et al*. 2007, found that TCTP activates *OCT4* and *NANOG* transcription in transplanted human somatic cell nuclei (Hela cells). Both *OCT4* and *NANOG* are key pluripotency genes (Boyer *et al*., 2005). The developmental function of TCTP was determined by down regulation of the gene. It was found that down regulation of *TPT1* results in significantly reduced *OCT4* transcription (Koziol *et al*., 2007). Also, NT experiments have shown that over-expression of *TPT1* mRNA increases expression of *OCT4*. These results taken collectively suggest that TCTP plays a major role in regulation *OCT4* expression. A reduction in *NANOG* transcription was also observed after a down-regulation of *TPT1* in *Xenopus* oocytes. It was shown that TCTP binds to the *OCT4* promoter but not directly to the *NANOG* promoter, suggesting TCTP regulates *OCT4* directly, whereas its regulation of *NANOG* occurs in an indirect way, such as binding to a distant site (Koziol *et al*., 2007).

Tani, Shimada *et al*. (2007) discovered that bovine oocytes which have a high ability to reprogram somatic cells contain a phosphorylated protein identified to be TCTP. The presence of this protein in oocytes with a high reprogramming potential was discovered using 2D polyacrylamide gel electrophoresis (PAGE), where a unique spot at the 23 KDa marker was observed. The amino acid sequence of this protein was then determined and shown to match the human TCTP protein. When enucleated MII oocytes were injected with the phosphorylated TCTP protein, their potential to develop to blastocyst was not increased, however, it was demonstrated that there was a beneficial effect on *in vivo* development (Tani *et al*., 2007).

1.3.2 BRG 1

Hansis, Barreto *et al*. (2004) developed a protocol to carry out experiments in which adult mammalian cells (human 293T kidney cells) are exposed to *Xenopus* egg extract to induce reprogramming. To test for nuclear reprogramming of the kidney cells, mRNA expression of *OCT4* and *GCAP* was measured using RT-PCR. *OCT4* and *GCAP* are pluripotency markers in human and mouse (Matsui *et al*., 1992; Thomson *et al*., 1998). It was found that in kidney cells exposed to *Xenopus* egg extract, the *OCT4* and *GCAP* pluripotency markers were up regulated. An immunodepletion screen was carried out using antibodies to deplete chromatin regulators and transcription factors thought to be involved in reprogramming. The depletion of the chromatin remodelling ATPase brahma-related gene 1 *(BRG1)* consistently inhibited *OCT4* up regulation in the 293T kidney cells. Competencies of egg extracts of different stages were also tested for their ability to up regulate pluripotency markers in the 293T cells. It was found

that early blastula (stage 8) *Xenopus* egg extracts only slightly up regulated *OCT4* and so it was concluded that reprogramming ability of the extract is lost at the midblastula transition (MBT). Zygotic transcription in *Xenopus* embryos is initiated during the MBT. This zygotic transcription may activate genes which repress pluripotency (Hansis *et al*., 2004).

Immunocytochemistry of two cell stage mouse embryos shows that BRG1 dissociates from the chromatin at mitosis (Egli *et al*., 2008). This makes the BRG1 protein an ideal candidate to analyse in my experiments, as mitotic zygotes (in metaphase) will be used as recipients in the NT experiments.

1.3.3 HDAC1

Kim, Liu *et al*. (2003) suggested that oocytes used as NT recipients repress a specific gene expression program by histone deacetylation. HDAC1 is involved in the deacetylation of histones and was found to be meiosis specific (localising to the chromatin during meiosis but not mitosis) (Kim *et al*., 2003).

Also, HDAC1 may be associated with the development of a transcriptionally repressive state in early mouse embryos (Zeng and Schultz, 2005). Ma and Schultz (2008) reported that HDAC1 regulates histone acetylation, development and gene expression in pre-implantation mouse embryos. The HDAC1 protein was shown to associate with chromosomes at the metaphase I and metaphase II stages of the mouse oocyte, and also with the pronucleus of the early zygote. Inverse correlations between the immunostaining intensity of histone H4K5

acetylation and HDAC1 nuclear staining suggest that HDAC1 is responsible for a reduction in the acetylation of histone H4K5. RNAi knockdown of HDAC1 was shown to result in hyperacetylation of histone H4K5. This deacetylation of the H4K5 histone induces a transcriptionally repressive state in the embryo, as deacetylation tends to give a tighter chromatin configuration. The developmental ability of HDAC1 depleted embryos is compromised, with significantly smaller numbers developing to the blastocyst stage. This may be due to the over expression of $p21^{Cip1/Waf}$, which leads to cell cycle arrest (Ma and Schultz, 2008). Epigenetic mechanisms such as histone modifications may play a major role during the normal development of the embryo (Fulka, 2008). Fulka (2008) examined the role of histone modifications relating to the efficiency of SCNT in mouse oocytes, at different stages of maturation and also after the activation of the NT reconstructs. Their results indicated that the ability of the oocyte cytoplasm to deacetylate the donor cell chromatin disappears soon after activation. Also discovered was that that some reprogramming activity appears in the oocyte cytoplasm following germinal vesicle breakdown (GVBD) which then disappears following activation (Fulka, 2008). This could explain why pre-activated cytoplasts (PACs) are unsuitable as recipients for SCNT (Wakayama *et al*., 2000). Using PACs as NT recipients fails to deacetylate the donor chromatin of the somatic cell (Wakayama *et al*., 2000).

By exposing mouse NT embryos to HDAC inhibitors, development can be significantly improved. Kishigami, Mizutani *et al*. (2006) showed *in vitro* development of mouse NT embryos was markedly increased when exposed to 5 – 50 nM concentrations of TSA for a 10 hour period following activation.

Blastocyst development was increased between two and five fold (depending on the donor cell type) compared to those NT embryos not exposed to TSA. *In vivo* development of TSA treated embryos was also improved (6% live offspring produced) as compared to the control group not treated with TSA (1% live offspring produced) (Kishigami *et al*., 2006). This increase in development may be due to reduced DNA hypermethylation or increased histone acetylation in the early embryos (Kishigami *et al*., 2006).

1.4 Aims and objectives

This study aims to produce blastocysts using metaphase arrested zygotes as recipients for NT of metaphase arrested somatic donor cells in bovine. Egli, Rosains *et al*. (2007) produced blastocysts from NT of mitotic somatic cells into metaphase arrested mouse zygotes, but did not attempt to generate any live cloned offspring. Conditions and procedures for my experiments will need to be optimised specifically for bovine. As a control experiment, metaphase arrested donor cells will be used for NT into standard MII oocyte recipients. A two-tailed Fisher exact test will be used to determine the significance of these results.

As using TII zygotes as NT recipients result in higher *in vivo* cloning efficiency aim to find out about the abundance and localisation of the previously mentioned candidate reprogramming factors mentioned in Interphase, Telophase II and Metaphase stage bovine zygotes as well as in bovine MII oocytes. Abundance of candidate reprogramming factors *BRG1* and *TPT1* will be measured using reverse transcriptase (RT) real-time PCR, following the isolation of mRNA (and subsequent conversion to cDNA) from the various samples. Statistical analysis in

the form of a two-tailed t-test will be performed on these results to reveal any significant differences in the levels of these genes between the different samples.

Localisation of the reprogramming factors in zygotes and cells will be observed using immunocytochemistry performed on the various samples. Antibodies will be used to stain the samples for the TPT1, BRG1 and HDAC1 proteins, then stained with a fluorescent secondary antibody allowing the stain to be viewed using fluorescence microscopy. Confocal imaging of the stained samples will also be used where necessary. Antibodies will be tested on bovine and human cells prior to the staining of the zygote samples, to ensure the antibody will recognise the specific antigen in these samples.

METHODS

2. Methods

2.1 General Methods

2.1.1 Preparation of culture media for bovine cells (DMEM/F12 + 10%FCS)

DMEM/F12 base media (89.5 ml) was aliquoted into a measuring cylinder. 500 µl Sodium Pyruvate (1mM) and 10 ml FCS were added to the base media. The solution was then poured into a beaker and filtered through a 0.2 µm vacucap filter into a 250 ml Schott bottle using an aspiration line connected to a vacuum pump. Following filtration, the bottle was labelled and placed in a 4°C refrigerator.

2.1.2 Thawing cells

DMEM/F12 + 10% FCS cell media was warmed in a 38°C water bath prior to the cells being thawed. The cells were stored in liquid nitrogen and so handling required care and speed. A small amount of liquid nitrogen was poured into a polystyrene container, the vile of cells placed in the liquid nitrogen and transported to the lab. Cells were thawed in a water bath (~38°C) and the vile swabbed with ethanol before being placed in a laminar flow hood. 5-10 ml culture media was added to a 15 ml conical tube. Once the cell sample was completely thawed, the solution was pippetted into the tube containing media (whilst mixing) and centrifuged (Biofuge Primo 75005181) at 1000 rpm for 3-5 minutes (this centrifuge was used for all cell work). The supernatant was removed using an unplugged pipette and the appropriate volume of tissue culture media was added

to the pellet. To disperse the cells, the bottom of the tube was tapped lightly on the bench. Media and cells were mixed well and transferred to a tissue culture dish. The dish was labelled with the date, cell line, passage number and number of cells. Cells were incubated at 38°C until they reached 70-90% confluency. When cells were thawed onto cover slips, sterile small round cover slips were placed on the bottom of the tissue culture dish before thawing the cells.

2.1.3 Passaging of cells

Cell culture media (DMEM/F12 + 10% FCS) was aspirated off the cells which were then washed with the appropriate volume of PBS (38°C). PBS was aspirated off and a small volume of 0.25% trypsin at 38°C (just enough to cover the cells) was added to the dish. The cells immersed in trypsin were then incubated in a 38°C incubator for 3-5 minutes until cells dislodged from the dish. A plugged Pasteur pipette was used to aspirate the media and cells up and down to help break up any sheets of cells. Cells were then transferred to a conical tube and the appropriate volume of culture media added to the tube before mixing thoroughly. The tube containing the cells was then centrifuged for 3-5 minutes at 1000 rpm. Once the centrifuge had stopped, the tube was remove and the supernatant aspirated off and discarded then the pellet loosened by gently tapping the tube. Following this, the desired amount of culture media was added to the tube and the solution transferred into a tissue culture dish. Cells were incubated at 38°C until they were 70-90% confluent.

2.1.4 Freezing of cells

When cells were 70-90% confluent, they were passaged and the total number of cells counted using a haemocytometer. Cells were then centrifuged at 1000 rpm for 3-5 minutes. The cryo-protectant was made during centrifugation by adding 1 ml DMSO (drop wise) to 4 ml FCS and filtering. This creates a 20% DMSO solution. Freezing media consisted of 50% culture media and 50% cryo-protectant solution, giving a final DMSO concentration of 10%. To the pellet of cells, half the total volume required in DMEM/F12 + 10% FCS cell culture media was added, followed by the remaining volume in cryo-protectant solution (added very slowly). This solution was mixed to ensure uniformity and aliquoted into 1 ml cryovials on ice. Vials were labelled with the date, passage number, cell ID and number of cells then placed in a freezing box in the -80°C freezer. After 24 hours, the vials of cells were transferred in to liquid nitrogen.

2.1.5 Aspiration of ovaries

Ovaries were collected from slaughtered cows on site at the abattoir and removed from the reproductive tract using scissors before being placed in warm saline in a thermos. The ovaries were then transported back to the lab within 2-4 hours. Prior to aspiration, ovaries were removed from the thermos, washed twice in sterile 0.9% saline (30°C) and stored in a large thermos flask (containing saline). Warm aspiration media was placed in sterile 15 ml conical tubes (approximately 2 ml of media per tube). The aspiration needle (18 gauge) bung was then placed into the tube and a blunt needle pushed through the bung and connected to the aspiration line. A negative pressure (approximately -50 mmHg) from the aspiration machine was used to generate a vacuum and follicles 3 – 10 mm in diameter were

aspirated. Approximately four oocytes per ovary can be expected. Full tubes were then searched for oocytes in the embryology lab.

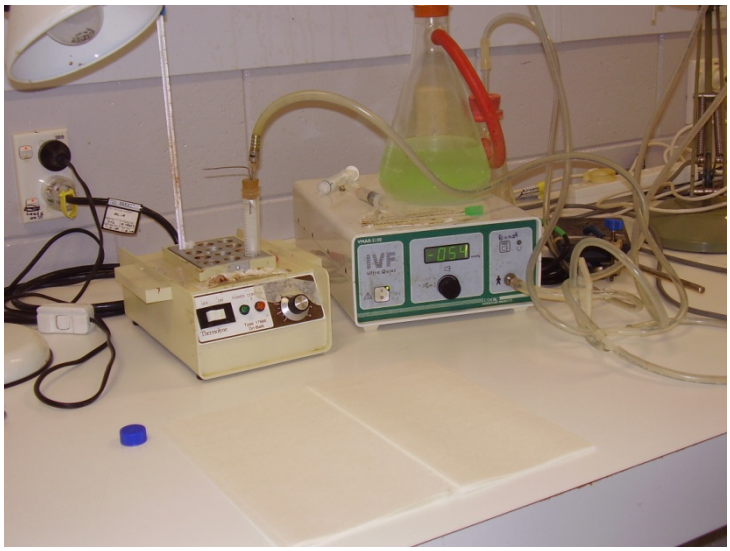


Fig 2.1 – Set-up for the aspiration of ovaries

2.1.6 In vitro maturation (IVM)

IVM plates (one per 120 oocytes expected) were prepared at least two hours prior to IVM in a laminar flow hood. 12 x 40 µl drops of maturation media with cysteamine added were placed into a 6 cm petri dish, using a dropper pipette. Drops were then overlaid with 8 ml of gassed mineral oil. Petri dishes were labelled with "IVM", date and user name and equilibrated for a minimum of 2 hours in a 5% CO_2 incubator at 38.5°C. Ovaries were then aspirated as per the ovary aspiration protocol (section 2.1.5). Tubes were placed in a test tube rack and the contents allowed to settle. Aspiration media was added to a 9 cm petri dish (just enough to cover the bottom of the dish) and a 2 ml syringe with a glass Pasteur pipette attached was used to draw up approximately 1 ml of sediment to add to the dish containing aspiration media. The dish was placed in a grid plate

and the contents searched for cumulus-oocyte complexes (COCs) with a roller pipette attached to a glass capillary. Oocytes lacking cumulus cells, or possessing a large spotted cumulus were left behind as these oocytes are too mature and this can lead to poor development to blastocyst. COCs were transferred to a 3 cm petri dish containing H199 + 10% FCS media, then washed in a second 3 cm plate containing H199 + 10% FCS. Oocytes were then washed a third time in a 3 cm dish containing B199 + 10% FCS media. From this dish, 10 oocytes in 10 µl of the B199 + 10% FCS medium were then transferred to an IVM drop (giving the drop a total volume of 50 µl). This was repeated until all selected oocytes were in IVM drops. The plate was labelled with the number of oocytes and the time the plate was filled before being placed into a 5% CO_2, 38.5°C incubator for 22-24 hours. All work was performed on a warm stage at 38.5°C.

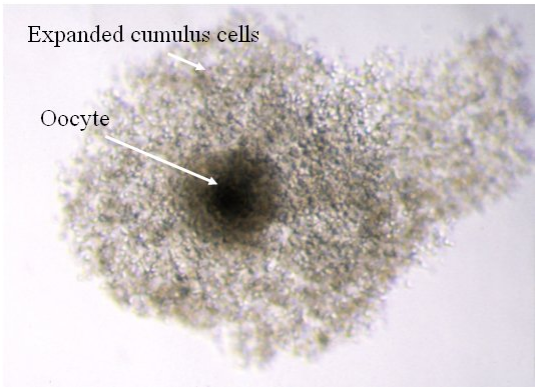


Fig 2.2 – Bovine cumulus oocyte complex (From archimede.bibl.ulaval.ca)

2.1.7 In vitro Fertilisation (IVF)

2.1.7.1 IVF plate preparation

IVFSOF (sterile) + 1 μM pyruvate + 1 μM penicillamine + 2 μM hypotaurine + 10 μg/ml heparin (IVF + PPHH) medium was made up in a laminar flow hood. These agents were added to the IVF media to increase the developmental efficiency of the zygotes. Pyruvate was added as it is metabolised by the early embryo increasing its developmental potential. Heparin was added to capacitate the sperm and increase the chances of fertilisation and penicillamine and hypotaurine were used to maintain sperm motility. Using a dropper pipette, two 60 μl wash drops of IVF + PPHH medium were placed at the top of each plate, and 12 x 30 μl drops placed throughout the dish (a plate with 12 drops holds up to 60 oocytes). The drops were overlaid with 8 ml of sterile warm mineral oil. IVF plates and the remainder of the IVF + PPHH medium was placed in a 38.5C, 5% CO_2 incubator and allowed to equilibrate for at least 2 hours. The cap on the bottle of IVF + PPHH medium was loosened to allow pH equilibration during incubation. Hepes-buffered synthetic oviduct fluid (HSOF) was also placed in the incubator to warm (with the cap tightened).

2.1.7.2 Sperm preparation

Redigrad™ gradients were prepared in a laminar flow hood by pipetting approximately 1.5 ml of 45% Redigrad™ into a 15 ml sterile centrifuge tube, and carefully under-laying with 1.5 ml of a 90% Redigrad™ solution. The Redigrad™ solution was used for creating a gradient to separate the motile fraction of sperm from the semen. A semen straw (from a standard IVF bull) was selected from the

liquid nitrogen tank and thawed for ~ 30 seconds in a 30°C water bath. As a general rule, one semen straw (0.25 ml, containing approximately 1 x 10^8 spermatozoa/ml) was used to fertilise approximately 150 oocytes. One gradient should accommodate two straws of semen. The straw was then dried with tissue paper in a laminar flow and wiped with 70% ethanol. To avoid contaminating the ends, the straw was handled in the middle from here on. One end of the straw was cut off and placed against the inside of the centrifuge tube containing the gradient. The other end of the straw was then cut and the sperm allowed to flow into the tube and settle on the gradient. Following this, the Redigrad™ gradient and sperm was centrifuged (Biofuge primo 75005181 centrifuge) at 700 g for at least 20 minutes. Sperm was recovered as soon as the centrifuge stopped to prevent motile sperm swimming up the gradient. Using a sterile glass Pasteur pipette and syringe, the sperm pellet was removed from the bottom of the tube and re-suspended in 1 ml HSOF medium. This was then centrifuged (Biofuge primo 75005181 centrifuge) at 200 g for 5 minutes. While this centrifugation was in progress, 190 µl of water was pipetted into a glass tube. Immediately after the centrifuge had stopped, the supernatant was removed and the sperm pellet resuspended in 200 µl of equilibrated IVF + PPHH medium. This was mixed gently and a 10 µl aliquot of the solution was added to the 190µl of water (a 1:20 dilution). The remaining volume of sperm solution was measured in the tube using a Pasteur pipette attached to a 1 ml syringe. While calculating sperm concentration, the solution was placed in a dark place, as U.V light is damaging to the sperm DNA.

2.1.7.3 Calculating sperm concentration

Cover glass was placed over the support shoulders of a haemocytometer. 10 µl of the 1 in 20 sperm dilution (from the glass tube) was pipetted against the edge of the cover glass until the grid was filled. Both sides of the haemocytometer were filled while taking care not to overfill. Sperm was counted in the 25 large squares that make up the grid on the haemocytometer (both sides). The following formula was used to get a final concentration of 1 million sperm/ml:

$$\frac{\text{Volume measured (A) x average number of sperm count (B)}}{25} = \text{Total volume (C)}$$

Total volume (C) – volume measured (A) = volume of IVF + PPHH medium to add to the sample (D). Volume (D) was added slowly so that the sperm did not suffer dilution shock. 10 µl of the diluted sperm sample was added to each 40 µl drop containing oocytes in the IVF plates. Sperm were checked to ensure they were motile. The plates were placed in a 5% CO_2 incubator for 6 hours. After 6 hours, zygotes were stripped (cumulus cells removed) in HSOF + 0.1% hyaluronidase (an enzyme which digests the cumulus cells) and placed in group culture IVC plates.

2.1.8 Removal of cumulus from metaphase II oocytes

Two 3 cm petri dishes containing H199 + 10% FCS, and a third 3 cm dish with H199 + 3 mg/ml BSA were poured. The dish containing H199 + 3 mg/ml BSA was labelled “3”, and the dishes containing H199 + 10% were labelled “1” and “2”. Oocytes were removed from IVM and placed into the dish labelled “1”. Then, taking as little media as possible, the oocytes were transferred to an eppendorf tube containing a 500 µl aliquot of 0.1% Hyaluronidase in H199. This was then vortexed for three minutes at 2200 rpm and spun down for a few seconds

in a Labnet Spectrafuge mini C1310 (2000 *g* maximum speed). Using a 200 µl pipette, all liquid along with the pellet of oocytes was removed from the tube and placed back into the first wash dish. Oocytes were swirled into the middle of the dish and transferred to the second wash dish (labelled "2"). The oocytes were washed again by swirling the dish and transferred to the third dish containing H199 + 3 mg/ml BSA. A mouth pipette was used to search oocytes for the presence of a polar body. In the dish, the oocytes were separated into two groups; those with a polar body and those without. Those with a polar body (extruded set of chromosomes to produce a haploid oocyte) were classed as being in the "Metaphase II" stage and were used for nuclear transfer (NT). Oocytes without a polar body were discarded. If the oocytes were to be zona-free, they were incubated in a drop of pronase (5 mg/ml dissolved in Ca and Mg free PBS) until the zona-pellucida disappeared, then washed out of the pronase in H199 + 10% FCS.

