Inhibition of ERK and GSK3 signalling pathways promotes development and expression of pluripotent markers in bovine blastocysts

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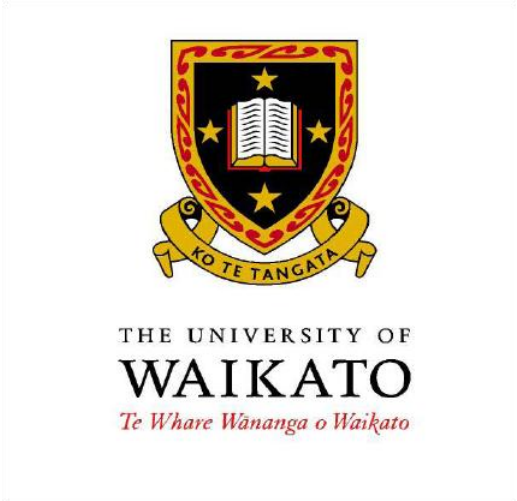

Abstract

Embryo-derived pluripotent stem cells (ePSCs) naturally derive from different founder tissues, namely the early and late epiblast, that vary in their cellular characteristics. Recent evidence in mice suggests these sources of starting material correspond with two states of pluripotency referred to as 'naïve' and 'primed', respectively. Pluripotency is defined as the ability of a cell to give rise to any foetal or subsequent adult cell type. Naïve and primed ePSCs have different fundamental properties and only naïve cells can give rise to all cell types of an animal, including functional gametes (Nichols & Smith 2009). Until recently, naïve ePSCs (also known as embryonic stem cell or ESCs) were only available in mouse. After almost 30 years, the first non-mouse ESCs were derived in rat using a combination of specific inhibitors that block the FGF-ERK differentiation pathway and simultaneously maintain self-renewal through inhibition of GSK3 (Buehr et al. 2008; Ying et al. 2008). The three inhibitor combination, or 3i, included inhibitors of the FGF receptor (PD173074), downstream ERK activity (PD0325901) and the GSK3 pathway (CHIR99021). The dual inhibition of PD0325901 and CHIR99021, termed 2i, was found to be effective for ESC culture; therefore the requirement for PD173074 became dispensable. If pluripotent mechanisms are conserved between species, we hypothesise that these inhibitors would aid the derivation of naïve ESCs in livestock species i.e. cattle.

The aim of this study was to assess the effects of these inhibitors on the developing bovine embryo. We hypothesised that the use of the inhibitors in culture medium would enhance the pluripotent characteristics of blastocysts for subsequent ESC culture. To test this idea we cultured in vitro fertilised bovine embryos in the presence of the inhibitors to initially assess the effect on embryo development. To assess embryo quality, blastocysts were analysed to evaluate the total number of cell nuclei. Additionally, mRNA and protein analyses were undertaken to characterise the expression of core pluripotent genes and assess if the inhibitors had an effect on their expression.

We found that culturing bovine embryos in either 3i or 2i did not influence the total number of embryos that progressed to the blastocyst stage. A significant decrease was observed in the proportion of early vs. late stage blastocysts in 2i, which suggested acceleration in the rate of development. In 2i medium, the number of cell nuclei in D7 blastocysts was significantly increased in both the inner cell mass (ICM) and the outer trophectoderm (TE). Additionally, embryos cultured in 2i showed a >2-fold increase in *NANOG*, a transcription factor at the core of the pluripotency network. The ICM-specific pluripotency marker *SOX2* was also up-regulated in the presence of 2i. Both these genes were found to be significantly enriched in the ICM of bovine blastocysts. Overall, we conclude that bovine blastocysts differ from mouse in their response to 2i signal inhibition and molecular control of pluripotency.

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List of Abbreviations

(v/v)	Volume per Volume
(w/v)	Weight per Volume
2i	Two Inhibitors (PD0325901 & CHIR99021)
3i	Three Inhibitors (PD173074, PD325901 & CHIR99021)
B	Blastocyst
B199	Bicarbonate-buffered M199
BMP	Bone Morphogenetic protein
bp	Base pairs
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribose Nucleic Acid
COC	Cumulus-Oocyte Complex
CP	Crossing Point
D7	Day Seven
DAKO	Dakocytomation fluorescent mounting medium
DEPC	Di-ethyl Pyrocarbonate
DMSO	Di-methyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide Triphosphate
EB	Early Blastocyst
EDTA	Ethylene-diamine-tetra-acetic Acid
ERK	Extracellular signal-Regulated Kinases
EpiSC	Epiblast Stem Cell
ePSC	Embryo-derived Pluripotent Stem Cells
ESC	Embryonic Stem Cells
ESOF	Early Synthetic Oviduct Fluid
FCS	Foetal Calf Serum
FGF	Fibroblast growth factor
GP	Guinea Pig complement
GSK3	Glycogen Synthase Kinase 3
H199	Hepes-buffered M199
HB	Hatched Blastocyst
HSOF	Hepes-buffered Synthetic Oviduct Fluid

ICC	Immunocytochemistry
ICM	Inner Cell Mass
IVC	In vitro Culture
IVF	In vitro Fertilisation
IVM	In vitro Maturation
IVP	In vitro Production
LCMM	LightCycler® Master Mix
LIF	Leukaemia Inhibitory Factor
LSOF	Late Synthetic Oviduct Fluid
M199	Medium 199
MAPK	Mitogen-activated Protein Kinases
mRNA	Messenger Ribonucleic Acid
MQ-H_20	Milli-Q water
NTC	No Template Control
Osm	Osmolarity
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGC	Primodial Germ Cells
PFA	Paraformaldehyde
PVA	Polyvinyl Alcohol
RαB	Rabbit anti-Bovine serum
RU	Relative Units
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse Transcriptase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SOF	Synthetic Oviduct Fluid
TB1-2	Total Blastocyst Grade 1-2
TB1-3	Total Bastocyst Grade 1-3
TE	Trophectoderm
THSOF	Transfer Hepes-buffered Synthetic Oviduct Fluid
XB	Expanded Blastocyst

Introduction

Embryo-derived Pluripotent Stem Cells (ePSCs) are the unicellular equivalent to a whole animal derived from either pre-(embryonic stem cells or ESCs) or post-(epiblast stem cells or EpiSCs) implantation embryos (Nichols & Smith 2009). These cells are capable of indefinite self renewal and have the ability to generate all adult cell types (Keefer et al. 2007; Nichols & Smith 2009; Blair et al. 2011). First isolated in mice in 1981 (Evans & Kaufman 1981; Martin 1981), these cells launched the stem cell revolution that was awarded a Nobel Prize in 2007. Until very recently, authentic ESCs could only be derived from a few inbred mouse strains (Nagy & Vintersten 2006; Ying et al. 2008). Unsuccessful derivation in all other mammals had been attributed to interspecies differences in early embryonic development and a lack of understanding in the mechanisms that drive the pluripotent cell state (Munoz et al. 2008b; Talbot & Blomberg 2008).

In 2008, Ying et al. used small molecular weight inhibitors in ESC culture medium to stabilize the pluripotent state whilst eliminating the requirement for previously fundamental culture resources, namely feeder cells and serum. The successful combination of inhibitors of different signalling pathways (3i/2i, for 3 and 2 inhibitors, respectively) allowed for the first derivation of bona fide ESCs from rat (Buehr et al. 2008), previously unpermissive mice strains (Ying et al. 2008) and helped establish human ESCs with greater resemblance to their mouse counterparts (Hanna et al. 2010). The indicative reliance on similar cellular pathways in these species suggested more widely conserved mechanisms of pluripotency and the potential to enhance the stability of pluripotency in other mammalian species (Blair et al. 2011).

It has been documented in mice that embryo culture in 3i/2i at the beginning of cleavage allows for ESC derivation at high efficiencies as cells are shielded from differentiation-inducing stimuli (Nichols et al. 2009). The aim of this thesis was to characterise pluripotency in cattle (bovine) embryos in response to 3i/2i supplementation during culture. I endeavoured to explore the possibility that the inhibitors mentioned above also promote pluripotency in cattle, a species previously considered as non-permissive for ESC derivation.

The first chapter of this thesis will discuss, in context, literature surrounding embryology and stem cell biology, and provide insight to both the progress and problems in the field over the past 30 years. Chapter Two will provide an account of the methodology, equipment and experimental design used for this work. Subsequent chapters present all findings of experiments using 3i/2i supplementation, including morphological and molecular data, as well as in-progress protein work. Lastly, the thesis will conclude with a comprehensive discussion of these results and conclusions obtained during the course of this research.

Chapter 1: Literature Review

1.1 Pre-implantation Embryo Development

Reproduction in any mammal is still very much an unknown science yet it is fundamental to our existence and central to breeding technologies in domestic livestock; an important source of income and food. The process of development from a single cell to a multicellular organism has many crucial events. Following a fixed pattern of continuous growth, one event sets the foundation for the next, each phase increasing in structural complexity as the organism develops. Any aberration can severely alter the succession of events (Arora 2009). Pre-implantation development refers to the period prior to an embryo's implantation into the uterus of an animal. During this period, there are a multitude of changes in the physical appearance and molecular characteristics of the embryo and this phase is considered highly regulative (Yamanaka et al. 2006).

One of the earliest events in mammalian development is the first lineage segregation marked by the formation of the blastocyst. Cells of the developing embryo (commonly termed blastomeres) mitotically divide following successful fertilisation. The division process is known as cleavage and blastomeres will proceed to exponentially produce smaller cells until the embryo compacts through increased cell to cell contact. Figure 1 displays the cleavage stage embryos as they lead up to the formation of the blastocyst. The blastocyst is characterised by the formation of the blastocoel; a fluid-filled cavity surrounded by the two anatomically distinctive lineages; trophectoderm (TE) and inner cell mass (ICM). Once the blastocyst has formed, it will progress to expand in size causing the zona pellucida (the membrane encasing the embryo) to rupture and disappear (Arora 2009). Following blastulation, the ICM further differentiates into the hypoblast on the blastocoelic surface, whilst the remaining internal cells form the epiblast and what will become the embryo proper. As development proceeds the trophectoderm and hypoblast contribute exclusively to extra-embryonic tissues such as the placenta and primitive endoderm, respectively. Concurrently, the

epiblast will give rise to the three primary germ layers of the developing foetus; ectoderm, mesoderm, and endoderm (Johnson 2007; Kuijk et al. 2008).

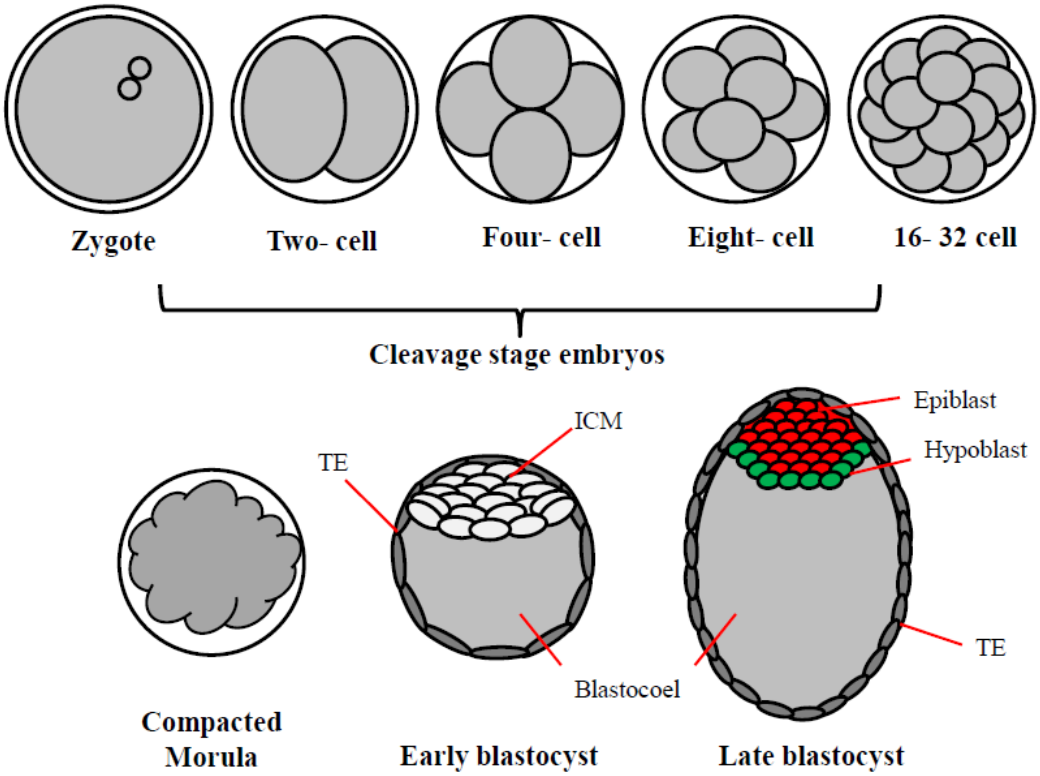

Figure 1. Stages of pre-implantation bovine development. Cleavage stage embryos are displayed along the top and subsequent divisions will lead to morula compaction (bottom left). Two distinct lineages are formed at the blastocyst stage: Inner Cell Mass (ICM) and trophectoderm (TE). The ICM then differentiates into Epiblast and Hypoblast in the late stage blastocyst. Modified from Yamanaka et al. 2006.

Before lineage specification, blastomeres up to the 8-cell stage in mice are considered totipotent, referring to the differentiation potential of these cells. Totipotent cells have the ability to give rise to both extra-embryonic tissues as well as a live foetus. On the other hand, pluripotent cells have the sole ability to give rise to all cell types of the developing embryo, including the gametes, and subsequent adult animal. These cells no longer have the capacity to derive the

extra-embryonic lineages (Renard et al. 2007; Nowak- Imialek et al. 2011). The nascent epiblast, formed at the late blastocyst stage of development, is where pluripotent cells first develop and is therefore functionally (and molecularly) distinct from blastomeres and early ICM (Nichols & Smith 2009).

The rate of development is variable amongst different species and blastulation occurs post-fertilization at approximately day seven in cattle (Roach et al. 2006), day four in mice (Nagy & Vintersten 2006) and between days five to six in human embryos (Arora 2009). In cattle, the blastocyst remains suspended in the uterine environment for more than a week before attachment to the uterine wall, observed around day 20 post-fertilisation (Wathes & Wooding 1980). This differs from the murine (mouse) embryo, which immediately starts to implant after entry into the uterus at day 4.5 post-fertilisation (Nagy & Vintersten 2006). As mentioned, the early events in lineage segregation take place prior to implantation in mice, however in late implanting species, such as cattle, there is little known about the precise timing of lineage specification. Berg et al. (2011) demonstrated convincingly that regulation of certain genes controlling lineage specification is different between cattle and mice (section 1.4). This had previously been implied, yet unconfirmed, by discrepancies in gene expression patterns. This finding showed that, at least between cattle and mice, lineage commitment is not necessarily chronologically equivalent and that not all developmental pathways are analogous. It has been speculated that this diversity in rate of development (as a result of evolution) may explain other cellular differences observed between these species and why extrapolation of experimental findings should be cautiously considered (Munoz et al. 2008a). Regardless, the mouse model is still the most well documented and efficient system for gaining information on new hypotheses and will continue to influence research in other species. However, these findings have highlighted the importance of taking species differences into account when formulating research conclusions.

1.2 IVP of Bovine Embryos

Since the early 1970's, it had become evident that the large pool of oocytes produced in female ovaries had many promising applications for exploitation. Only a tiny fraction of oocytes residing in the ovary generate live offspring, the rest are lost to atresia and are in essence a wasted resource. Techniques such as in vitro production (IVP) of embryos have allowed for the utilisation of these reservoirs and have become instrumental for manipulation experiments, and the subsequent acquisition of knowledge on the physiological and molecular functioning of early development. Nevertheless, extensive research is still required to increase efficiencies to those achieved by in vivo counterparts (Galli & Lazzari 1996; Hansen 2006).

IVP consists of three biological steps; in vitro maturation (IVM), in vitro fertilisation (IVF) and embryo culture (IVC). IVC is a critical step as the developing embryos are susceptible to a number of perturbations, which can ultimately affect the developmental competence of the resulting blastocyst and subsequent offspring (Wagtendonk-de Leeuw et al. 2000). Following fertilisation, the proportion of presumptive zygotes that reach the blastocyst stage in culture varies and is dependent largely on the intrinsic qualities of the oocyte. However, different laboratory procedures and culture conditions, including media supplementation, have a large role in determining embryo quality in vitro. Obviously, a key research objective regarding IVP is focused on suitable medium formulations that enhance the developmental competency of embryos (Feugang et al. 2008).

Most commonly, a two step culture system is used for embryo culture using synthetic oviductal fluid (SOF) medium (Tervit et al. 1972). In vivo, the embryo migrates from the oviduct to the uterine lumen, subsequently entering a different micro-environment. It is therefore beneficial for embryo development to mimic these changes in vitro. The necessity for a larger consumption of amino acids is included in the second step of culture as well as the change in glucose for energy requirements, as at blastulation energy demands are high compared with cleavage stage embryos (Feugang et al. 2008). For these reasons, change in culture medium

from an early SOF to late SOF occurs at day five in cattle when the embryo is reaching compaction. The embryo spends a further two days in culture following this switch in medium.

A frequent measure of embryo quality is blastocyst rate, measured at day seven when embryos are expected to be at least at an early blastocyst stage in development (Galli et al. 2003). Lindner & Wright, Jr (1983) found that embryo stage alone was not a good predictor of success, whereas embryos classified by excellent, good, fair and poor morphology had resulting pregnancy rates of 45%, 44%, 27% and 20%, respectively. Parameters often used to evaluate embryo quality include shape, colour, number and compactness of cells, size of the perivitelline space, and number of extruded and degenerated cells. These parameters together with an embryo's stage of development offer a good indicator of expected success in establishing pregnancies (Lindner & Wright. Jr 1983). The measure of embryo quality is subjectively based on individual assessment and guidelines from different laboratories. However, as a standard measure of embryo success in vitro it has proved reliable as it is not always possible to perform the definitive test of deriving a live animal.

In vitro production is an attractive technique for generating abundant low-cost embryos for embryo analysis, diagnostics, and basic research processes (Hoshi 2003). It has become well established in cattle, as pioneering systems in the mouse proved useful yet inadequate for bovine embryo needs (Thompson et al. 2000). Additionally, IVP has become a reliable alternative to conventional techniques as working in vivo with bovine embryos is both expensive and laborious (Munoz et al. 2008b). There have been an ever increasing number of in vitro produced embryos used worldwide to establish pregnancies, representative of the potential it has for selective breeding schemes that aim to improve productive/ reproductive traits in the next generation (Hansen 2006).

Additionally, the ability to acquire abundant bovine blastocysts through IVP has provided scientific researchers with the fundamental means for obtaining valuable information on early development and the enigmatic phenomenon of pluripotent stem cells; cells that have the capability of giving rise to an entire animal (Talbot

& Blomberg 2008). The use of IVP permits a larger number of time-efficient and cost-effective studies to be undertaken to uncover the complexities of these cells.

1.3 Embryo-derived Stem Cells; Naive vs. Primed

Pluripotent cells can be immortalised in vitro by isolating epiblast tissue from the developing blastocyst. This was first achieved in 1981 in mice (Evans & Kaufman 1981; Martin 1981) and thus announced the birth of embryonic stem cells (ESCs). Aside from being a population of undifferentiated cells, a further key characteristic of ESCs is their capability of unlimited self-renewal (Nagy & Vintersten 2006; Munoz et al. 2008a; Nowak- Imialek et al. 2011). The idea of deriving cell lines that indefinitely exhibited pluripotency was an incentive to provide a number of technological platforms (i.e. biomedical, transgenic and reproductive) with cells which could be reprogrammed effectively with no past cellular history (Keefer et al. 2007; Renard et al. 2007; Talbot & Blomberg 2008).

The problem is that these cells have only been successfully derived in mice, and most recently in rat (Buehr et al. 2008). Human ESCs have been reported since 1998 (Thompson et al, 1998) however, given that they cannot be functionally characterised, there is speculation about whether these represent true ESCs (Nichols & Smith 2009). Derivation in ungulates, especially domestic species such as cattle, has been attempted but with limited success. This is not unexpected as almost every other mammalian species is still non-permissive to true ESC derivation. Instead, ESC-like cells have been established in many species. Although these cells are to some extent morphologically and molecularly similar to bona fide ESCs, they cannot be propagated indefinitely in culture and do not exhibit the functional requirements of ESCs (Talbot & Blomberg 2008). The most stringent criterion for ESC identification is the production of chimaeric animals. A chimaera is a single organism that is made up of cells from different embryonic origins (Nagy & Rossant 2001). Injection of true ESCs into a cleavage-stage host embryo will ultimately result in full ESC contribution to the tissues, and most importantly the germ line of the developing foetus (Nagy & Vintersten 2006).

Embryo-derived pluripotent stem cells (ePSCs) encompass a number of cell populations derived from different sources that subsequently vary in their cellular characteristics. ESCs are derived from pre-implantation embryos. Alternatively, cells derived from the late epiblast of post-implantation embryos have been accordingly named epiblast stem cells or EpiSCs (Nichols & Smith 2009). These EpiSCs express all the core transcriptional pluripotency markers (*Nanog*, *Oct4*, and *Sox2*) and differentiate into multi-lineages in vitro, but in contrast to ESCs have a 'flattened' morphology, limited capacity for chimera formation, and a change in regulation of a number of specification markers associated with differentiation (Nichols & Smith 2009; Blair et al. 2011; Huang et al. 2011). Another key finding is that female EpiSCs have undergone X chromosome inactivation, an epigenetic modification that further suggests these cells are in a fundamentally distinct 'state' of pluripotency from ESCs. For these reasons, EpiSCs represent a 'primed' state as opposed to the 'naive' state present in ESCs. EpiSCs represent a more differentiated state than ESCs; therefore representing a step further on the developmental track which is irreversible without genetic manipulation. The previous point provides a strong argument as to why these two states are distinctively different and represent a true differentiation event. A summary of characteristics for the naive and primed pluripotent states is illustrated in Figure 2.

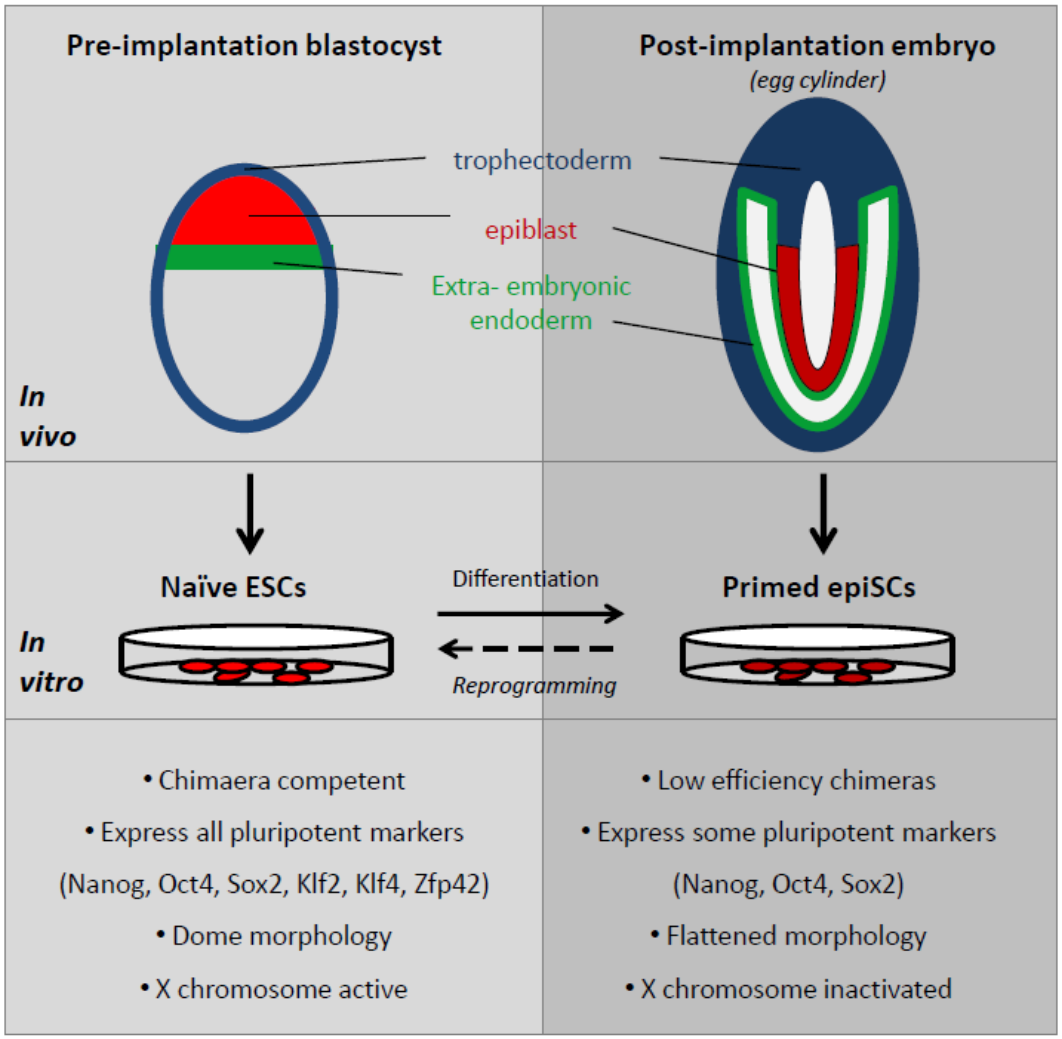

Figure 2. Comparative schematic of naive and primed pluripotent stem cells. Modified from Nichols & Smith, (2009), and Blair et al., (2011).

Species in which ESC-like cells have been derived, e.g. cattle, show characteristics argued to closely resemble EpiSCs. This is perhaps puzzling given that effort is made to derive these cells at equivalent stages of development when pluripotency is first initiated (Nichols & Smith 2009). As mentioned before, there are a number of differences in development to consider as to why very few species have had ESCs successfully derived.

1.4 Intrinsic Control of Pluripotency: Transcription Factors

Cell fate is ultimately driven by transcription factors that act as a switch to effectively turn on and off molecular pathways. However, signalling pathways that segregate the lineages remain poorly defined (Lu et al. 2008). In all mammals, with lesser extent in mice, there is still a huge amount of research required into the cellular pathways, and underlying gene expression, which drive lineage development. Conventional lineage markers in mice, especially those associated with pluripotency, are unspecific for some species (Keefer et al. 2007; Munoz et al. 2008b). This is an obvious hurdle that must be resolved to further characterise the states of pluripotency and perhaps aid in derivation of naive ESCs in other species. The nomenclature used in the following sections adheres to general guidelines (Society for the Study of Reproduction, 2008). Genes/transcripts are presented in italics compared to non-italicised protein names. Moreover, mouse gene and protein names are in lowercase (except the first letter) as opposed to uppercase for all other species mentioned.

1.4.1 Pluripotent epiblast markers

In the mouse, analyses of the molecular network that regulates pluripotency have identified three core transcription factors; Nanog, Oct4 and Sox2. All these factors become exclusively expressed in the epiblast of murine blastocysts as well as undifferentiated ESCs, and are found to be co-localised. An enhancer, which contains both Oct4 and Sox2 binding motifs, is highly active in undifferentiated cells and has been found to regulate a huge number of pluripotent-related genes (including *Nanog*) and *Oct4-Sox2* themselves, suggestive of a positive feedback loop. Knock-out experiments for each gene have resulted in early embryonic fatality due to a failure to maintain pluripotent cells. These experiments have provided further confirmation of their importance in early lineage segregation (Masui et al. 2007).The following looks at each of these factors individually, as well as identifying other factors involved in early gene expression.

1.4.1.1 NANOG

The most fundamental transcription factor associated with pluripotency is Nanog. Nanog is a highly divergent homeodomain-containing protein that is exclusively expressed in pluripotent cells. Silva et al. (2009) experimentally showed that in murine embryos Nanog expression is specific to the epiblast tissue. Additionally, ICMs of *Nanog*-null blastocysts failed to properly establish. ICM cells including pluripotent epiblast that could not up-regulate *Nanog* would degenerate through differentiation and apoptosis. Intriguingly, without Nanog the hypoblast also failed to establish. This suggests either a role of Nanog in specification of hypoblast or that survival of the hypoblast is dependent on paracrine signalling from the epiblast (Silva et al. 2009). This implicates *Nanog* at the heart of pluripotent signalling in mouse as the specification factor for epiblast.

In cattle, NANOG expression has been located to the ICM at the blastocyst stage suggesting some functional conservation in relation to findings in the mouse. However, it has a mosaic pattern at day seven and little is known of its locality after this point (Kuijk et al. 2008). Yang et al. (2011) detected transcripts of *NANOG* in bovine embryos at the morula stage with statistically significant up-regulation as the embryo proceeded through to late blastocyst. This is consistent with the detection of *Nanog* at the late morula stage in mouse. Interestingly, in porcine (pig) embryos *NANOG* transcripts have been located from as early as the 4-cell stage through to the late blastocyst, and even some organs (Wolf et al. 2011). The NANOG protein, however, was reported as undetectable in porcine blastocysts at day eight (Kuijk et al. 2008), but present in the epiblast of day 11-12 late blastocysts. The expression varies between individual embryos and is sometimes co-expressed with markers of the hypoblast (Wolf et al. 2011). Consequently, NANOG may not play as supportive a role in initial lineage development in pig and demonstrates once more why obtaining information regarding species-specific signalling pathways is vitally important.

1.4.1.2 OCT4

Oct4 (encoded by the gene POU5F1) has had a long history of association with early lineage development and is considered one of the core pluripotent factors. It

is a POU domain transcription factor which recognises an 8-bp DNA site, hence its name Oct4 (Chambers & Tomlinson 2009). Its expression in murine embryos becomes progressively restricted to the pluripotent epiblast after initially signifying the ICM at blastulation. Trophectoderm cells lose Oct4 expression, and alternatively express the marker Cdx2. In mice, Oct4 and Cdx2 are always reciprocally expressed as they inhibit one another's expression in the blastocyst specifying the two lineages; ICM and TE, respectively. Oct4 is required to establish the epiblast compartment as *Oct4*-null embryos will still develop into blastocysts but their ICM lineages are not maintained, and consequently differentiate (Nichols et al. 1998; Nichols & Smith 2009). Niwa et al. (2000) used transfected ESCs with an inducible *Oct4* transgene to modify the quantity of Oct4 and examine its effect. They found that a critical amount of expression was required for self- renewal of mouse ESCs (mESCs). An increase in expression resulted in differentiation into primitive endoderm and mesoderm, whilst a repression in Oct4 caused differentiation into trophectoderm. Overall, this established Oct4 as a key regulator for pluripotency in mouse (Niwa et al. 2000).

Surprisingly, while Oct4 plays an important role in mouse development, its significance in other species, remains ill-defined. In cattle, OCT4 is not restricted to the ICM but instead has been reported as ubiquitously expressed throughout day seven blastocysts. Up until recently there were a lot of contradictory results surrounding expression in later development stages. Berg et al. (2011) showed that by day 11, *OCT4* mRNA expression in the TE was greatly reduced, reflected also by the localisation of OCT4 protein seen restricted to the epiblast tissue at the equivalent period. This, however, was not due to repression by CDX2 (as seen in the mouse). The finding indicated that OCT4 regulation in cattle is not analogous to mice in respect to its role setting up the first lineage specification. However, its restricted presence in the epiblast at day 11 suggests it may still be important in the pluripotent network.

Figure 3 outlines key differences in *NANOG*/*Nanog* and *OCT4*/*Oct4* mRNA and Nanog/NANOG and Nanog/OCT4 protein expression between bovine, human and murine blastocysts and ESCs (or ESC-like cells). Note that all ESC cultures

express both transcription factors uniformly, whereas the corresponding blastocyst stage exhibits differential expression between the three species.

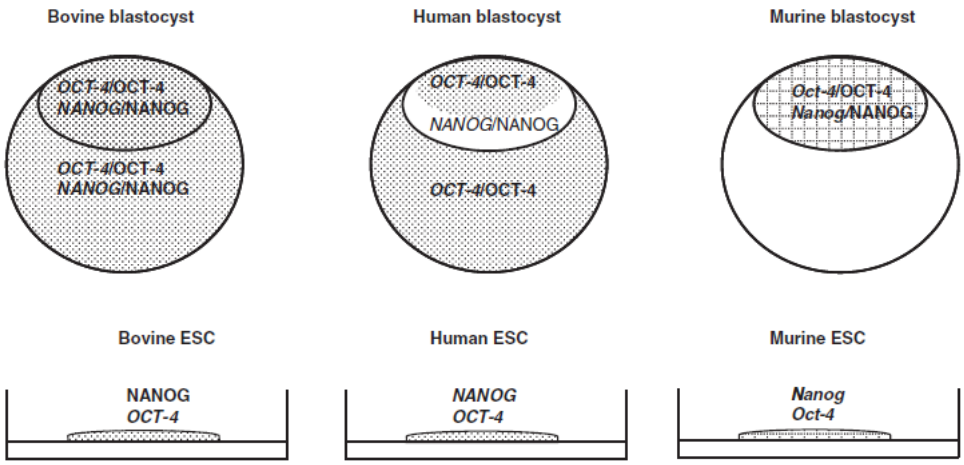

Figure 3. Differences of *OCT4/Oct4* and *NANOG/Nanog* mRNA and OCT4 and NANOG protein expression between bovine, human and murine blastocysts and embryonic stem cells (Munoz et al., 2008a).

1.4.1.3 SOX2

Sox2 is a member of a superfamily of proteins that all possess a High Mobility Group (HMG) box DNA binding domain. It binds the *Oct4-Sox2* enhancer synergistically with Oct4, and is also considered a key member in the transcriptional network of pluripotent ESCs. *Sox2*-null embryos die soon after implantation, as attachment sites lack epiblast and extra-embryonic ectoderm components (Keramari et al. 2010). Until recently, the activity of Sox2 in ESCs had yet to be experimentally confirmed. The model predicted Sox2 as an essential regulator due to its close proximity to the Oct4 binding site on the enhancer element, indicating they must bind as a complex to activate transcription. However, no direct evidence had been acquired. Masui et al. (2007) showed that not only is Sox2 required in ESCs for its enhancer activity, but also as a crucial regulator of multiple factors that affect *Oct4* expression.

Furthermore, Kermari et al. (2010) addressed the question of *Sox2* contribution at an earlier stage in development. They used small-interfering RNA (siRNA) to remove all transcripts during pre-implantation development. Intriguingly, depletion of *Sox2* transcripts at the 2-cell stage resulted in over 76% of embryos halted at the morula stage or earlier with a down-regulation of TE-associated markers. Their conclusion surmised that the initial function of *Sox2* expression is to facilitate the formation of the TE lineage. This is also coincident with evidence showing Sox2 is the earliest marker of ICM formation in mouse (Guo et al. 2010). The importance of these pluripotent markers therefore goes beyond establishment of the ICM and ensuing epiblast, but also as key regulators of a wider range of functions in the developing embryo.

The expression of *SOX2* in bovine embryos remains fairly ambiguous. *SOX2* transcripts have been reportedly detected in day seven bovine blastocysts within the ICM, but also faintly detectable in TE cells. It becomes gradually restricted to epiblast tissue of the later stage embryo between days 12-17 (Degrelle et al. 2005). Other reports have recognized that SOX2 protein is specifically localised in the ICM in day seven blastocysts, (Prasanna Kallingappa, personal communication) yet this finding has not been published to my knowledge. Consequently, SOX2 is acknowledged as an important transcription factor in lineage determination in cattle, but its exact functions and interactions with other factors are still elusive.

1.4.1.4 DPPA3

Dppa3 (otherwise referred to as *stella* or *Pgc7*) is a gene that expression has been identified as a key marker of pluripotent cell types in both human and mice (Bowles et al. 2003; Payer et al. 2003). Sato et al (2002) first described and isolated the novel gene *Pgc7*, that was expressed specifically in early pre-implantation embryos, primordial germ cells (PGCs) and oocytes. The authors found that Pgc7 was expressed in both TE and ICM of murine blastocysts just prior to implantation (D3.5), which disappeared post-implantation until the emergence of PGCs. Studies by Saitou et al (2002) also convincingly showed that the gene's expression was restricted in post-implantation embryos to the PGC

population and therefore defined these cells from those that gave rise to somatic cells. The fact that this gene was specifically expressed in a number of key pluripotent cell types has implicated it as an important regulator in lineage specification.

Furthermore, gene knock-out studies in mice have shown that murine embryos deficient in *Dppa3* cleave but development is severely compromised past the 2 to 4-cell stage. Some embryos failed to reach the 8-cell stage, and embryos that developed further failed to initiate compaction (Bortvin et al. 2004). Bortvin et al (2004) disagree that *Dppa3* is required for germ cell specification as indicated by their results using knock-out mice, yet they believe that it is important in the early pre-implantation embryos.

Reports on *DPPA3* expression in bovine are scarce. One study documented that the mRNA expression of a *stella* variant progressively decreased in expression throughout development from the immature oocyte to the blastocyst stage (Thelie et al. 2007). However, specific details on the expression in blastocysts were not included. This emphasizes a requirement to further characterise *DPPA3* expression in early development in bovine.

1.4.2 Extra-embryonic markers

1.4.2.1 CDX2

The caudal-type homeodomain protein Cdx2 is well known as a marker of the TE lineage in mouse. Cdx2 expression is restricted solely to the TE in mouse blastocysts and similarly absent in any ESC culture. The loss of *Cdx2* in embryos through genetic knock-out causes lethality around the time of implantation due to failure in epithelial integrity. Additionally, the repression of *Nanog* and *Oct4* in TE does not occur in embryos devoid of *Cdx2*, thus implicating *Cdx2* as an important regulator for the TE lineage in mouse (Strumpf et al. 2005).

Kuijk et al. (2008) documented CDX2 protein expression specific to the TE lineage in bovine embryos. Similarly, Berg et al. (2011) found a 2-fold increase in *CDX2* mRNA located in the TE respective to the ICM. Interestingly, *CDX2*

expression was considerably lower compared with *OCT4*, whereas mouse blastocysts contain equivalent levels. This posed the question of whether *CDX2* had a role in *OCT4* repression as seen in the mouse. Experiments involving CDX2 knockdown embryos resulted in normal OCT4 expression at the blastocyst stage, indicating that in cattle CDX2 does not play a role in OCT4 down-regulation.

1.4.2.2 GATA4 & GATA6

Equally important markers of the extra-embryonic lineage hypoblast are two members of the GATA family of proteins; Gata4 and Gata6. These DNA binding proteins are vertebrate zinc fingers that play a crucial role in not only embryogenesis but also cell differentiation in later development. In mouse inactivation of either *Gata4* or *-6* genes result in developmental arrest or early embryonic lethality, and 30-40% of *Gata4*-null embryos fail to even gastrulate (Nemer & Nemer 2003). ESCs devoid of *Gata4* do not differentiate into primitive endoderm suggesting a key role in extra-embryonic regulation. Conversely, over expression of both closely related factors is sufficient to transform ESCs into primitive endoderm. Gata6 expression in early mouse blastocysts has a mosaic distribution pattern within the ICM, which is restricted to the hypoblast layer at 4.5 days post fertilisation (Chazaud et al. 2006). This indicates that the lineage potential of individual cells becomes restricted at an early stage, when spatial determination is yet to be established.

Similarly, GATA6 displayed a mottled expression pattern in the ICM of bovine blastocysts. In some blastocysts, GATA6 expression was restricted to a set of cells that aligned one side of the ICM in a fashion similar to hypoblast in mouse. This finding suggests a conserved role for GATA6 in early lineage development in cattle (Kuijk et al. 2008). Published information for the localisation of GATA4 in cattle is limited. Although, Yang et al (2011) recently reported the localisation of GATA4 in the ICM of D8 bovine blastocysts with an increase in *GATA4* transcripts at the expanded and hatched blastocyst stages.

Cell fate and maintenance, including pluripotency, can be viewed as a coordinated network of positive and negative regulation of gene expression rather than the sequential on/off molecular events (Rossant 2004). It is therefore important to

continue identifying the many players involved and how they interact to organize normal embryonic development. It is also central for the derivation of ESCs, to understand differences in the underlying factors involved in specification of pluripotent epiblast within species.

1.5 Extrinsic Control of Pluripotency: Signalling Pathways

1.5.1 In-vitro

Nagy & Vintersten (2006) insisted that ESCs do not exist in vivo but that they are entirely a tissue culture artefact dictated by the environment they grow in. This notion has been challenged recently by Nichols & Smith (2009) who believe that nascent epiblast tissue in intact embryos is set in a poised "ground state" of pluripotency equivalent to ESCs in culture. Simply put: it is the shielding from differentiation-inducing stimuli that determines the existence of these cells. That is, the ability of pluripotency is already present and not acquired, hence the name 'ground state' (Ying et al. 2008). The validity of the previous hypothesis awaits the derivation of true ESCs in other species, but introduces the novel idea behind using inhibitors of differentiation to enhance derivation of naïve ESCs.

Mitogen-activated protein kinase (MAPK) pathways are crucial for the transmission and response of cellular cues including those associated with cell proliferation, growth and differentiation. The large group of proteins are highly conserved throughout eukaryotic systems and incorporates the signalling cascade 'extracellular signal-regulated kinases' or ERKs (Madan et al. 2005) outlined in Figure 4. The importance of ERK signalling activated by fibroblast growth factors (FGF) is well established in murine early development (Lanner & Rossant 2010). Fgf4 is a member of the FGF family of proteins which is expressed by undifferentiated ESCs (Kunath et al. 2007). Kunath et al. (2007) looked into the relationship between Fgf4 and ERK signalling and its influence on mESCs. They showed that interference with Fgf or ERK activity by chemical inhibition or genetic manipulation limited the ability of mESCs to differentiate, maintaining

their pluripotent state. It was therefore concluded that Fgf is an auto-inductive signal for ESCs to exit their self-renewal programme and become responsive to differentiation.

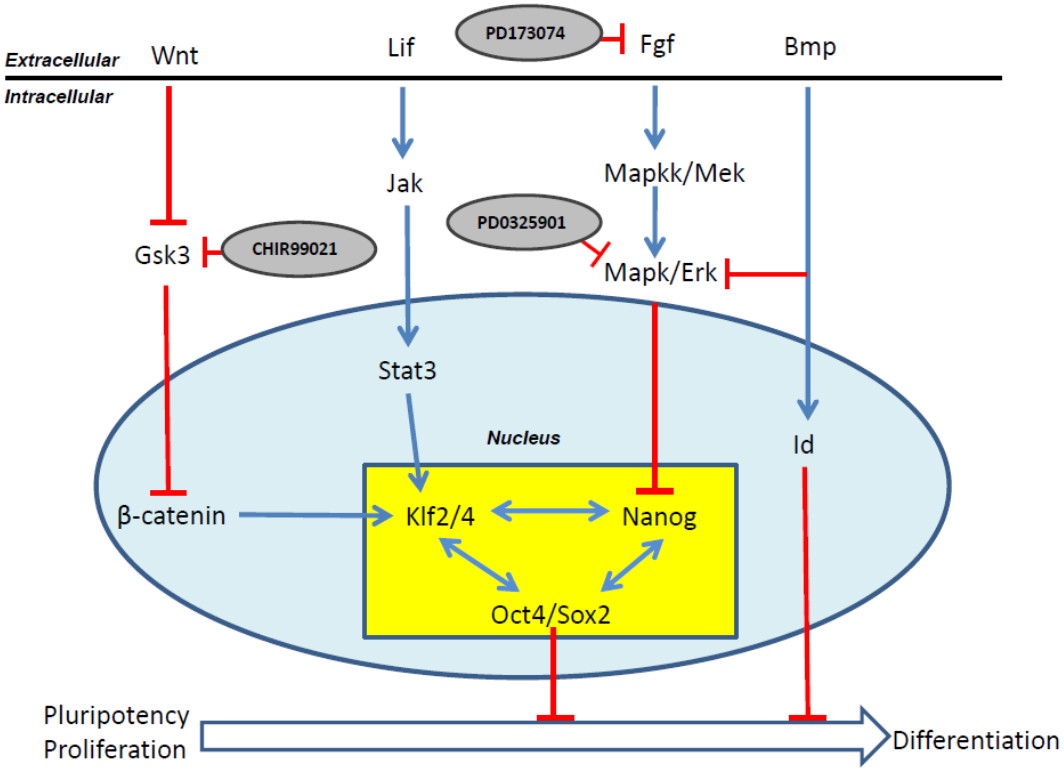

Figure 4. Pluripotent signalling pathways of Wnt, Lif, Fgf and Bmp. The succession of effects within the cascade is shown by either activation (blue arrows) or inhibition (red bars). Selective inhibition by PD173074, PD0325901 or CHIR99021 is illustrated.

The experiments outlined in Ying et al. (2008) built on the concept of ERK inhibition. The importance of this study was the discovery that the use of specific FGF/ERK inhibitors, as well as an inhibitor of general biosynthetic activity, allowed for the dispensability of undefined culture conditions. Traditionally, mESCs had been maintained in culture by a feeder layer of mitotically inactivated fibroblasts and foetal calf serum (Evans & Kaufman 1981; Martin 1981). These materials could be the source of a number of key influences for ESC growth. As a result, further research was necessary to define the exact components required for

ESC survival. It was later found that feeder cells could be substituted with the cytokine leukaemia inhibitory factor (LIF). Like many cytokines, LIF activates JAK proteins, which further mediate the phosphorylation of Stat3 and results in the signalling of cells into a self-renewal programme (Kisseleva et al. 2002; Ying et al. 2008). Serum was replaced with bone morphogenetic protein (BMP), which activates inhibitor of differentiation (Id) proteins as well as exerts an inhibitory effect on MAPK/ERK in the mouse embryo (Figure 4), yet how it does so is unknown (Qi et al. 2004). However, Ying et al (2008) found that neither LIF nor serum/ BMP blocked the activation of ERK in mESCs. They hypothesised that these factors work downstream from the ERK cascade and hence specific inhibitors of this particular pathway could, if successful, effectively replace the need for undefined additives to culture medium. Supplementation of ESC culture medium with these inhibitors resulted in propagation of mESCs at a doubling rate comparable with standard culture conditions with 90% of cells remaining undifferentiated. These authors showed for the first time that mESCs could be efficiently propagated and maintained in conditions free from supplementary cells, serum or cytokines, thus provoking the hypothesis of 'ground state' pluripotency (Ying et al. 2008). The potential of these inhibitors for naïve ESC derivation in other mammals was reinforced after successful derivation of germ-line competent ESCs in rat (Buehr et al. 2008).

The three inhibitor combination, or 3i, included two inhibitors of the FGF receptor and ERK activity (SU5402 and PD184352, later substituted with a more specific generation of inhibitor; PD173074 and PD0325901) respectively, and an inhibitor of the glycogen synthase kinase-3 (GSK3) pathway (CHIR99021). Figure 4 indicates where each inhibitor exerts its effect along their corresponding signalling pathway. The inclusion of a GSK3 inhibitor was necessary to revive the loss of cells to apoptosis and allow for greater survival at high clonal densities (Ying et al. 2008). The GSK3 pathway was initially discovered for its role in glycogen metabolism. Amongst a diverse array of other functions, recently GSK3 has been implicated in early development as a signalling pathway involved in specification of cell fates (Cohen & Frame 2001) and a key participant in the Wnt signal transduction pathway. GSK3 phosphorylates β-catenin leading to its degradation and the blocked transmission of Wnt signals to the nucleus (Figure 4).

Proper regulation of Wnt signalling has been correlated to normal embryo growth and development. The inhibition of GSK3 is therefore also important to mitigate the loss of correct regulation of Wnt signalling (Renard et al. 2007; Aparicio et al. 2010). Inhibition of the GSK3 pathway had previously been described by Sato et al. (2004) who used the pharmacological inhibitor bromoindirubin-3′-oxime (BIO) in human and mouse ESC cultures. ESC propagation was reportedly enhanced and the maintenance of undifferentiated cells that expressed pluripotent markers (including Nanog and Oct4) were sustained with BIO addition (Sato et al. 2004). CHIR99021, on the other hand is a more selective inhibitor of the GSK3 pathway, but also has some non- neural differentiation- inducing properties. As this is undesirable, combination with the dominant effects of the ERK inhibitors results in completely undifferentiated colonies that can be efficiently expanded (Ying et al. 2008). The dual inhibition of PD0325901 and CHIR99021, termed 2i, has been found to be a very effective combination for mESCs, and induced pluripotent cells (Silva et al. 2008). It has ultimately made the requirement for the FGF receptor inhibitor PD173074 redundant.

1.5.2 3i/2i in-vivo

Following the success of 3i/2i in mESCs, Nichols et al (2009) speculated whether culturing intact pre-implantation embryos in 3i/2i would elicit similar cellular responses as ESC culture in-vitro. To test this notion, they cultured embryos in 3i/2i to examine the relationship and identity between mESCs and pluripotent epiblast of the mouse blastocyst. 3i/2i culture from the 8-cell stage for three days did not impede formation to the blastocyst nor did it restrain development of the ICM (seen in Figure 5.C; Oct4 expression). The number of cells expressing the epiblast marker Nanog increased in 2i culture at the expense of hypoblast tissue, indicated by the diminished Gata4 expression. Figure 5 summarises these findings both in 3i (A & B) and in 2i (C & D).