2.1.9 Removal of cumulus from IVF zygotes

Zygotes were removed from IVF and placed in a 3 cm petri dish containing HSOF. The zygotes were then taken from the dish using a pipette (carrying over minimal media so as not to dilute the hyaluronidase) and placed in an eppendorf tube containing 0.1% hyaluronidase in HSOF. Following this, the tube containing the zygotes was vortexed for two minutes at 2000 rpm and spun down using a mini centrifuge (Labnet Spectrafuge C1301) for a few seconds. After vortexing, the entire content of the tube was removed using a 200 µl pipette and expelled into a second 3 cm petri dish containing HSOF. Embryos with cumulus cells removed were washed through a third dish containing HSOF before being placed

into IVC. Zygotes were then placed in a drop of pronase (5 mg/ml dissolved in Ca and Mg free PBS) until the zona-pellucida disappeared (observed under a microscope) before placing in IVC (if zona-free zygotes were required).

2.1.10 Nuclear transfer (NT) procedure

NT plates were made the previous day and held in the fridge overnight. Plates included: Hold plates (for holding the zygotes and oocytes before enucleation), a stain plate with 5 μg/ml Hoechst 33342 (Invitrogen, NZ), a cytoplast plate (for holding enucleated zygotes and MII oocytes), a lectin (200 μg/ml in H199 + 3 mg/ml BSA) plate for sticking cells to the cytoplasts, and a post-fusion plate for holding the fused NT reconstructs. All plates were made by placing 30 μl drops in a 6 cm petri dish and overlaying with 8 ml mineral oil. Chromosomes of recipients (MII oocytes or metaphase zygotes) were stained with 5 μg/ml Hoechst 33342 in H199 for approximately 5 minutes prior to enucleation. Enucleation was carried out using a blunt aspiration pipette (25–30 μm in outer diameter) and separation needle (100-150 μm in outer diameter) with a closed fire-polished tip. No holding pipette was used as the recipients were zona-free. Oocytes and zygotes were then enucleated in a drop of H199 + 3 mg/ml BSA under oil at 100 × total magnification. All tools were made from thin wall borosilicate capillaries (CG100T-15, Harvard Apparatus Ltd., UK) using a horizontal puller (P-87, Sutter Instruments, CA) and a microforge (MP-9, Narishige, Japan) (Oback and Wells, 2003). Mitotic nuclei were removed from zygotes and metaphase II (MII) oocytes (those which have extruded a polar body). The MII oocyte group was used as a control. Cytoplasts were stuck to metaphase arrested donor cells (cell line LJ801 ~ passage 5) in H199 + 3 mg/ml BSA containing lectin. Couplets were then

electrically fused, using two pulses of 450 V with a 30 μs pulse length in a parallel plate fusion chamber filled with 205 osm fusion buffer. Fused couplets (where the donor cell has moved inside the oocyte) were placed into a post-fusion plate before activation.

2.1.11 Activation of fused reconstructs

Cycloheximide (CHX) plates were made the morning before activation and placed in a CO_2 gassed incubator. Each CHX plate consisted of a 6 cm Petri Dish containing three 40 μl wash drops at the top of the plate and 35 x 5 μl drops throughout the plate overlaid with 8 ml of mineral oil. The concentration of CHX used was 10 μg/ml (diluted in ESOF medium). Immediately after fusion had been scored (for the metaphase NT experiments), fused reconstructs were placed in a 3cm petri dish containing HSOF + 1 mg/ml BSA for approximately 15 minutes. 5 ml of a 5 μM ionomycin solution in HSOF + 1 mg/ml BSA was made up and placed in a 3 cm petri dish. Ionomycin was used as it is a calcium ionophore, allowing an influx of Ca^{2+} into the oocyte to mimic the process of normal fertilisation. Reconstructs were activated by being placed in the ionomycin solution for exactly 4.5 minutes before immediately transferring them to a 3 cm petri dish containing HSOF + 30 mg/ml BSA media where they were left for at least three minutes. The embryos were then washed three times in the wash drops of CHX plates and individually placed in the 5 μl drops. CHX was used for activation as it allows for extrusion of the second polar body, another activating agent commonly used; 6-dimethylaminopyridine (6-DMAP) does not. After six hours in CHX, the reconstructs were washed out of the cycloheximide and transferred to IVC.

2.1.12 In vitro culture (IVC)

All plates for IVC were prepared in a laminar flow hood. For group culture plates (zona-intact embryos) a 3 cm petri dish was used. Using a dropper pipette, two central wash drops of 40 µl size were placed into the petri dish and surrounded by six 20 µl drops of Early Synthetic Oviduct Fluid (ESOF) medium. The drops were overlaid with 3 ml of mineral oil. Plates were then placed in a modular incubator chamber with a small amount of milli-Q water in the base. The O-ring surrounding the lid of the chamber was greased with a small amount of lubricant, and the modular incubator chamber gassed for 5 minutes with 5% CO_2, 7% O_2, and 88% N_2 to allow the plates to equilibrate (for at least two hours). Following this, the chamber with the plates was then placed in a "dry" (non-gassed) 38.5°C incubator. A plate of B199 + 10% FCS was placed into the chamber as an indicator (this should always remain a salmon pink colour) to ensure the chamber remains gassed. Using a mouth pipette, the zygotes were washed through both the central wash drops of the IVC plates, and 10-15 zygotes placed in each of the six 20 µl drops in the plate. Plates remaining in the incubator chamber continued to be gassed whilst working with other plates. On day 5, embryos were changed over to fresh drops of Late Synthetic Oviduct Fluid (LSOF) medium. LSOF plates were made the same way as the ESOF plates, and also equilibrated for at least two hours in a gassed modular incubator chamber. On day 5 during the change over, cleavage (embryos which have progressed passed the one cell stage) and the number of tight morulae (TM)/compacted morulae were recorded. During day 7, embryos were graded for blastocyst development (appendix A: Criteria for grading of bovine blastocysts).

For the single culture of zona-free zygotes, the same fundamental protocol applies, however plates were made differently. 6 cm petri dishes were used, with three 40 µl wash drops of ESOF/LSOF medium at the top of the plate. 30 x 5 µl drops of ESOF/LSOF medium were then placed in rows throughout the plate (three rows of eight drops, and the final row of six drops). The drops were then overlaid with 8 ml of mineral oil.

2.1.13 Isolation of mRNA

All steps of this procedure were carried out on ice. The appropriate volume of trizol was added to a cell sample or embryo sample. 1 µl ms2 RNA (200 ng/µl) and 5 µl alpha globin (1 pg/µl) was then added to the sample. Ms2 RNA preferentially sticks to the sides on the PCR tube to prevent any loss of RNA from the sample. Alpha globin spikes the sample with a known amount of a specific RNA. Samples were then mixed by pipetting and spun in a mini centrifuge (Eppendorf minispin plus 5453) for a few seconds. The appropriate volume of chloroform was added (to give a 1:5 ratio) and again mixed by pipetting. This was then spun at 5000 *g* (Biofuge fresco 75005521 centrifuge) at 4°C for 10 minutes and the upper aqueous phase removed then transferred to a new tube. 2 µl linear acrylamide (5 µg/ml) was then added, this aids in the sterilisation of the precipitate. An equal volume of isopropanol (~50 µl) was also added then the solution mixed briefly using a vortex. The samples were then incubated for 10 minutes at room temperature, spun at 14000 rpm for 30 minutes at 4°C (Biofuge fresco 75005521 centrifuge), with care taken to ensure tubes were orientated correctly so the small pellet was not lost. Following centrifugation, the supernatant was decanted and the pellet washed with ~70 µl of 70% ethanol then

spun again (Biofuge fresco 75005521 centrifuge) at 14000 rpm for 10 minutes (tubes orientated correctly). The resulting supernatant was again decanted and the pellet allowed to air dry. Once dry, the pellet was resuspended in 8 µl of DEPC water, centrifuged (Eppendorf Minispin plus 5453) for a few seconds then incubated for 5 minutes at 65°C before being placed on ice for 30 minutes.

DNase treatment involved adding 1 µl of 10X DNase buffer (Invitrogen, NZ) and 1 µl of DNase (Invitrogen, NZ). Samples were then spun down and incubated at 37°C for 60 minutes. 1 µl EDTA (25mM, Invitrogen, NZ) was added, and the samples were spun down again and incubated for 10 minutes at 65°C. 1.5 µl Sodium Acetate along with 45 µl 100% ethanol was then added and left at -80°C for 1 hour or -20°C overnight. After freezing, the samples were spun at 14000 rpm for 30 minutes (4°C) with tubes orientated correctly. The supernatant was then aspirated off, the pellet washed in 70% ethanol and spun at 13000 rpm (4°C) for a further 10 minutes. Ethanol was removed and the pellet allowed to air dry. The pellet was then resuspended in 11 µl of DEPC water, spun down and redissolved by incubating at 90°C for 2 minutes. Samples were left on ice for 2 minutes before beginning the cDNA synthesis.

1 µl dNTP mix (10mM, Invitrogen, NZ) was added to the 11 µl RNA sample along with 1 µl of random hexamers (Invitrogen, NZ) and this was incubated at 65°C for 5 minutes. Samples were then left on ice for 1 minute following the incubation and spun down in the mini centrifuge (Eppendorf Minispin plus 5453). 10 µl of master mix (2 µl 10X First strand buffer, 4 µl $MgCl_2$ (25mM), 2 µl DTT, 1 µl RNase OUT, and 1 µl Superscript III – not added to no RT control. All

Invitrogen, NZ) was added to each RNA sample. Once the master mix was added, samples were vortexed and incubated for 10 minutes at 25°C, 50 minutes at 50°C and finally 5 minutes at 85°C before being placed on ice. 1 µl of RNase H (Invitrogen, NZ) was then added and the samples incubated for 20 minutes at 37°C. Following RNase treatment, cDNA samples were stored at -80°C. The no RT control is a sample of mRNA that has not been transcribed to cDNA, and so should not be amplified in the PCR. This control was used as an indicator of any nuclear DNA contamination.

2.1.14 Real-time PCR

All pipettes and the work space were wiped with ethanol before use, and gloves worn at all times during this procedure. Roche LightCycler® Master Mix (LCMM) was made by adding 14 µl of the Roche LightCycler® enzyme mix (tube labelled 1a) to the Roche LightCycler® reaction mix (tube labelled 1b).

Samples were thawed, vortexed and centrifuged briefly (Eppendorf minispin plus 5453) before use. For each run, an RT –ve control and NTC (no template control, containing only DEPC water instead of cDNA) were included in the analysis. A master mix containing 0.4 µl of each primer (forward and reverse), 2 µl LCMM and 5.2 µl of water was made for each gene to be analysed. Note that the volumes used are per sample i.e. – if 10 samples were to be run, 10 times the volumes was used.

All primers used for PCR analyses were obtained from Invitrogen NZ Ltd.

8 µl of the master mix was added to each capillary along with 2 µl of the cDNA template, making total volume per capillary 10 µl. The maximum number of samples one can run on a LightCycler® is 32.

Capillaries were loaded into the Roche LightCycler® carousel by vertically dropping each capillary into the appropriately numbered well. Once all capillaries were loaded, each one was pushed down using straight vertical pressure until it locked into place.

Samples were spun down in a Roche LightCycler® carousel 2.0 centrifuge. The carousel containing the samples was placed into the rotor and the rotor placed into the centrifuge with the Roche symbol facing upwards. Capillaries were then spun in the centrifuge at 15000 rpm for 15 seconds. Once finished, the carousel was removed from the rotor and placed into the LightCycler® machine (locking the rotor into place). LightCycler® software was used to start a new experiment. A self-test was performed prior to carrying out the run if the machine had not been used previously that day. Run conditions for the experiment were specified, as was the number of samples. The run was started and samples named appropriately in the software. In some cases, capillaries were frozen at -20°C following the run in order to load the samples on to a gel in future.

2.1.15 Immunocytochemistry of cells

The cell line used for immunocytochemistry (ICC) was passaged onto a 3 cm tissue culture petri dish, containing ~ 6 small round cover slips. Once the cells were 70 - 90% confluent, they were fixed in a 4% paraformaldehyde (PFA) solution at 4°C for 30 minutes. After the cells were fixed, they were washed twice

in phosphate-buffered saline (PBS) and then placed in a 50 mM solution of NH_4Cl for 10 minutes. This improves the specificity of the staining. The cells were then permeabilised in a 0.1% Triton X-100 solution for 10 minutes. Cells (on cover slips) were washed out of TX-100 3 times in PBS, then left in blocking buffer (3% BSA dissolved in Milli-Q Water) at 4°C for 30 minutes. Cover slips were then removed from blocking buffer and placed in the primary antibody solution (diluted in blocking buffer). This was achieved by placing them face down onto 20 µl drops of the primary antibody on Parafilm™ laid on damp filter paper inside a wet chamber. The wet chamber was sealed around the edges with Parafilm™ and placed in the fridge overnight. Cells were washed out of the primary antibody by washing 3 times in PBS. Following washing, the cover slips were placed in the secondary antibody solution in blocking buffer, also containing Hoechst 33342 dye in another wet chamber and incubated at 37°C for 30 minutes. Cells were then washed out of the secondary antibody by washing 3 times in PBS and once in Milli-Q water before being mounted on slides in 5 µl of DAKO™ mounting medium. Slides were placed in fridge and sealed with nail polish. Slides were viewed under fluorescence using an Olympus BX-50 microscope with attached mercury lamp and camera.

2.1.16 Immunocytochemistry of oocytes and embryos

Oocytes/embryos were fixed in a 4% paraformaldehyde (PFA) solution at 4°C for 30 minutes. Once fixed, they were washed twice in 0.1 mg/ml PBS/PVA and placed in a 50 mM solution of NH_4Cl for 10 minutes to improve the specificity of the staining. Fixing and washing steps were done in 30 µl drops in a 6 cm petri dish overlaid with 8 ml of mineral oil. Quenching in 50mM NH_4Cl was carried

out in 30 µl drops in a 6 cm dish also containing 30 µl drops of blocking buffer, overlaid with mineral oil. Following quenching in NH_4Cl, oocytes/embryos were permeabilised in the 0.1% TX-100 solution in a 3 cm petri dish for 10 minutes. Permeabilised samples were washed once in a 3cm petri dish of PBS/PVA (0.1 mg/ml) before being placed in drops of the blocking buffer in a 6 cm petri dish. The oocytes/embryos were left in the blocking buffer for 30 minutes at room temperature then placed in the fridge for a further 60 minutes. After removal from the blocking buffer samples were then incubated in the primary antibody (diluted to the appropriate concentration in blocking buffer) overnight in a refrigerator. As a negative control some oocytes/embryos were incubated only in blocking buffer (not containing the primary antibody). The following morning, the oocytes/embryos were washed out of the primary antibody three times in PBS/PVA then incubated with the secondary antibody/Hoechst 33342 (5 µg/ml) for 30 minutes at 37°C. Finally, after removal from the secondary antibody, they were washed three times in PBS/PVA and once in milli-Q water before being mounted in 5 µl of DAKO™ mounting medium under a cover slip and sealed with nail polish. Double staining was carried out on embryos by incubation in two primary antibodies diluted in blocking buffer followed by incubation in the two appropriate secondary antibodies. Slides were viewed under fluorescence using an Olympus BX-50 microscope with attached mercury lamp and camera. Confocal imaging of embryos was carried out using an Olympus FluoView FV1000 confocal microscope.

2.1.17 Statistical analysis

Statistical analysis was carried out using Microsoft Excel®. A two-tailed t-test was used for the real-time RT PCR results, with the assumption that the samples have unequal variances. This statistical analysis reveals if there are any significant differences between the concentrations of the target genes in the various samples relative to *18S*, and also between the "MII Equivalents" of the samples. The accepted level of significance was $P < 0.05$.

For the statistical analysis of developmental data, a two-tailed Fisher Exact test was used to determine the significance of results i.e. – if the developmental results (to blastocyst) of two groups differ significantly. The accepted level of significance was again $P < 0.05$.

2.2 Metaphase Zygote NT Methods

2.2.1 Metaphase zygote NT procedure

The morning of NT at approximately 8:30am, zygotes were moved from ESOF medium to ESOF + nocodazole medium (0.5 μg/ml nocodazole concentration). Zygotes were retained in the ESOF + nocodazole medium for 4.5 hours. When enucleation was due to be commenced, the zygotes were removed from the ESOF + nocodazole medium and incubated in pronase to remove the zona pellucida. Any 2-cell embryos were discarded at this stage. Zygotes were then placed in drops of H199 + 3mg/ml BSA + nocodazole. All media from this point on contained nocodazole at a concentration of 0.5 μg/ml, to prevent zygotes or donor cells exiting mitosis during the NT procedure. The zygotes were then stained with Hoechst 33342 dye (5 μg/ml) for approximately 5 minutes prior to enucleation. Zygotes were selected on the microscope stage during enucleation for metaphase chromosomes (Fig 2.3) and enucleated under U.V illumination. Successfully enucleated metaphase zygotes (zygotic cytoplasts) were held in drops of H199 + 3mg/ml BSA – nocodazole. The cytoplasts were then stuck to metaphase donor cells using lectin (200 μg/ml) before being fused to the metaphase arrested donor cells and placed into IVC. The basic procedure for these experiments is shown in fig 2.4.

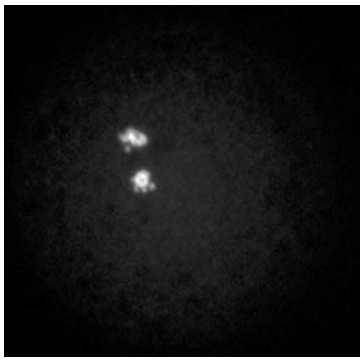


Fig 2.3 – Zygote arrested in metaphase showing condensed chromosomes

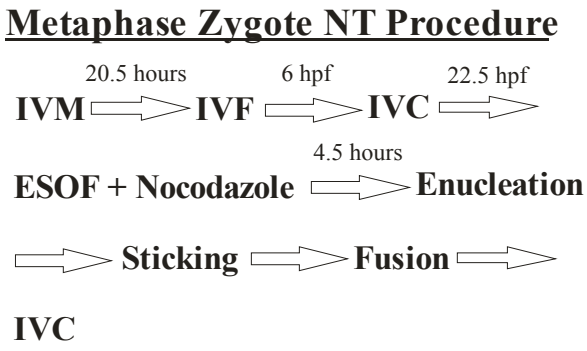


Fig 2.4 – Basic schematic of metaphase zygote NT experiment

2.2.2 Control NT experiments using MII oocyte recipients

Control NT experiments using MII oocytes as recipients of metaphase donor cells were carried out using the standard NT procedure as described in section 2.1.10. However, couplets (enucleated MII oocyte stuck to a metaphase donor) were kept in media containing nocodazole from the point they were stuck to the metaphase arrested donor cell in lectin. This prevents the donor cell from exiting mitosis during the NT procedure (fig 2.5).

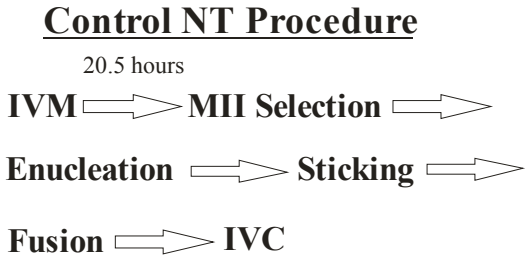


Fig 2.5 – Basic schematic of control NT experiments

2.2.3 Metaphase arrest of somatic donor cells

LJ801 (approximately passage 5) cells were thawed into three wells of a four well plate. The cells were seeded at a density of 5 x 10^4 cells per well in 1ml DMEM/F12 + 10% FCS media, and left to grow overnight. Prior to NT, cells were treated with nocodazole (0.5 µg/ml) by adding nocodazole to the media in the wells. The cells were then incubated for two hours at 38.5°C. Following this, the plate was tapped to dislodge the metaphase arrested cells and the media and cells were centrifuged (Biofuge primo 75005181) then the cell pellet resuspended in 250 µl H199 + 0.5% FCS + nocodazole. 40µl drops of the cells in H199 + 0.5% FCS + nocodazole were then on a 3cm petri dish and overlaid with mineral oil before selection of the cells for NT. Cells were assessed as being in metaphase by morphology and clarity. Only large, round, transparent donor cells were classed as metaphase and used as donors for the NT experiments.

2.2.4 Chromatin analysis of early embryos

Embryos at time points of pre fusion (couplets), immediately post fusion, two hours post fusion (hpf), four hpf, 19 hpf and 24 hpf were fixed in 4% Paraformadehyde (PFA) for 30 minutes at 4°C. The embryos were then stained with Hoechst 33342 dye and the distribution of chromatin analysed by exposure of the stained embryos to U.V light.

2.2.5 Cytochalasin B (CB) treatment of embryos

IVC was carried out on the embryos/NT embryos according to the procedure described in section 2.1.12. The ESOF medium used for the IVC contained CB (7.5 μg/ml) to prevent cleavage of embryos. Embryos were kept in the ESOF medium + CB for periods of either four or 17 hours, after which time they were washed out in ESOF medium not containing CB for approximately 30 minutes and allowed to cleave. Embryos which did not cleave were discarded. Once cleaved, the embryos were placed into IVC, or fixed in 4% PFA for 30 minutes at 4°C. The embryos fixed in 4% PFA were stained with Hoechst 33342 and viewed under U.V for analysis of their chromatin distribution. Embryos placed into IVC were cultured to day 7 and their development to blastocyst was assessed. A control group containing embryos in ESOF - CB (ESOF + 1.5 μl/ml DMSO) was also present in these experiments.