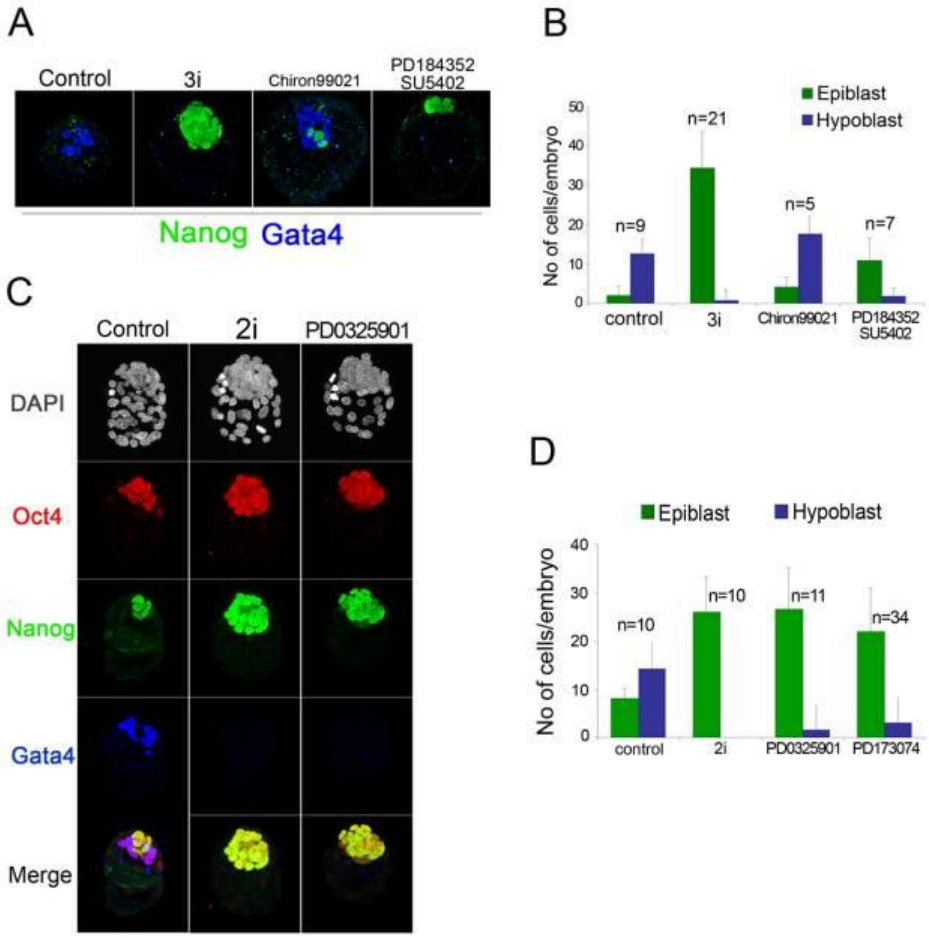

Figure 5. Effect of FGF/ ERK and GSK3 inhibition on inner cell mass development. A) Confocal images of mouse blastocysts grown from the 8- cell stage for 3 days in 3i, medium supplemented by CHIR99021 alone, or supplemented with PD184352 and SU5402. Embryos were immunostained using antibodies raised against Nanog (green) and Gata4 (blue). B) Bar chart showing cell numbers of epiblast (Nanog positive, green) and hypoblast (Gata4 positive, blue) of embryos cultured in conditions of A. Bars indicate the mean ± s.d. C) Confocal images of mouse blastocysts grown from the 8- cell stage for 2 days in 2i, or medium supplemented with PD0325901. Embryos were immunostained using antibodies raised against Nanog (green), Oct4 (red) and Gata4 (blue). Nuclei were counterstained with DAPI. D) Bar chart showing cell numbers of epiblast (Nanog positive, green) and hypoblast (Gata4 positive, blue) of embryos cultured in conditions of C. Bars indicate the mean ± SD. (Nichols et al. 2009).

The identity and pluripotency of the epiblast was confirmed by the production of chimeras with full contribution including germ line transmission. It was concluded that mESCs and pluripotent epiblast exhibit identical properties, and hence represent equivalent ground states of pluripotency (Nichols et al. 2009).

Conclusions were affirmed in studies by Lu et al. (2008) who also used an inhibitor of differentiation (PD98059) in culture medium of intact mouse embryos. PD098059 is the less specific equivalent to PD0325901 which also blocks ERK signalling. This study found that inhibition of ERK activity caused a considerable delay in development, as well as a marked attenuation of Cdx2 expression. The reduction in Cdx2 was also observed by Nichols et al. (2009). Together these two studies proposed that culturing embryos in 3i/2i has significant positive effects for deriving pluripotent stem cells. The real test will be whether using these inhibitors in other species will uncover similar findings.

1.6 My Research

The aim of this study was to assess the effects of inhibitors PD173074, PD0325901 and CHIR99021 on the developing bovine embryo. We hypothesised that the use of these inhibitors in culture medium would enhance the pluripotent characteristics of blastocysts for subsequent ESC derivation.

Firstly, in vitro fertilised (IVF) bovine embryos were generated and cultured with and without the presence of 3i/2i in culture medium. Day-seven embryos (blastocysts) were graded on morphological quality and developmental stage recorded for later statistical analysis. As a measure of blastocyst quality, total cell numbers in the ICM and TE lineages were analysed by differential staining. Blastocysts were also analysed for the expression and localisation of core pluripotent transcription factors and were assessed for whether 2i had any effect on these markers. At present molecular tools specific for bovine embryos are scarcely available (e.g. antibodies). Therefore, some trailing of tools was performed to ensure validity of results prior to testing. Molecular techniques used included real-time qPCR and immunocytochemistry to observe gene and protein expression patterns, respectively.

Results obtained will add to the growing knowledge of mechanisms underlying pluripotency in cattle. It will also assist future work in reproductive technologies for the New Zealand agricultural sector, as manipulation of embryos in farm animals, especially dairy and beef cattle, is becoming increasingly important due to a rising demand in agricultural products worldwide. Desirable genotypes (e.g. speciality milk, disease resistance, fertility, or adaptation to particular environments) from genetically screened embryos could be effectively captured through multiplying a few embryonic cells into potentially unlimited numbers of pluripotent stem cells. These could then be converted into stem cell-derived animals capturing unique genetics for breeding, transgenic and biomedical applications (Bjorn Oback, personal communication). Being able to access numerous high quality oocytes from females and monopolising subsequent embryos would increase genetic output by superior individuals. Ultimately, this would lead to an increase in the overall performance within a breeding population (Feugang et al. 2008; Galli & Lazzari 2008) and subsequently benefit associated agricultural sectors.

Chapter 2: Materials and Methods

2.1 In Vitro Production (IVP) of Bovine Blastocysts

Standard operating procedures were followed for the in vitro production (IVP) of bovine embryos in the embryology laboratory at Agresearch Ltd, Ruakura, Hamilton. Modifications to the standard procedure were dependent upon conditions of the current experiment. An additional section (2.1.7) is included on the manipulation of the procedure for the specific work covered in this thesis. Note that all plates were made previously in a sterile laminar flow hood (Westinghouse Pty Ltd, NSW) and gassed in a 38°C humidified 5% CO_2 incubator for 2 h prior to in vitro maturation (IVM) and in vitro fertilisation (IVF), or gassed with 5% CO_2, 7% O_2, and 88% N_2 within a humidified modular incubation chamber (QNA International Pty Ltd., Australia) at 38°C for 2 h prior to in vitro culture (IVC). For further details on reagents, solutions and media composition see Appendix 1. Information from each day of IVP was recorded using a production sheet (Appendix 2) and kept in a personal laboratory book for future reference.

2.1.1 Aspiration - Day -1

Ovaries were collected from local abattoirs and transported to the aspiration laboratory, Ruakura, Hamilton in a thermos flask filled with 29.5- 30.5°C, 0.9% saline. The time taken between the removal of ovaries and aspiration was no longer than 3 h. Cumulus-oocyte complexes (COC's) were recovered from cow ovaries by aspiration of follicles between 3 to 10 mm using an 18 g needle under vacuum (48 mmHg). The needle was penetrated into the follicle and moved around slightly to aid the dislodgment of the oocyte within the follicle. The aspirated follicular contents were collected in a 15 ml conical tube filled with 2 ml of aspiration media.

2.1.2 In vitro Maturation - Day -1

After completion of aspiration, tubes were transported to the main embryology laboratory and searched. Searching consisted of transferring the pellet from the conical tubes into a 90 mm petri dish containing additional aspiration media. A 35 mm petri dish containing H199 medium was prepared for collected COCs. All collected COC's were transferred to a second dish containing H199 and then once more into a 35 mm petri dish containing B199 medium. Ten COC's were pipetted up in 10 µl of B199 and placed into 40 µl drops of IVM medium located in IVM plates. Each 60 mm plate contained 12 drops overlaid with mineral oil, therefore holding 120 COC's. After all COC's were placed into the plates, they were returned to the 38°C incubator for a further 22-24 h to allow the oocytes to mature.

2.1.3 In vitro Fertilisation – Day 0

Freshly thawed additives were dissolved into the sterile IVF medium during plate preparation; 10 µl of heparin, 10 µl of pyruvate and 100 µl of penicillamine/ hypotaurine per every 10 ml of medium. Each 60 mm plate contained 12 x 30 µl drops and 2 x 60 µl wash drops overlaid with mineral oil. Hepes-buffered synthetic oviduct fluid (HSOF), used as a transitional/wash medium, required warming before use to 38°C.

In vitro fertilisation occurred in two phases, the first being oocyte preparation. Plates containing oocytes from the previous IVM were taken out of the incubator and oocytes were removed into a small petri dish with HSOF media. Cumulus cells of the COC's were loosened by pipetting them up and down several times. However, caution was taken not to entirely remove the cumulus cells. A second wash was required in another dish of HSOF before a third transfer of the oocytes into a small dish of sterile IVF media. Lastly, five oocytes were pipetted up in 10 µl of the IVF media and transferred into the IVF plate drops.

Sperm preparation was the second phase of IVF and requires the capacitation of frozen semen from 0.25 ml straws. A Percoll Plus gradient was set up in a laminar flow hood for sterility. The gradient consisted of 2 ml of 45% Percoll Plus

underlaid with 2 ml of 90% Percoll Plus within a 15 ml conical tube. The gradient was used to separate the sperm from the semen by centrifugation. The appropriate number of straws required for fertilisation, previously determined by experiments on the specific bull's sperm quality (1-2 straws per run is generally required), was thawed from liquid nitrogen storage. Straws removed from liquid nitrogen were thawed for 30 s in 30-35°C water. Once removed from the water, the straw tips were wiped with ethanol and cut by sterile scissors at the clamped end. The straw contents were dispensed into the tube carefully so as to lie gently atop the 45% gradient. The tube was then centrifuged for 20 minutes at 2200 rpm (700xg).

After 20 min, the pellet was aspirated into a sterile glass pipette preloaded with 1 ml of HSOF. The mixture of sperm and HSOF was placed into a fresh 15 ml conical tube and again centrifuged for 5-10 min at 1200 rpm (200xg). This time the supernatant was removed by a sterile glass pipette following centrifugation, leaving a small pellet of sperm behind. Both centrifugation steps were done as fast as possible to ensure that the motile sperm did not start swimming up through the supernatant. Next, 200 µl of IVF media was added very gently to the pellet. Subsequently, 10 µl was removed and added to 190 µl of sterile H_2O. The remaining sperm solution was measured (A) and placed back into an incubator. The addition of sperm to the H_2O dilutes it 1:20, but also kills the sperm. A further 10 µl is then transferred to a haemocytometer for an accurate sperm count under a light microscope. Sperm were counted within the 25 central squares of the haemocytometer on both sides to obtain an average cell count (B). The calculation is as follows:

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

2) Total volume (C) – Volume measured (A) = Volume of IVF to add to tube

The total volume (C) required gives a final concentration of 1 million/ml when the sperm is added at a 1:4 dilution (volume of sperm: volume of eggs in 40 µl IVF drop). IVF was completed by administering 10 µl of the sperm medium into each

drop of five oocytes in the IVF plates. The plates were then returned to the incubator for 18-24 h.

2.1.4 In vitro Culture – Day 1

On day one post-fertilisation, early synthetic oviduct fluid (ESOF) plates were made and placed into an incubation chamber to equilibrate for 2 h. Cumulus cells were removed by manual stripping by pipette or by vortexing embryos at 1800 rpm in hyaluronidase for 2 min. The bare zygotes were then washed through two dishes of HSOF. A finely drawn glass pipette (mouth pipette) with a bore size slightly larger than the zygote was used to transfer 60 embryos into each IVC plate. All embryos were put through a first and second wash drop in each plate, then divided up into groups of 10 and transferred into the 6 x 20 µl early synthetic oviduct fluid (ESOF) culture drops. Once all zygotes were placed in culture, the incubation chamber was gassed with 5% CO_2, 7% O_2, and 88% N_2 for 5 min at high pressure. After 5 min, all valves were shut off and the chamber was placed into a 38°C incubator for 5 d.

2.1.5 LSOF change of medium– Day 5

On day five, late synthetic oviduct fluid (LSOF) plates were made and placed into the same incubation chamber to equilibrate for 2 h. During the change over, embryos were transferred by mouth pipette to a LSOF plate. Once more, the embryos were placed first into the wash drop, then the second wash drop and divided into groups of 10 in each of the six culture drops. However, to avoid detrimental effects as a consequence of group culture, embryos which had not cleaved or looked degenerative at this stage were separated out. The number of embryos to cleave was recorded at this stage. Plates were then returned to the incubation chambers for re-gassing and a further two days of incubation.

2.1.6 Grading – Day 7

Embryos were graded at day seven based on their stage of development and morphological quality (grade). The total number, stage of development and grade of blastocysts were recorded for personal records as well as for developmental

analysis. The criterion for assessment is shown in Appendix 3. Blastocysts were processed for either differential staining, RNA extraction or protein analysis as outlined in subsequent sections. For a few downstream applications that required D8 blastocysts, 5 µl of FCS was added to the 20 µl culture drops following D7 grading to assist a further overnight incubation.

2.1.7 3i/2i supplementation

The basic experimental concept for this thesis relied on supplementation of bovine embryo culture media (ESOF & LSOF) with inhibitors of differentiation and general biosynthetic activity; PD0325901 and CHIR99021, respectively (Stemgent, USA). Collectively, the two inhibitors are referred to as '2i'. A third inhibitor (PD173054, Sigma) was used in initial experiments and in combination with the previous inhibitors constitutes '3i'. Table 1 outlines details of each inhibitor, including the working concentrations used in all experiments.

These concentrations are identical with those used in the mouse system (Nichols et al., 2009) and remained consistent throughout these experiments as a comparable measure.

Table 1. Inhibitor details for 3i/2i

		Inhibitor	Pathway	Stock	Dilution	Working	Solvent
3i		PD173074	FGFTK	1 mM	1/10000	100 nm	DMSO
	2i	PD0325901	MEK	10 mM	1/25000	0.4 µM	DMSO
		CHIR99021	GSK3	10 mM	1/3333	3 µM	DMSO

Plates containing culture media supplemented with 3i/2i were made 2 h before addition of embryos. Inhibitors were diluted to the correct concentration in ESOF or LSOF (whichever applicable) to a total volume of 1 ml. The solvent of all inhibitors, di-methylsulphoxide (DMSO), was used as a control and diluted at the

equal volume to the combined concentration of inhibitors. Culture plates were made according to the standard operating procedure (1 plate of 200 µl = 6 x 20 µl, 2 x 40 µl drops). Plates were gassed in an incubation chamber with 5% CO_2, 7% O_2, and 88% N_2 for 5 min at high pressure and kept in a 38°C incubator until required. Any remaining media containing 3i/2i was kept at 4°C and used within 3 d, otherwise fresh aliquots were made weekly.

During transfer of embryos into culture, separate glass pipettes were used for each treatment to avoid cross contamination of media. Figure 6 outlines the experimental design for 3i tests and later refined experiments with 2i. IVF was considered as day zero (D0), therefore embryo culture and subsequently the start of specific treatments began at day one (D1) post-fertilisation. The treatments of 3i, 2i or DMSO from D1 were cultured in ESOF + 3i/2i/DMSO for 4 d and LSOF + 3i/2i/DMSO for the last 2 d. Embryos cultured in 3i from day three (D3) were cultured in standard ESOF for 2 d, followed by ESOF + 3i for 2 d and then LSOF + 3i for 2 d. Notably, inhibitors were refreshed as a consequence of medium change at D5 in the previous treatment groups. The treatment that included the addition of 3i at D5 was cultured in standard ESOF up until the D5 changeover, followed by culture in LSOF + 3i for the remaining 2 d.

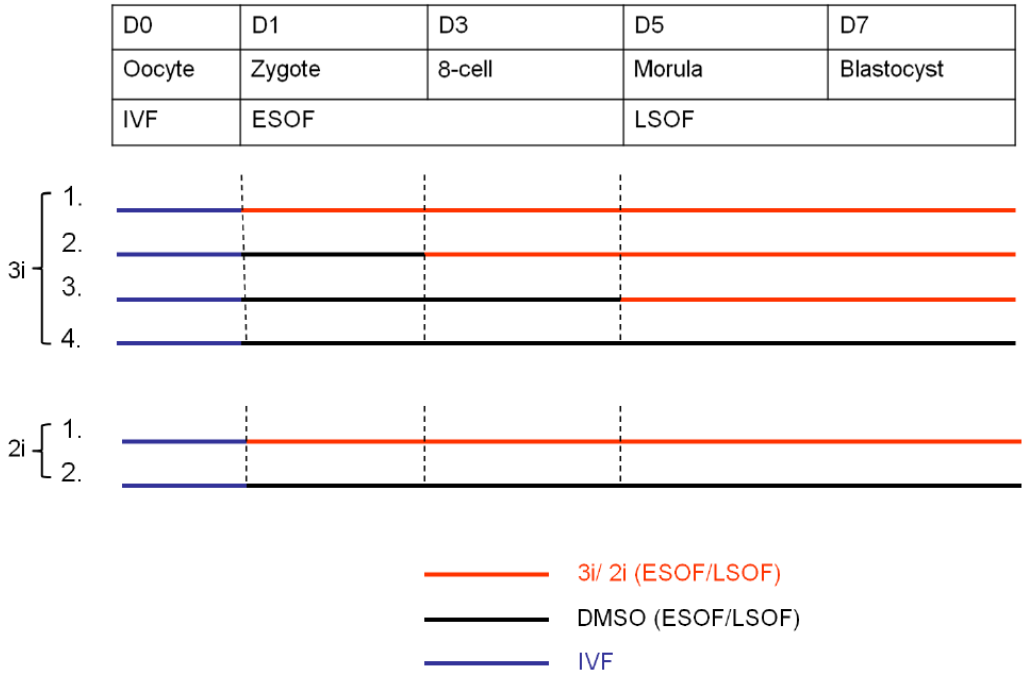

Figure 6. Experimental design for embryo culture in 3i/2i. Day (0, 1, 3, 5 and 7) post-fertilisation is displayed at the top of the figure, as well as corresponding development stage and base culture medium for each time period. Treatments for 3i are 1) 3i @ D1, 2) 3i @ D3, 3) 3i @ D5 and 4) DMSO control. Treatments for 2i are 1) 2i @ D1 and 2) DMSO control.

2.2 Differential Staining

Working solutions of anti-bovine serum developed in rabbit (RαB, Sigma B3759 Missouri, USA) and guinea pig compliment serum (GP, Sigma S1639 Missouri, USA) were prepared previously. Transfer hepes-buffered synthetic oviduct fluid without bovine serum albumin (THSOF -BSA) was added to RαB aliquot at a 1:4 dilution. A DNA stain (Hoechst 33342, 1 mg/ ml) and propidium iodide (1 mg/ml) were added at 40 µl /ml, to a GP aliquot diluted 1:4 by THSOF -BSA. Plates were prepared with 20 µl drops overlaid with mineral oil.

Expanded blastocysts were placed in pronase to remove the zona pellucida then washed in HSOF before commencing. Embryos were further washed multiple times in THSOF –BSA/ + polyvinyl alcohol (PVA, 0.1 mg/ml), a protein-free

medium, to prevent transfer of protein into the RαB serum. Embryos were washed in RαB drops twice before being left in a third drop for 45 min.

Subsequently, embryos were washed again in THSOF –BSA/ +PVA thoroughly and transferred to a GP plate for 15 min. At this stage it was crucial to have embryos out before the specified time otherwise cells lyse and become difficult to handle for mounting. After a brief HSOF wash, the embryos were mounted onto glass slides using 1.3 µl of Dakocytomation fluorescent mounting medium (DAKO, Med-Bio Ltd., S3023, New Zealand) and 9 mm circular cover slips. Images were taken immediately using an epi-fluorescence microscope (Olympus BX50, Japan).

Analysis of images was undertaken using Scion Image software (Beta 4.0.2, 2000). Inner cell mass (ICM) and trophectoderm (TE) cells were counted as indicated by blue and pink coloration, respectively. Results were recorded in an Excel spreadsheet for further statistical analyses (referred to in section 2.9).

2.3 Embryo Bisection

For acquiring separate samples of ICM & TE cells embryos were bisected by mechanical means. Expanded blastocysts of high morphological quality (grade 1 & 2s) were selected for embryo bisection as the ICM was compact and tidy, therefore making it easier to visualise and separate during the bisection. Blastocysts were placed in 20 µl pronase to remove the zona pellucida and then washed twice in phosphate buffered saline (PBS) + $MgCl_2$:$6H_2O$ (1.3 mg/L)/ $CaCl_2$:$6H_2O$ (1 mg/L)/ PVA (0.1 mg/ml). Embryos were transferred in small groups (< 5) in 20 µl drops of THSOF -BSA in a 60 mm petri dish. The protein-free medium caused embryos to settle to the bottom of the dish and become ‘stuck’. Aid was given to assist embryos to settle in the correct orientation for a splitting blade, mounted on a micromanipulator (MO-188, Nikon Narishige, Japan), to cut each blastocyst into two portions. One portion contained TE cells and the other contained the ICM and overlying TE cells (see Figure 7). Bisected pieces of TE and ICM/TE were held on ice in separate dishes containing HSOF

for TRIZOL® collection. Bisected embryos were held at this colder temperature to slow down any cell degradation that may occur.

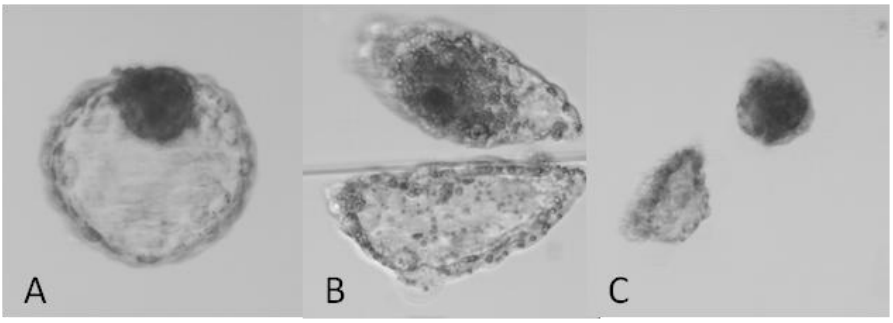

Figure 7. Bisection procedure for D7/D8 blastocysts. A) Correct orientation of embryo in dish, B) 'Splitting' embryo by mechanical blade C) Bisected pieces of ICM/TE and TE cells.

2.4 Immunosurgery

Working solutions of anti-bovine serum developed in rabbit (RαB, Sigma B3759 Missouri, USA) and guinea pig compliment serum (GP, Sigma S1639 Missouri, USA) were prepared previously. THSOF -BSA was added to the RαB and GP aliquots at a 1:4 dilution. Solutions were centrifuged for 10 min, and plates were prepared with 20 µl drops overlaid with mineral oil.

Expanded blastocysts were placed in pronase to remove the zona pellucida then washed in HSOF before commencing. Embryos were further washed multiple times in THSOF –BSA/ + polyvinyl alcohol (PVA, 0.1 mg/ml), a protein-free medium, to prevent transfer of protein into the RαB serum. Embryos were washed in RαB drops twice before being left in a third drop for 45 min. Subsequently, embryos were stringently washed again in THSOF –BSA/ +PVA and transferred through three drops in GP. Embryos were left in the GP plate for a further ~40 min. Embryos were then removed from the GP and placed into HSOF to dislodge

the lysed TE cells. A small bore glass pipette was most effective at removing the TE cells from the ICM. The remaining ICM was placed into TRIZOL®.

2.5 TRIZOL® Collection

Whole or bisected embryos were added in a droplet of HSOF media on the side of an RNase/DNase-free 0.65 ml tube containing 50 µl TRIZOL® (Invitrogen #15596-018, Life Technologies, USA). Contents were mixed, by flicking the tube lightly, and settled by a brief centrifuge spin. Samples in TRIZOL® were stored at -80°C until required for RNA extraction.

2.6 mRNA Quantification: RT- PCR

All reagents, unless otherwise stated, were sourced from Invitrogen, Life Technologies, USA.

2.6.1 RNA extraction

RNA was isolated by firstly adding 1 µl MS2 RNA (200 ng) and 5 µl α globin (5 pg/µl, Sigma) to each thawed TRIZOL® sample. Chloroform was added at one fifth the volume of TRIZOL® (10 µl), mixed, and centrifuged at 4°C for 10 min at 7000 rpm. The resulting aqueous phase (~40 µl) was transferred to a fresh tube. Isopropanol (40 µl) and 1 µl linear acrylamide (10 µg/µl) were added, followed by a 10 min incubation at room temperature. Samples were then centrifuged at 4°C for 30 min at 13,000 rpm. The supernatant was removed and the pellet washed in sterile 70% ethanol (100 µl) for 10 min at 13,000 rpm. The supernatant was again removed and the pellet left to air-dry. To finish, the pellet was re-solubilised in 8 µl DEPC treated H_2O.

2.6.2 DNAse treatment

RNA samples of 8 µl were treated with 2 µl DNase (1 U/ µl) and 1 µl 10X DNase buffer for 60 min at 37°C. The reaction was halted by 65°C incubation with 1 µl of EDTA (25 mM) for 10 min. To precipitate out RNA, 1.5 µl sodium acetate and

45 µl sterile 100% ethanol were added and the sample left at -80°C for at least 60 min. Samples were then centrifuged at 13,000 rpm for 30 min to pellet RNA and washed with 70% ethanol for a further 10 min spin. Ethanol was aspirated off and the RNA pellet re-suspended in 11 µl DEPC-treated H_2O.

2.6.3 cDNA synthesis

For first-strand complementary DNA (cDNA), 1 µl random hexamers (50 ng/µl) and 1 µl of 10 mM dNTP mix were added to 11 µl total RNA sample and incubated for 5 min at 65°C, followed by immediate quenching on ice for 1 min. Reverse transcription was performed by the addition of 10 µl master mix containing 2 µl 10X buffer, 4 µl of 25 mM $MgCl_2$, 1 µl RNaseOUT (40 U/µl) and 1 µl Superscript III reverse transcriptase (200 U/µl). The reaction mixture was first incubated for 10 min at 25°C, 50 min at 50°C, and then terminated by heating to 85°C for 5 min. Immediately, cDNA was quenched on ice for 1 min followed by storage at -80°C or PCR. To determine the presence of contaminating genomic DNA, reverse transcriptase was omitted in one sample each time a batch of samples was processed for cDNA synthesis ("-RT").

2.6.4 RT-PCR

The PCR was performed using a Mastercycler gradient (Eppendorf, Germany) with primer sequences shown in Table 2. Primers were originally designed by Bjorn Oback in LightCycler® Probe Design 2.0 or NCBI/Primer-BLAST. A total of 25 µl of reaction mix was added to 1 µl of cDNA template in a PCR tube. The PCR reaction mixture for each RNA sample consisted of 0.5 µl dNTP mix (10 mM), 0.5 µl of each primer (10 µM), 2.5 µl 10X PCR buffer (incl. $MgCl_2$), 0.2 µl Taq polymerase (5 U/µl), and 20.8 µl DEPC-treated H_2O. The following conditions were used: 1) denaturation for 5 min at 95°C, 2) amplification cycle of 94°C for 15 s, 56°C for 30 s followed by 72° for 1 min was repeated 34 times, 3) final extended amplification phase of 7 min at 72°C, and 4) cooling to hold at 4°C.

Table 2. RT- PCR and qPCR Primers

Gene	Sequence (5'-3')	RT- PCR annealing (°C)	qPCR melting peak (°C)	Ampli-con size (bp)
18S	F:GACTCATTGGCCCTGTAATTGGAATGAGTC	56	84	87
	R: GCTGCTGGCACCAGACTTG			
NANOG	F: CACCCATGCCTGAAGAAAGT	56	87	295
	R: TGCATTTGCTGGAGACTGAG			
SOX2	F: CTATGACCAGCTCGCAGA	60	90	152
	R: GGAAGAAGAGGTAACCACG			
OCT4	F: GGTTCTCTTTGGAAAGGTGTTC	60	90	333
	R: TGGCGACGGTTGCAAAACCA			
DPPA3	F: TGCAAGTTGCCACTCAACTC	60	83	158
	R: TCTTACCCCTCTCCGCCCTAT			
CDX2	F: AGACAAATACCGGGTCGTGTACA	60	88	162
	R: TTTGCTCTGCGGTTCTGAA			

2.6.5 Gel electrophoresis analysis

PCR products were loaded into 0.8-1.0% (w/v) agarose gels containing SYBER safe™ DNA gel stain (S33102, Invitrogen). Samples were mixed with loading dye prior to loading into the gel. Electrophoresed PCR products were run simultaneously with a 1 kb+ ladder (10787-018, Invitrogen) for determining their molecular sizes.

2.7 mRNA Quantification: Real-time qPCR

2.7.1 LightCycler® system

The LightCycler® system (Roche Diagnostics, USA) was used for qPCR amplification and data analysis. All consumables (excluding primers) and equipment were sourced from Roche Diagnostics, USA. All reactions were

performed with the LightCycler® FastStart DNA Master Plus SYBR Green 1 kit within LightCycler® capillaries (20 µl). Primer sequences are shown in Table 2.

For each sample a reaction mix consisting of 2 µl LightCycler® Master Mix, 0.4 µl of each primer (10 µM) and 5.2 µl DEPC-H_2O was prepared and added to 2 µl of cDNA template in a LightCycler® capillary. Samples were prepared in duplicate or triplicate, where possible given very low initial volumes, and repeated with at least three different samples of the same treatment. Samples were centrifuged (LightCycler® Carousel centrifuge) for 15 s to ensure contents were located at the end of each capillary before the reaction commenced. The following program was used: 1) denaturation (10 min at 95°C); 2) amplification and quantification (20 s at 95°C, 20 s at 56–60°C, followed by 20 s at 72°C with a single fluorescent measurement repeated 45 times); 3) melting curve (95°C, then cooling to 65 °C for 20 s, heating at 0.2°C s-1 to 95°C while continuously measuring fluorescence); and 4) cooling to 4°C.

Product identity was verified by gel electrophoresis and melting curve analysis. Results were analysed using generated standard curves to express concentration relative to the concentration of a housekeeping gene (18S). The analysis holds the assumption that our treatments had no effect on the concentration of the housekeeping gene.

2.7.2 Melting curve analysis

Melting curves were analysed in LightCycler® Software (4.1.1.21) after each run. A primer pair should have the same melting peak for each sample (except negative controls) and be consistent with previous experiments (see Table 2 for melting peaks of primers used). If this did not occur it was either due to primer dimers or contaminating genomic DNA. Results were discarded if the melting peak was not ‘clean’ as primers had previously been optimized, and therefore could only suggest a problem with the cDNA sample or specific reaction. A non-template control containing no cDNA was always included in every run and as a control should have a different melting peak to the samples. An example melting curve analysis is shown in Figure 8 for *CDX2* primers. All samples have a

melting peak temperature of 88°C, which is consistent with the optimisation results for the particular primer. As expected, the non-template control (no cDNA) is different at 79°C. One peak is present at the correct temperature, and therefore it can be confirmed that the amplified DNA product in our samples is *CDX2*.

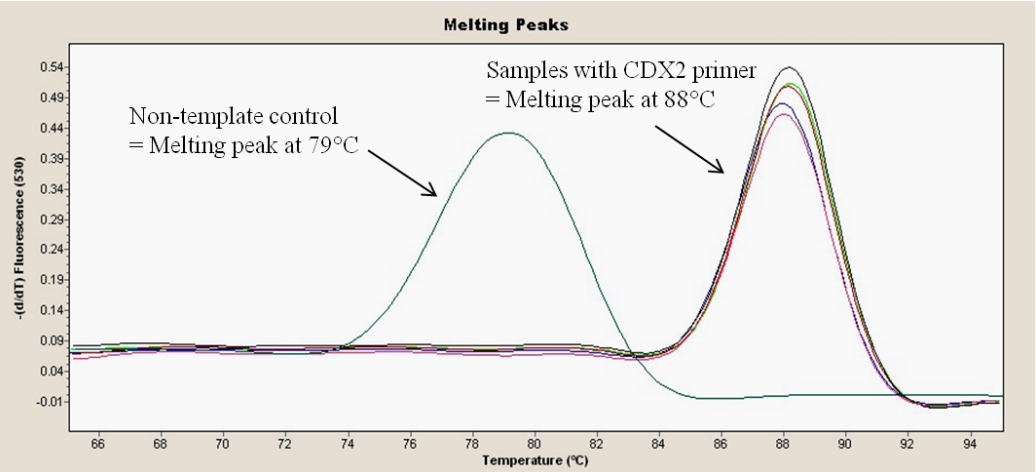

Figure 8. LightCycler® Melting Curve Analysis. Example of melting peaks obtained during a real-time qPCR run with *CDX2* primers.

2.7.3 Standard curve generation

RNA was extracted and cDNA produced from a large quantity of blastocysts (~60). The subsequent cDNA sample or DNA obtained by gel extraction was used to generate standard curves from serial 5-log dilutions. Dilutions for each gene were run in duplicate and triplicate at higher dilution ranges dependent on initial starting concentrations. The higher the dilutions, the greater number of replicates were performed to eliminate bias as the sensitivity of the PCR machine decreased. Standard conditions described in section 2.6.1 were used. Curves were generated using the LightCycler® Software (4.1.1.21) and information exported to Excel. R values and trend line equations were obtained from graphed curves. One high efficiency curve (1.90-2.10) was saved for each target gene and the subsequent equation used for relative quantification.

2.7.4 Gel extraction for standard curves

Many of the target genes investigated are expressed in very low abundance at the blastocyst stage, therefore gel extraction was used to obtain enough starting material for standard curves. End-point PCR was initially used to amplify the target sequence and products were run on a 1% agarose gel at low voltage for greater resolution. Products were visualised under UV and bands of correct size were cut out using a sterile scalpel blade and placed in pre-weighed tubes. Tubes were re-weighed and weight of gel calculated. The E.Z.N.A Gel extraction kit (D2500-01) was used to isolate pure DNA. The band of interest was cut from the gel, dissolved in binding buffer and applied to a HiBind DNA spin-column. DNA was then washed and eluted ready for subsequent applications. The DNA concentration was measured using a Nanodrop Spectrophotometer. DNA was measured by wavelength absorbance at 260 nm of 1 µl of sample to give a final concentration of ng/µl. A 260/280 ratio of 1.8 or greater was desired to confirm the purity of the DNA sample. The sample could then be utilised for generating real-time qPCR standard curves.

2.7.5 Statistical analysis of results using standard curves

Standard curve equations were used to formulate relative units (RU) of a given target gene for each sample. The crossing point (CP) was the quantitative unit acquired for all samples in a real-time qPCR run. To gain the relative concentration of any given sample, the CP was incorporated along with the equation generated from a target gene's standard curve (x & y values) into the following:

RU = EXP (-(CP value – x value)/ y value)

The resulting value was then divided by the RU from 18S to obtain the relative ratio of target gene to 18S for each sample. Ratios between treatments could then be averaged from the total number of samples, compared and graphed accordingly. Ratio averages obtained after all repeated trials had statistical students t-tests performed to determine statistical significance ($P < .05$).

2.8 Protein Quantification

2.8.1 Fixation

Pronase was used to remove the zona pellucida from blastocysts before being placed in 65°C depolymerised fixative for 30 min at 4°C. The fixed embryos were stored in PBS/PVA drops at 4°C until required. If embryos were processed later than two weeks after the first fixation, a re-fixation was essential.

2.8.2 Embryo immunocytochemistry

The immunocytochemistry (ICC) procedure with embryos was carried out in a conical 96-well plate. A mouth pipette with a 45-90° bend was necessary for transferring embryos between wells. Permeabilisation occurred in 0.1% Triton X-100 in PBS/PVA for 15 min. The embryos were placed into 5% donkey serum block for 90 min to prevent non-specific binding of the primary antibodies. The embryos were subsequently exposed to a primary antibody (Table 3), or combination of, diluted in block solution overnight at 4°C. Negative control embryos remained in block solution without any antibody addition. Embryos were washed twice in PBS/PVA for 15 min each and incubated in their appropriate secondaries for 30 min at 38°C (Alexor Fluor® 488 or 568 donkey anti-mouse, -rabbit, -goat or -rat, all Invitrogen). DNA was counterstained simultaneously with 5 µg/ml Hoechst 33342 stain (Sigma, USA). The embryos were transferred through two 15 min washes in PBS/PVA and a final brief wash in H_2O.

Embryos were mounted onto slides by 1.3 µl of DAKO and 9 mm circular cover slips. The slides were observed under an epifluorescence microscope (Olympus BX50, Japan) to observe protein localization and photographed using Spot Basic software. Slides were stored in the dark at 4°C.

Table 3. ICC Primary Antibodies

Antigen	Dilution	MAb/PAb	Secondary	Supplier & Cat. No
NANOG	1:1000	Poly	Rabbit	Peprotech, 500- P236
NANOG	1:50	Poly	Rabbit	Thermo Scientific, PA1-817
NANOG	1: 30	Poly	Rabbit	Abcam, #21603
NANOG	1:100	Poly	Rabbit	Abcam, #80892
NANOG	1:100	Poly	Rabbit	Abcam, #47102
NANOG	1:100	Poly	Goat	Abcam, #77095
NANOG	1:520	Mono	Mouse	Abcam, #62734
OCT4	1:200	Poly	Mouse	Santa Cruz, Sc-5279
SOX2	1:30	Poly	Goat	R&D Systems, AF2018

2.8.3 Cell immunocytochemistry

Cells were previously seeded in dishes containing sterile 9 mm circular cover slips. Cover slips (with attached cells) were fixed in depolymerised 4% fixative for 30 min at 4°C, washed three times in PBS and quenched in 50 mM NH_4Cl in PBS for 10 min. Permeabilisation occurred in 0.1% Triton X-100 in PBS for 10 min, followed by 30 min at room temperature in block solution (5% donkey serum/ PBS). On parafilm, cover slips were placed atop 30 µl drops of primary antibody (see Table 3), diluted in block solution. Cells were left to incubate overnight at 4°C in large (10 cm) petri dishes with a lining of filter paper soaked in sterile H_2O to maintain humidity. The cover slips were washed three times in PBS for 10 min the following day, then incubated in 30 µl drops of corresponding 488 or 568 Alexor Fluor® donkey anti-mouse, -rabbit, -rat or -goat secondary IgG antibodies (all Invitrogen) for 30 min at 38.5°C. DNA was counterstained simultaneously with 5 µg/ml Hoechst 33342 stain (Sigma, USA).

Negative controls were processed the same way, however instead of primary antibodies the cells were incubated in block solution overnight. Lastly, cover slips were washed three final times in PBS for 10 min, and once in H_2O before cover slips were mounted on slides with 3 µl of DAKO. Cells were observed under an epifluorescence microscope (Olympus BX50, Japan) to observe protein localization and photographed using Spot Basic software. Slides were stored at 4°C.

2.9 Plasmid Vector Cloning

2.9.1 Plasmid vector construct

To obtain a cell line that over-expressed bovine *NANOG* for testing of antibodies a Tet-On® 3G system was used (Clontech Laboratories Inc, USA). The bovine *NANOG* sequence was obtained from GeneBank and synthesized into a plasmid vector containing a doxycycline-inducible TRE3G promoter by GENEART, Germany. The plasmid additionally contained an ampicillin resistance gene and cherry fluorescent marker. A second plasmid was required for transfections, which contained the Tet-On 3G transactivator protein responsible for initiating induced gene expression at the TRE3G promoter. The Tet-On 3G transactivator protein, compared with its earlier predecessors, has increased sensitivity to doxycycline. The pTRE3G-bovineNANOG-mcherry and pEF1α-TET3G plasmid maps are shown in Figure 9, A & B respectively. Plasmids were amplified via bacterial transformation and purified by DNA Maxi-preparation for cell transfections at a later date. Transfections were carried out by Andria Green.

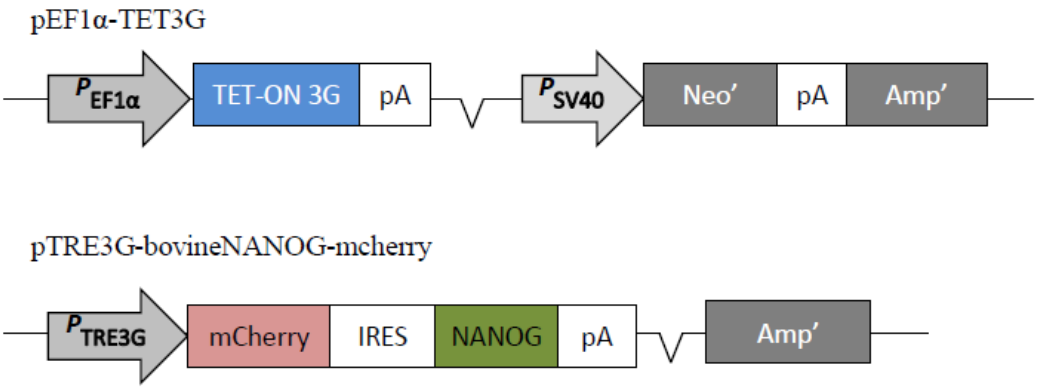

Figure 9. Linear plasmid maps for A) pTRE3G-bovineNANOG-mcherry and B) pEF1α-TET3G.

2.9.2 Bacterial transformation

Plasmid vector dilutions (25 ng/µl) were prepared previously, along with LB broth and LB agar plates containing antibiotic (ampicillin). LB broth and agar plates were made to manufacturer's specifications on the container, and ampicillin was added at a final concentration of 100 µg/ml. The competent bacteria DH5α were slowly thawed on ice for 10-20 min. To every 100 µl of bacteria, 1 µl of plasmid vector solution was added. Bacteria/plasmid mixture were incubated at room temperature for 1-2 min, followed by heat shock of 1 min at 42°C, immediately followed by cooling on ice for 1 min. The bacteria was then diluted 1:10 in LB broth and incubated at 37°C for 30-60 min. Bacteria were spread evenly on LB agar plates and incubated overnight in a 37°C dry incubator. The vector contained ampicillin resistance so only colonies that took up the vector would survive. Colonies were selected the following day at the optimal growth period. Further incubation proceeded in LB broth on a shaker at approx 180 rpm in a 37°C incubator to aerate bacteria. The culture time in the incubator was dictated by the volume of bacteria required (For a DNA Maxi-preparation and larger volume required= overnight (~12 h)).

2.9.3 DNA maxi-preparation

A Purelink™ HiPure Plasmid Filter Maxi-prep Kit (K2100-74, Invitrogen) was used to isolate pure plasmid DNA from bacterial cultures according to the manufacturer's instructions. Bacterial cells were harvested by centrifugation (4,000 xg) for 10 min. All media was removed and replaced with 10 ml resuspension buffer with RNase A. The cells were then mixed until homogenous. Next, 10ml of lysis buffer was added to the mixture and incubated at room temperature for 5 min before addition of 10 ml of precipitation buffer. The precipitated lysate was transferred into a Maxi-Column and allowed to filter through by gravity flow. Approximately 50 ml of wash buffer was added to rinse the column and DNA of previous buffer residuals. This step could be repeated if necessary. To elute the DNA, 15 ml of elution buffer was added to the column and collected in a new tube. Subsequently, 10.5 ml of Isopropanol was added to the eluted DNA and left to incubate for 2 min at room temperature. DNA was precipitated out of solution by centrifugation at 15,000 xg for 30 min at 4°C. The supernatant was discarded and 6 ml of 70% ethanol was added. A further 30 min centrifugation was required before the supernatant was again removed and discarded. The resulting pellet was left to air-dry followed by re-suspension in 300 µl of either TE buffer or DepC-H_20. Lastly, to ensure all DNA was dissolved before quantification the re-suspended DNA was placed into a 37°C dry incubator for 10-15 min.

2.9.4 DNA quantification

Plasmid DNA concentrations were measured using a Nanodrop spectrophotometer. DNA was measured by light absorbance at 260 nm of 1 µl of sample to give a final concentration in ng/µl. A 260/280 ratio of 1.8–2.0 was desired to confirm the purity of the DNA sample.

2.9.5 Restriction digest

To confirm the identity of the cloned plasmid a restriction digest was performed. The following was combined in a small tube for linear digestion; 0.5 µl restriction enzyme, 2 µl 10X buffer (of greatest compatibility with the enzyme used), 1µg DNA. The remainder was made up of H_2O to a total volume of 20 µl. An additional 0.5 µl of a second restriction enzyme (substituted with H_2O) was added for double digests as long as the same buffer could be used optimally for both enzymes. The restriction enzyme/s used depended on the restriction site/s present within the plasmid construct. Tubes were incubated in a 37°C water bath for 1 h before products were loaded in a 0.8-1.5% agarose gel. Molecular sizes were determined using a 1 kb+ ladder (10787-018, Invitrogen).

2.10 Statistical Analyses

Statistical analyses were carried out using Microsoft Excel®. For the analysis of developmental data, a two-tailed Fisher Exact test for independence in 2 x 2 tables was used to determine the significance of results. A two-tailed Students t-test with equal variance was used to determine significance for gene expression data. Statistical significance was accepted at $P < 0.05$.

Chapter 3: Embryo Development Results

3.1 Embryo development in 3i

3.1.1 Introduction

Blastocysts were produced following the standard operating procedure in the PC1 embryology laboratory at Agresearch Ltd, Ruakura. Embryos were separated into groups of approximately 50-60 and cultured for seven consecutive days in their respective treatments unless otherwise stated. Grading occurred accordingly at day seven (D7) post-fertilisation where the morphological quality and stage of development was assessed. Quality was ranked on a scale from 1-3, with grade 1 embryos being of highest quality (Criterion for quality assessment is outlined in Appendix 3). Blastocysts were also defined in one of four developmental categories shown in Table 4.

Table 4. Blastocyst developmental categories for D7 morphological assessment

Blastocyst category	Description
Early Blastocyst (EB)	Blastocoel cavity visible, however is less than 50% of entire embryo
Blastocyst (B)	Blastocoel cavity is greater than 50% of entire embryo with a compact, dark ICM present. Zona pellucida is unchanged
Expanded Blastocyst (XB)	Blastocoel cavity is greater than 75% of entire embryo. Embryo size has increased and zona pellucida thinning is visible
Hatched/ Hatching Blastocyst (HB)	Same properties as expanded blastocyst, but zona pellucida has disappeared completely or embryo is currently hatching from it

Initial experiments using 3i looked at the effect of length of exposure on the development of bovine embryos. As inhibitors are diluted in DMSO, it served as a solvent control for all experiments. Preliminary experiments therefore compared embryo culture without DMSO (no DMSO/ control) and the addition of DMSO to culture medium (DMSO). These experiments were undertaken to justify that any change we would encounter with the inhibitors was not a consequence of DMSO supplementation. As a result, no significant difference was observed between the control and DMSO.

The developmental data (n= 3 runs) is plotted in Figure 10, including the total number of embryos that cleaved (i.e. ≥ 2-cell). Both control and DMSO groups averaged an approximate 60% cleavage rate indicating that DMSO had no effect on initial cleavage. The graph also illustrates the total number of blastocysts present at day seven of quality grade 1-3 (TB1-3) and blastocysts of the total that represent grade 1-2 (TB1-2). The results are presented as a percentage of all embryos that were transferred into culture (nIVC). Although DMSO produced fewer TB1-3 at day seven, 26% compared to 35% of the control group, the difference was not significant at $P < 0.05$. Moreover, both groups had similar numbers of TB1-2 with 21.4% and 24.1% for DMSO and control, respectively. From these results we acknowledged that DMSO had no direct positive or negative effect on embryo culture, and could therefore validate its use as a solvent control for inhibitor experiments.

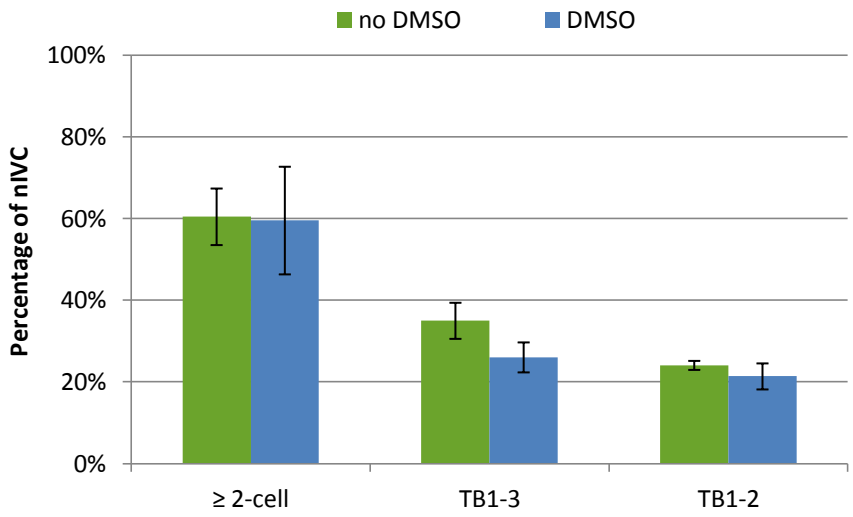

Figure 10. Development results of no DMSO (control) vs. DMSO. Percentage of embryos that cleaved and are ≥ 2-cell, total blastocysts grade 1-3 (TB1-3) and grade 1-2 (TB1-2) from all embryos that were placed in culture (nIVC). Error bars indicate ± SD.

Table 5 summarises all embryo data recorded during no DMSO vs. DMSO embryo culture and subsequent grading, including exact numbers of blastocysts referred to in Figure 10. The TB1-3 that developed from those that cleaved (≥2-cell) is also shown.

Table 5. Embryo development: no DMSO (control) vs. DMSO culture

Culture condition	**N**	**nIVC**	**≥2-cell**	**TB1-3**	**TB1-2**	**TB1-3/ ≥2-cell (%)**
Control	3	220	133	77	53	57.9
DMSO	3	215	128	56	46	43.8

N= number of independent IVF experiments; nIVC= number of embryos analysed.

3.1.2 3i culture

Inhibitors (dissolved in DMSO) were introduced into culture at either day one (3i @ D1), day three (3i @ D3), or day five (3i @ D5) post-fertilisation. On the day of inhibitor culture, inhibitor solutions were added directly to culture media prior to making plates. DMSO (no inhibitors) was added at equal volumes to 3i and served as a control for the following experiments. No significant variation was observed between each treatment group and the DMSO control for embryos that cleaved, or the total number and quality of blastocysts obtained at day seven (Figure 11).