2.2.6 Nocodazole washout treatment of metaphase zygote NT embryos

Before metaphase zygote NT embryos were placed into IVC, they were washed out of nocodazole containing media in H199 + 3mg/ml BSA – nocodazole for periods of either 30 minutes (group 1) or 60 minutes (group 2). Following their washout they were placed into IVC as according to the procedure in section 2.1.12.

2.3 mRNA Analysis Methods

2.3.1 Interpretation of Real-time PCR results

All real-time PCR reactions were carried out on a Roche LightCycler® system using the LightCycler® FastStart DNA Master Plus SYBR Green I kit.

Real-time PCR amplifies DNA (or cDNA) and can quantify it by attaching a fluorescent probe (in this case SYBR green) to the DNA and measuring the fluorescence intensity (Valasek and Repa, 2005). The PCR reaction only amplifies DNA up to a certain threshold efficiently, before a plateau phase is reached. Real-time PCR measures DNA amplification efficiently in the exponential phase (the steep part of the amplification curve where the product increases exponentially) (Valasek and Repa, 2005). The point where the product begins to amplify exponentially is termed the crossing-point (or Cp value). Dissociation or "melting" curves produced at the end of the PCR run show whether a specific PCR product was created. These should show a single sharp peak at a specific temperature for the product which differs from the melting points shown for the NTC and RT –ve controls (Valasek and Repa, 2005). An example of a real-time PCR output is shown in figures 3.4 and 3.5.

2.3.2 Standard curve derivation

To generate a standard curve, a known amount of cDNA was used as the starting material. Dilutions were done in 1/10 amounts as the concentrations covered the same range of Cp's as the samples. An example of dilutions performed for deriving a standard curve is shown in table 2.1.

Table 2.1 – Example of standard curve dilutions

Conc.	Dilution	Sample Amount	Water or TE Amount	Total Volume (µl)
1	1	2µl	0	2
0.1	1/10	1µl	9µl	10
0.01	1/100	1µl of 1/10	9µl	10
0.001	1/1000	1µl of 1/100	9µl	10
0.0001	1/10000	1µl of 1/1000	9µl	10

Before being added to the capillary, each sample was vortexed and spun down in the mini centrifuge (Eppendorf minispin plus 5453) then loaded into the capillaries as per the Real-time PCR protocol in the section 2.1.14. Each sample was done in duplicate. Once the run was complete, the melting curve analysis was checked to ensure the No Template Control (NTC) had a different melting peak from the samples. Absolute quantification analysis was done using the LightCycler® software, with "standard" selected as the sample type and the sample concentration specified to produce the standard curve. The standard curves were then exported into the Excel program and a scatter plot produced fitted with a logarithmic regression. Using the regression equation and the formula [concentration] = $e^{(-(Cp\text{-intercept})/slope)}$, Cp values were able to be converted to a relative concentration.

2.3.3 Quantification of gene expression

Absolute gene expression values were gained by converting Cp values to an absolute concentration (in relative units) using the equation gained from the standard curve graph. Relative gene expression was calculated by dividing the absolute concentration of the target gene by the concentration of the house-keeping gene (*18S*) in the samples. *18S* was used as the normalising or house-keeping gene in these experiments. The quantity of *18S* is not expected to change under the experimental conditions and can therefore act as an internal standard (Valasek and Repa, 2005). However it has been discovered that levels of house-keeping genes may be highly variable (Dheda *et al*., 2004), and this may affect the results.

2.3.4 18S mRNA analysis

All real-time PCR analysis for 18S was carried out according to the real-time RT-PCR method (section 2.1.14). The primers for 18S were:

Forward Primer: GAC TCA TGG CCC TGT AAT TGG AAT GAG GC

Reverse Primer: GCT GCT GGC AAC AGA CTT G

Annealing temperature: 56°C

Fragment size: 87bp

2.3.5 TPT1 mRNA analysis

All real-time PCR analysis for TPT1 was carried out according to the real-time RT-PCR method (section 2.1.14). The primers for TPT1 were:

Forward Primer: TCT ACA AGA TCC GGG AGA TCG

Reverse Primer: GAC ACC AGT GAT TAC TGT GCT

Annealing temperature: 60°C

Fragment size: 161 bp

2.3.6 BRG1 mRNA analysis

All real-time PCR analysis for BRG1 was carried out according to the real-time RT-PCR method (section 2.1.14). The primers for BRG1 were:

Forward Primer: GAC AAG CGC CTG GCC TA

Reverse Primer: ACC AGC TCC GTG AGG TTA

Annealing Temperature: 60°C

Fragment Size: 165 bp

2.4 ICC Methods

2.4.1 Selection of zygotes

Zygotes were generated using the standard IVF procedure (section 2.1.7) and stripped of cumulus cells. The zygotes were then incubated in pronase until zona-free and stained in H199 + 3 mg/ml BSA + Hoechst 33342 dye (5 µg/ml). Telophase II zygotes were selected under U.V fluorescence (to observe telophase nuclei) at 4.5 hpf (hours post fertilisation). Interphase zygotes were selected under U.V fluorescence 22.5 hpf, and metaphase zygotes selected under U.V fluorescence at 27 hpf (after 4 hours in ESOF + 0.5 µg/ml nocodazole).

2.4.2 TCTP ICC of somatic cells, oocytes and zygotes

All ICC carried out on cells, oocytes and zygotes using the TCTP antibody, was done so according to the procedures in sections 2.1.15 and 2.1.16. The TCTP antibody (MBL, Japan) was used at a concentration of 1:1000. This concentration was found to be optimal after trialling the antibody at concentrations of 1:250, 1:500 and 1:1000. The secondary antibody was the anti-rabbit Alexa-Fluor 568 (Molecular Probes, Invitrogen, NZ), used at a concentration of 1:1000. When double staining of zygotes using the TCTP and lamin (nuclear proteins, to see if TCTP associates with nuclear material) antibodies was carried out, the lamin antibody was used at a concentration of 1:100. The secondary antibody used for lamin was anti-goat rhodamine (Jackson ImmunoResearch Inc. PA, USA) which was used at a concentration of 1:300. The secondary antibody used for TCTP in the double-staining experiments was anti-rabbit Cy2 (Jackson ImmunoResearch

Inc. PA, USA). Samples were also stained with Hoechst 33342 dye in order to visualise the nuclear DNA.

2.4.3 BRG1 ICC of somatic cells and zygotes

All ICC carried out on cells using the BRG1 antibody (Santa Cruz, CA, USA) was done so according to the procedures in sections 2.1.15 and 2.1.16. The primary antibody (BRG1) was used at a concentration of 1:50 (the strongest concentration recommended by the manufacturer). The secondary antibody used in these experiments was anti-goat rhodamine (Jackson ImmunoResearch Inc. PA, USA), which was used at a concentration of 1:300. Samples were also stained with Hoechst 33342 dye in order to visualise the chromosomes.

2.4.4 HDAC1 ICC of somatic cells, oocytes and zygotes

All ICC carried out on cells, oocytes and zygotes using the HDAC1 antibody (Millipore, MA, USA), was done so according to the procedures in sections 2.1.15 and 2.1.16. The HDAC1 primary antibody was used at a concentration of 1:250. This concentration was found to be optimal, when concentrations of 1:250, 1:500 and 1:1000 were tested. The secondary antibody for these experiments was the anti-rabbit Alexa-Fluor 568 (Molecular Probes, Invitrogen, NZ), used at a concentration of 1:1000.

2.5 Reagents, solutions and media composition

M199

Medium 199 containing Earle's salts and $_{L}$-glutamine, but no $NaHCO_3$ (Life Technologies; cat. no. 31100-035)

B199

Bicarbonate buffered medium M199 with 25mM $NaHCO_3$ and 0.086 mM kanamycin monosulfate

IVM medium

B199 + 10% FCS with 10 μg/ml ovine follicle-stimulating hormone (FSH) (Ovagen; Immuno-Chemical Products [ICP], Auckland, New Zealand), 1 μg/ml ovine luteinising hormone (LH) (ICP), 1 μg/ml 17-β-estradiol and 0.1 mM cysteamine for *in vitro* maturation (de Matos et. al. 1995).

HSOF

Hepes-buffered synthetic oviduct fluid (SOF) with 107.7 mM NaCl, 7.15 mM KCl, 0.3 mM KH_2PO_4, 5 mM $NaHCO_3$, 3.32 mM sodium lactate, 0.069 mM kanamycin monosulfate, 20 mM Hepes, 0.33 mM pyruvate, 1.71 mM $CaCl_2.2H_20$, 3 mg/ml fatty-acid free bovine albumin (ABIVP;ICP) (Thompson et. al. 1990).

Aspiration media

H199 + 925 IU/ml Heparin (CP Pharmaceuticals Ltd., UK) + 20% (w/v) Albumin concentrate (ICP).

IVF SOF medium

107.7 mM NaCl, 7.15 mM KCl, 0.3 mM KH_2PO_4, 25 mM $NaHCO_3$, 3.32 mM sodium lactate, 1.71 mM $CaCl_2.2H_20$, 0.04 mM kanamycin monosulfate, 8 mg/ml fatty-acid free bovine albumin (ABIVP, ICPbio), supplemented with 0.33 mM sodium pyruvate, 0.001 mM Heparin, 0.2 mM penicillamine and 0.1 mM hypotaurine.

Redigrad™

Composition: Silica sol with covalently linked saline

Density (g/ml): 1.130 ± 0.005

Osmolarity (mOsm/kg H_20): max. 30

Viscosity (cP): max. 15 at 20°C

pH: 9.4 ± 0.5 at 20°C to 25°C

Endotoxin (EU/ml): max. 2

GE Healthcare Life Sciences, Level 1, 8 Tangihua Street, Auckland 1010, New Zealand.

Biphasic AgR SOF (ESOF)

Biphasic AgResearch Synthetic Oviduct Fluid medium (AgR SOF, AgResearch, Hamilton, New Zealand). AgR SOF is a modified formulation to that used by Gardner *et al*., (1994) containing 8 mg/ml BSA (Wells et. al. 2003).

Biphasic AgR SOF (LSOF)

LSOF is the biphasic AgR SOF (ESOF) medium, containing 10 μM 2,4-dinitrophenol which acts as an uncoupler of oxidative phosphorylation (Wells et. al. 2003).

Fusion buffer (205osm)

200 mM mannitol, 50 μM $CaCl_2$, 100 μM $MgCl_2$, 500 μM Hepes, 0.05% bovine albumin (ABIVP, ICP), pH 7.3 (Oback and Wells, 2003).

4% Paraformaldehyde (PFA) fixant

Depolymerised 4% (w/v) PFA, 4% (w/v) sucrose, phenol red indicator, 1 M NaOH, 1x Phosphate Buffered Saline (PBS). Final pH should be 7.0 – 7.5.

Phosphate-buffered saline (PBS)

1.9 mmol/l sodium dihydrogen orthophosphate 1-hydrate, 8.4 mmol/l disodium hydrogen orthophosphate 2-hydrate, 150 mmol/l sodium chloride.

DEPC-H_20

0.1% (v/v) diethyl procarbonate in milli-Q water. Mixed overnight then autoclaved for 30 minutes at 121ºC.

Cell lines

The LJ 801 cell line used in these experiments was derived from an ear punch taken from a limosine-jersey (LJ) cross-bred steer.

RESULTS

3. Results

3.1 Metaphase Zygote NT Results

3.1.1 Metaphase zygote NT

3.1.1.1 Introduction

For the metaphase zygote NT experiments, the overall objective was to carry out the standard NT procedure (as described in section 2.1.10) using zygotes arrested in the metaphase stage as recipients for SCNT. Metaphase arrested somatic cells were used as donor cells for these experiments. This work was carried out in an attempt to replicate the experiments of Egli, Rosains *et al*. (2007) in which metaphase-arrested mouse zygotes were used as recipients for SCNT. Control experiments using MII oocytes as recipients of metaphase arrested somatic donor cells were carried out in parallel with the metaphase zygote NT experiments.

3.1.1.2 Fusion parameter comparison

Several fusion parameters were tested, varying the amplitude, length between the two fusion pulses (pulse length) and osmolarity of the fusion buffer. This was done in order to find the optimal fusion parameters for the fusion of metaphase somatic donor cells into zygotes. In all trials, non-enucleated zygotes 22 hours post fertilisation (hpf) were used as recipients for the metaphase donors.

The optimal fusion buffer of the two buffers tested was that with an osmolarity of 205 osm (table 3.1 and table 3.2). From the early trails an amplitude of 450 V and two 30 µs pulses gave encouraging results. We trialled these parameters extensively and results show fusion of 60% of couplets was achieved. A later smaller trial using our standard fusion parameters for somatic G_0 or G_1 donors (600 V amplitude with two 10 µs pulses) revealed a higher rate of successfully fused couplets (76.9%) than all other fusion parameters tested (Table 3.2). This result, however, was not significant. The fusion parameters used for the metaphase zygote NT experiments described in the following section were 450 V amplitude with two 30 µs pulses.

Table 3.1 – Fusion Parameter comparison (271 osm buffer)

Amplitude (V)	Pulse Length (µs)	No. Fused	% Fused
300	30	0/20	0.0
375	30	5/20	25.0
450	30	3/20	15.0
525	30	4/20	20.0

Table 3.2 – Fusion Parameter comparison (205 osm buffer)

Amplitude (V)	Pulse Length (µs)	No. Fused	% Fused
375	30	17/40	42.5
450	10	8/20	40.0
450	30	48/80	60.0
450	50	6/20	30.0
525	30	7/20	35.0
600	10	10/13	76.9

3.1.1.3 Selection of zygotes in metaphase

Zygotes were classed as being in metaphase by staining with Hoechst 33342 dye and illuminating the chromosomes under U.V light on the microscope stage prior to enucleation.

During the second metaphase zygote NT experiment, 19 random karyoplasts (the enucleated portion of cytoplasm containing the chromosomes) were fixed for 30 minutes at 4°C in 4% PFA and later examined (by illumination under U.V) to determine if the zygotes selected were in fact in metaphase. It was found that 78.9% of those zygotes enucleated were in metaphase (Table 3.3). Metaphase zygote and MII oocyte chromosomes were distinguishable, as the metaphase zygote karyoplasts contained two chromatin masts, whereas the MII oocyte karyoplasts only contained one metaphase plate.

Table 3.3 – Selection efficiency of metaphase zygotes

n	Metaphase Zygote	Interphase Zygote	MII Oocyte	Other	% Metaphase
19	15	1	2	1	78.9

3.1.1.4 Enucleation of metaphase zygotes

To determine the volume of cytoplasm removed from the metaphase zygotes during the enucleation process, 24 random karyoplasts were retained from the third metaphase zygote NT trial. These karyoplasts were then photographed, the diameter measured and the karyoplast volumes calculated (Table 3.4) using the standard spherical volume calculation $4/3\ \pi\ r^3$ where r is the radius of the karyoplast. A non-enucleated metaphase zygote from the same experiment was

also retained and its volume calculated. The average volume removed was calculated and finally the average cytoplasmic volume removed from metaphase zygotes during enucleation, was found to be 6.5 ± 4.1%.

Table 3.4 – Volume of cytoplasm removed during enucleation

Karyoplast number	Diameter in cm (1.0 cm = 40 µm)	Actual Size (µm)	Volume (μm^3)
1	1.4	56.0	91952.3
2	1.4	56.0	91952.3
3	1.3	52.0	73622.2
4	1.4	56.0	91952.3
5	1.3	52.0	73622.2
6	1.2	48.0	57905.8
7	1.4	56.0	91952.3
8	0.8	32.0	17157.3
9	0.8	32.0	17157.3
10	0.8	32.0	17157.3
11	0.8	32.0	17157.3
12	1.2	48.0	57905.8
13	0.8	32.0	17157.3
14	0.8	32.0	17157.3
15	0.7	28.0	11494.0
16	1.6	64.0	137258.3
17	1.3	52.0	73622.2
18	1.2	48.0	57905.8
19	1.0	40.0	33510.3
20	0.8	32.0	17157.3
21	1.1	44.0	44602.2
22	1.4	56.0	91952.3
23	1.4	56.0	91952.3
24	1.4	56.0	91952.3
Karyoplast Averages	**1.1**	**45.0**	**57717.3**
Zygote	2.6	119.0	882347.2
Standard Deviation of Karyoplast volume	**0.3**	**11.2**	**35935.0**

Volume Removed = <u>6.5 ± 4.1%</u>

3.1.1.5 Metaphase zygote NT development

Table 3.4 shows a summary of results from the metaphase zygote NT experiments, and the control experiments using MII oocytes as recipients. The column labelled "n" signifies the number of repeats for each experiment.

The control experiments gave consistent development to blastocyst, with an average total development overall of 10.8%.

When enucleated metaphase zygotes were used as recipients of metaphase somatic donor cells for NT, no development to blastocyst was observed (Table 3.5, %TD). The resulting embryos did not progress past the 8 cell stage of development (Table 3.5, n > 8c).

Table 3.5 – Metaphase Zygote NT Development

Recipient	n	Fused	% Fused	nCHX	nIVC	nLSOF	n Cleaved	% Cleaved	n2-8c	n > 8c	B 1-3	%TD
MII Oocyte (Control)	5	293/371	79.0[a]	284	277	126	260	93.9[a]	N.D	N.D	30	10.8[a]
Metaphase Zygote	3	53/106	50.0[b]	N/A	53	21	44	83.0[b]	15	0	0	0.0[b]

a,b – Values with different superscripts are significantly different; N.D – Not Determined; CHX – Cycloheximide; IVC – *In vitro* culture; LSOF – Late synthetic oviduct fluid; B 1-3 – Blastocyst grade 1-3; 8c – 8 cell; %TD - % Total development.

3.1.1.6 Polar body extrusion of NT embryos

Approximately 24 hours post activation; control NT embryos were examined for extrusion of a pseudo polar body (PPB) (table 3.6). The metaphase zygote recipients (approximately 26 hours post fusion) were also examined for pseudo polar body extrusion. The proportion of control embryos which did extrude a pseudo polar body was 26.7%. None of the metaphase zygote NT embryos had extruded a polar body (table 3.6).

Table 3.6 – Polar body extrusion of NT embryos

Recipient	n(IVC)	n(PPB)	% (PPB)
MII oocyte	277	74	26.7[a]
Metaphase zygote	53	0	0.0[b]

a,b – values with different superscripts differ significantly

3.1.1.7 Analysis of early embryos for correct chromatin distribution

Early embryos were fixed and analysed at various time periods after fusion.

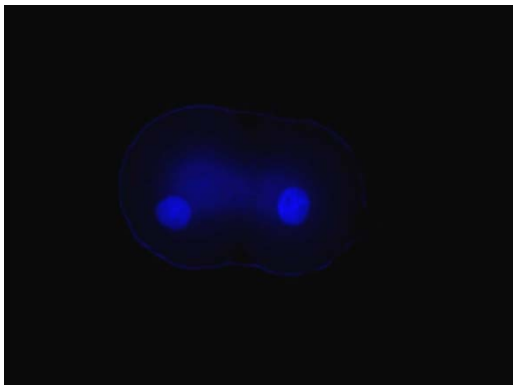


Fig 3.1 – MII control

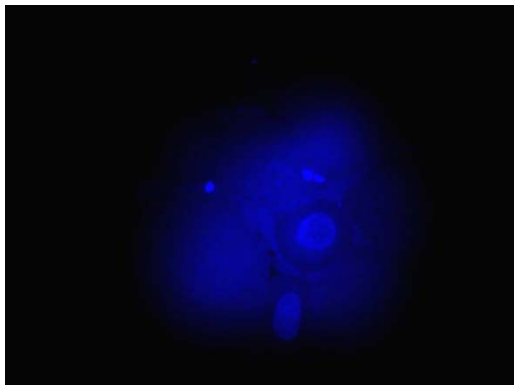


Fig 3.2 – Metaphase Zygote

Fig 3.1 shows the control recipient (metaphase II oocyte) 24 hours post fusion to a metaphase donor cell. The embryo is at the two cell stage and shows the correct chromatin distribution of one nucleus per blastomere. Blastomeres are cells

produced by mitotic division of the zygote following fertilisation. Fig 3.2 shows the situation 24 hours post fusion when a metaphase zygote is used as a NT recipient for a metaphase donor cell. It can be seen that the chromatin distribution in the metaphase zygote recipient (Fig 3.2) is erratic and uneven throughout the embryo. Embryos which had ≥ 2 interphase nuclei or > 2 metaphase chromatin masses in at least one blastomere (cells which make up the embryo) were classed as being abnormal. Quantification of the chromatin distribution in these early embryos (Table 3.7, below) shows none of the metaphase zygote NT embryos had the correct chromatin distribution 24 hours post fusion. Over half of the MII controls had the correct distribution of chromatin at the same time point.