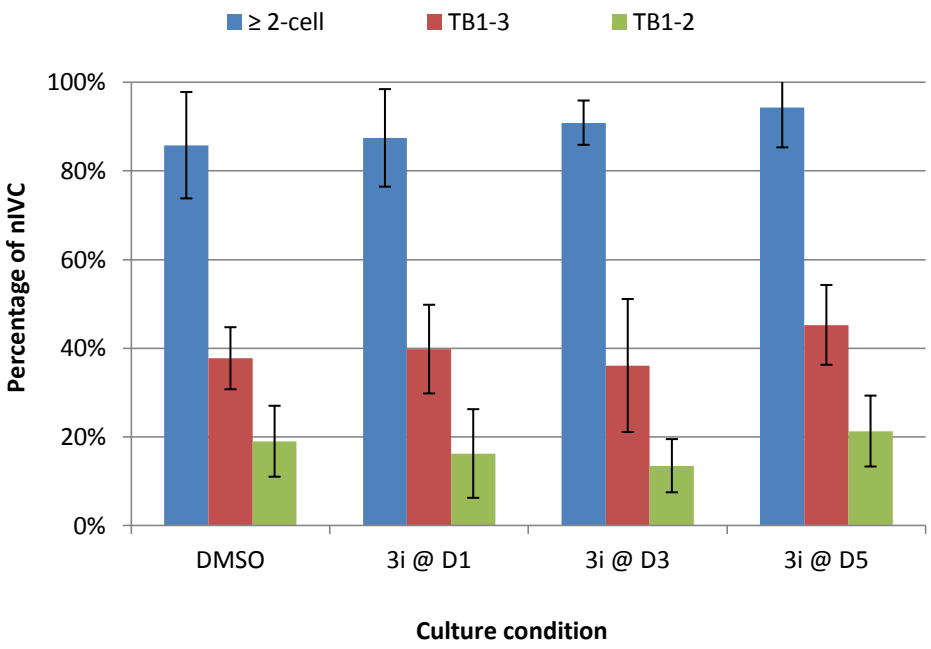

Figure 11. 3i development results. Comparison of all four treatments; DMSO control, 3i @ D1, 3i @ D3 and 3i @ D5. Percentage of embryos ≥2-cell, total blastocysts grade 1-3 (TB1-3) and grade 1-2 (TB1-2) from all embryos that were placed in culture (nIVC). Error bars indicate ± SD.

To observe an effect on the rate of development total blastocysts were further segregated into their respective stage of development categories. Figure 12 shows the proportion of blastocysts assessed in each developmental category (as dictated in Table 4). The percentage of blastocysts in each category (EB, B, and XB/HB) is given from the TB1-3 for each treatment (as shown in Table 6). For these results XB and HB's are classified together for all treatments, this is due to very low HB numbers as a consequence of the time of grading. Overall the largest difference was observed in both EB and B categories: 38% vs. 27% and 42% vs. 52% for DMSO and 3i @ D5, respectively. These results would imply an increase in rate of development with 3i as more embryos were assessed at the B stage than EB. Regardless, no difference was observed within the later stage XB/HB category, and the number of blastocysts within each category was not statistically significant between all treatments and the DMSO control.

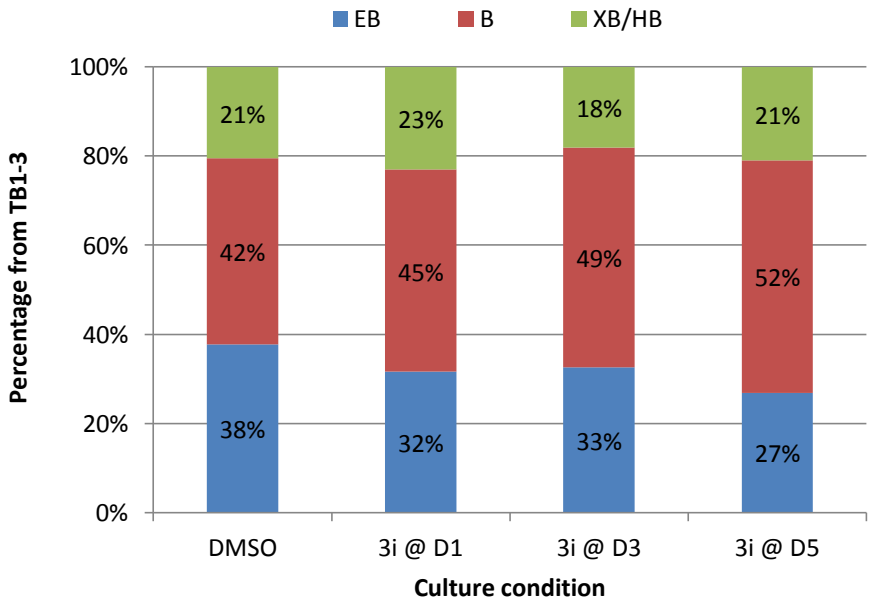

Figure 12. Stage of development results with 3i culture. The proportion of blastocysts graded within each developmental category (EB, B and XB/HB) from the total number of blastocysts grade 1-3 (TB1-3).

In all 3i conditions, expanded blastocysts had developed and in a few cases the blastocysts had hatched from the zona pellucida. This indicated that the inhibitors did not interfere with trophectoderm formation or differentiation.

Table 6 presents the embryo development data for 3i culture recorded for each treatment and the DMSO control. The exact numbers of TB1-3 and TB1-2, as well as the percentage of embryos that reached the blastocyst stage from those that cleaved (%TB1-3/Cleaved) are shown. The total number of blastocysts assessed in each developmental category is also displayed.

Table 6. Embryo Development: DMSO control vs. 3i @ D1/D3/D5

Culture condition	N	nIVC	≥2-cell	EB1-3	B1-3	XB/HB1-3	TB1-3	TB1-2	TB1-3/Cleaved
DMSO	6	387	332	55	61	30	146	62	44%
3i @ D1	6	382	334	48	69	35	152	62	46%
3i @ D3	4	230	209	27	41	15	83	31	40%
3i @ D5	4	263	248	32	62	25	119	56	48%

N= number of independent IVF experiments; nIVC= number of embryos analysed.

3.2 Embryo Development in 2i

3.2.1. Introduction

After 3i results were analysed the number of culture treatments was reduced to two (DMSO control and 2i) for a more comprehensive evaluation of embryo culture with these inhibitors. The reduction was determined to increase sample size per treatment, but additionally focus on the effect of the two inhibitors (PD0325901 and CHIR99021) on the entire culture period. There had been reported evidence that the PD173074 inhibitor was dispensable for culture (Silva et al. 2008) therefore, we eliminated its use for the following experiments. Taking into consideration that there was no detrimental morphological effect observed with long term culture from day one in 3i, we felt this was the best treatment to define the effect of 2i on other molecular aspects. Inhibitors were added at D1 of culture for the entire culture period. Blastocysts at D7 were morphologically assessed as stated for previous pilot experiments in section 3.1.1.

During the standard IVP procedure embryos are manually stripped to dislodge the majority of cumulus cells surrounding the zygote. Some cells remain attached, yet it normally has an unnoticeable effect on development and removal of cells is unnecessary for general IVP. When required, hyaluronidase is used to chemically remove all cumulus cells. The following results are divided into: 1) embryos that were manually stripped or 'cumulus-intact', 2) embryos that were chemically stripped or 'cumulus-free' and 3) pooled results of the two previous methods.

3.2.2 Cumulus-intact culture in 2i

Developmental results for cumulus-intact culture are displayed in Figure 13. Cleavage rates did not differ between DMSO and 2i, 56.3% and 55.6%, respectively. The DMSO control had slightly greater proportions of TB1-3 (29.6% vs. 25.6%) and TB1-2 (19.4% vs. 14.9%) compared with 2i. However, these were not significant at $P < .05$.

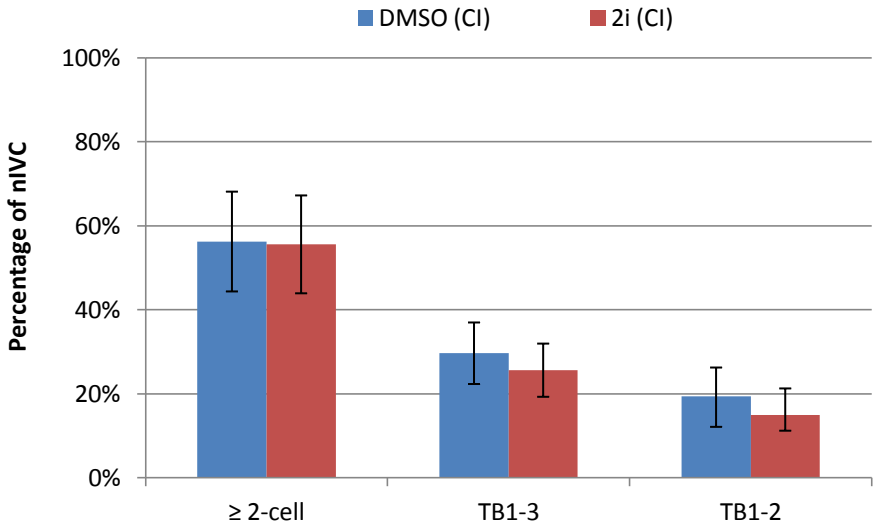

Figure 13. 2i cumulus-intact (CI) development results. Percentage of embryos that are ≥ 2-cell, total blastocysts grade 1-3 (TB1-3) and grade 1-2 (TB1-2) from all embryos that were placed in culture (nIVC). Error bars indicate ± SD.

During cumulus-intact culture observations were made regarding the morphology and behaviour of cumulus cells at the D5 medium changeover. Cumulus cells in 2i-supplemented medium appeared 'sticky' or 'flaky' and in some cases had stuck to the bottom of petri dishes. Several embryos were observed to remain encased in thick cumulus cell clumps and could not be dislodged. The occurrence of this morphology was only observed in the 2i treatment and across a number of different IVP experiments. In addition, a larger number of degenerate embryos were noticed in the 2i group. However, the number of degenerate embryos were not recorded consistently and therefore we do not have any evidence to support this statement.

The portion of blastocysts assessed in each developmental category at D7 is depicted in Figure 14. A minor increase in the proportion of blastocysts (B) was detected (44.5% vs. 40.2%) and a decrease in the number of early blastocysts (EB; 11.4% vs. 16.8%) in 2i. The difference was statistically insignificant in both categories. The proportion of expanded blastocysts (XB) remained similar (41.0%

vs. 41.2%), as did the proportion of hatched blastocysts (HB; 2.0% vs. 3.3%). All comparisons between DMSO and 2i developmental categories were not significant at $P < .05$.

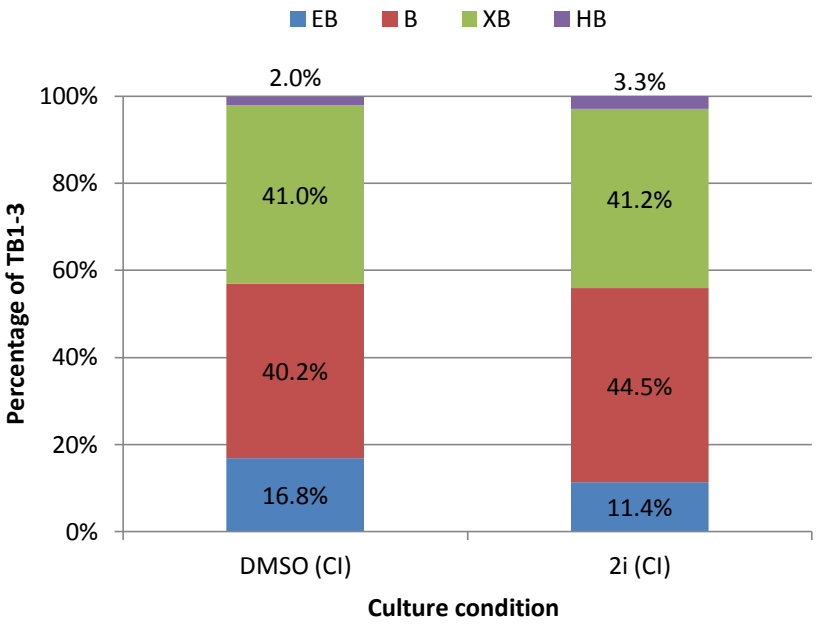

Figure 14. Stage of development results with cumulus-intact (CI) culture. Bars, representative of each group (DMSO control and 2i), are divided into the portion of blastocysts graded within each developmental category (EB, B, XB and HB) from the total number of blastocysts grade 1-3 (TB1-3).

3.2.3 Cumulus- free culture in 2i

We hypothesised that the previous observations of cumulus cell growth could perhaps be a factor influencing 2i embryo development and that we may see more profound effects if the cumulus cells were removed. To exclude this variable we therefore decided to remove cumulus cells prior to culture by a brief vortex in hyaluronidase.

Cumulus-free cleavage rates did not differ between DMSO and 2i, comparable to figures seen between the two groups in cumulus-intact culture (Figure 15). The

proportion of TB1-3 (34.0% vs. 35.6%) and TB1-2 (22.5% vs. 24.1%) are marginally greater in 2i compared with DMSO, which is in contrast to what was observed with cumulus-intact culture. However, the previous figures are not significant at $P < 0.05$.

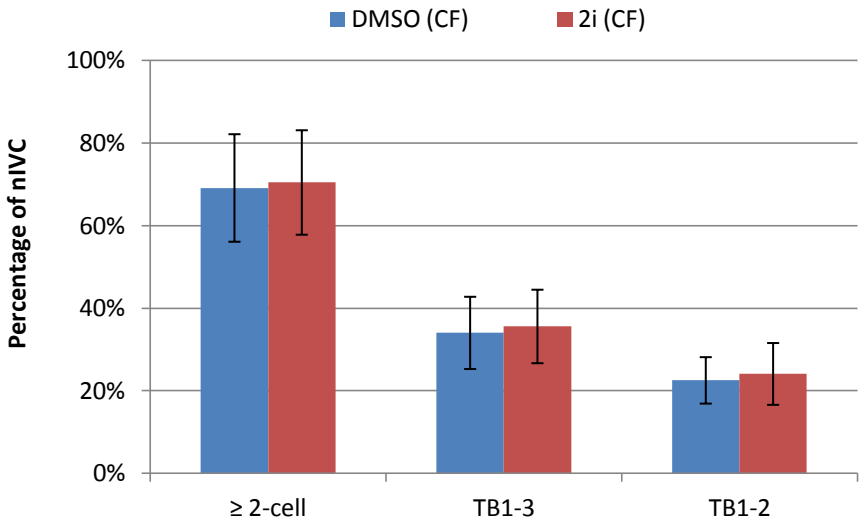

Figure 15. 2i cumulus-free (CF) development results. Percentage of embryos that are ≥ 2-cell, total blastocysts grade 1-3 (TB1-3) and grade 1-2 (TB1-2) from all embryos that were placed in culture (nIVC). Error bars indicate ± SD.

Total blastocysts grade 1-3 from cumulus-free culture were divided into their respective blastocyst development categories and findings displayed in Figure 16. The portion of HBs was significantly greater in 2i than DMSO, 4.4% vs. 1.6% at $P < 0.01$. Whereas, no significant difference was found for any other category. It may be worthy to note that within the early blastocyst categories (EB and B), DMSO had a greater proportion of blastocysts than 2i. However on the other hand, 2i had a greater proportion of later stage blastocysts (XB and HB) than DMSO.

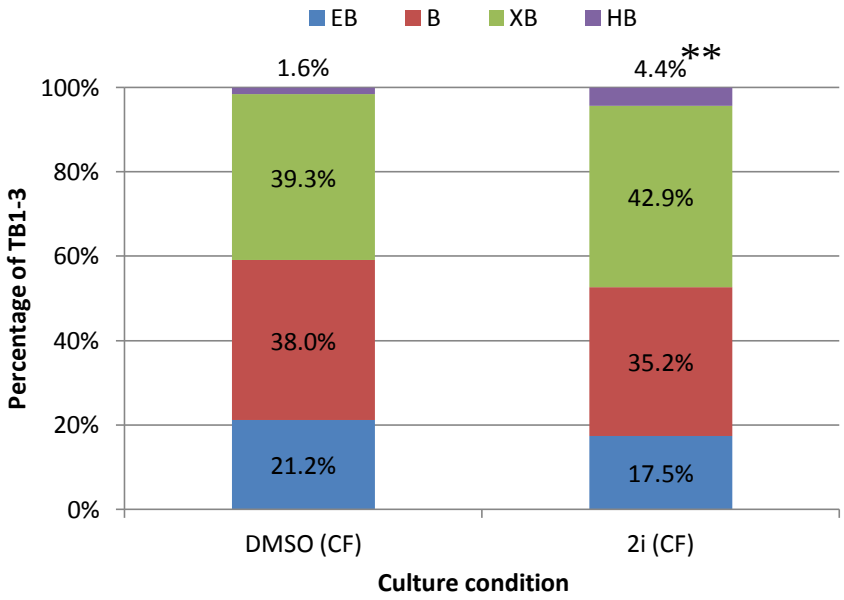

Figure 16. Stage of development results with cumulus-free (CF) culture. Bars, representative of each group (DMSO control and 2i), are divided into the portion of blastocysts graded within each developmental category (EB, B, XB and HB) from the total number of blastocysts grade 1-3 (TB1-3). ** = $P < .01$ compared with DMSO control.

3.2.4 Pooled 2i results

Figure 17 presents overall statistics (including both cumulus-intact and cumulus-free results) of the total percentage of embryos that cleaved (≥ 2-cell), TB1-3 and TB1-2. In total, 28 independent IVF runs were completed with morphology assessed on 868 and 870 blastocysts for DMSO and 2i, respectively. As evident, TB1-3 did not differ between the two groups, neither did the TB1-2. The results were confirmed by significance testing. Cleavage rates did not vary between treatments and were also not significant at $P < 0.05$.

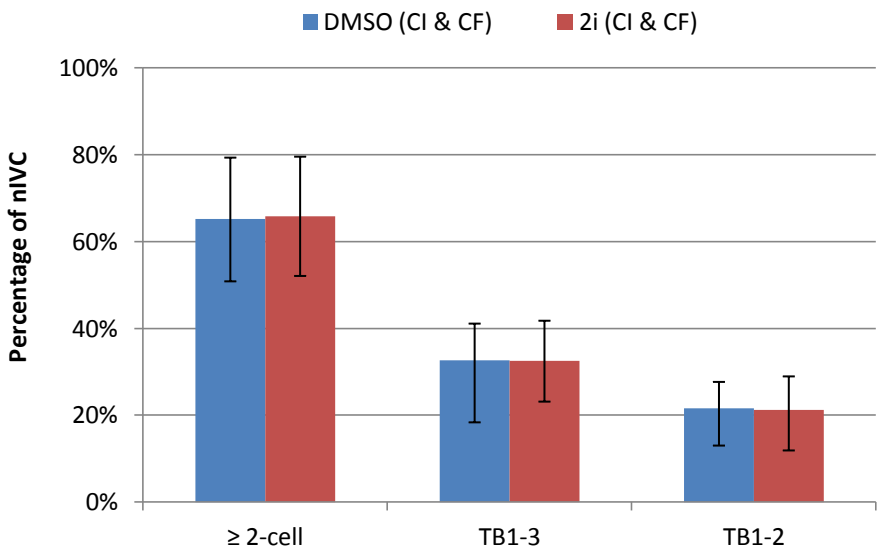

Figure 17. Overall development results for cumulus-intact and cumulus-free data (CI & CF). Percentage of embryos that are ≥ 2-cell, total blastocysts grade 1-3 (TB1-3) and grade 1-2 (TB1-2) from all embryos that were placed in culture (nIVC). Error bars indicate ± SD.

Stage of development results, for all 28 runs, are displayed in Figure 18. The total proportion of HBs was significantly greater in 2i than DMSO, 4.1% vs. 1.7% at $P < 0.01$. Additionally, the total proportion of EBs was significantly less in 2i than DMSO, 16% vs. 19.9% at $P < 0.05$. The major categorical groupings of B and XB remained insignificant with approximately 80% of all blastocysts attributed to either category. Together these results suggest that there is a subtle increase in the rate of development of embryos cultured in 2i.

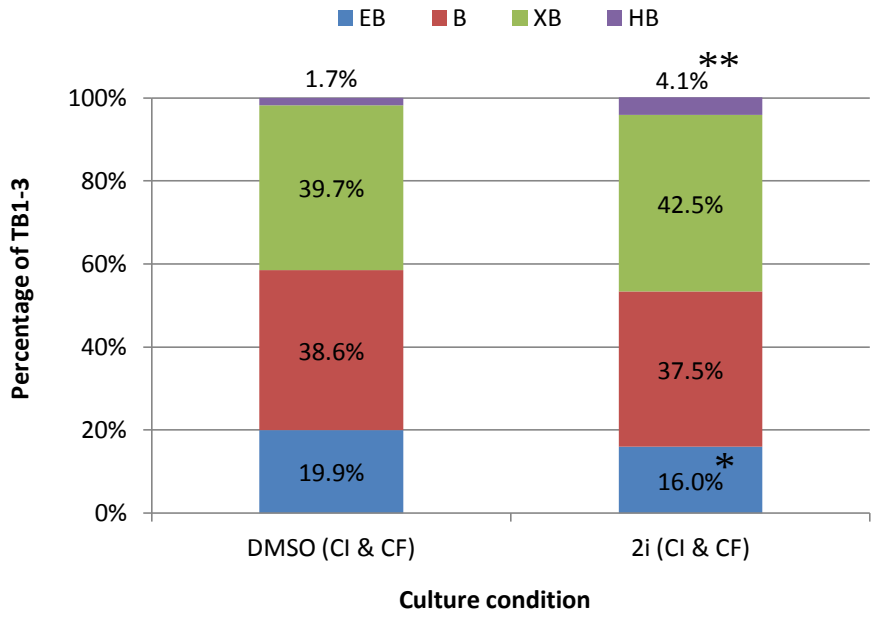

Figure 18. Stage of development result of overall CI and CF culture. Bars, representative of each group (DMSO control and 2i), are divided into the portion of blastocysts graded within each developmental category (EB, B, XB and HB) from the total number of blastocysts grade 1-3 (TB1-3). * = P < .05 and ** = P < .01 compared with DMSO control.

Observations recorded during day seven grading made reference to the size of 2i embryos that were irrespective of stage of development. A number of blastocysts in 2i were much larger than their DMSO counterparts, which was most obvious for the expanded blastocyst category when the zona pellucida first permits the expansion of the developing embryo.

Table 7 presents all developmental data collected during 2i embryo culture. This includes both cumulus-intact and cumulus-free results over the entire experimental period.

Table 7. Embryo development: DMSO control vs. 2i

Culture condition		N	nIVC	≥ 2-cell	EB1-3	B1-3	XB1-3	HB1-3	TB1-3	TB1-2	TB1-2/TB1-3 (%)	TB1-3/cleaved (%)
Cumulus-intact	**DMSO**	10	823	463	41	98	100	5	244	160	65.6	52.7
	2i	10	824	458	24	94	87	7	211	123	58.3	46.1
Cumulus-free	**DMSO**	18	1835	1268	132	237	245	10 ***a***	624	413	66.2	49.2
	2i	18	1853	1305	115	232	283	29 ***b***	659	446	67.7	50.0

N= number of independent IVF experiments; nIVC= number of embryos analysed; ***ab*** rows with these different superscripts within a column differ $P < 0.01$.

3.3 Blastocyst Quality Assessment: Total Cell Nuclei

3.3.1 Introduction

To further investigate the morphological differences between DMSO and 2i blastocysts we used differential staining to quantify cell numbers. The technique allowed for the number of putative ICM, TE and total nuclei in blastocysts to be measured. Expanded blastocysts grade 1-2 were used as these gave the most accurate results during differential staining and produced the greatest clarity under the microscope. ICM and TE cells were identified by blue (Hoechst DNA stain) and pink (propidium iodide) coloration, respectively, and cell numbers were quantified the same day using Scion Image software (Beta 4.0.2, 2000). Blastocysts were sourced from three individual IVF experiments and differential staining commenced directly following day seven grading.

3.3.2 Differentially stained TE and ICM in bovine blastocysts

The average ICM, TE and total cell nuclei (± SD) in DMSO control and 2i expanded blastocysts are presented in Table 8. The number of embryos analysed (n) was 27 and 37 for DMSO and 2i, respectively. DMSO control embryos had on average significantly less ICM (43 vs. 57, $P < .01$) and TE cell numbers (69 vs. 89, $P < 0.001$). As a result, a significant increase in the average total number of cells was observed in 2i cultured embryos (143 vs. 110, $P < 0.001$). The ICM: Total cell ratio, was equivalent between the two treatments which indicates a proportional allocation in cell nuclei to ICM and TE. The increase in cell numbers is therefore not specific to one lineage, and represents an overall increase in cell nuclei of blastocysts cultured in 2i.

Table 8. Total nuclei numbers and allocations of nuclei (mean ± SD) to ICM and TE in DMSO control vs. 2i expanded blastocysts.

			No. of nuclei			
Culture condition	***n***	***N***	**ICM**	**TE**	**Total**	**ICM: Total**
DMSO	3	27	43 ± 15^{a}	69 ± 18^{a}	112 ± 28^{a}	$0.39 \pm .11$
2i	3	37	57 ± 24^{b}	89 ± 24^{c}	147 ± 46^{c}	$0.40 \pm .12$

n= number of independent IVF experiments; *N*= number of embryos analysed; ^{ab} rows with these different superscripts within a column differ $P < 0.01$; ^{ac} rows with these different superscripts within a column differ $P < 0.001$.

Figure 19 displays representative photographs of both DMSO control and 2i cultured embryos as viewed directly following the differential staining procedure. Photographs were taken on an epifluorescence microscope with an RGB filter at 200x magnification (Olympus BX50, Japan).

3.4 Embryo Development Summary

Culture in 2i did not have any significant effects on cleavage, and the total number of embryos to reach the blastocyst stage. Additionally, no significant difference in morphological quality was observed between 2i and the DMSO control blastocysts at D7. Overall however, there was a significant increase in the proportion of hatched blastocysts at the expense of the early blastocysts in 2i cultured embryos. This effect was more profound with the absence of cumulus cells from culture. The number of cells in blastocysts cultured in 2i was found to be significantly greater than the DMSO control, with an increase in cells contributing to both TE and ICM lineages.

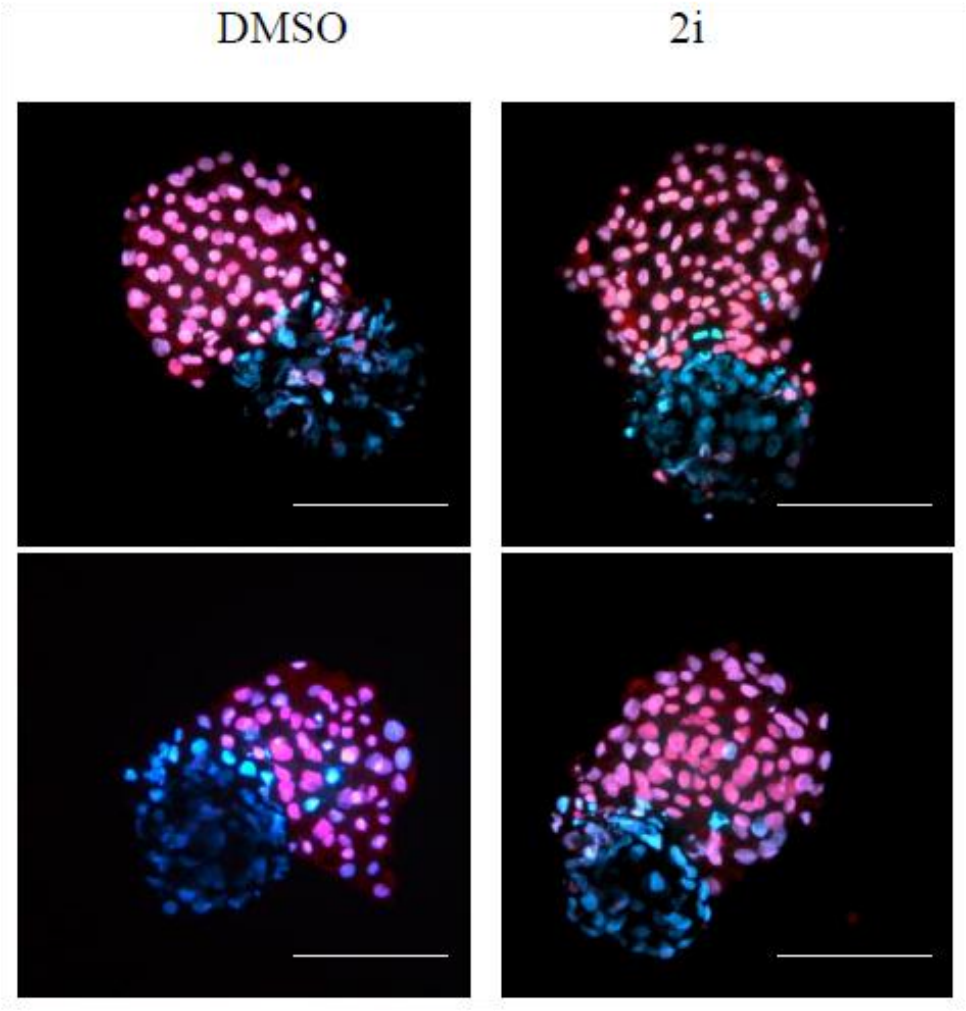

Figure 19. Differential staining of DMSO and 2i expanded blastocysts. DMSO and 2i embryos presented in the left and right columns, respectively. All expanded blastocysts were grade 1-2. Blue (Hoechst) and pink (propidium iodide) colours represent ICM and TE cells, respectively. Scale bar = 100 µm.

Chapter 4: Gene Expression Analyses

4.1 Introduction

In contrast to the available information on mouse embryos, little is known of the markers that govern the events of pre-implantation development in cattle. This is partly due to the unavailability of tools and methods that specifically work in bovine, but also driven primarily by the species-specific differences in the temporal and spatial expression of the transcription factors usually associated with lineage specification in mice. As epiblast markers are simultaneously key indicators of the establishment of the pluripotent cell population, it is fundamental to the derivation of true ESCs in cattle to identify specific lineage markers (Munoz et al. 2008b).

Our objective was to improve characterization in bovine embryos of a few of the core pluripotency-maintaining markers associated with mice; *NANOG, OCT4*, *SOX2* and *DPPA3*. As we had observed a significant increase of the TE cells in 2i blastocysts, we were also curious to look into expression of the TE marker; *CDX2*. We initially focused on intact blastocyst samples to determine if gene expression was detectable and whether 2i culture had any effect on overall transcription. These experiments were followed by a study into mRNA expression of these genes within the specific lineages, ICM and TE, using bisected blastocyst samples (section 4.2). We initially used embryos cultured without the inhibitors to optimise the bisection method and also to provide us with control results once our method worked well. Subsequently, we aimed to assess whether these markers were differentially expressed in either TE or ICM. We then examined the expression profiles of bisected blastocysts cultured in 2i to compare the effect of the inhibitors.

4.2 Effect of 2i on Pluripotent Marker Gene Expression in Intact Blastocysts

The embryos for RNA extraction were sourced from IVF experiments at D7 post-fertilisation. Blastocysts grade 1-3 from the three treatment groups; no DMSO (control), DMSO (solvent control) and 2i were placed into TRIZOL® and stored at -80°C until required for the extraction process. Following RNA extraction and cDNA synthesis, the cDNA samples were amplified by RT-PCR with bovine 18S primers, and subsequently run on a gel. If a single DNA band was observed at 87 bp, the sample was used for real-time quantitative PCR (qPCR) experiments. Samples were removed from analyses if cDNA synthesis had not been successful (no band).

In all experiments gene expression was normalised to the housekeeping gene, 18S, to produce a relative ratio for each sample presented in relative units (RU). Results presented in figures below are representative of the average relative ratio of gene expression from a number of individual IVF experiments (*n*) with *N* corresponding to the total number of blastocysts analysed. Blastocyst samples were analysed by qPCR in duplicates where possible given the very small amount of starting material. For all primer details refer to Table 2, section 2.6.4.

4.2.1 Relative gene expression of pluripotent markers

The relative expression ratio of *NANOG, OCT4, SOX2, DPPA3*, and *CDX2* in no DMSO, DMSO and 2i intact blastocysts is displayed in Figure 20. *NANOG* expression was found to be significantly increased in 2i cultured blastocysts compared to both control groups (no DMSO and DMSO) at $P < 0.01$. This corresponded to a 2.3- and 2.7-fold increase in the abundance of *NANOG* transcripts from the no DMSO and DMSO controls, respectively. No significant difference was observed between the no DMSO and DMSO treated embryos for *NANOG* transcription.

SOX2 expression was interesting as we observed a significant difference ($P < 0.05$) between the DMSO control and both the no DMSO and 2i blastocysts. Surprisingly, 2i maintained expression at levels equivalent to the no DMSO control. This result indicates that the use of DMSO has a negative effect on *SOX2* transcription, which is seemingly recovered in the presence of 2i.

In contrast, no significant differences were detected between no DMSO, DMSO and 2i blastocysts for *OCT4* expression. Similarly, no significant difference was detected in *DPPA3* and *CDX2* expression between no DMSO, DMSO and 2i treated blastocysts.

The finding that *NANOG* is consistently up-regulated in 2i supports our hypothesis that 2i does enhance pluripotent characteristics in the bovine blastocyst. Based on our experimental results, the effect of 2i is limited to *NANOG* and possibly *SOX2* transcription as all other genes were seemingly unaffected.

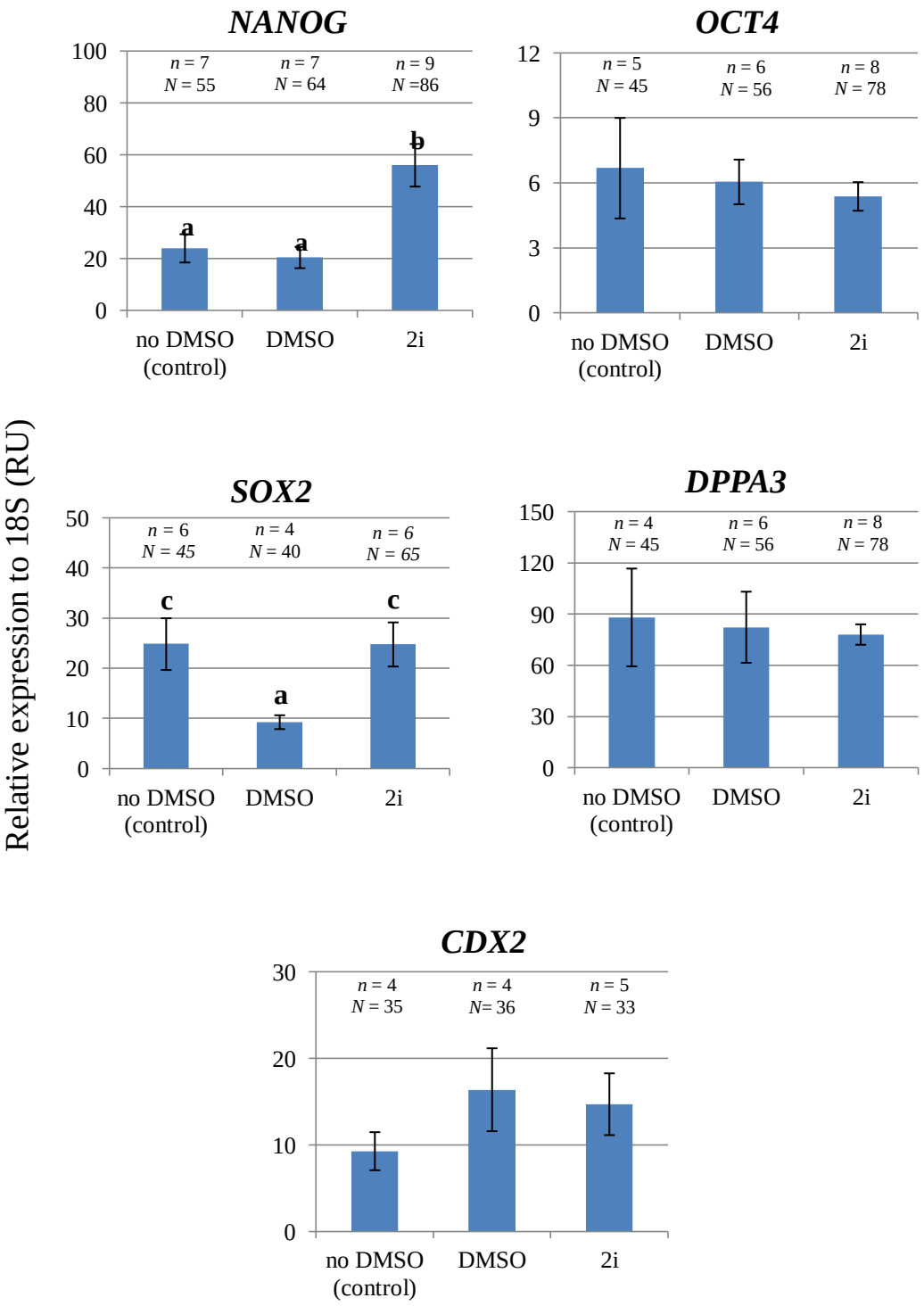

Figure 20. Relative gene expression analysis for *NANOG*, *OCT4*, *SOX2*, *DPPA3* and *CDX2* in no DMSO, DMSO and 2i cultured D7 embryos. Data are presented relative to 18S mRNA abundance. Different superscripts denote differences (**ab** = $P < 0.01$, **ac** = $P < 0.05$) between culture conditions. Error bars indicate ± SEM. *n* = independent IVF experiments, *N* = total number blastocysts.

4.3 Lineage Specificity of Pluripotent Marker Gene Expression

The next objective was to assess and quantify the expression of *NANOG*, *OCT4*, *SOX2*, *DPPA3* and *CDX2* in the ICM and TE lineages independently. As little information exists on the localisation of these genes, we aimed to measure and confirm whether they were differentially expressed in either the TE or ICM of bovine blastocysts. We initially analysed bisected blastocysts that were cultured without DMSO or inhibitors to optimise the bisection technique. Subsequently, experiments including the DMSO control and 2i treatment groups were performed.

The bisection of blastocysts was carried out to obtain representative samples from the ICM and TE lineages. It was impossible to isolate the ICM without some contaminating cells from the overlying polar TE, however to avoid confusion these samples are still referred to as ICM. In contrast, the TE samples will comprise purely TE cells. Regardless, this method will still allow us to obtain the relative information we require as the majority of cells in the ICM/TE samples will belong to the ICM lineage.

The embryos for all bisections were sourced from IVF experiments between D7 and D8 post-fertilisation. Expanded blastocysts grade 1-2 were used as these had clear ICM and TE boundaries. Bisections were carried out by Bjorn Oback using a splitting blade mounted on a micromanipulator. Bisected pieces were placed into separate tubes of TRIZOL® and stored at -80°C until required for the RNA extraction process (the bisection method is illustrated in Figure 7, section 2.3). Samples were verified as previously described (section 4.2) by RT-PCR before being used for real-time qPCR experiments.

4.3.1 Pluripotent marker gene expression in untreated bisected blastocysts

The results presented here are from samples obtained from untreated bovine blastocysts. The number of independent IVF experiments (*n*) = 3 (TE) and 5 (ICM), and total number of blastocysts (*N)* = 25 (TE) and 64 (ICM). Two ICM samples were obtained by immunosurgery. These samples will not contain TE as the procedure involves the chemical removal of the TE cells. We chose not to continue with this method for subsequent experiments as all TE cells were sacrificed in the process, therefore we could not obtain TE and ICM samples that corresponded to the same blastocysts. Nonetheless, these are valuable ICM samples and are included in the results below.

The relative expression ratio for *NANOG, OCT4, SOX2, DPPA3* and *CDX2* in TE and ICM from bisected blastocysts is presented in Figure 21. We found that *NANOG* and *SOX2* transcripts were barely detectable in the TE and alternatively were enriched in the ICM. Both results were statistically significant and indicate that these genes are ICM-specific. The expression of *DPPA3* mRNA was detectable in both ICM and TE, although the ICM had over a 2-fold greater relative expression than observed in the TE. However, *DPPA3* expression in the ICM compared to the TE was insignificant ($P < 0.053$). *OCT4* was likewise expressed in both lineages and accordingly the relative ratios of both ICM and TE were similar. On the other hand, *CDX2* transcripts were detected at slightly greater abundance in the TE but these results were not significant.

These results establish that *NANOG* and *SOX2* are potential markers of the ICM in bovine blastocysts. *DPPA3*, to a lesser extent, also provides us with evidence to suggest that it could be somewhat restricted to the ICM at D7/D8. Protein analyses will be required to assert if these genes subsequent proteins are specific to the ICM.

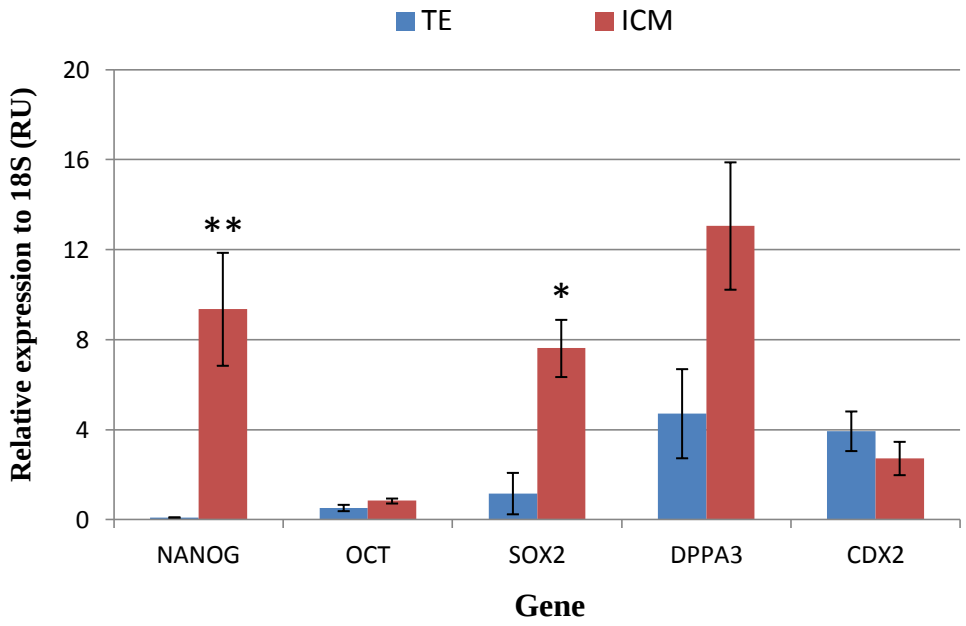

Figure 21. Relative gene expression of *NANOG, OCT4, SOX2, DPPA3* and *CDX2* in bisected bovine blastocysts. Data are presented relative to 18S mRNA abundance. Error bars indicate ± SEM. ** = $P < 0.001$, * = $P < 0.01$.

4.3.2 Effect of 2i on pluripotent marker gene expression in bisected blastocysts

Bisected samples were collected as described previously from blastocysts cultured with DMSO and 2i. The following results illustrate the relative gene expression of *NANOG, OCT4, SOX2, DPPA3* and *CDX2* in the TE and ICM from these bisected blastocysts. No significant differences were observed between the untreated bisected blastocysts (previous results) and DMSO controls. Significance testing was excluded from *SOX2* and *CDX2* results as sample numbers were limited (*n*= 1). Therefore, as further samples are required to assert differences between treatments we report preliminary results for these genes from experiments to date.

The relative expression of *NANOG, OCT4, SOX2, DPPA3* and *CDX2* in the TE and ICM from DMSO and 2i bisected blastocysts is shown in Figure 22. *NANOG* transcripts are detected in greater quantity in the ICM of blastocysts in both DMSO and 2i, which is the same trend we observed between TE and ICM in

untreated blastocysts. The abundance of *NANOG* transcripts in the ICM of 2i blastocysts significantly ($P < 0.05$) exceeded the relative expression observed in the control group (DMSO), and likewise the untreated blastocysts (Figure 21). However, no significant difference was observed between the two groups for *NANOG* expression in the TE. These results provide further evidence that *NANOG* is an ICM-specific marker in cattle and that 2i elicits an up-regulation in the ICM, which is consistent with the findings we observed in intact blastocysts.

Bisected blastocysts cultured in 2i exhibited a slight increase in *OCT4* expression in the ICM compared to the TE. However, given that we had observed no difference between treatments in the intact blastocysts, this finding may be due to a low *n*. Overall, no significant difference is present between TE and ICM in 2i or between treatments.

The DMSO control and 2i treatment had a very limited number of samples for *SOX2* analyses, therefore results remain speculative. However, it is worthy to note that *SOX2* transcripts were detected in greater abundance in the ICM of both treatments providing further support that *SOX2* could be a potential ICM-specific marker. Both DMSO and 2i showed the same trend and similar values of relative expression.

The relative expression of *DPPA3* shows an increase in the ICM of both DMSO and 2i bisected blastocysts compared to their respective TE counterparts. These values were not significant, but also follow the same trend we observed in untreated bisected blastocysts.

Unfortunately, we were only able to analyse one sample from each of the DMSO control and 2i for *CDX2* expression in the TE and ICM. However, there was a noticeable difference between the TE and ICM expression with *CDX2* transcripts detected in greater quantity in the TE in both DMSO and 2i. These results support the observation that *CDX2* is a marker of TE in cattle, and therefore we would expect to observe small quantities in the ICM samples as these also contain some TE cells. We additionally identified no difference between the DMSO control and 2i in relative abundance of *CDX2* transcripts.

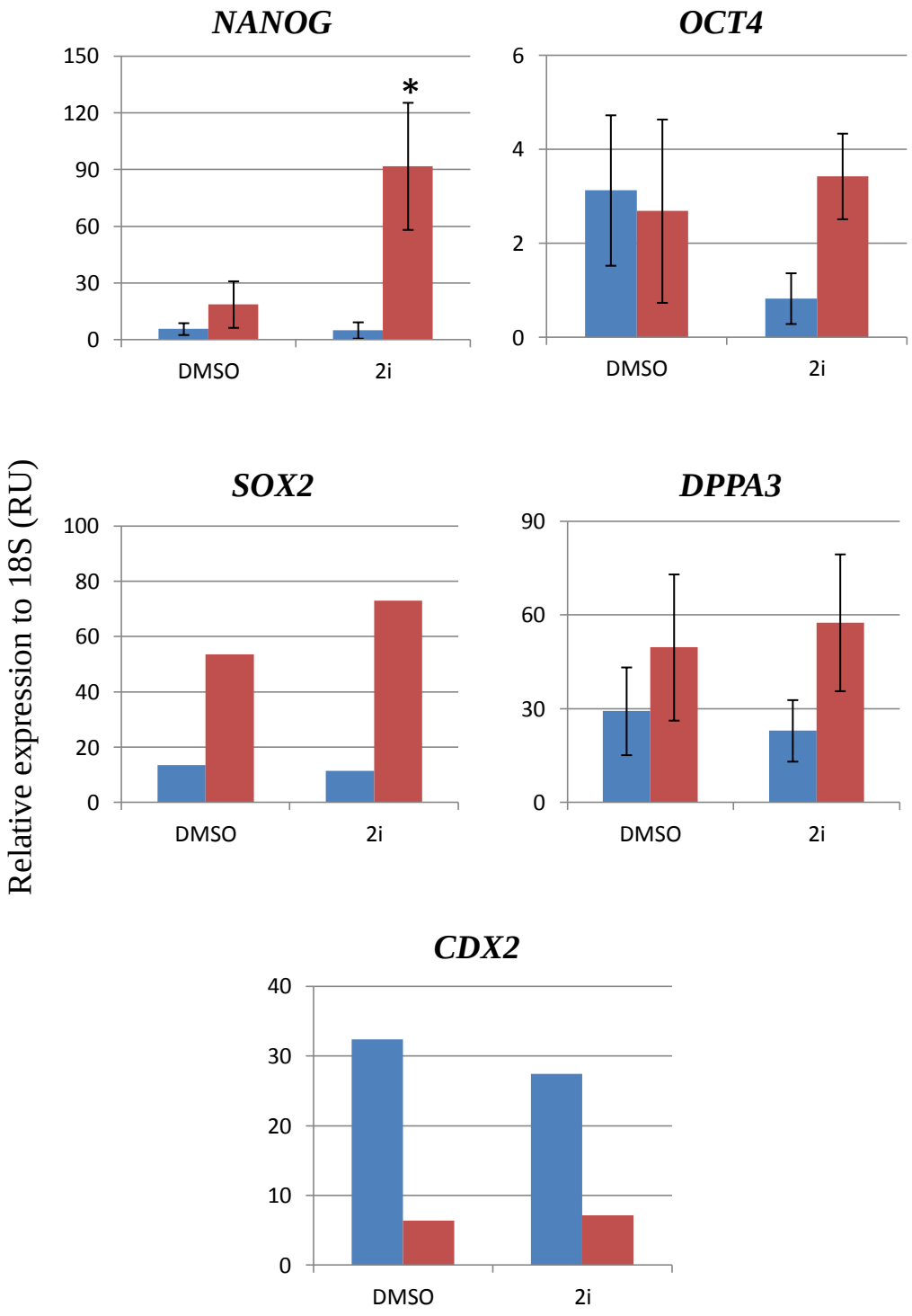

Figure 22. Relative gene expression analysis for *NANOG* in TE (blue) vs. ICM (red) of DMSO and 2i cultured D7/D8 bisected blastocysts. Data are presented relative to 18S mRNA abundance. * = $P < 0.05$ compared against control ICM. Error bars represents ± SEM.

4.4 RNA Analyses Summary

In summary, we found that *NANOG* was significantly up-regulated in 2i and that this was specific to the ICM of bovine blastocysts. *SOX2* was additionally found to be restricted to the ICM in blastocysts and was affected by DMSO, while blastocysts cultured in 2i restored expression to normal levels (no DMSO control). All other genes (*OCT4, DPPA3* and *CDX2*) transcription levels were unaffected by the presence of 2i.

Interestingly, in the bisected blastocyst samples the factor *DPPA3* was also found to be differentially expressed and in greater abundance in the ICM than the TE. The trend reversed for *CDX2*, which was shown to be expressed in greater abundance in the TE. In contrast, *OCT4* was the only gene we observed to be equally expressed in both the TE and ICM of bisected blastocysts. Ongoing experiments will help to further validate these observations.

Chapter 5: Protein Localisation

5.1 Introduction

In this chapter we investigate the localisation of NANOG and SOX2 proteins through a method known as immunocytochemistry (ICC). ICC is a common laboratory technique which involves using primary antibody preparations to detect specific cellular antigens (or proteins). A second fluorescent-labelled antibody is then used to bind with the primary antibody to amplify detection and signal the protein of interest under a microscope (Holmseth et al. 2006). This technique would enable us to observe where our specific proteins were located in the bovine blastocysts.

Our intentions were to explore the translational profiles of bovine blastocysts with reference to what information we had already obtained from the mRNA quantification and whether culture in 2i would affect protein expression. We decided to limit our study to investigate the transcription factors NANOG and SOX2 as both were identified as being transcribed specifically in the ICM. Moreover, we had observed a significant increase in *NANOG* transcripts in 2i blastocysts and therefore wanted to distinguish whether this subsequently resulted in a correlated increase in the NANOG protein.