Table 3.7 – Chromatin distribution in early embryos

Recipient	Hours Post Fusion	n Embryos	n Correct Distribution	% Correct Distribution
Metaphase Zygote	24	17	0	0.0[a]
MII (Control)	24	12	7	58.3[b]

a,b – values with different superscripts differ significantly

3.1.1.8 Cleavage of metaphase zygote NT reconstructs

Metaphase zygote NT reconstructs were placed into IVC and assessed for cleavage 3.5 hours after fusion. In all three metaphase zygote NT experiments, 62.3% of the zygotes had cleaved 3.5 hours post fusion (Table 3.8). None of the control NT embryos (MII oocyte recipients) had cleaved 3.5 hours post fusion (table 3.8). My hypothesis was that this early cleavage of the zygotes may be contributing to the zygotes having an incorrect distribution of chromatin and so delaying cleavage may be beneficial to the development of the embryos.

Table 3.8 – Cleavage of Metaphase Zygote NT reconstructs

Recipient	Hours Post Fusion	n	n Cleaved	% Cleaved
Metaphase zygote	3.5	53	33	62.3
MII Oocyte	3.5	277	0	0.0

3.1.2 Cytochalasin B treatment of embryos

3.1.2.1 Introduction

Embryos were treated with Cytochalasin B (CB) post fusion in order to delay cleavage of the zygote and potentially achieve correct chromatin distribution in the embryos. CB delays cell cleavage by inhibiting formation of the actin filaments (MacLean-Fletcher and Pollard, 1980). IVP controls were generated in order to find if CB affected the chromatin distribution of standard IVP embryos. The embryos were analysed after periods of 4 hours and 17 hours in CB. The metaphase zygote NT embryos were also treated with CB post fusion, before washout and placement in to IVC. This was done as a further attempt to achieve development to blastocyst using the metaphase arrested zygotes as recipients.

3.1.2.2 CB Treatment of IVP embryos

Embryos (22 hpf) were fixed and stained with Hoechst 33342 after time periods of 4 h and 17 h in CB (the +CB group). These embryos were then analysed for correct chromatin distribution under U.V exposure. – CB controls (with media containing the DMSO solvent CB is dissolved in) were also included in this analysis. Before washout from CB, all embryos were at the one cell stage. 30 minutes after washout from CB in ESOF medium, almost all of the embryos had

cleaved. This shows CB is effective for delaying embryo cleavage, and that this delay in cleavage is reversible. Section 2.2.5 explains the full method for these experiments. Percentage figures in brackets indicate the proportion of embryos with the correct chromatin distribution of one nucleus (or two condensed chromatin masses) per blastomere. In a number of cases, it was unclear whether embryos did or did not have a correct chromatin distribution. The group kept in CB for the 4 hour period shows a larger proportion of embryos with the correct chromatin distribution as compared to the other treatment groups (Table 3.9, +CB 4 h). In the group treated for 4 h, a greater number of embryos were at the one and two cell stages, while the group treated for 17 h had a larger number of embryos at the ≥ 3 cell stages. This is expected due to the age of the embryos, i.e. – at 17 h, the embryos would normally be at a more advanced stage.

Table 3.9 – CB treatment of IVP embryos

Treatment	n	1c	2c	3c	4c	≥8c	Unclear
+CB 4h	25	4 (50%)	15 (67%)	1 (0%)	1 (0%)	1 (0%)	3 (12%)
-CB + DMSO 4h	19	4 (25%)	8 (63%)	4 (0%)	2 (0%)	0 (0%)	1 (5%)
+CB 17h	14	3 (33%)	6 (50%)	1 (100%)	1 (0%)	0 (0%)	3 (21%)
-CB + DMSO 17h	25	1 (0%)	7 (43%)	6 (0%)	6 (33%)	1 (0%)	4 (16%)

3.1.2.3 Effect of CB on development

Metaphase zygotes used as recipients for NT were treated with CB for the 4 h period. Again in these experiments, no cleavage of the embryos was observed prior to washout from CB. Also included in this group are the MII oocyte NT controls and IVP controls. These controls were included in case development to

blastocyst was observed in the metaphase zygote group, as the development could be compared to standard the control groups. The IVP controls are shown for both the 4 h +CB treatment, and the –CB (+ DMSO) treatment (Table 3.10). Development to blastocyst was observed in the control MII recipient group. The IVP +CB group shows higher development to blastocyst than the IVP –CB group (Table 3.10), however this result is not significant. There was still no development past the 8-cell stage in the Metaphase Zygote +CB NT group (Table 3.10).

Table 3.10 – CB treatment of metaphase zygote NT embryos

Treatment	nIVC	nLSOF	n Cleaved	% Cleaved	n2-8c	n > 8c	B 1-3	%TD
+CB IVP	30	10	26	86.7	N.D	N.D	5	16.7[a]
-CB IVP	30	5	27	90.0	N.D	N.D	2	6.7[a]
+CB NT (M-zygote)	17	4	15	88.2	4	0	0	0.0[a]
-CB NT (MII oocyte)	44	13	32	72.7	N.D	N.D	5	11.4[a]

N.D – Not determined; a,b – values with different superscripts differ significantly

3.1.3 Nocodazole washout of metaphase zygote NT +CB embryos

3.2.3.1 Introduction

Nocodazole is a drug which arrests the zygotes and cells in metaphase by depolymerisation of microtubules (Jordan *et al.*, 1992). Washing the NT embryos out of nocodazole and therefore allowing a longer time for the nuclear spindle to reform correctly before being placed CB may be beneficial for the embryos. For these experiments, NT was carried out in the same manner as in section 3.1.2.3. However, post fusion reconstructs were washed out of nocodazole for a period of either 30 minutes (group 1) or 60 minutes (group 2). This would allow observation of whether washing the reconstructs out of nocodazole would be

beneficial for the embryos and result in development to blastocyst in the metaphase zygote NT +CB group.

3.1.3.2 Nocodazole washout (w/o) data for metaphase zygote NT +CB embryos

Embryos produced from the metaphase zygote NT were washed out of nocodazole in drops of H199 + 3 mg/ml BSA - nocodazole. The first group was washed out of nocodazole for a period of 30 minutes, and the second group for a period of 60 minutes. Washing the embryos in this media allows the nuclear spindle an extra 30 – 60 minutes to reform in the absence of nocodazole and CB. This did not lead to development of the embryos past the 8-cell stage (Table 3.11). In this experiment, no embryos developed to a stage worthy of being transferred to the LSOF medium on day 5 of IVC.

Table 3.11 – Nocodazole washout of metaphase zygote NT embryos +CB

Treatment	nIVC	nLSOF	n Cleaved	% Cleaved	n2-8c	n > 8c	B 1-3	B 1-2	%TD
30min w/o	26	0	21	80.8	21	0	0	0	0.0
60min w/o	31	0	25	80.6	25	0	0	0	0.0

3.2 mRNA Analysis Results

3.2.1 Introduction

The relative mRNA content of *TPT1* and *BRG1* in zygotes of various stages, as well as MII oocytes was analysed using real-time RT PCR. This was carried out to observe any differences in the abundance of mRNA between the oocyte and zygote samples. It was proposed that those zygote/oocyte stages with a higher mRNA concentration of *TPT1* or *BRG1* would have a greater ability to reprogram somatic nuclei. All standard curves were generated using cDNA from LJ801 bovine fibroblast cells.

3.2.2 18S mRNA analysis

A standard curve was derived for the 18S primers to calculate the relative concentration of *18S* mRNA in a sample (sections 2.3.2 and 2.3.3).

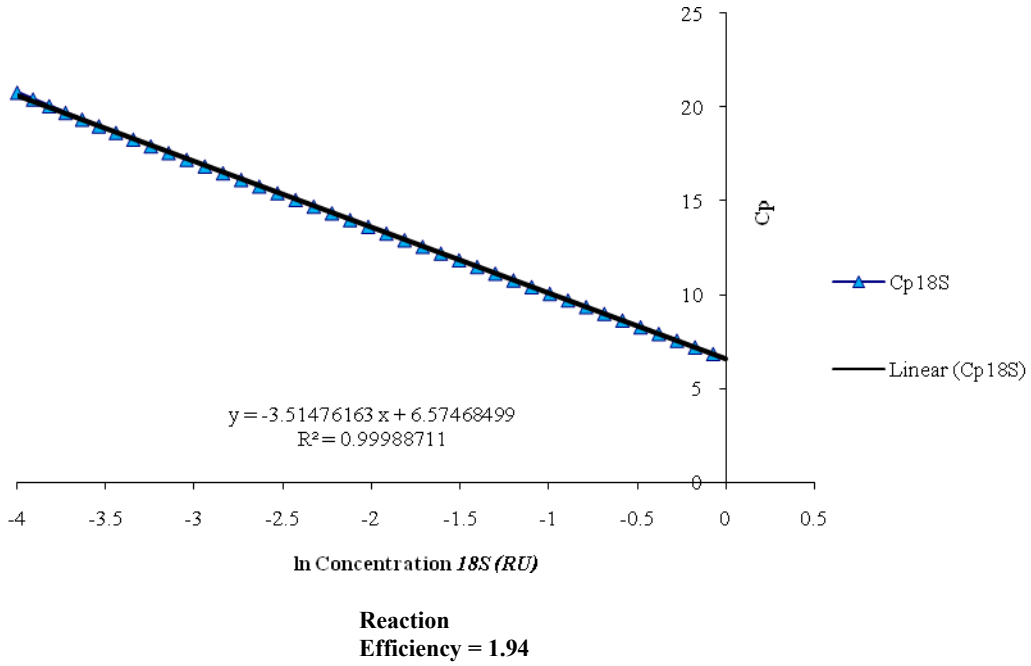


Fig 3.3 – 18S standard curve. Cp values are plotted and fitted with a logarithmic regression.

Fig 3.3 shows the standard curve for analysis of *18S* mRNA. The equation gained from this standard curve (y = -3.51476163 x + 6.57468499) was used to determine the relative concentration of *18S* mRNA in the samples (Tables 3.12 and 3.13). The relative concentration of the mRNA from the gene of interest (*TPT1* or *BRG1*) was then normalised against the concentration of *18S* mRNA in the samples. *18S* is therefore the "house-keeping" gene, as levels of *18S* are assumed to remain fairly constant throughout all samples allowing detection of any real differences in the levels of the target gene in the samples.

3.2.3 TPT1 mRNA analysis

Fig 3.4 shows the amplification curves for *TPT1* and *18S* run simultaneously using real–time PCR. The exponential phase (steep portion of the graph) and plateau phase (the flat portion at the top of the graph) are clearly visible. Crossing point values (Cp's) were taken from this real-time PCR run and placed into table 3.12.

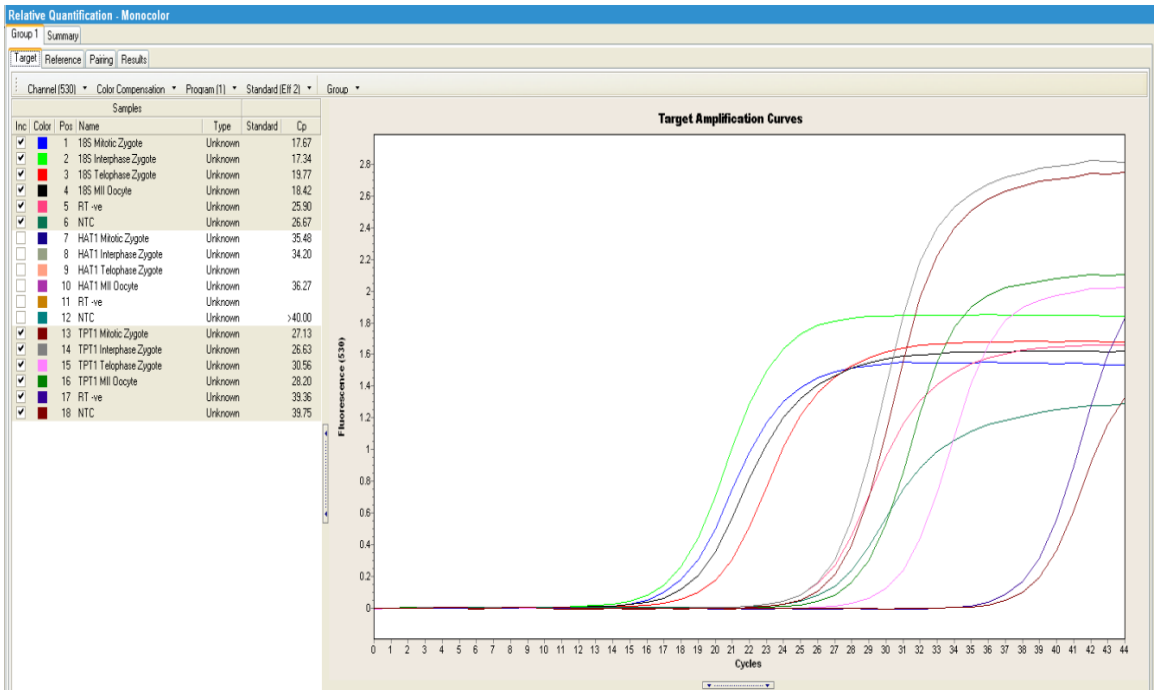


Fig 3.4 – Amplification plot of TPT1/18S

Fig 3.5 shows the dissociation curves gained for *TPT1* and *18S* when run simultaneously using real-time PCR. It can be seen that there are specific melting curves for the products, whereas melting curves for the NTC and RT –ve differ vastly from these. The curves confirm a specific product has been produced using

these primers. Melting points for *18S* range between 83.09 – 83.38°C and melting points for *TPT1* range from 88.15 – 88.47°C.

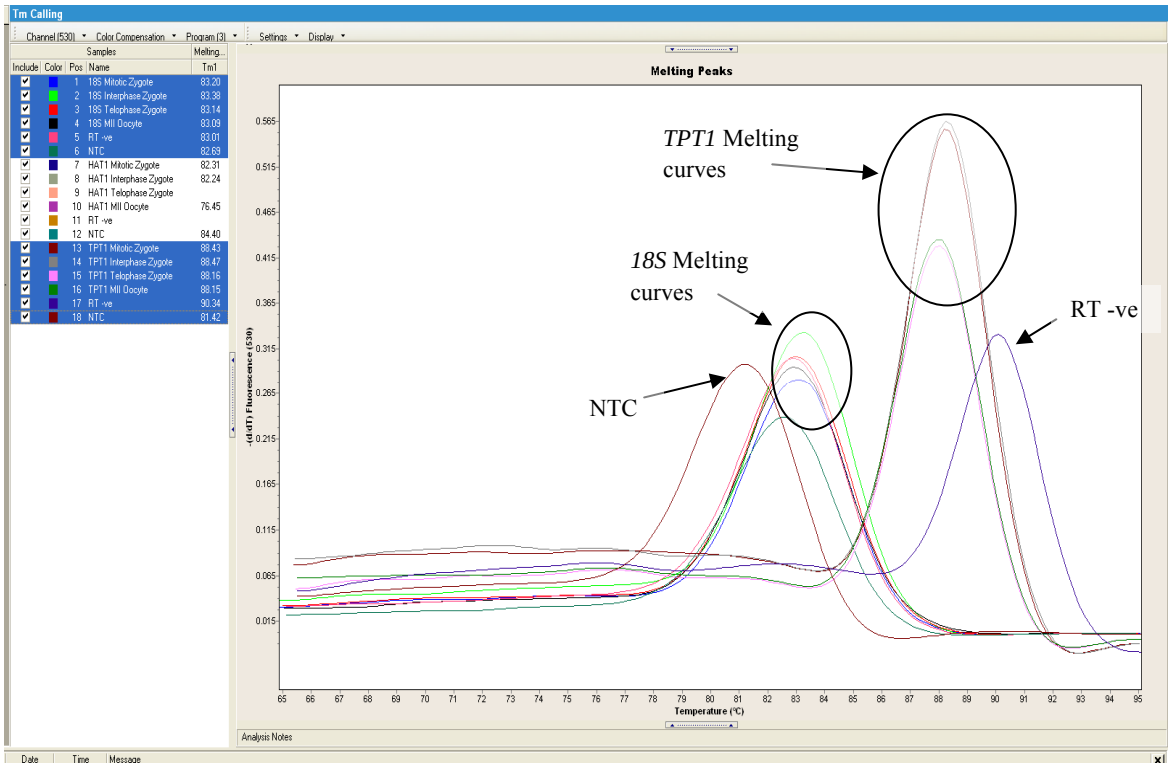


Fig 3.5 – Dissociation curves for TPT1/18S

A standard curve was derived for *TPT1* (fig 3.6) to calculate the relative concentration of *TPT1* in the samples.

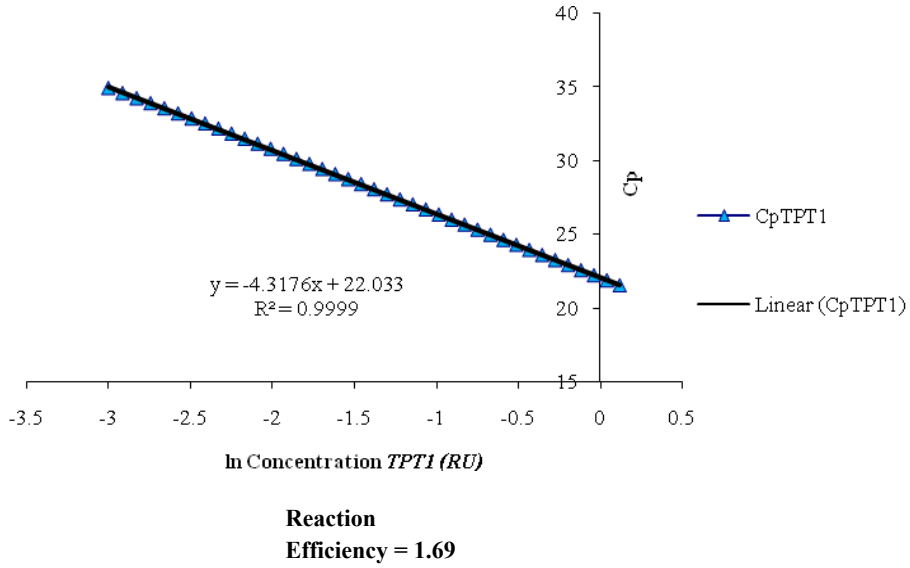


Fig 3.6 – TPT1 standard curve. Cp values are plotted and fitted with a logarithmic regression.

Fig 3.6 shows the standard curve graph for the *TPT1* mRNA analysis. Using the equation gained from this graph (y = -4.3176x + 22.033), the relative concentration of *TPT1* mRNA in the samples analysed was able to be calculated (sections 2.3.2 and 2.3.3).

Table 3.12 shows the data gained from the two real-time PCR reactions carried out using the cDNA isolated from the zygote and oocyte samples. All reactions for the *TPT1* primers were carried out simultaneously with *18S* (the house-keeping gene). For each run, the relative concentration of the *TPT1* mRNA from each zygote stage has been compared to the amount in an MII oocyte and placed in the table as the "MII Equivalent" ("MII Equi" in table 3.12). The MII equivalent was calculated as a ratio of the relative *TPT1* mRNA concentration in

the zygote to the relative *TPT1* mRNA concentration in the MII oocyte; hence the "MII Equi" value for the MII oocyte in table 3.12 shows 1.00.

Table 3.12 – mRNA analysis of TPT1

Group	**nPCR (*TPT1*)**	**Cp*TPT1***	**[*TPT1*] (RU)**	**nPCR (*18S*)**	**Cp*18S***	**[*18S*] (RU)**	***[TPT1]/[18S]***	**MII Equi**
MII	1	26.58	0.05	1	15.98	0.002	24.82	1.00
	2	28.20	0.02	2	18.42	0.000	42.91	1.00
TII	1	26.96	0.04	1	16.49	0.002	27.09	1.09
	2	30.56	0.00	2	19.77	0.000	22.47	0.52
Interphase	1	26.49	0.06	1	15.79	0.002	23.23	0.94
	2	26.63	0.05	2	17.34	0.001	58.57	1.36
M-Zygote	1	29.84	0.00631	1	18.94	0.000	20.82	0.84
	2	27.13	0.03663	2	17.67	0.001	52.56	1.22

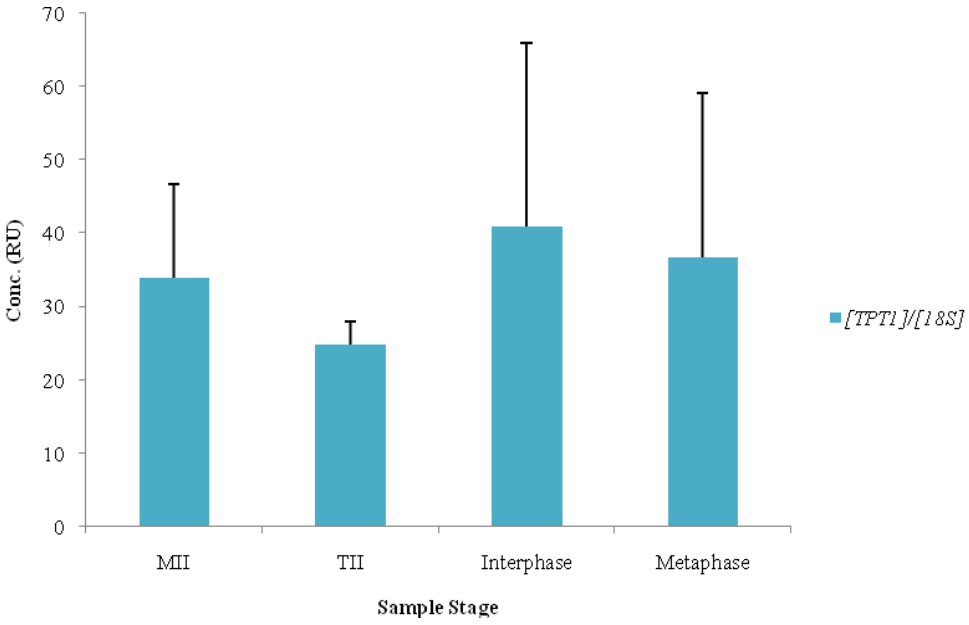


Fig 3.7 – Relative concentration of TPT1 in different stages of embryo development. Error bars represent the standard deviation from the two real-time PCR reactions. A statistical analysis carried out on this data in the form of a two-tailed t test reveals the data is not significant (values do not differ $P < 0.05$).