5.2 NANOG Antibody Optimisation

The NANOG protein was especially crucial to detect after mRNA results had shown a convincing up-regulation in 2i cultured blastocysts. However, no primary antibody was currently commercially available that was specific to the bovine NANOG antigen. A number of primary antibodies were stated to detect mouse and human full length antigens or specific segments within their antigens. Therefore, we initially compared homology of protein sequences between mouse, human and cattle to define our search for potential antibody candidates.

Table 9 shows the potential candidates that were screened, the immunogen recognised, and the stated reactivity in other species. Additionally, sequence identity (%) is an approximate calculation based on amino acid differences between the immunogen and bovine NANOG sequence. The Ab80892 antibody was ordered later after Ab21603 was permanently discontinued from the supplier. Consequently, this is the reason it was excluded from initial trials.

Table 9. NANOG primary antibodies

Supplier & Catalogue #	Immunogen	Reactivity	Sequence identity (%)
Abcam, 21603	Full-length of Ms	Ms, Hu, Mk, Rat, Pig, Bv	52
Abcam, 80892	Full-length of Ms	Ms, Hu, Mk	52
Abcam, 47102	aa 1-50 of Hu	Ms, Hu, Mk, Gt	68
Abcam, 77095	aa 144-147 of Hu	Hu	86
Abcam, 62734	Full-length of Hu	Hu	69
PeproTech, 500-P236	Recombinant full-length Hu	Hu	69
Thermo Scientific, PA1-817	aa 135- 149 Hu	Hu	100

aa = amino acid, Hu = human, Ms = mouse, Mk = monkey, Gt = goat, Bv = bovine.

5.2.1 NANOG staining in murine and human ESCs

As a positive control, all antibodies were initially tested on murine ESCs. Murine ESCs were cultured and fixed in 4% PFA by Andria Green. Subsequently, we carried out ICC on these cells. Of the six antibodies to arrive and be tested, four gave positive signals for the mouse Nanog protein (Figure 23 & 24). Ab47102 and Ab62734 were both deemed negative in relation to the negative controls. This result indicated that either the antibodies had been somehow compromised or that they did not recognise the mouse antigen as they are both directed against the human NANOG protein. However, Ab47102 was stated to positively cross-react with the mouse protein. The Peprotech and Ab77095 antibodies gave the clearest results. This was interesting as both were stated to specifically recognise sequences of the human NANOG antigen and worked better than Ab21603 which should have recognised the full-length mouse antigen. The DNA stain was accidentally excluded and therefore phase contrast photographs were taken to display individual nuclei instead.

We concluded based on these results that Ab77095, Ab21603, Peprotech and Thermo NANOG antibodies positively detected the mouse NANOG antigen.

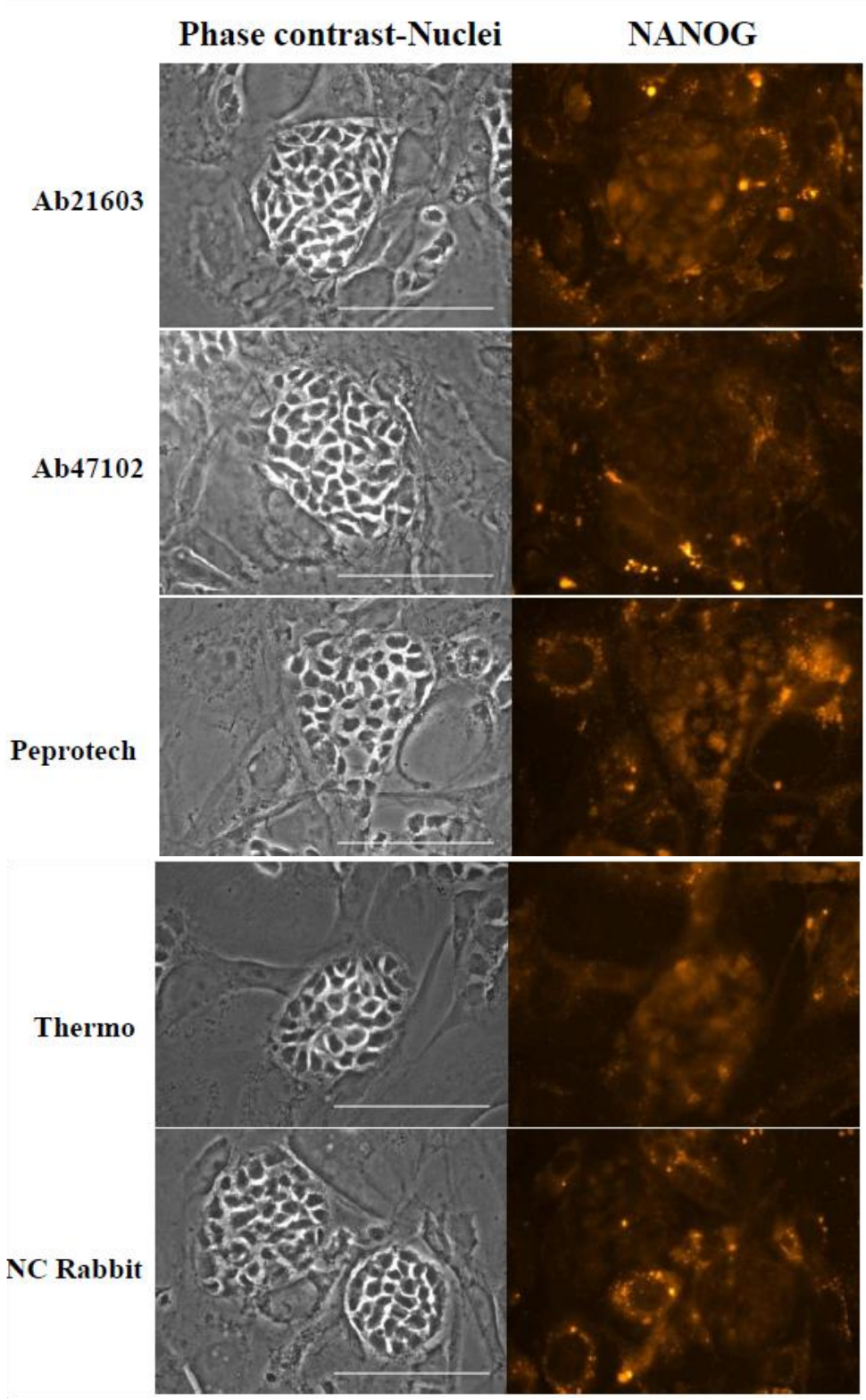

Figure 23. Positive control staining of NANOG antibodies on mESCs. Phase contrast of cell nuclei (left) and NANOG staining (right) for antibodies raised in rabbit. NC = negative control. Scale bar = 100 µm.

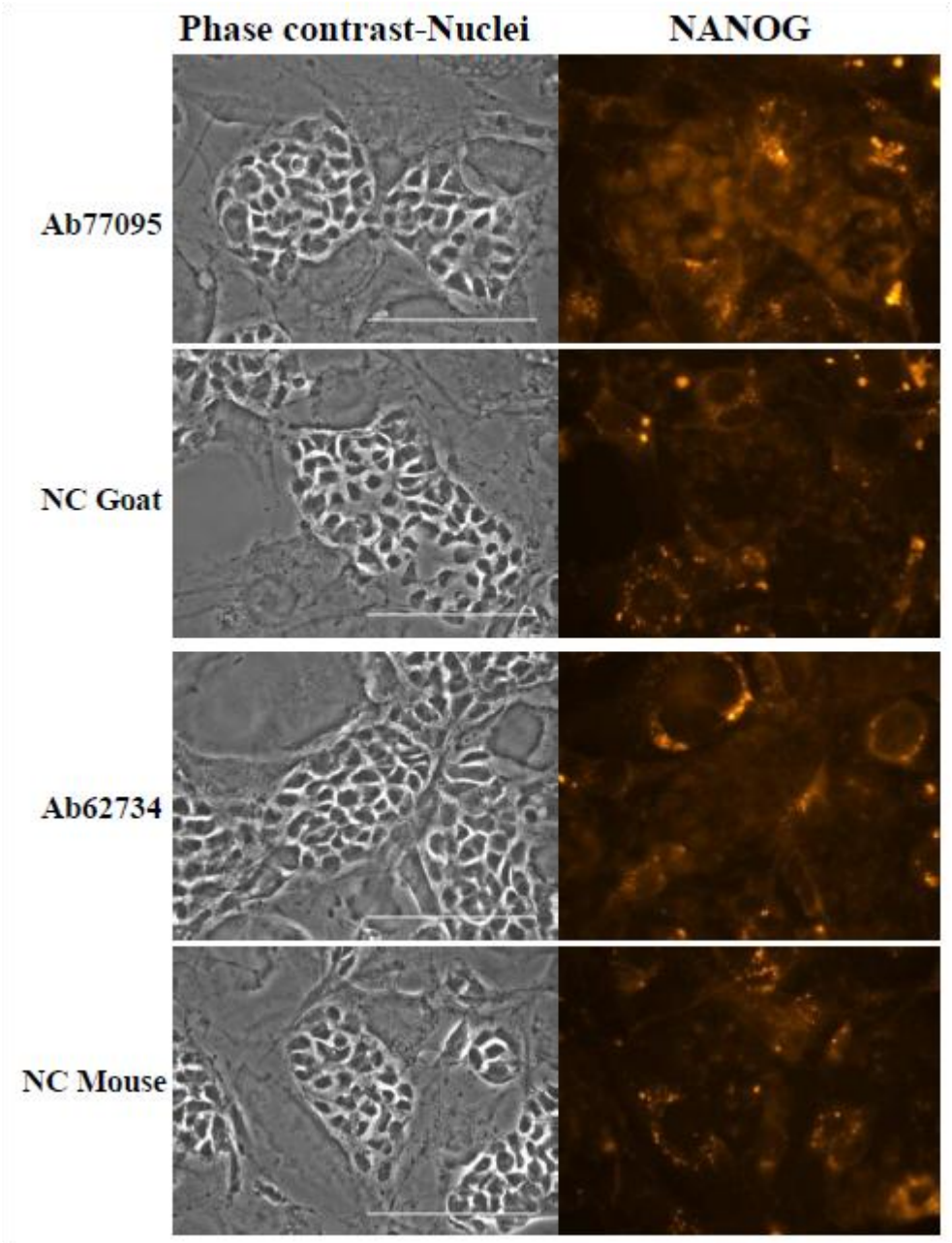

Figure 24. Positive control staining of NANOG antibodies on mESCs. Phase contrast of cell nuclei (left) and NANOG staining (right) from antibodies raised in goat (Ab77095) and mouse (Ab62734). NC = negative control. Scale bar = 100 µm.

To test the two unsuccessful antibodies in mESCs staining (Ab47102 and Ab62734), and confirm which antibodies would detect the human NANOG antigen, we used human ESCs (H1, Wavell Research Institute, Wisconsin, USA). To ensure the human ESCs stained were undifferentiated colonies, a primary antibody known to stain positively in human ESCs was included as a further control (OCT4, Sc-5279). In human ESCs, NANOG and OCT4 have been reported to co-occupy many target genes and their expression is lost from differentiated cells (Boyer et al. 2005).

We found of all the antibodies tested (excluding Ab80892) that the Peprotech NANOG antibody worked extremely well (Figure 25). The staining was specifically located in the nucleus of undifferentiated cells within human ESC colonies. All other antibodies tested failed to produce a positive result, which was interesting considering they all (except Ab21603) were raised against the human NANOG protein.

From these results we can only conclude that the Peprotech NANOG antibody detected the human antigen. We can exclude the possibility that the NANOG protein was not present in these cells as OCT4, a marker of undifferentiated colonies, was used as a double stain. Additionally, the fact that the Peprotech NANOG and OCT4 stained positively verifies that the method and secondary antibodies used work well, and are therefore not a factor in the negative results.

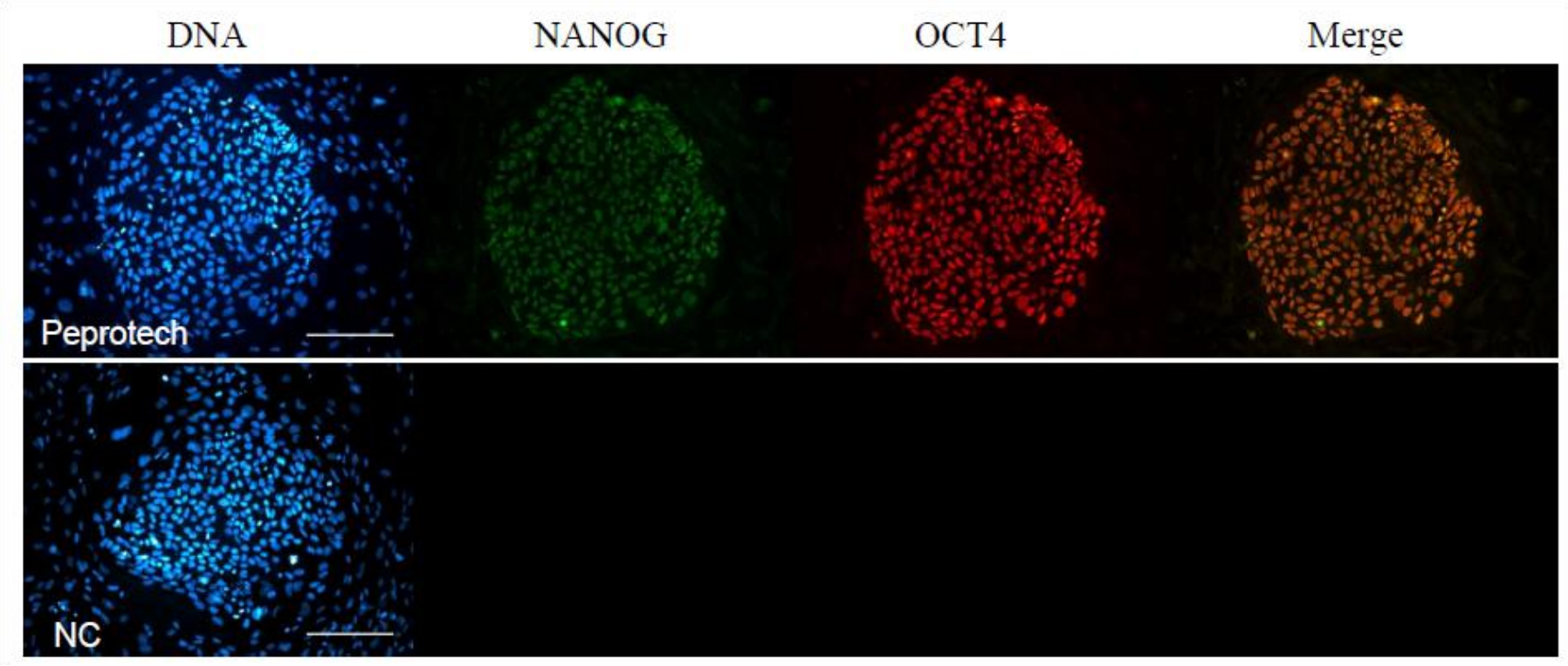

Figure 25. Positive control staining of Peprotech 500-P536 NANOG and OCT4 in hESCs. H33342 DNA stain displays cell nuclei (blue) with NANOG (green) and OCT4 (red) staining and NANOG/OCT4 merge. NC = negative control. Scale bar = 100 µm.

5.2.2 NANOG staining in transiently transfected fibroblasts

To conclusively validate that the antibodies found to work in either murine or human ESCs would detect bovine NANOG, bovine embryonic fibroblast cells were transfected with pTRE3G-bovineNANOG-mcherry and pEF1α-TET3G plasmids (for plasmid details refer to section 2.9.1). This would enable the testing of each NANOG antibody on cells that were capable of over-expressing bovine NANOG in the presence of doxycycline. Plasmids were transformed into DH5α competent bacteria and purified using a maxi-preparation kit. All techniques used are outlined in section 2.9. Cell culture, transfections, and ICC of the transfected cells were carried out by Andria Green.

Purified plasmid DNA was identified by an analytical restriction digest. A linear digest of pTRE3G-bovineNANOG-mcherry was performed with Xho1 which resulted in a 5.6 Kb fragment. In addition, a double digest was performed using EcoRV and BamH1 enzymes and resulted in two fragments 4.7 Kb and 902 bp, respectively. The pEF1α-TET3G was linearised by the enzyme EcoR1 (7.9 Kb). Moreover, a double digest was performed with EcoR1 and HindIII, which resulted in two fragments at 1.2 Kb and 6.7 Kb, respectively. Digested samples were run on a 1.5% agarose gel alongside undigested plasmid to visualise subsequent products (Figure 26). We observed the correct number of bands for the linear and double digests and accordingly the bands ran at the appropriate size for each plasmid. Additionally, the undigested plasmid was different to the linearised plasmid as you would expect to observe. The resolution of the gel (especially for the larger fragments) was not the greatest, however, the restriction digest confirmed the identity of our plasmids, and we therefore proceeded to use them for cell transfection.

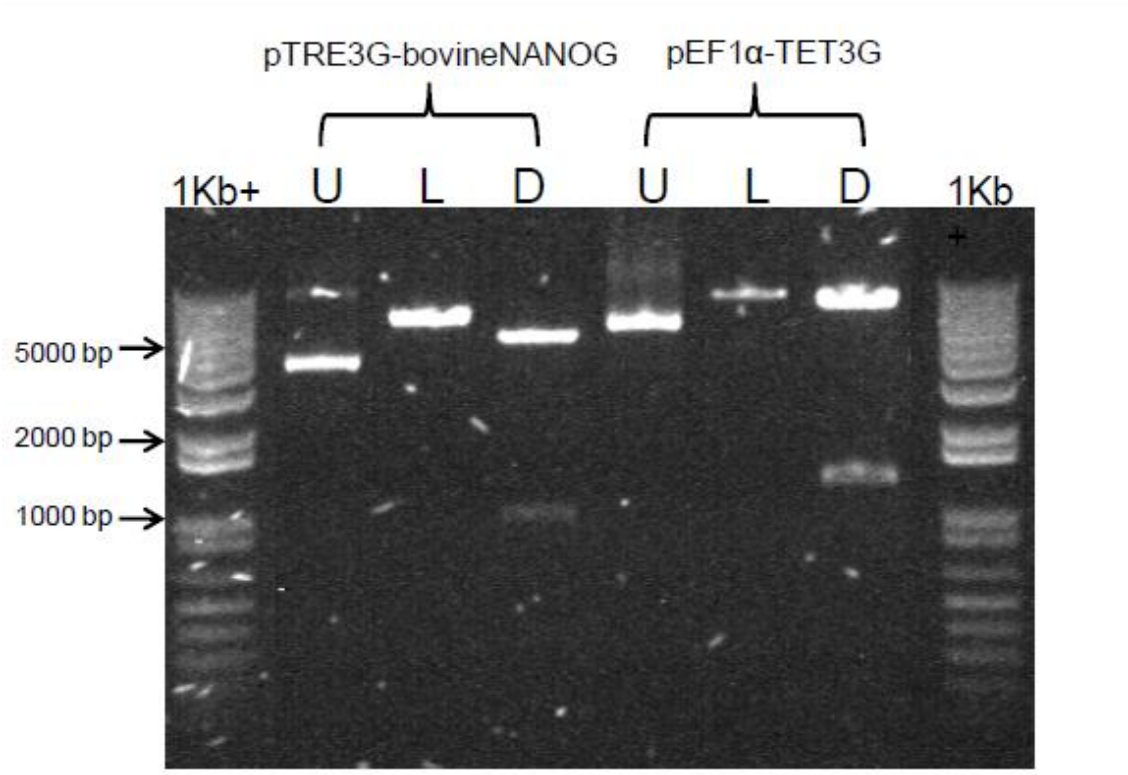

Figure 26. Analytical restriction enzyme digest of pTRE3G-bovineNANOG-mcherry and pEF1α-TET3G. U = undigested plasmids, L = linear digestion, and D = double digest.

Following cell transfections, cells were stained with all NANOG antibodies (excluding Ab21603 which was discontinued). We found that of all antibodies tested, on cells that had successfully incorporated the plasmids (red cherry marker), that four were positive for the detection of bovine NANOG (green, Figure 27). Moreover, the Ab80892, Ab47102 and Peprotech antibodies worked the best with the strongest stainings detected. Ab62734 antibody had a very faint signal, but was still positively stained. All positive stains were specific to the cell nucleus, distinct from the red cherry marker which was cytoplasmic. Unfortunately, a negative control is unavailable, but there was a clear distinction between cells that had no primary antibody incubation and those that were positive for the NANOG protein shown in Figure 27.

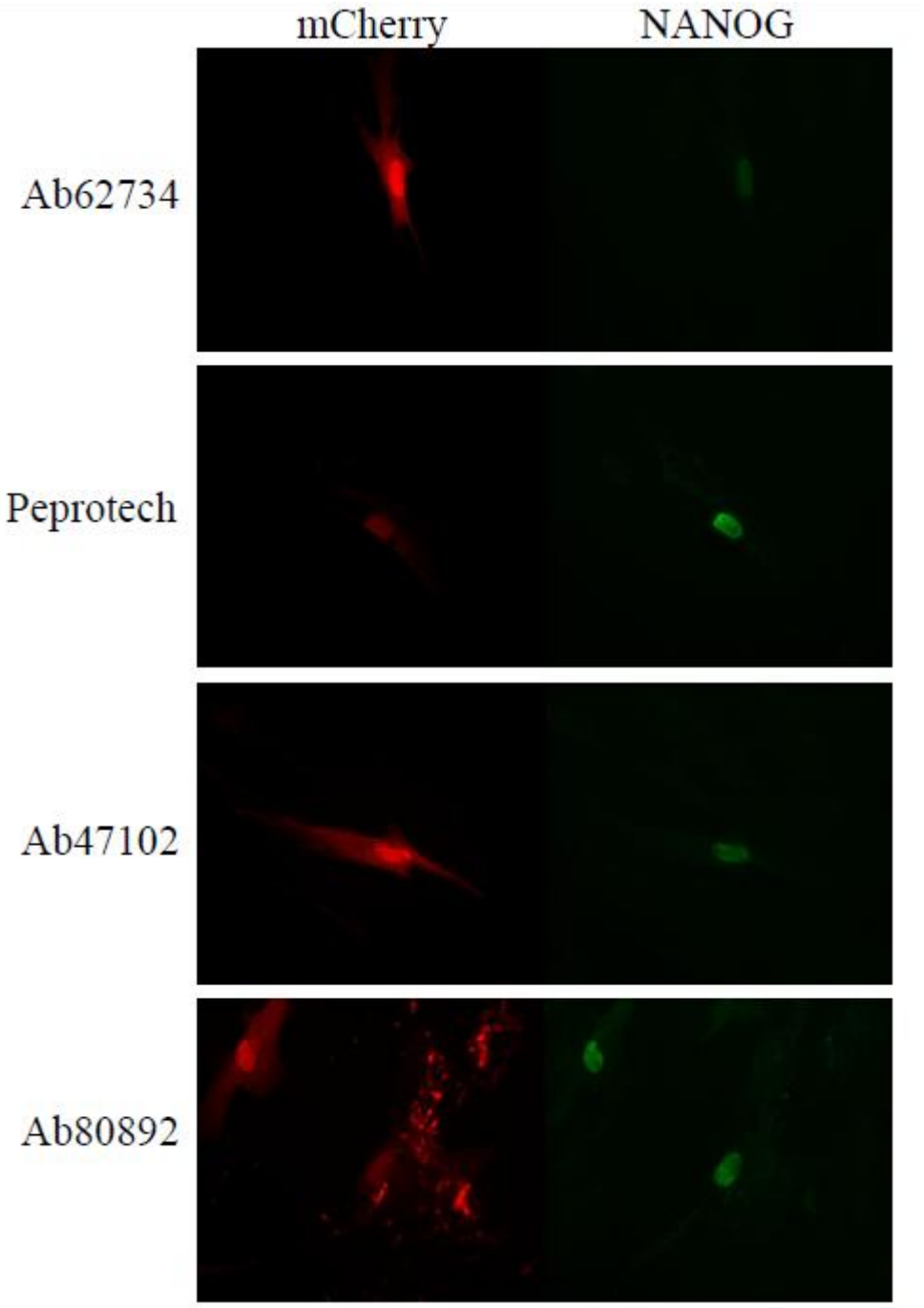

Figure 27. NANOG antibody testing on transiently transfected bovine embryonic fibroblasts with pTRE3G-bovineNANOG-mCherry. Cells positive for the plasmid express the cherry fluorescent marker (red). NANOG protein (green) detection with the antibodies Ab62734, Peprotech, Ab47102 and Ab80892.

Table 10 summarises all positive control testing with the NANOG antibodies in mouse ESCs (mESCs), human ESCs (hESCs) and the NANOG-transfected cells.