3.2.4 BRG1 mRNA analysis

A standard curve was derived for the *BRG1* primers (fig 3.27) to calculate the relative concentration of *BRG1* mRNA in the samples analysed.

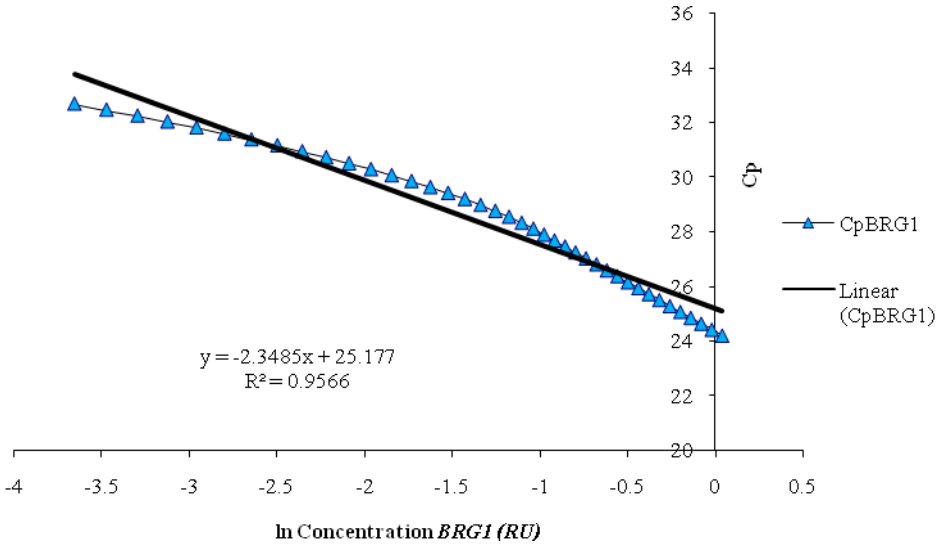


Fig 3.8 – BRG1 standard curve. Cp values are plotted and fitted with a logarithmic regression.

Fig 3.8 shows the standard curve generated for the *BRG1* mRNA analysis. The equation of this curve (y = -2.3485x + 25.177) was used to determine the relative concentration of *BRG1* in the samples analysed (sections 2.3.2 and 2.3.3).

Table 3.13 shows the same information as shown in table 3.12, but for the *BRG1* gene. As for the *TPT1* mRNA analysis, the real-time PCR carried out on the *BRG1* gene was normalised using the house-keeping gene *18S*. The relative concentration of *BRG1* in the zygote samples was compared to the amount in a

standard MII oocyte and the MII equivalent ratio calculated for all samples as done for the *TPT1* gene. All PCR experiments were carried out using cDNA from the same isolation procedure.

Table 3.13 – mRNA analysis of BRG1

Group	**Replicate (*BRG1*)**	**Cp*BRG1***	**[*BRG1*] (RU)**	**Replicate *18S***	**Cp*18S***	**[*18S*] (RU)**	***[BRG1]/[18S]***	**MII Equi**
MII	1	25.01	1.11	1	13.59	0.010	110.43	1.00
	2	28.85	0.09	2	18.42	0.000	216.42	1.00
TII	1	26.81	0.35	1	15.04	0.004	88.80	0.80
	2	31.06	0.02	2	19.77	0.000	124.94	0.58
Interphase	1	25.15	1.02	1	14.00	0.008	131.91	1.19
	2	29.84	0.05	2	17.34	0.001	56.11	0.26
M-Zygote	1	29.84	0.00631	1	18.94	0.000	20.82	0.84
	2	27.13	0.3663	2	17.67	0.001	52.56	1.22

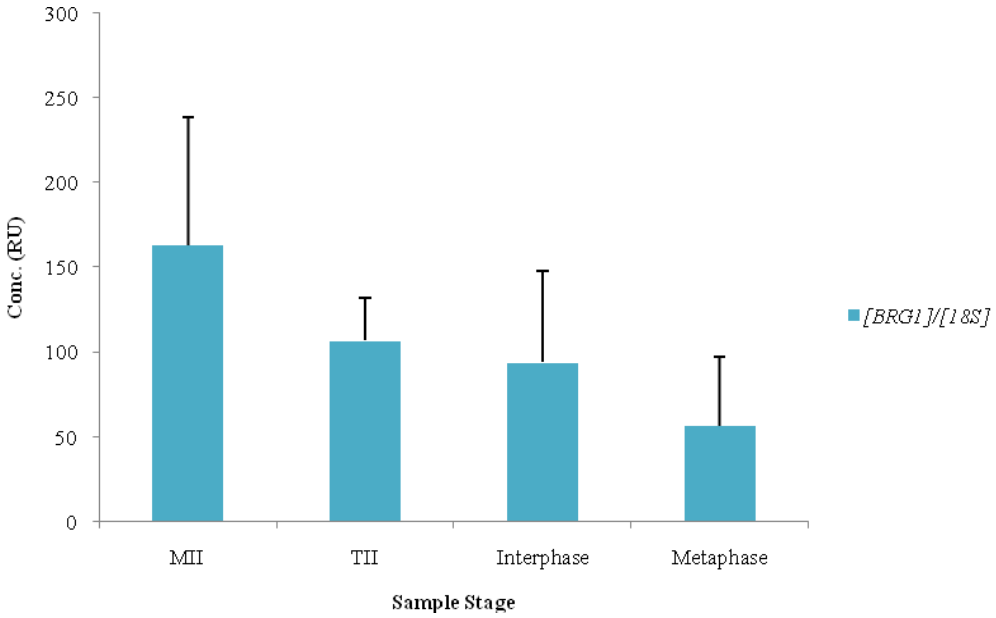


Fig 3.9 – Relative concentration of BRG1 in different stages of embryo development. Error bars represent the standard deviation from the two real-time PCR reactions. A statistical analysis carried out on this data in the form of a two-tailed t test revealed the data was not significant (values do not differ $P < 0.05$)

3.3 Immunocytochemistry Results

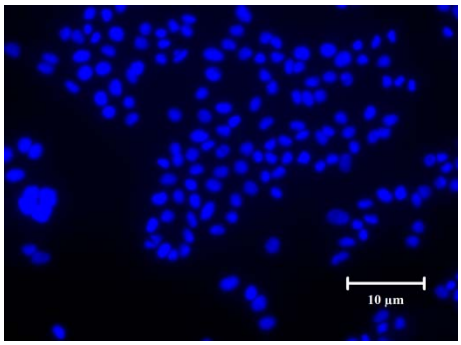
3.3.1 TCTP Immunocytochemistry

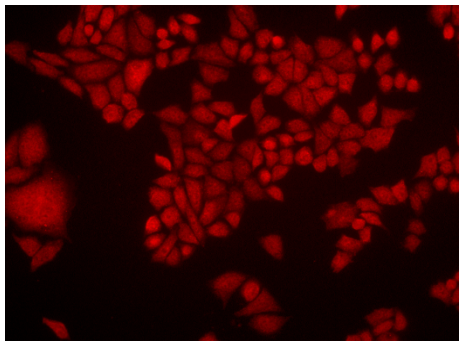
3.3.1.1 Introduction

TCTP was identified as a putative reprogramming factor, as it has been found to activate transcription of key pluripotency genes (*OCT-4* and *NANOG)* in transplanted Hela cell nuclei (Koziol *et al*., 2007). TCTP was also found to be present in bovine oocytes with a high potential to reprogram somatic cells (Tani *et al*., 2007). Cells, oocytes and zygotes of various stages were stained with the TCTP primary antibody and a fluorescent secondary antibody in order to analyse its presence in these samples. The hypothesis was that TCTP would localise to the pronuclei of interphase zygotes.

3.3.1.2 Hela cells

A) ***B)***





C) ***D)***

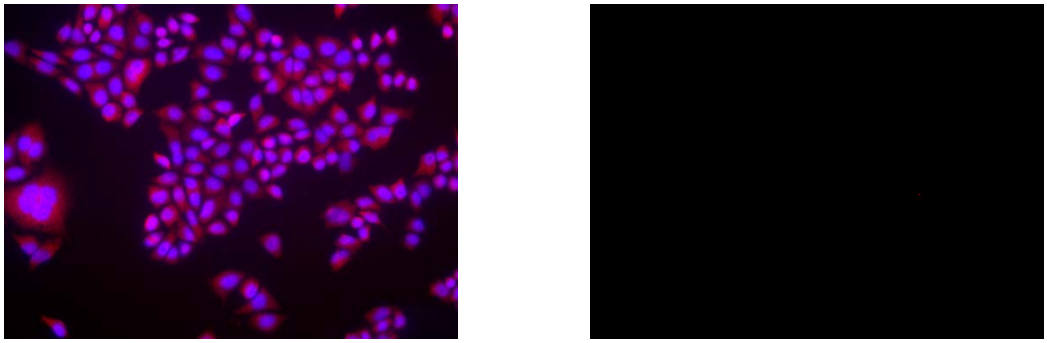
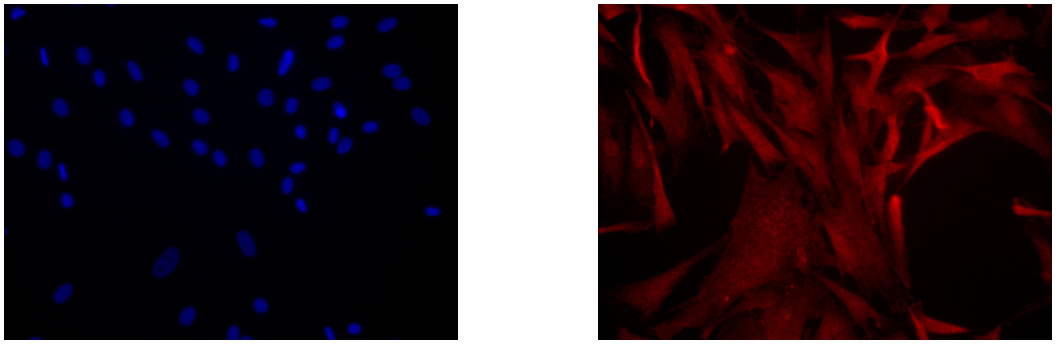


Fig 3.10 – TCTP ICC on Hela cells. A) Hoechst 3342 nuclear stain. B) TCTP antibody stain. C) Merge of figs A and B. D) Negative control.

Reactivity of the TCTP antibody with Hela cells was tested. Hela cells are immortal human epithelial cervical cancer cells. The TCTP antibody has been tested to cross react with human species. Results gained here show a positive stain for TCTP and a cytoplasmic, as well as nuclear distribution of the TCTP protein (figs 3.10 C and 3.10 B). The negative control for this ICC is shown in fig 3.10 D) and is clearly negative.

3.3.1.3 CCL-44 bovine fibroblast immortalised cell line

A) ***B)***



C) ***D)***

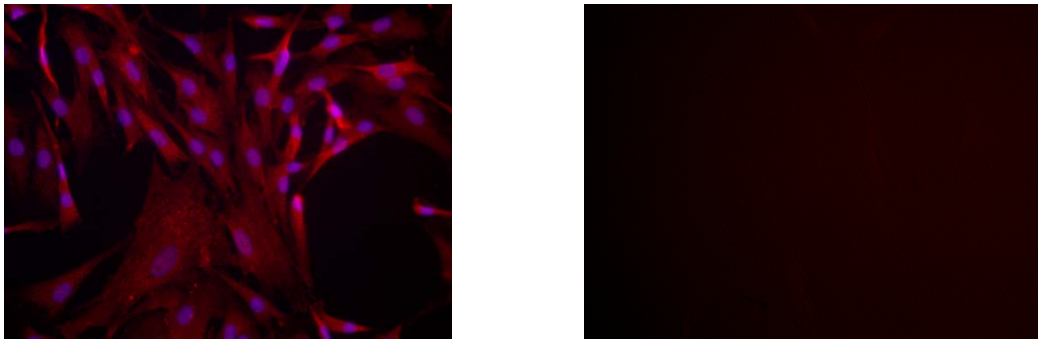


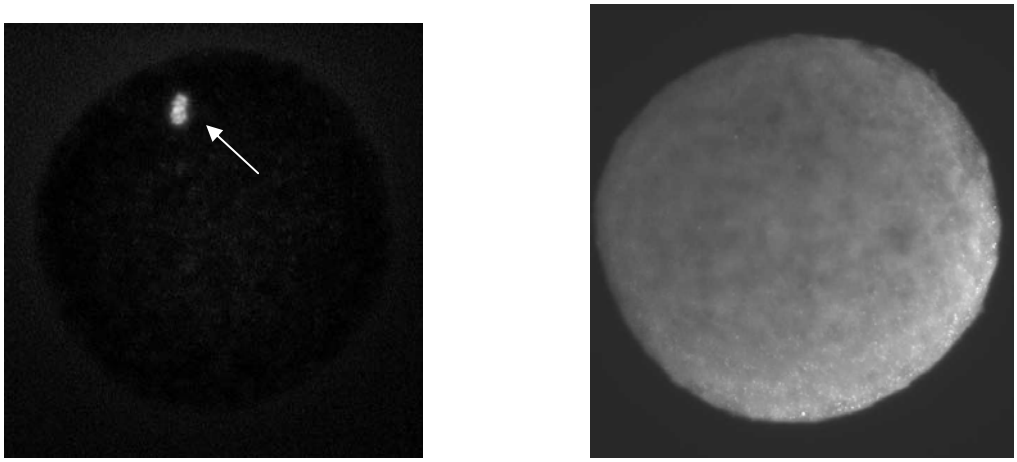
Fig 3.11 – TCTP ICC on CCL-44 cells. A) Hoechst 33342 nuclear stain. B) TCTP antibody stain. C) Merge of figs A and B. D) Negative control

The TCTP antibody was tested for cross-reactivity with the bovine species by carrying out immunocytochemistry on the immortalised bovine fibroblast cell line CCL-44 (embryonic bovine trachea cells). A positive result was obtained in these samples, showing the antibody does indeed cross react with bovine cells. From the image, it is clear that TCTP is distributed in the cytoplasm and nuclei of the randomly proliferating CCL-44 cells (figs 3.11 B and 3.11 C).

3.3.1.4 Oocytes and Zygotes

3.3.1.4.1 MII oocyte

A) ***B)***



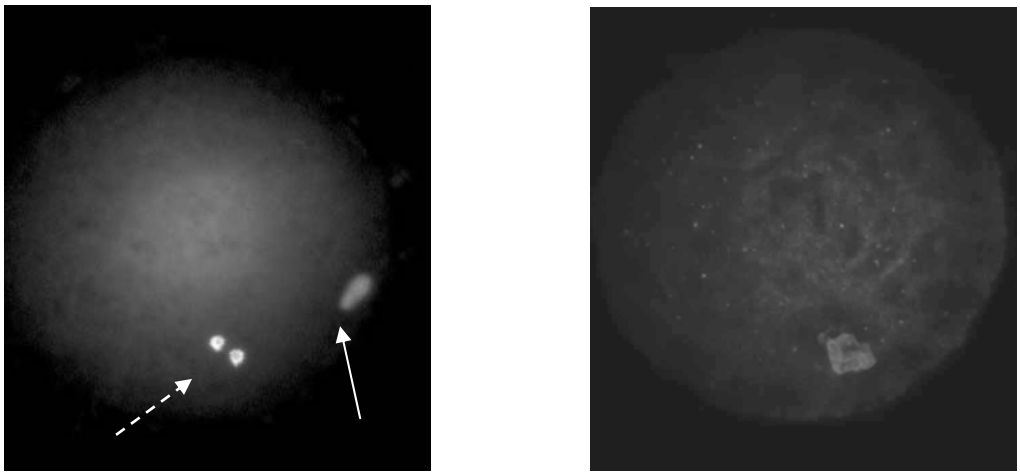
C)

Fig 3.12 – TCTP ICC on MII oocyte. A) Hoechst 33342 nuclear stain. B) TCTP antibody stain. C) Negative control.

Fig 3.12 A) shows the Hoechst 33342 staining of the MII oocyte, with the condensed metaphase II plate visible (solid arrow). MII oocytes have a uniform staining of TCTP throughout, clearly indicating that TCTP is present in the MII oocyte (fig 3.12 B).

3.3.1.4.2 Telophase II zygote

A) ***B)***



C) ***D)***

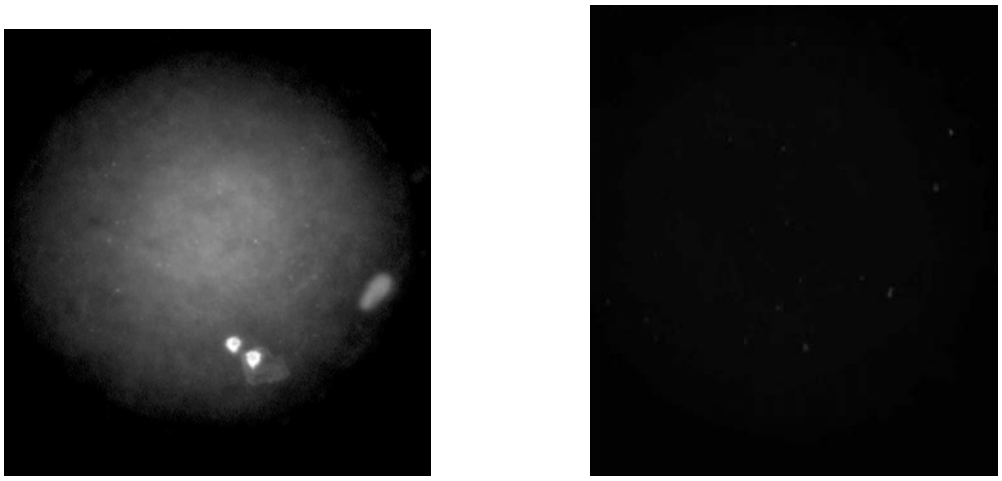


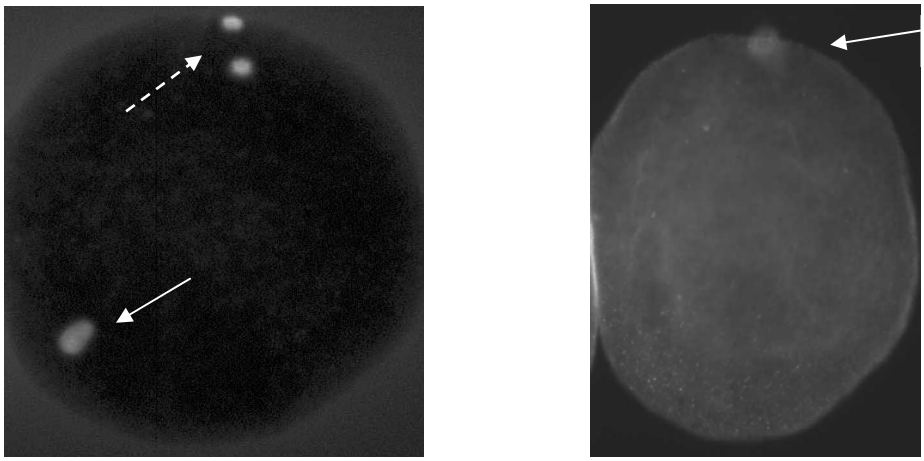
Fig 3.13 – TCTP ICC on TII zygote. A) Hoechst 33342 nuclear stain. B) TCTP antibody stain. C) Merge of figs A and B. D) Negative control.

The Hoechst 33342 stain of the TII zygote (fig 3.13 A) shows the two maternal chromatin masses (dashed arrow) and the paternal sperm DNA (solid arrow). It can be seen the TCTP stain of the same TII zygote (fig 3.13 B) shows a clear localisation of TCTP to the maternal chromatin. There is no staining visible

around the paternal chromatin. When figs 3.13 A) and 3.13 B) are merged (fig 3.13 C) it can be seen that the TPT1 localises to only one of the two maternal chromatin masses

Further images of TII zygotes were analysed. Figs 3.14 A) and 3.14 B) show a TII zygote mounted in the correct orientation on the slide, so that the extruded second polar body (PB) is clearly visible (fig 3.14 B). Hoechst 33342 staining shows the two maternal chromatin masses (dashed arrow) and the paternal chromatin (solid arrow). TCTP staining of the zygotes (fig 3.14 B) shows that TCTP protein localises to the second polar body of the TII zygote (as there is a more intense signal).

A) ***B)***



C)

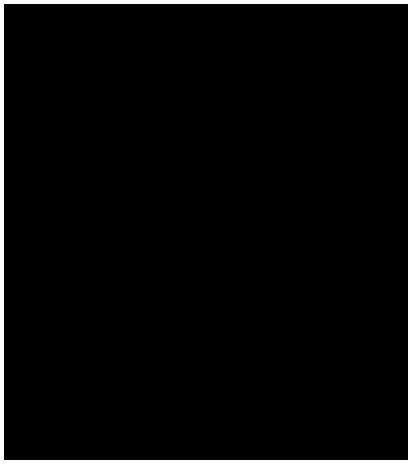


Fig 3.14 – TCTP ICC on TII zygotes showing 2nd PB. A) Hoechst 33342 nuclear stain. B) TCTP antibody stain. C) Negative control.

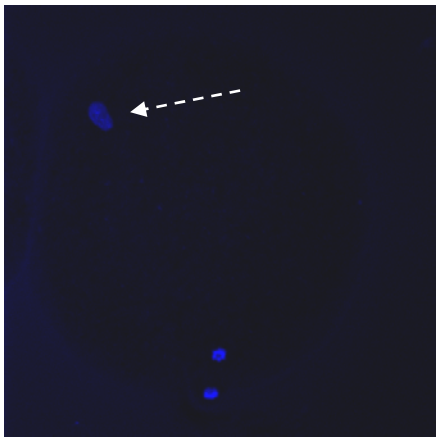
Quantification of the number of TII zygotes with a TCTP localisation to the second polar body is shown in table 3.14 below. It can be seen that from 14 TII zygotes stained with the TCTP antibody where the second polar body is visible, nearly 80% showed a clear localisation of TCTP to the polar body. None showed clearly no localisation to the polar body and three which were analysed it was unclear whether or not there was a localisation of TCTP to the polar body.

Table 3.14 – Quantification of TII TCTP localisation

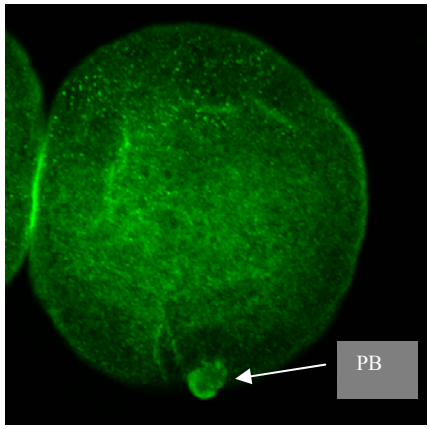
n	n Positive	n Negative	n Inconclusive	% Positive
14	11	0	3	78.6

3.3.1.4.3 Confocal imaging of TII zygotes

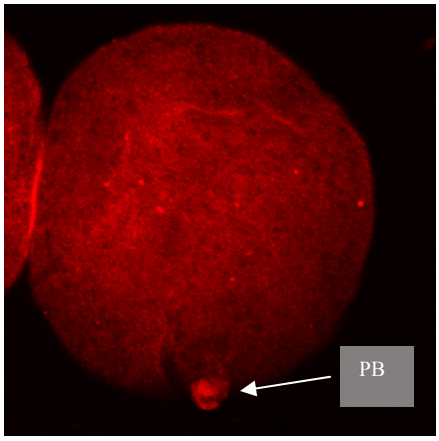
A)



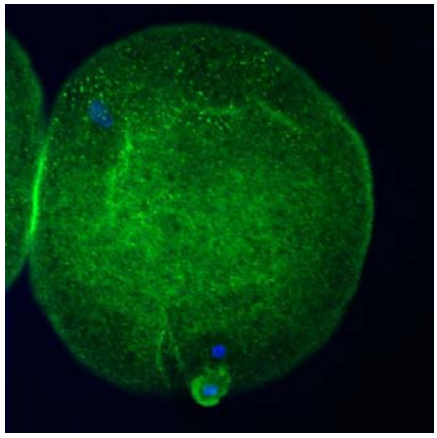
B)



C)



D)



E) ***F)***

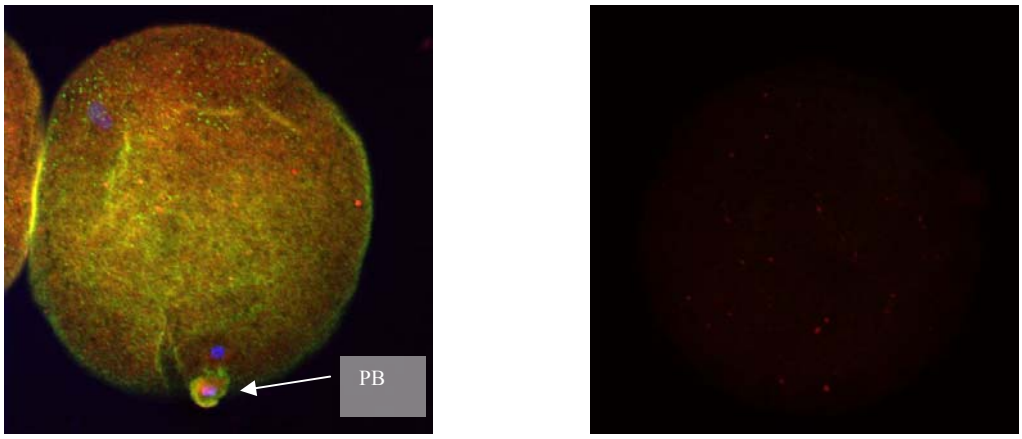
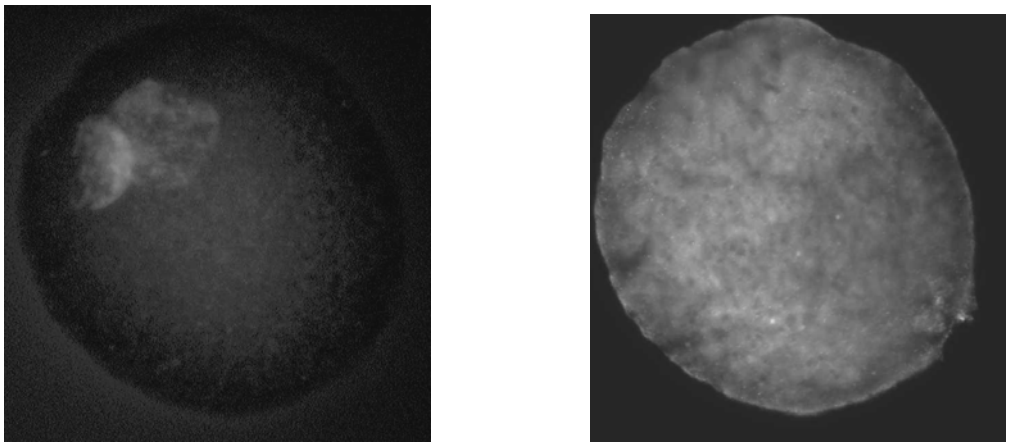


Fig 3.15 – Confocal images of a TII zygote showing 2nd PB. A) Hoechst 33342 nuclear stain. B) TCTP antibody stain. C) Lamin antibody stain. D) Merge of figs A and B. E) Merge of figs A, B and C. F) Negative control.

Confocal microscopy was also used to analyse TII zygotes mounted in the correct orientation on the slide so the second polar body (PB) is visible. This high resolution analysis confirms that TCTP localises to the second polar body (figs 3.15 B and 3.15 D). Double staining with Lamin (red) shows that TCTP also co-localises with lamin (fig 3.15 C). Paternal DNA from the sperm (dashed arrow) can be seen from the Hoechst 33342 stain (fig 3.15 A). When merged with the image of the TCTP stain (fig 3.15 B), it can be seen that TCTP does not localise to the paternal chromatin of the TII zygote (fig 3.15 D). The negative control (fig 3.15 F) shows some red background staining from the lamin antibody (red), but is essentially negative. Fig 3.15 E) shows the TCTP stain co-localises with lamin in the second polar body.

3.3.1.4.4 Interphase zygote

A) ***B)***



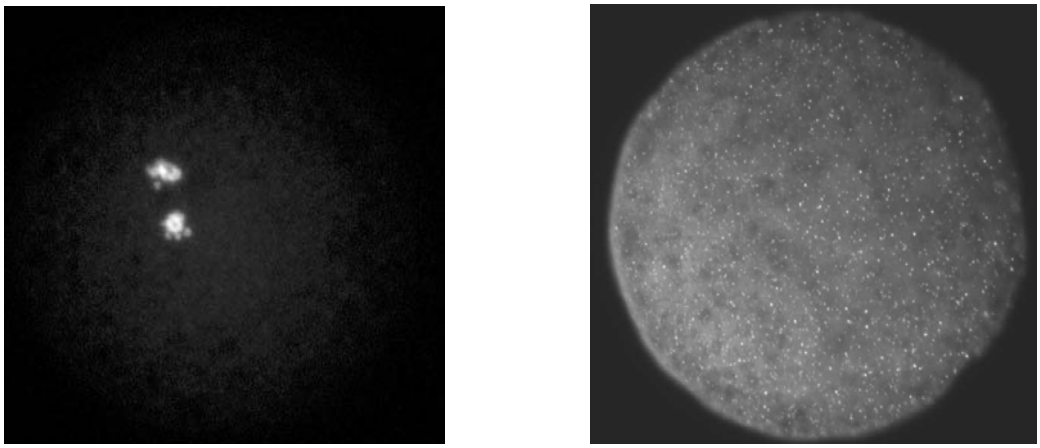
C)

Fig 3.16 – TCTP ICC on interphase zygote. A) Hoechst 33342 nuclear stain. B) TCTP antibody stain. C) Negative control.

Fig 3.16 A) shows the Hoechst 33342 stain of an interphase zygote, with the two large pronuclei clearly visible. From fig 3.16 B) it can be seen the interphase zygote stains positively for TCTP, with a small localisation to the plasma membrane (possibly an artefact).

3.3.1.4.5 Metaphase zygote

A) ***B)***



C)

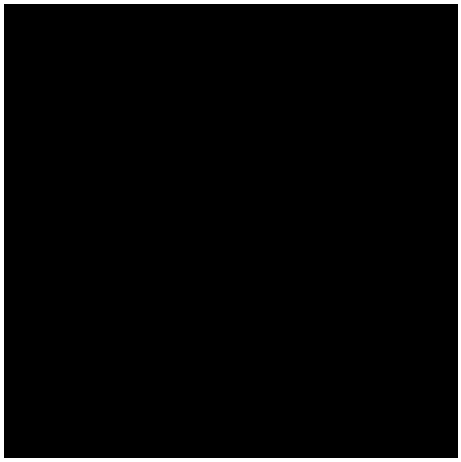


Fig 3.17 – TCTP ICC on metaphase zygote. A) Hoechst 33342 nuclear stain. B) TCTP antibody stain. C) Negative control.

Fig 3.17 A) shows the Hoechst 33342 stain of a metaphase zygote with the two distinct condensed chromatin masses. The metaphase zygote stains positive for TCTP (fig 3.17 B). There looks to be a uniform distribution of the protein, with a small localisation to the plasma membrane, which may be an artefact of the staining.

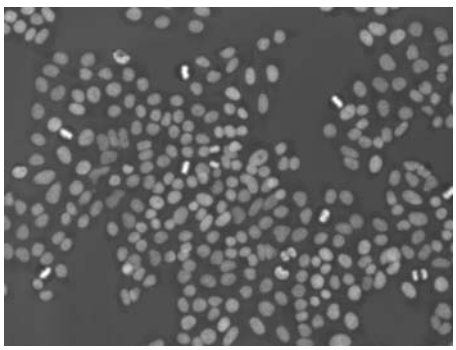
3.3.2 BRG1 Immunocytochemistry

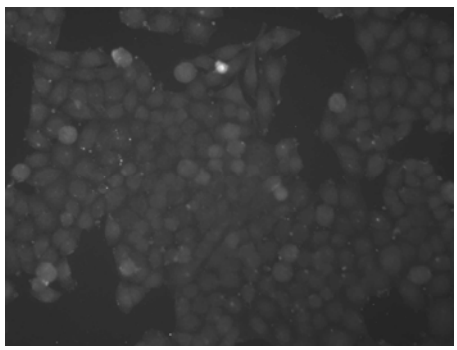
3.3.2.1 Introduction

BRG1 has been identified as necessary for reprogramming human somatic cells using *Xenopus* egg extract. Pluripotency markers (*OCT4* and *GCAP*) were up regulated in somatic cells in the presence of BRG1 (Hansis *et al*., 2004). Immunocytochemistry of BRG1 was carried out on Hela cells, bovine cells, oocytes and zygotes of various stages to analyse the presence of the protein in these samples. The hypothesis was that like TCTP; BRG1 would also localise to the pronuclei of interphase zygotes.

3.3.2.2 Hela cells

A) ***B)***





C)

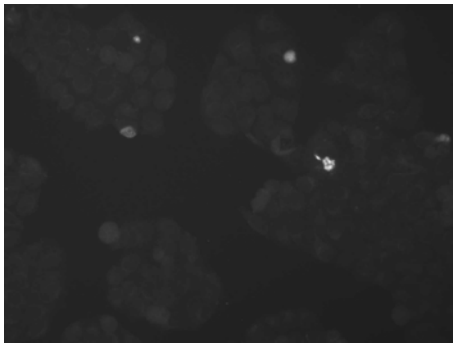
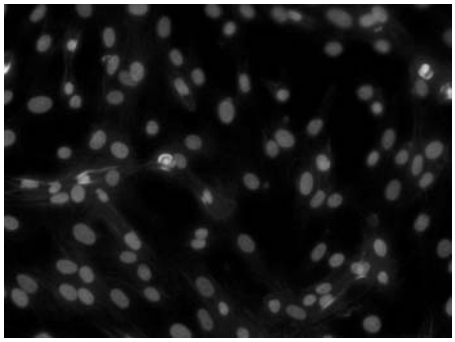


Fig 3.18 – BRG1 ICC on Hela cells. A) Hoechst 33342 nuclear stain. B) BRG1 antibody stain. C) Negative control.

The BRG1 antibody was tested on Hela cells as a positive control to confirm the antibody reacts with human cells (a species on which the antibody has been tested). Fig 3.18 B) confirms the antibody does react with the Hela cells, showing a weak stain throughout the cells. Although this staining is weak and non-specific, it is significantly different from the negative control (fig 3.18 C), where only weak background staining was observed.

3.3.2.3 CCL-44 cells

A) ***B)***



C)

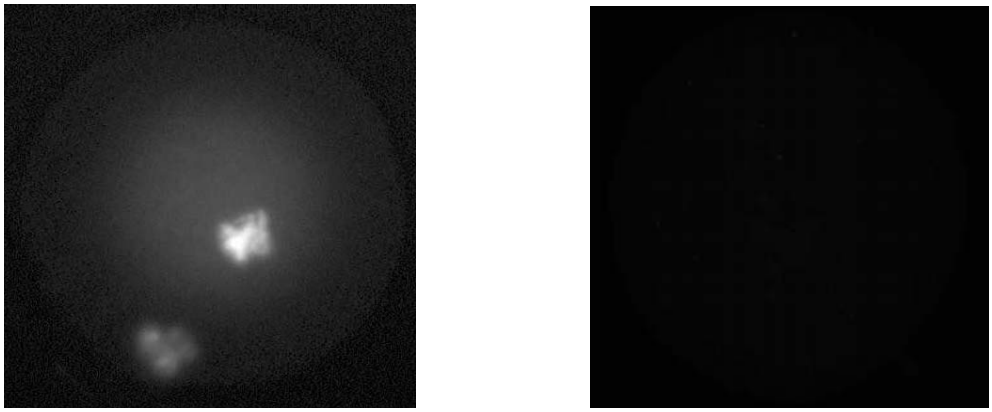
Fig 3.19 – BRG1 ICC on CCL-44 cells. A) Hoechst 33342 nuclear stain. B) BRG1 antibody stain. C) Negative control.

The antibody was tested on the bovine fibroblast cell line CCL-44 (fig 3.19 B) to observe if the antibody cross-reacts with the bovine species. It can be seen there is no significant difference between the BRG1 antibody stain on the CCL-44 cells (fig 3.19 B) and the negative control (fig 3.19 C), as both are essentially black.

3.3.2.4 BRG1 ICC on bovine embryos

The BRG1 antibody was also tested on a bovine metaphase zygote (fig 3.20 A) to confirm the antibody does not react with the bovine species. It can be clearly seen that the results for the BRG1 ICC on the bovine zygote are also negative. The antibody stain (fig 3.20 B) does not differ from the negative control (fig 3.20 C).

A) ***B)***



C)

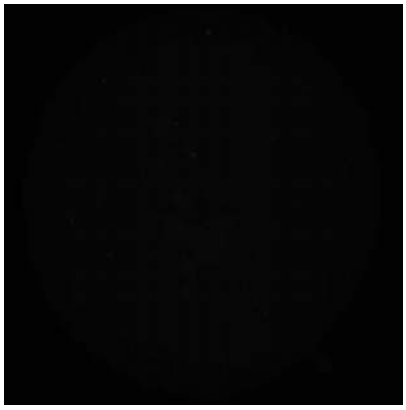


Fig 3.20 – BRG1 ICC on bovine zygotes. A) Hoechst 33342 nuclear stain. B) BRG1 antibody stain. C) Negative control.

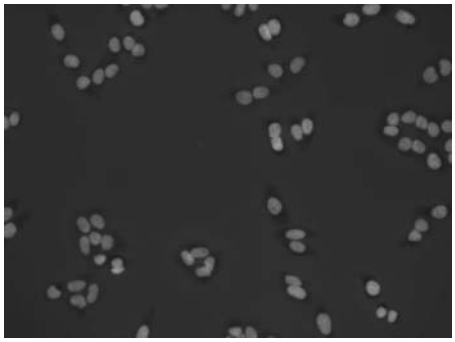
3.3.3 HDAC1 Immunocytochemistry

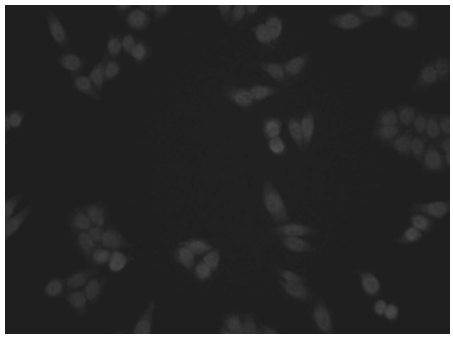
3.3.3.1 Introduction

Histone Deacetylase 1 (HDAC1) has been shown to have important affects on the development of preimplantation mouse embryos, by acting as a repressor of transcription in 2-cell mouse embryos (Ma and Schultz, 2008). HDAC1 immunocytochemistry was carried out on Hela cells, bovine cells, oocytes and zygotes of various stages and its presence in these samples was analysed. HDAC1 has been shown to localise to the chromosomes of MII mouse oocytes as well as the nucleus and pronucleus of mouse zygotes (Ma and Schultz, 2008). Due to these observations, a similar nuclear localisation was expected in the bovine cells.

3.3.3.2 Hela cells

A) ***B)***





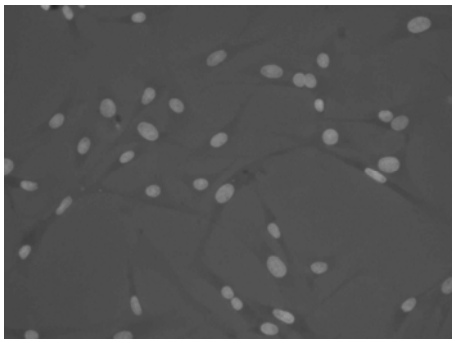
C)

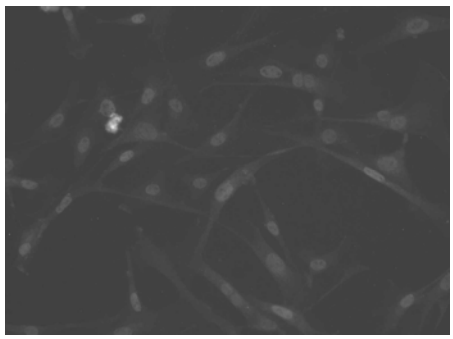
Fig 3.21 – HDAC1 ICC on Hela cells. A) Hoechst 33342 nuclear stain. B) HDAC1 antibody stain. C) Negative control.

The staining of Hela cells shows a clear positive result for the presence of HDAC1 (fig 3.21 B). Localisation of the staining looks to be essentially nuclear for the HDAC1 antibody.

3.3.3.3 CCL-44 cells

A) ***B)***





C)

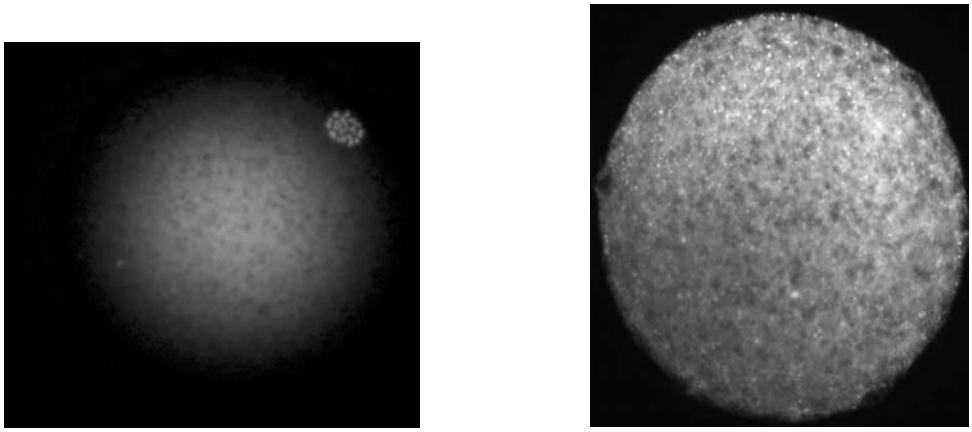
Fig 3.22 – HDAC1 ICC on CCL-44 cells. A) Hoechst 33342 nuclear stain. B) HDAC1 antibody stain. C) Negative control.

Fig 3.22 A) shows the Hoechst 33342 stain on a population of randomly proliferating CCL-44 bovine cells. The HDAC1 antibody stain fig (3.22 B) shows there is the same nuclear staining present in the CCL-44 bovine cells as seen in Hela cells. This confirms that the antibody reacts with both the bovine and human species, and is therefore suitable to use on bovine zygotes and oocytes.

3.3.3.4 Oocytes and Zygotes

3.3.3.4.1 MII oocyte

A) ***B)***



C)

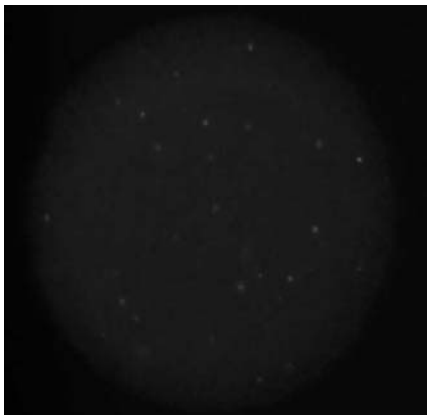
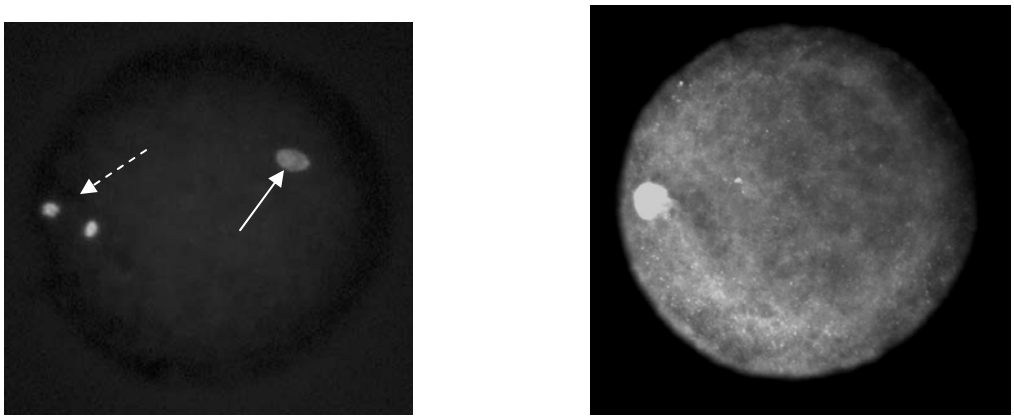


Fig 3.23 – HDAC1 ICC on MII oocyte. A) Hoechst 33342 nuclear stain. B) HDAC1 antibody stain. C) Negative control.

Fig 3.16 A) shows the Hoechst 33342 stain of a metaphase II oocyte, with the condensed metaphase plate clearly visible. From the respective HDAC1 antibody stain of this oocyte (fig 3.23 B), it can be seen the HDAC1 stain is very similar to the TCTP stain (fig 3.12 B). Both have a strong positive signal throughout the oocyte.

3.3.3.4.2 Telophase II zygote

A) ***B)***



C)

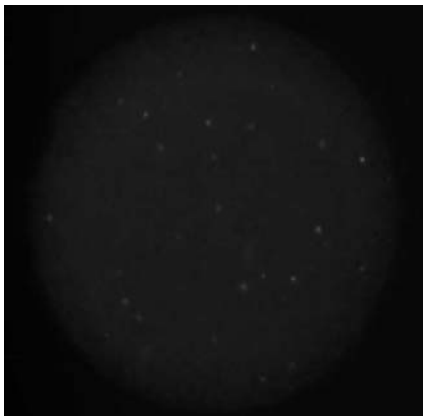
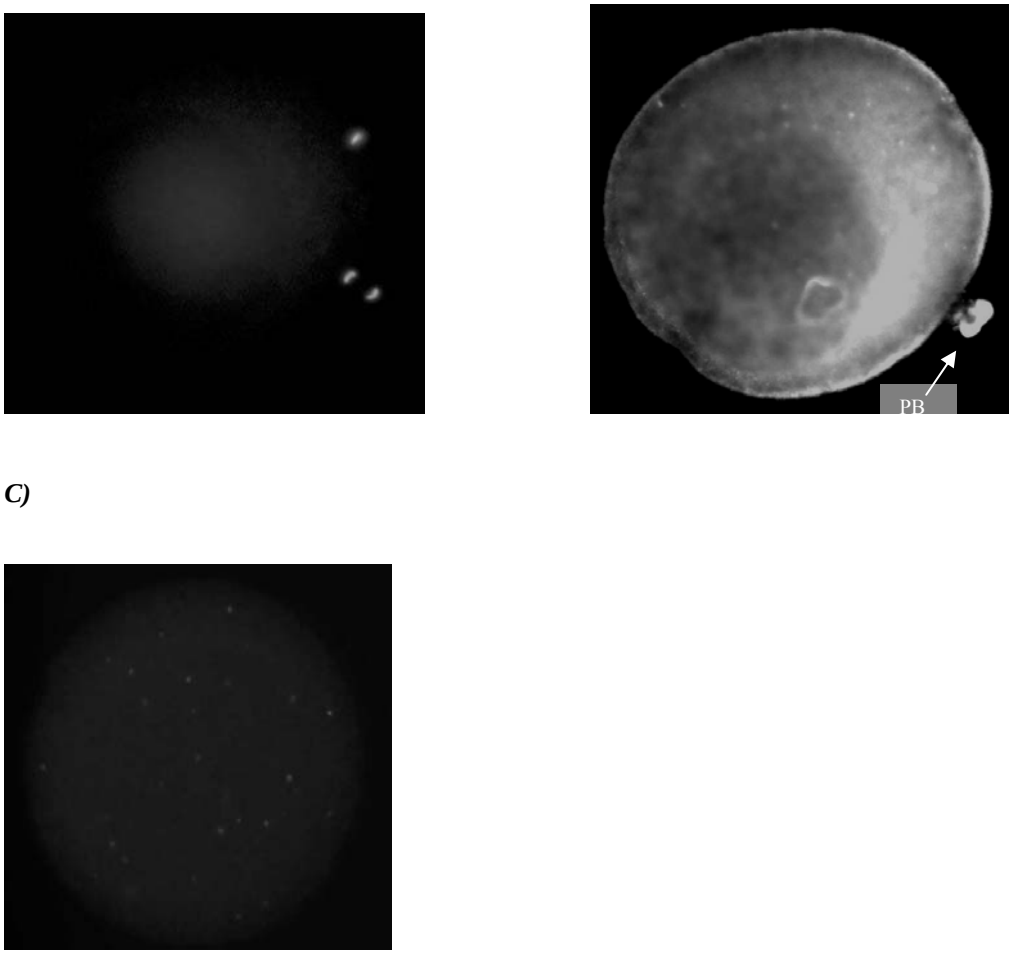


Fig 3.24 – HDAC1 ICC on TII zygote. A) Hoechst 33342 nuclear stain. B) HDAC1 antibody stain. C) Negative control.

Fig 3.24 A) shows the Hoechst 33342 stain of a TII zygote, with two maternal chromatin masses (dashed arrow), and the paternal sperm genome (solid arrow). The respective HDAC1 antibody stain of this zygote can be seen in fig 3.24 B). It can be seen there is a strong staining of HDAC1 which localises to one of the maternal chromatin masses of the zygote (which could be the extruded second polar body). Also, the HDAC1 protein is clearly absent from the paternal DNA.

This is almost identical to the TCTP staining observed in the TII zygotes (fig 3.14 B). To confirm if the HDAC1 protein does localise to the second polar body of the TII zygote, zygotes mounted in the correct orientation so that the second polar body can be seen were analysed (below).

A) ***B)***



C)

Fig 3.25 – HDAC1 ICC on TII zygote showing 2nd PB. A) Hoechst 33342 nuclear stain. B) HDAC1 antibody stain. C) Negative control.

Fig 3.25 A) shows the Hoechst 33342 stain of a TII zygote, with the second polar body (PB) is clearly visible. The respective HDAC1 stain of this zygote (fig 3.25

B) shows there is a strong localisation of the HDAC1 protein to the second polar body of the TII zygote. Also there seems to be some localisation to the plasma membrane (possibly an artefact). This is similar what was observed for the TCTP ICC performed on TII zygotes with an extruded second PB (fig 3.14 B).

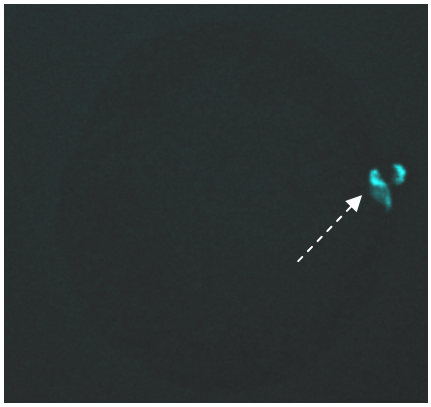
As with TCTP, numbers of TII zygotes with a HDAC1 localisation to the second polar body were recorded. Results are shown in table 3.15 below. From 9 TII zygotes stained with the HDAC1 antibody where the second polar body is visible, 7 showed a clear localisation of HDAC1 to the polar body. There were none where no localisation to the second polar body was evident, and two where it was unable to be concluded whether there was a localisation or not.

Table 3.15 – Quantification of TII HDAC1 localisation

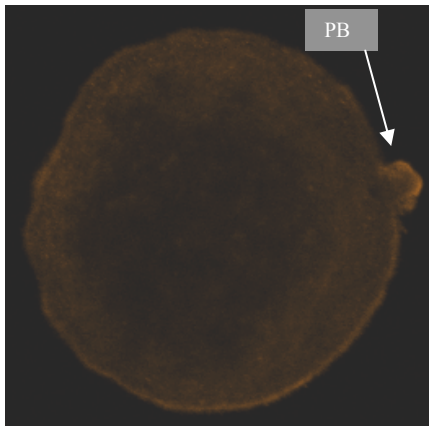
n	n Positive	n Negative	n Inconclusive	% Positive
9	7	0	2	77.8

3.3.3.4.3 Confocal imaging of TII zygotes

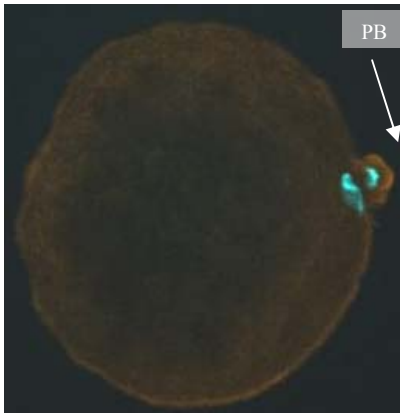
A)



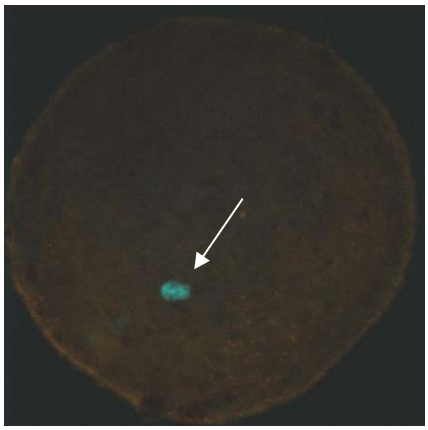
B)



C)



D)



E)

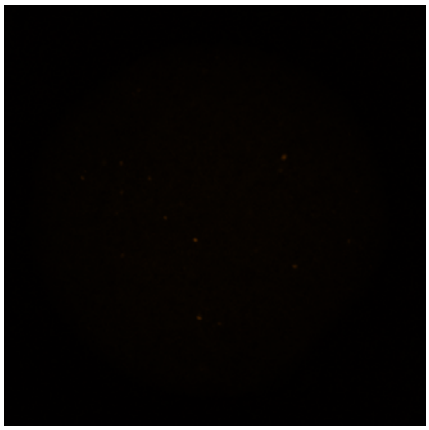
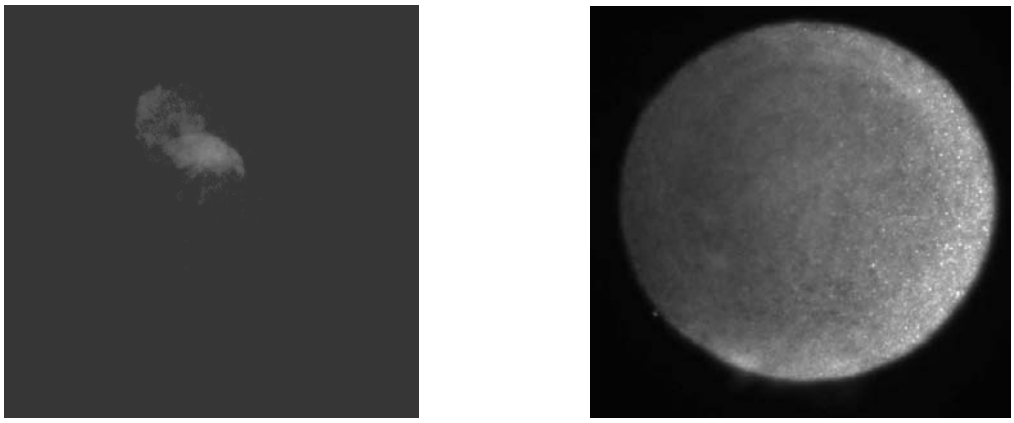


Fig 3.26 **-** Confocal images of a TII zygote showing 2nd PB. A) Hoechst 33342 nuclear stain (maternal chromatin). B) HDAC1 antibody stain. C) Merge of figs A and B. D) Hoechst 33342 nuclear stain (paternal chromatin) and HDAC1 stain. E) Negative control.

Figs 3.26 A) – E) show images of a TII zygote viewed under the confocal microscope. In figs 3.26 B) and C), the extruded second polar body is clearly visible. The Hoechst 33342 stain of the TII zygote with the two maternal chromatin masses clearly visible (dashed arrow) is shown in fig 3.26 A). Fig 3.26 B) shows the respective HDAC1 antibody stain of the zygote in fig 3.26 A). It can be seen that there is a stronger staining signal (orange) in the extruded second polar body of this zygote. A merge of figs 3.26 A) and 3.26 B) shows the stronger staining signal of the HDAC1 is found around the second maternal chromatin mass of the zygote which is extruded as the polar body. Fig 3.26 D) shows a merged image of the paternal chromatin (solid arrow) from a TII zygote (stained with Hoechst 33342) and the respective HDAC1 stain of this zygote. It can be seen from this image that the HDAC1 protein does not localise to the paternal chromatin (there is no HDAC1 signal around the paternal chromatin). This is identical to the results from the TCTP staining (fig 3.13 B) of the TII zygote, which shows TCTP does not localise to the paternal chromatin.

3.3.3.4.4 Interphase zygote

A) ***B)***



C)

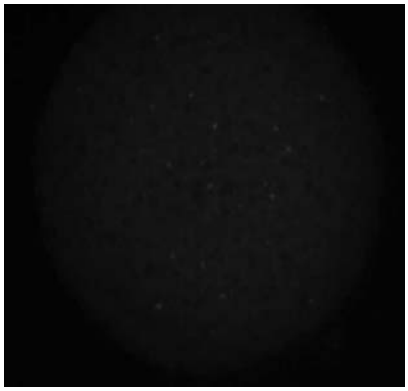
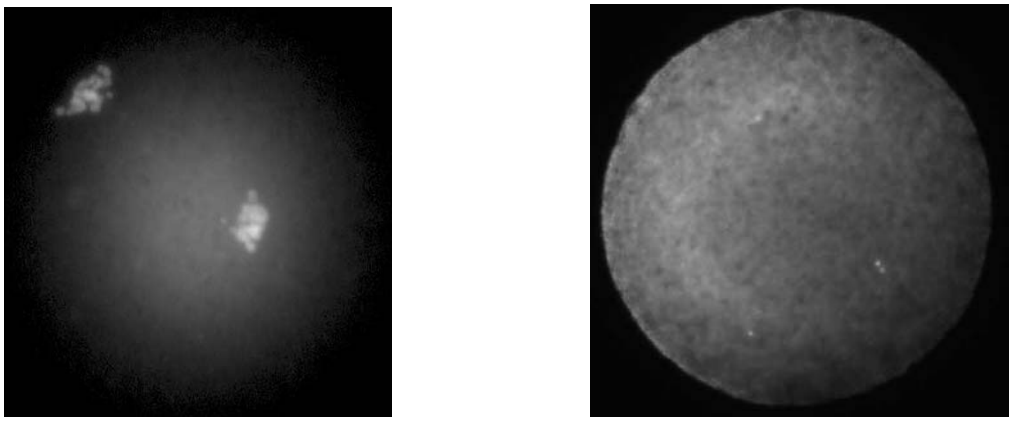


Fig 3.27 **–** HDAC1 ICC on interphase zygote. A) Hoechst 33342 nuclear stain. B) HDAC1 antibody stain. C) Negative control.

Fig 3.27 A) shows the Hoechst 33342 stain of an interphase zygote, with two pronuclei (cloudy in appearance) visible. There seems to be a fairly uniform stain of HDAC1 throughout the interphase zygote (fig 3.27 B). HDAC1 staining of the zygote is similar to the TCTP stain (fig 3.16 B) with a small apparent localisation to the plasma membrane observed.

3.3.3.4.5 Metaphase zygote

A) ***B)***



C)

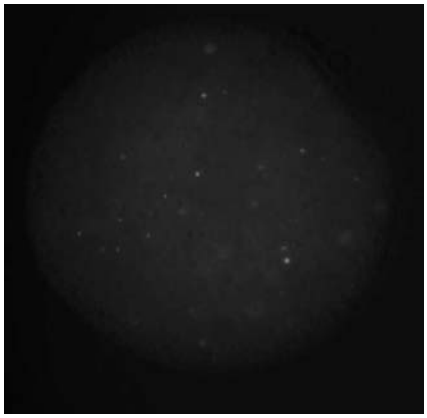


Fig 3.28 – HDAC1 ICC on metaphase zygote A) Hoechst 33342 nuclear stain. B) HDAC1 antibody stain. C) Negative control.

Fig 3.21 A) shows the Hoechst 33342 stain of a metaphase zygote with the two condensed chromatin masses. The staining of a metaphase zygote with HDAC1 (fig 3.28 B) is similar to the staining of TCTP observed in the metaphase zygote (fig 3.17 B). There is a clear positive stain evident with an apparent small localisation to the plasma membrane.

DISCUSSION

4. Discussion

4.1 Metaphase zygote NT experiments

This study demonstrates that blastocysts can be produced by NT of a metaphase donor cell into an enucleated metaphase II oocyte. Tani, Kato *et al*. (2001) also produced blastocysts using somatic mitotic donor cells for NT, with an average efficiency of ~ 30%; three times higher than development achieved in my experiments (10.8%). This may be due to the different methodologies used by Tani, Kato *et al*. (2001). In their experiments, fused NT embryos were activated using a combination of an electrical pulse (rather than a calcium ionophore) and exposure to CHX. Also, Tani and colleagues used bovine cumulus cells as donors, rather than LJ 801 fibroblasts used in my experiments. It is also unclear from the Tani, Kato *et al*. (2001) study if all media used during the NT procedure contained nocodazole to ensure the donor cells would not exit metaphase. This could mean some of the observed development may have resulted from recipients fused to a donor cell of a stage other than metaphase. Differences in techniques of the operator carrying out the procedure (between myself and Tani, Kato *et al*. 2001) may have also contributed to these observed differences in results.

Tani, Kato *et al*. (2001) discovered that whether or not the early embryo extruded a second polar body, had no effect on the *in vitro* developmental potential of that embryo. However, those embryos which did not extrude a second polar body were found to be tetraploid and therefore could not develop into live calves (Tani *et al*.,

2001). Five diploid embryos produced using metaphase arrested donor cells were transferred to recipient cows, and one live calf was produced. This proves that NT using metaphase donor cells can produce cloned embryos which have the potential to develop into normal calves (Tani *et al*., 2001). In my experiments using MII oocytes as NT recipients for metaphase donor cells, polar body extrusion was found to be low (~ 27%), but the result is similar to the rate of second polar body extrusion seen by Tani, Kato *et al*. (2001) (26.5%). Karyotyping of 20 embryos (data not shown) indicated 18 of the blastocysts produced in my experiments were clearly not diploid and therefore may not have the potential to develop into normal live cloned offspring (Booth *et al*., 2003).

Several fusion parameters were trialled in order to find the optimal conditions for fusion of metaphase arrested somatic donor cells into metaphase zygotes. Despite this, fusion rates for metaphase arrested somatic cells into enucleated metaphase zygotes were low. The fusion rates for metaphase arrested somatic cells into MII oocytes were higher, and similar to the results achieved by Tani, Kato *et al*. (2001). However, the fusion efficiency achieved in my control NT experiments is still considerably lower than fusion efficiency for control NT experiments in the Schurmann, Wells *et al*. (2006) paper which used serum starved donor cells (Schurmann *et al*., 2006). This may be due to the larger size of the metaphase donor cells used in my experiments. The larger cytoplasmic volume removed from metaphase zygotes during enucleation as compared to MII oocytes (Oback *et al*., 2003) may also have contributed to the drop in fusion efficiency (Zakhartchenko *et al*., 1997). Membrane changes which occur in the oocyte

during fertilisation and subsequent activation (Runft *et al*., 2002) may also be responsible for the lower observed fusion efficiency.

During the second metaphase zygote NT experiment, the selection efficiency of metaphase zygotes was analysed. It was found approximately 80% of all NT reconstructs produced in these experiments used a metaphase zygote recipient. This figure may be influenced by my method of selecting the zygotes, and one can assume that over time my selection efficiency of metaphase zygotes would improve. The remaining 20% were either MII oocytes, interphase zygotes or the stage of the recipient could not be determined from the appearance of the chromatin. It can be assumed enucleated interphase zygotes mistakenly used as recipients would not develop (Wakayama *et al*., 2000) and therefore not produce a false positive result. However, MII cytoplasts used as recipients for these experiments could develop to blastocyst, giving a misleading result. If this was the case, it would be expected at least some development or polar body extrusion would be observed. Numbers of embryos placed into IVC from these experiments may have been too small to observe any potential development in the metaphase zygote recipient NT group.

From the third metaphase zygote NT experiment, the average volume of cytoplasm removed from the zygotes during enucleation was calculated. This volume was found to be considerably more than the volume removed from MII oocytes during the standard zona-free (zona-pellucida removed) NT procedure. Oback, Wiersema *et al*. (2003) found the average volume removed from MII

oocytes during standard zona-free enucleation to be 1.8 ± 0.3% (Oback *et al*., 2003). It is possible the higher volume of cytoplasm removed when enucleating the metaphase zygotes (as compared to the MII oocytes) may have contributed to halting development of the embryos at the 8-cell stage. Reducing the volume of bovine oocytes used has recipients for NT by 50% has been shown to decrease development to blastocyst (Westhusin *et al*., 1996), however, reducing the volume of the oocyte by 5% was shown to have no significant effect on development to blastocyst (Westhusin *et al*., 1996). In my experiments, although the average volume of cytoplasm removed from the embryos was less than 50%, it was greater than 5% and so this reduction may have compromised development of the resulting embryos. Westhusin, Collas *et al*. (1996) also found a significantly lower cell number was present in blastocysts produced from oocytes with 50% of their cytoplasm removed. Again, a 5% reduction in the cytoplasmic volume of NT recipient cytoplasts shows no significant difference in blastocyst cell number compared to the controls. The cytoplasmic volume removed when enucleating zona-free metaphase zygotes is more than three times the volume removed when enucleating zona-free MII oocytes, and is also much more variable. The rationale that a reduction in cytoplasmic volume of bovine oocytes decreases development may also apply to bovine zygotes.

Blastocysts could not be produced by NT of a metaphase donor cell into an enucleated metaphase zygote. Delaying cleavage of the embryos by exposure to CB for four hours was successful and appeared to have a beneficial effect on the chromatin distribution in the early embryos. Exposure of embryos to CB for a period of four hours did not have any significant affect on total development to

blastocyst. Development in both the + and – CB groups was observed to be low, which may be due to the low number of embryos produced in these experiments. Another potential reason as to why no development to blastocyst was observed may have been due to exposure of the embryos to nocodazole throughout the NT procedure, which ensures the zygotes and cells remain in metaphase. Nocodazole arrests cells in metaphase by depolymerisation of the microtubule spindles of the cell (Jordan *et al*., 1992). Washing the reconstructs out of nocodazole and therefore allowing more time for the spindle to reform in the presence of CB may be beneficial to embryo development. No development was observed past the 8c stage in embryos washed out of nocodazole before treatment with CB and culture.

All development in the metaphase zygote recipient NT experiments was halted at the 8-cell stage. Possibly the main reason behind this may be due to the early cleavage of the NT zygotes post fusion, which would not allow for correct chromatin organisation. Egli, Rosains *et al*. (2007) observed correct chromosome segregation and cleavage within 90 – 150 minutes in the mouse. In my experiments over 60% of the reconstructs had cleaved 3.5 hours post fusion, however correct chromosome segregation was not observed, therefore these events post NT seem to occur differently in bovine. Although CB was effective in delaying cleavage, it may also have inhibited reformation of the nuclear spindle. Ploidy errors resulting from no extrusion of a pseudo polar body may also explain why no development was observed past the 8-cell stage.

Embryonic genome activation occurs at the 8 – 16-cell stage in bovine embryos (Camous *et al*., 1986) and this may not have occurred in these experiments, meaning no new transcription would be initiated in the early embryo. Another potential explanation as to why no development was observed past the 8-cell stage may be due to the exposure of the zygotes to U.V light during the enucleation procedure. In the Egli *et al*. 2007 paper in which blastocysts were produced using metaphase zygotes as recipients for NT, the microtubule spindle was visualised for enucleation using either Hoffman modulation contrast (which allows phase gradients to be visualised) or optical birefringence (where the double refraction of light allows chromosomes to be visualised). Visualising chromosomes by staining with Hoechst 33342 dye and exposure to U.V was found to compromise development (Egli *et al*., 2007). In my experiments the chromosomes were visualised for enucleation by staining with Hoechst 33342 dye and illumination under U.V light. Exposure times to U.V of greater than 30 seconds have been shown to affect membrane integrity, methionine incorporation and therefore to alter protein synthesis in bovine oocytes (Smith, 1993).

4.2 mRNA analyses of candidate reprogramming factors

TPT1 mRNA was found to be expressed in all samples analysed. This is in agreement with current findings that *TPT1* is expressed in all mammalian tissues analysed thus far (Sanchez *et al*., 1997). It has also been previously described that *TPT1* mRNA is present in bovine oocytes from the germinal vesicle (GV) to MII stage as well as during preimplantation development stages from the pronucleus to blastocyst (Tani *et al*., 2007). From the mRNA analysis of the *TPT1* gene, it

was unable to be concluded if this gene would have a major influence on the reprogramming ability of the oocytes and zygotes analysed. No significant differences were observed in the relative concentrations of *TPT1* mRNA between the various samples**.**

BRG1 mRNA was also found in all samples analysed by real-time PCR, which is in agreement with current knowledge. *BRG1* is known to be expressed in mouse oocytes (Bultman *et al*., 2000) and was also found to be expressed in zygotes (Bultman *et al*., 2006). However, results from this analysis of *BRG1* mRNA were also inconclusive.

Results gained from real-time PCR reactions for the *TPT1* and *BRG1* genes may be inconclusive due to the relatively low reaction efficiencies gained when producing the standard curves for the primers. The ideal reaction efficiency for a PCR reaction is 2.0 (meaning the amount of transcript is exactly doubled each cycle). My reaction efficiencies for standard curves were consistently less than 2.0, which could lead to errors being produced when calculating the relative concentrations of the genes in the samples. Recent evidence suggests house-keeping genes may vary between samples (Dheda *et al*., 2004) and so variability in the concentration of *18S* in these samples may also have contributed to errors in the calculated relative concentrations.

Statistical analysis was carried out on these results in the form of a two-tailed t test. This test revealed no significant differences in the relative concentrations of the target genes between the samples. The differences in the MII equivalents between the samples were also not significant. Although mRNA transcript levels of *TPT1* remained constant throughout the various samples, some stages of recipients used for NT have differing abilities to reprogram an introduced somatic donor cell. This may be due to a different localisation of the TCTP protein between the NT recipients. Analysis of HDAC1 mRNA was not carried out as primers for this gene were not acquired.

4.3 ICC Analyses of cells, MII oocytes and zygotes

Immunocytochemistry performed on the TCTP, BRG1 and HDAC1 proteins allowed observation of the distribution of the protein the gene translates to throughout the various sample stages. Bovine cells and zygotes stained with the BRG1 antibody produced negative results. One explanation for this may be that the mRNA present in the zygote and oocyte samples (as shown from real-time RT-PCR results) is not translated to the BRG1 protein in the bovine cells or embryos. The positive result for BRG1 on human Hela cells shows the BRG1 mRNA is translated in this sample. Another explanation for the negative result of BRG1 ICC on bovine samples is that the chosen antibody does not cross-react with the bovine species. This is plausible as the portion of the protein the antibody reacts with (first 15 amino acids of the N-terminus) in human is not identical to the bovine equivalent (94% homology).

Results from ICC of TCTP on human and bovine cells as well as show a cytoplasmic distribution of the TCTP protein, with some nuclear localisation observed (certainly not absent from all cell nuclei). These results are in agreement with the description that TCTP is a cytoplasmic protein (Li *et al*., 2001), although some nuclear localisation has been observed (Yubero *et al*., 2008). Tani, Shimada *et al*. (2007) found that phosphorylated TCTP is present in bovine oocytes before GVBD until the MII stage and was seen to decline gradually after activation by ionomycin and CHX or 6-DMAP. My results also show a clear presence of TCTP in the bovine MII oocyte. Localisation of TCTP in bovine zygotes has not been previously described. These results show a positive stain for TCTP in all stages of zygote, with a clear localisation of the protein to the maternal chromatin in the second polar body of TII zygotes.

MII mouse oocytes stained with an HDAC1 antibody show a localisation of the protein to the metaphase chromosomes (Ma and Schultz, 2008). This result is contradictory to my observations on bovine MII oocytes, where a uniform stain of HDAC1 can be seen throughout the entire oocyte. ICC performed on mouse zygotes by Ma and Schultz (2008) shows that following fertilisation, the HDAC1 protein remains present in the pronucleus and nucleus, up to the blastocyst stage. My results show no nuclear localisation of HDAC1 to the pronuclei of interphase zygotes or to the chromosomes of metaphase zygotes in bovine. In the TII zygotes, however, there is a localisation of the HDAC1 protein to the maternal chromatin of the second polar body. This finding has not been described previously, possibly as zygotes at the TII stage are seldom examined.

Both the TCTP and HDAC1 proteins have very similar patterns of staining throughout the oocytes as well as the various zygote stages. In interphase and metaphase zygotes there is a uniform stain throughout the zygote, with a slight localisation to the plasma membrane. This observed localisation to the plasma membrane may however been an artefact due to an artificially high concentration of the antibody present around the edges of the sample ("edge effect"). Both proteins were shown localise to the maternal chromatin of TII zygotes, and remain absent from the paternal chromatin. More specifically, they seem to localise to the maternal chromatin mass which is extruded from the zygote as the second polar body. Almost all TII zygotes which were mounted on the slide in such a way as to allow clear viewing the extruded second polar body, showed a clear staining intensity in this region. Confocal microscopy of these samples confirmed this result, and double staining zygote samples with lamin and TCTP revealed that TCTP also co-localises with lamin (main components of the nuclear envelope) in the second polar body of TII zygotes. The ultimate result from these findings is that these putative reprogramming factors (TCTP and HDAC1) localise to the second polar body of TII zygotes, which is then extruded from the zygote. Although there were no significant differences in the relative concentrations of the *TPT1* gene between the zygote stages, the localisation of the protein produced by the translated mRNA is clearly unique in the TII zygotes.

The result from immunocytochemistry performed on the TII zygotes is surprising, as it is known that enucleated TII zygotes used as recipients for NT results in improved *in vivo* development of offspring (Schurmann *et al*., 2006). Since TCTP (potentially required for correct reprogramming of the donor nucleus) localises to

the second polar body, this candidate reprogramming factor would be enucleated and discarded during the IVF-NT procedure used by Schurmann, Wells *et al*. (2006). One would assume that this loss of this potentially beneficial reprogramming factor would result in poorer development of the IVF-NT embryos. However the observed *in vivo* development results from Schurmann, Wells *et al*. (2006) contradict this. One reason why this occurs may be due to TCTP becoming obsolete once fertilisation has occurred. Once the sperm has entered the oocyte and a zygote is formed, activation of the *POU5F1* and *NANOG* pluripotency genes by TCTP (Koziol *et al*., 2007) may no longer be required. This theory may also confirm the suggestion that artificial activation of NT embryos contributes to poorer *in vivo* development (Schurmann *et al*., 2006), as artificial activation of the reconstructs may lead to incorrect localisation or extrusion of TCTP. This means the introduced donor nucleus may not be reprogrammed correctly to an embryonic state. Removal of HDAC1 from the TII zygote during the IVF-NT procedure may be beneficial for development of the resulting embryos. HDAC1 is known to initiate a transcriptionally repressive state in preimplantation mouse embryos (Ma and Schultz, 2008). This may mean repressing transcription of pluripotency genes such as *POU5F1* and *NANOG*, and therefore not allowing correct epigenetic reprogramming of the donor genome. Exposure of NT embryos to the HDAC inhibitor TSA, results in greater development of the embryos (Kishigami *et al*., 2006) which confirms HDAC is inhibitory to their development and justifies that removal of HDAC1 may be beneficial to embryo development.

4.4 Conclusions

These results suggest metaphase zygotes are not suitable as recipients for SCNT using the current methodologies. Use of a longer wavelength dye to visualise the chromatin or employing an alternative enucleation technique may lead to success in future experiments. This study showed there were no significant differences between the relative concentrations of *TPT1* and *BRG1* mRNA in zygotes or MII oocytes. The ICC analysis of the zygotes and oocytes revealed TCTP and HDAC1 proteins seemed to be evenly distributed throughout the MII oocytes, metaphase zygotes and interphase zygotes. However these proteins clearly showed a localisation to the second polar body of telophase II zygotes. This has never been described previously. Future work on these candidate reprogramming factors must be carried out in order to unearth the significance of this finding.

4.5 Future work

Future metaphase zygote NT experiments conducted to achieve development in the metaphase zygote recipient group may include the use of a longer wavelength fluorochrome (such as SYBR 14) to visualise the chromosomes of metaphase zygotes during enucleation. Staining chromosomes of oocytes with longer wavelength fluorochromes results in greater development to blastocyst when compared with Hoechst 33342 staining followed by U.V illumination (Dominko *et al*., 2000). An alternative enucleation technique such as chemical enucleation (Moura *et al*., 2008) could be employed to reduce the amount of cytoplasm removed during the procedure. These experiments should also use larger numbers

of fused reconstructs, approximately the same number as used in the MII control NT experiments.

Analysis of the *HDAC1* mRNA could be carried out on the different zygote stages as well as the MII oocytes in order to observe any differences in the levels of the *HDAC1* gene between the samples. This would allow comparison of the gene expression pattern of *HDAC1* to those of *BRG1* and *TPT1*. Gene expression levels of other candidate reprogramming factors such as nucleoplasmin (Betthauser *et al*., 2006; Tamada *et al*., 2006), germ cell proteins FRGY2a and FRGY2b (Gonda *et al*., 2003) and the nuclear protein N1/N2 (Kleinschmidt *et al*., 1986) could also be analysed in zygotes and oocytes.

In continuation of the ICC analysis, western blotting could be performed on the various stages of zygote as well as the MII oocytes to quantify any differences in the abundance of protein between the samples. ICC could be carried out on isolated second polar bodies of the TII zygotes, as well as the first polar bodies of the MII oocytes. It is not known if the TCTP and HDAC1 proteins also localise to the first polar body of MII oocytes. Western blotting could be carried out on the isolated polar bodies also.

Telophase II nuclear transfer (TNT) experiments could be trialled in an attempt to increase the concentration of candidate reprogramming factors in NT embryos, leading to greater cloning efficiency. TNT experiments would involve removing the maternal chromatin (including the second polar body) from TII zygotes by enucleation. The intact karyoplasts would be retained and fused into MII oocytes.

Once the candidate reprogramming factors had dissociated into the cytoplasm, the remaining chromatin would then be enucleated and a somatic donor cell fused into the oocyte as per the standard NT procedure. A control group of standard NT embryos would also be generated and the development to blastocyst of each group compared.

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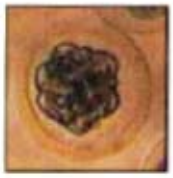
APPENDICES

Appendix A: Criteria for grading of bovine blastocysts

Cycle day: 6
Stage Code: 3
Quality Code:1
Comments: a



Cycle day: 6.5
Stage Code: 3
Quality Code:1
Comments: a

Cycle day: 6.5
Stage Code: 3
Quality Code: 2
Comments: a, b

Cycle day: 6.5
Stage Code: 3
Quality Code: 2
Comments: a, b

Cycle day: 6.5
Stage Code: 4
Quality Code: 1
Comments: b,c,d

Cycle day: 7
Stage Code: 4
Quality Code: 1
Comments:

Cycle day: 7
Stage Code: 4
Quality Code: 1
Comments:

Cycle day: 7
Stage Code: 4
Quality Code:1
Comments: d

Cycle day: 7
Stage Code: 4
Quality Code: 1
Comments: d

Cycleday: 7
Stage Code: 4
Quality Code: 1
Comments: d

Cycle day: 7
Stage Code: 4
Quality Code: 2
Comments: b

Cycle day: 7
Stage Code: 4
Quality Code: 2
Comments: b

Cycle day: 7
Stage Code: 4
Quality Code: 2
Comments: b

Cycle day: 7
Stage Code: 4
Quality Code:2
Comments: b, e

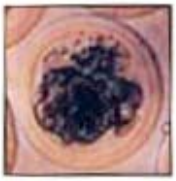
Cycle day: 7
Stage Code: 4
Quality Code: 2
Comments: b

Comments:

a. If this embryo is collected on day 7 or later, the stage is not consistent with the expected stage of development and, therefore, should be lowered one quality code.
b. Large cells that were extruded from the embryo mass prior to the 16-cell stage easily make up more than 15% of the total cellular material through stage 5 embryos.
c. Large individual blastomeres indicate compaction is not complete and is an early stage 4.
d. Single or small extruded blastomeres comprise less than 15% of the total cellular material and the embryo is consistent with the expected stage of development.
e. Sperm on zona pellucida.

Cycle day: 7
Stage Code: 4
Quality Code:2
Comments:

Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments:f,g



Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g

Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g

Cycle day: 7
Stage Code: 4
Quality Code:
Comments: f, g

Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments:f, g

Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g, h

Cycle day: 7
Stage Code: 5
Quality Code:1
Comments:

Cycle day: 7
Stage Code: 5
Quality Code: 1
Comments: d

Cycle day: 7
Stage Code: 5
Quality Code: 1
Comments:

Cycle day: 7
Stage Code: 5
Quality Code: 1
Comments: d, i

Cycle day: 7
Stage Code: 5
Quality Code: 2
Comments: e

Cycle day: 7
Stage Code: 5
Quality Code: 2
Comments:

Cycle day: 7
Stage Code: 5
Quality Code:2
Comments:

Cycle day: 7
Stage Code: 5
Quality Code:
Comments: g

Comments:

d. Single or small blastomeres comprise less than 15% of the total cellular material and the embryo is consistent with the expected stage of development.
e. Sperm on zona pellucida.
f. Embryos with many extruded cells or debris must be carefully rolled over to determine the presence and quality of any viable embryo mass.
g. Quality code 3 embryos have an embryo mass that is less than 50% of all cellular material within the zona pellucida.
h. This embryo has a nice but very small mass. If the embryo mass is less than 25% of all cellular material, it should be given quality code 4 (non-viable).
i. Irregular shape is a common variation in blastocoel development.

Cycle day: 7
Stage Code: 5
Quality Code:3
Comments:

Cycle day: 7
Stage Code: 6
Quality Code:1
Comments:

Cycle day: 7.5
Stage Code: 6
Quality Code: 1
Comments: k

Cycle day: 7.5
Stage Code: 6
Quality Code: 1
Comments: d,k

Cycle day: 7.5
Stage Code: 6
Quality Code: 2
Comments: k

Cycle day: 7.5
Stage Code: 7
Quality Code: 1
Comments:

Cycle day: 7.5
Stage Code: 7
Quality Code: 1
Comments:

Cycleday: 7.5
Stage Code: 7
Quality Code:1
Comments: j

Cycle day: 7.5
Stage Code: 7
Quality Code:1
Comments: j

Cycle day: 7.5
Stage Code: 7
Quality Code: 2
Comments: j, k

Cycle day: 8.0
Stage Code: 8
Quality Code: 1
Comments: j

Cycle day: 8.0
Stage Code: 8
Quality Code: 1
Comments: j

Cycle day: 7.0
Stage Code: 4
Quality Code: 2
Comments: l

Cycle day: 7.0
Stage Code: 4
Quality Code:1
Comments: m

Cycle day: 7.0
Stage Code: 4
Quality Code: 1
Comments: n

Comments:

d. Single or small blastomeres comprise less than 15% of the total cellular material and the embryo is consistent with the expected stage of development.
j. Collapsing of the blastocoel is considered a normal physiological process that does not lower the quality code.
k. Extruded cells in stage code 6, 7 and 8 embryos are often pressed against the zona pellucida and not obvious unless the embryo has collapsed due to normal physiological processes or when a cryoprotective additive is introduced.
1. This embryo has a flat (even concave) surface of the zona pellucida that can cause the embryo to stick to a petri dish or straw. This defect alone keeps the embryo from being classified as quality code 1 and should not be utilized in international commerce unless agreements allow for other than quality code 1 embryos.
m. Cellular debris on the surface of the zona pellucida shows that this embryo has not been washed by proper procedures.
n. This embryo has a cracked zona pellucida at the top of the picture. Embryos that do not have an intact zona pellucida should not be utilized in international commerce.

All images in Appendix A were obtained from the "Manual of the International Embryo Transfer Society" (3rd edition).

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