Table 10. Summary table of NANOG antibodies and positive control detection

Antibody	Immunogen	mESCs	hESCs	NANOG cells
Ab21603	Full-length of Ms	Yes	N/T	No
Ab80892	Full-length of Ms	N/T	N/T	Yes
Ab47102	aa 1-50 of Hu	No	No	Yes
Ab77095	aa 144-147 of Hu	Yes	No	No
Ab62734	Full-length of Hu	No	No	Yes
Peprotech	Recombinant Hu	Yes	Yes	Yes
Thermo	aa 135-149 Hu	Yes	No	No

Hu = human, Ms = mouse, N/T = not tested

5.3 NANOG Staining in Bovine Blastocysts

5.3.1 NANOG staining in untreated D7 blastocysts

Subsequently, the antibodies that could be verified with either mouse or human positive controls and/or recognised the bovine NANOG protein were tested on D7 bovine blastocysts. No staining above background levels could be detected for any of the blastocysts, regardless of the antibody used (Figure 28, 29 & 30). Figure 28 shows the staining of antibody Ab77095 with the appropriate control as this antibody was raised in goat. Figure 29 presents the antibodies; Ab21603, Ab47102, Thermo and Peprotech, which were all raised in rabbit and therefore required a different negative control. Ab80892 (raised in rabbit) and Ab62734 (raised in mouse) were processed at a later date as a double stain due to the limitations with blastocyst numbers at this time. Different fluorescent wavelengths were used to pick up each antigen and therefore the same blastocysts were able to be stained with both antibodies. These results are shown in Figure 30.

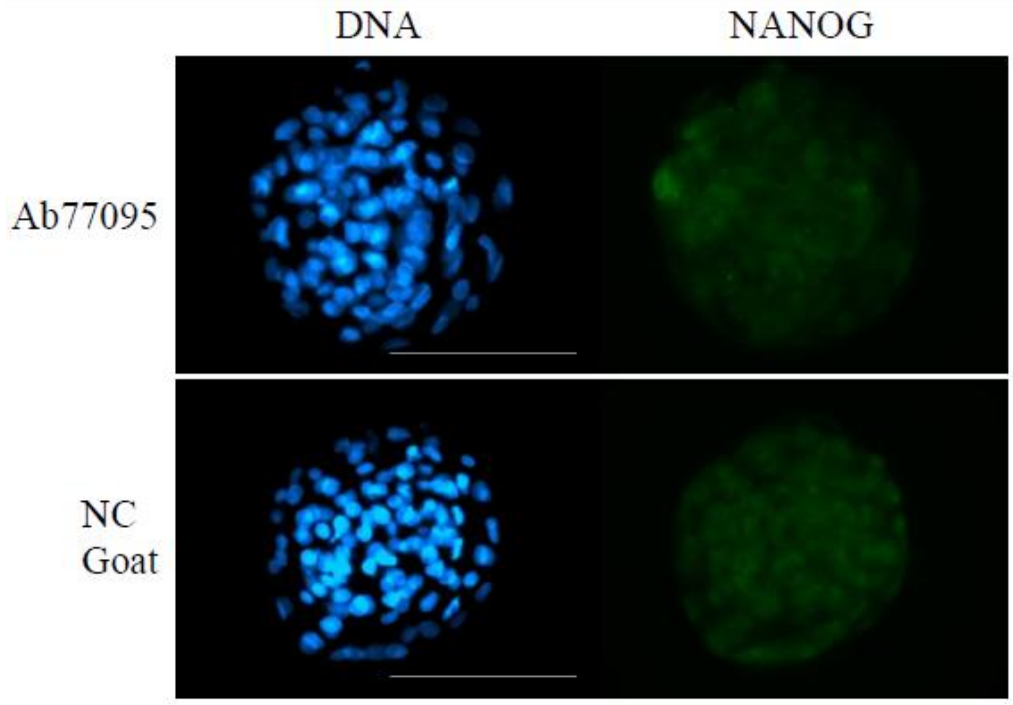

Figure 28. NANOG staining of Ab77095 (raised in goat) in D7 untreated bovine blastocysts. NC = negative control. Scale bar = 100 μm.

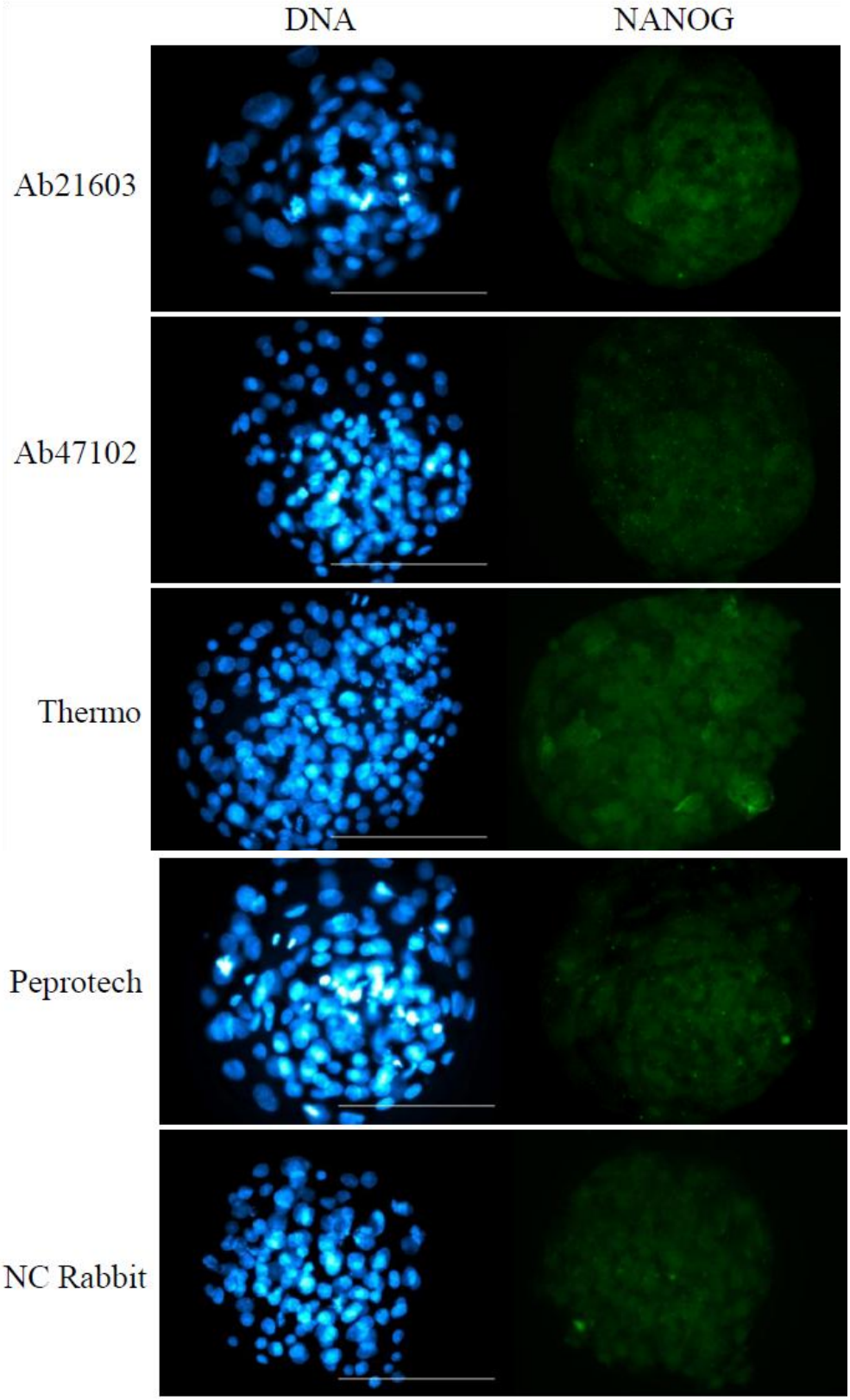

Figure 29. NANOG staining of antibodies raised in rabbit (Ab21603, Ab47102, Thermo and Peprotech) in D7 untreated bovine blastocysts. NC = negative control. Scale bar = 100 µm.

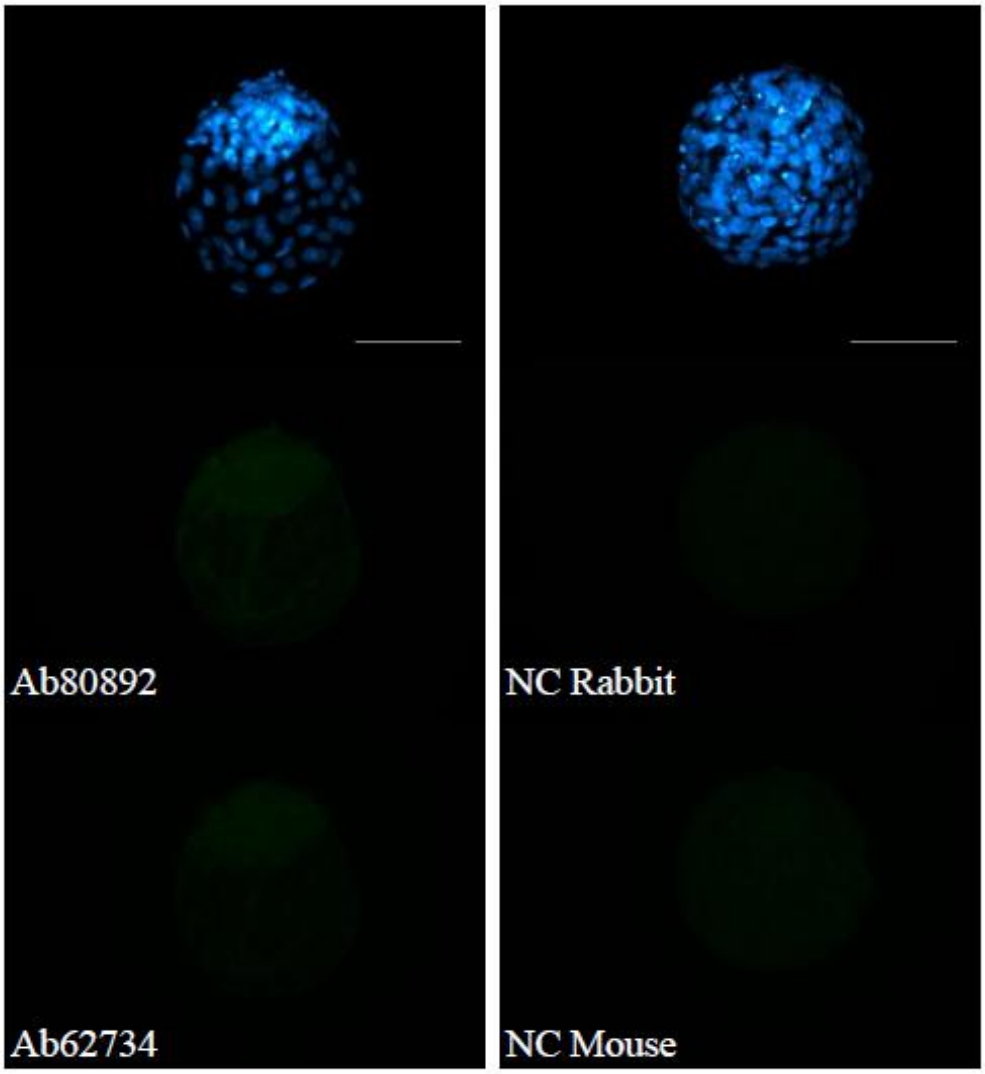

Figure 30. NANOG staining of Ab80892 (rabbit) and Ab62734 (mouse) in untreated D7 blastocysts. Hoechst DNA stain (blue) and NANOG (green). NC = negative control. Scale bar = 100 µm.

Our next trials included a double stain between the NANOG antibodies (Peprotech and Ab80892) and a SOX2 antibody which we previously found to work in bovine blastocysts. We did this as a control for the method to ensure all reagents were fine, but also to confirm the localisation of SOX2. The SOX2 staining was nuclear and very clearly defined in the ICM of stained blastocysts (Figure 31). This supports our mRNA data that SOX2 is an ICM-specific marker in bovine blastocysts. Unfortunately, the NANOG protein was still undetectable above background levels (NC), similar to previous experiments.

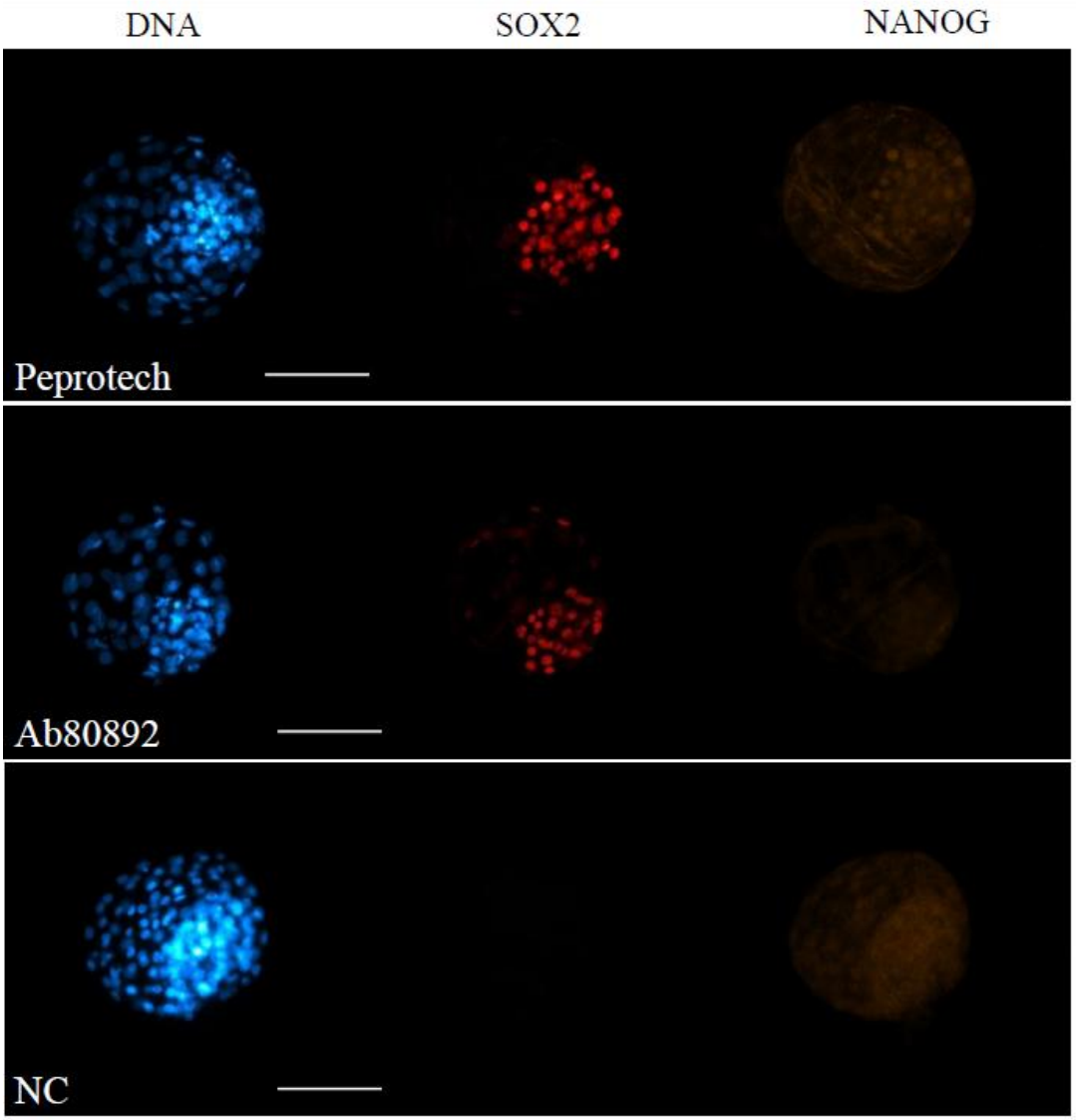

Figure 31. SOX2 and NANOG protein localisation in D7 bovine blastocysts. Hoechst DNA stain (blue), and SOX2 (red) stainings were included with either Peprotech or Ab80892 NANOG (orange). NC = Negative control. Scale bar = 100 µm.

5.3.2 NANOG staining in D7 and D8 blastocysts with 2i

Consequently we proceeded to repeat the NANOG antibodies on D7 and D8 bovine blastocysts cultured in 2i or the DMSO control. Since we identified an increase in *NANOG* mRNA transcripts of 2i cultured blastocysts, we hypothesised that staining these blastocysts may improve our chances of detecting the protein. This time each blastocyst was double stained with a combination of two NANOG antibodies: Ab41702 and Peprotech. These antibodies had both detected the bovine NANOG in transfected cells. The idea behind the double stain was to see if a stronger signal could be observed as antibodies may detect different sequences of the same protein.

Interestingly, we obtained a positive signal distinguishable in the TE in both DMSO and 2i blastocysts (Figure 32). No specific staining could be confirmed in the ICM of either group at D7 or D8. It is possible that these stainings are specifically detected in the nucleoli of the TE as seen by the spotted appearance in the NANOG stain that correlates with nucleus locations (Figure 33). Nonetheless, the stainings were very weak in both D7 and D8 blastocysts, and there was a lot of non-specific 'noise' included. Regardless, the level of NANOG expression was no different whether blastocysts were cultured in the DMSO control or 2i.

As we confirmed that these antibodies should recognise the bovine NANOG protein from previous control experiments with the transfected cells, we concluded that the levels of NANOG could overall be very low and therefore detection is poor using the immunocytochemistry method. Other methods with greater sensitivity may need to be utilised to determine the presence of NANOG in bovine blastocysts and whether 2i has any effect on protein expression levels.

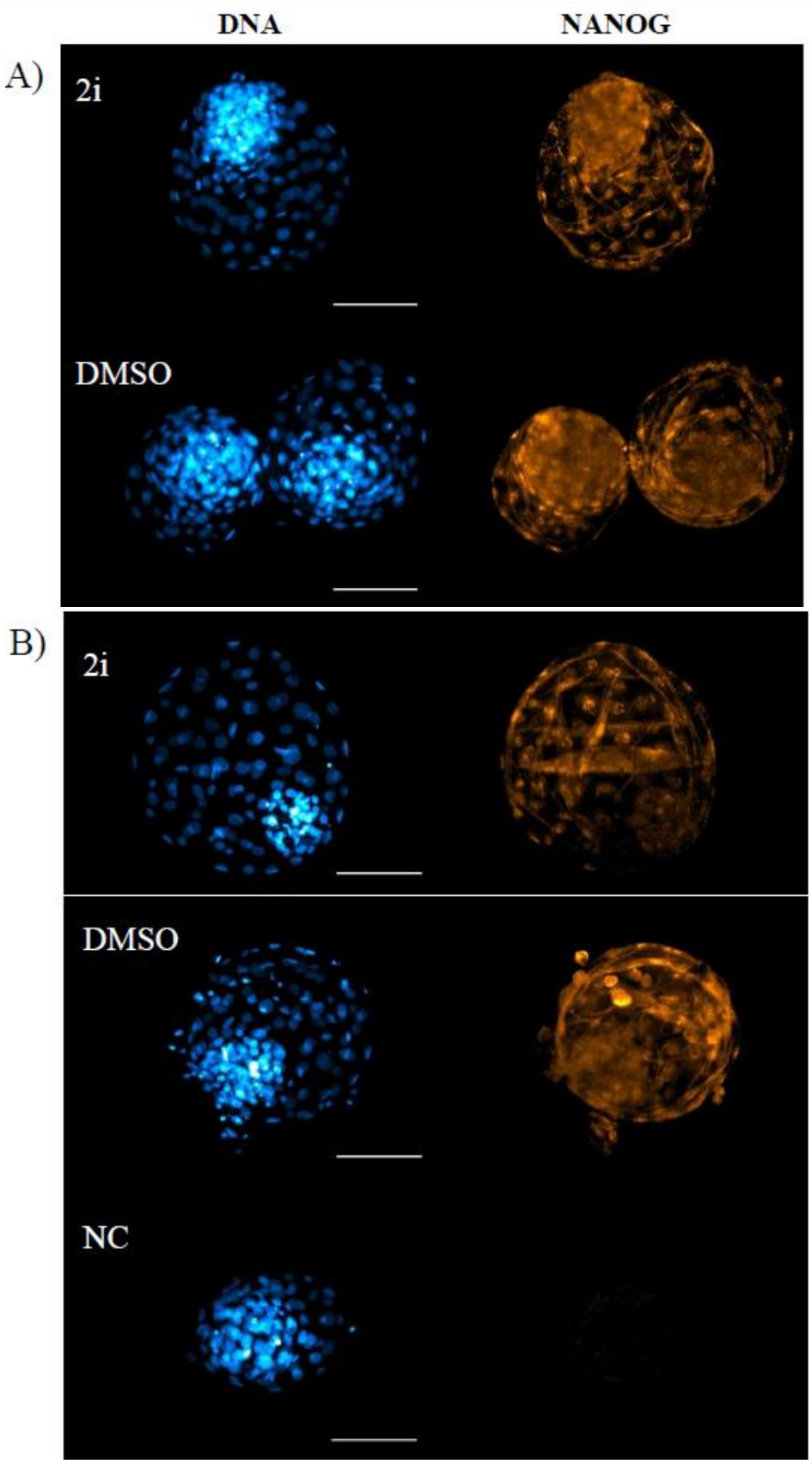

Figure 32. NANOG protein localisation in A) D7 and B) D8 bovine blastocysts in 2i or DMSO control. NC = negative control. Scale bar = 100 µm.

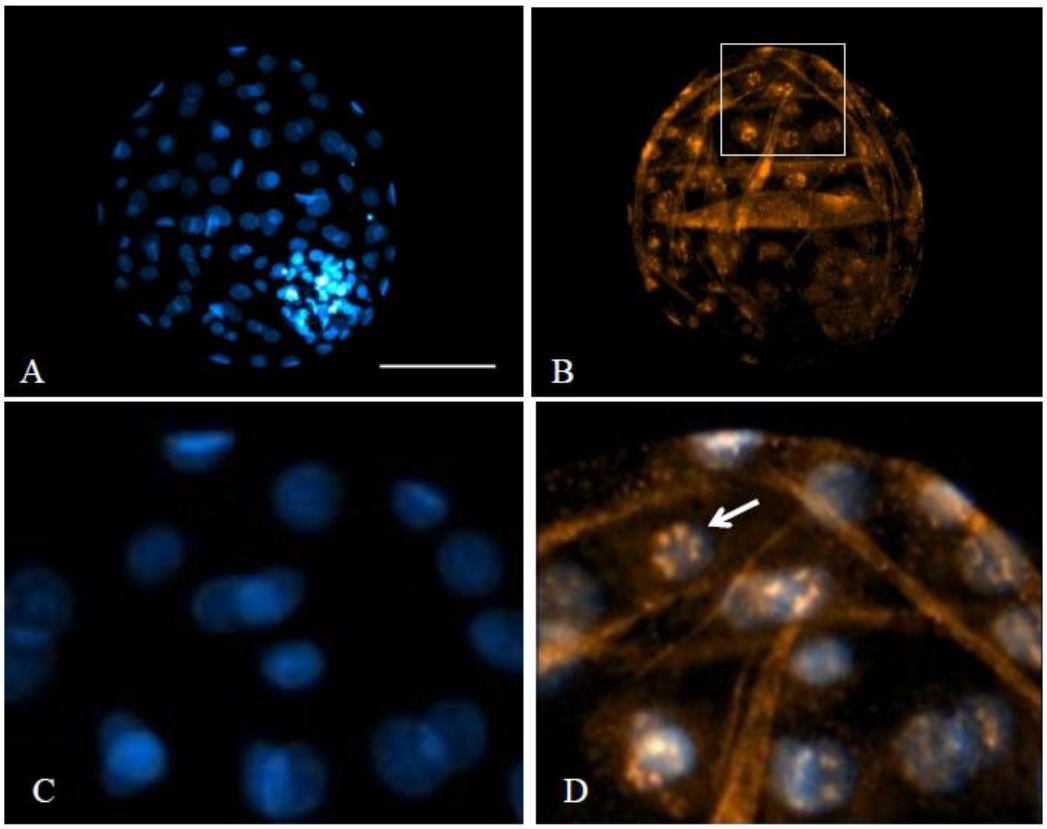

Figure 33. NANOG protein localisation in D8 bovine blastocyst cultured in 2i. A) Hoechst DNA stain, B) Combined NANOG stain with antibodies; Ab47102 and Peprotech, C) Hoechst DNA stain of inset in B, and D) Merged images of DNA stain and NANOG nucleoli stain from inset in B. White Arrow points to a speculated nucleoli staining. Scale bar = 100 µm.

5.4 SOX2 Staining in D8 Bovine Blastocysts with 2i

As we had observed ICM specificity in D7 blastocysts, we investigated the later stage D8 embryos cultured with and without the presence of 2i. At this particular period in experiments we were exploring the effect of the inhibitors alone and therefore blastocysts from the treatment with only the inhibitor PD0325901 were included in SOX2 staining. As there were limited numbers of blastocysts stained within the DMSO control group, we were unable to infer whether there were any comparable differences in SOX2 expression between the treatments (Figure 34). However, overall (across all treatments) the D8 SOX2 stainings were interesting

as the localisation of the protein still resided within the ICM, but there seemed to be a restriction in the expression to a subset of cells. Interestingly, the blastocyst depicted for the treatment PD0325901 displays a ring type staining similar to that described in mice for the appearance of Sox2-positive extra-embryonic ectoderm (Avilion et al. 2003), and still a few SOX2-positive cells in the inner region of the ICM (white arrow, Figure 34). These would speculate that bovine SOX2 expression is analogous to that observed in mice blastocysts.

These results taken together with D7 SOX2 stainings indicate that SOX2 is an ICM-specific marker in bovine, but the restriction of expression in blastocysts gives us reason to suspect that it could be a more specific marker of pluripotent epiblast and/or extra-embryonic ectoderm.

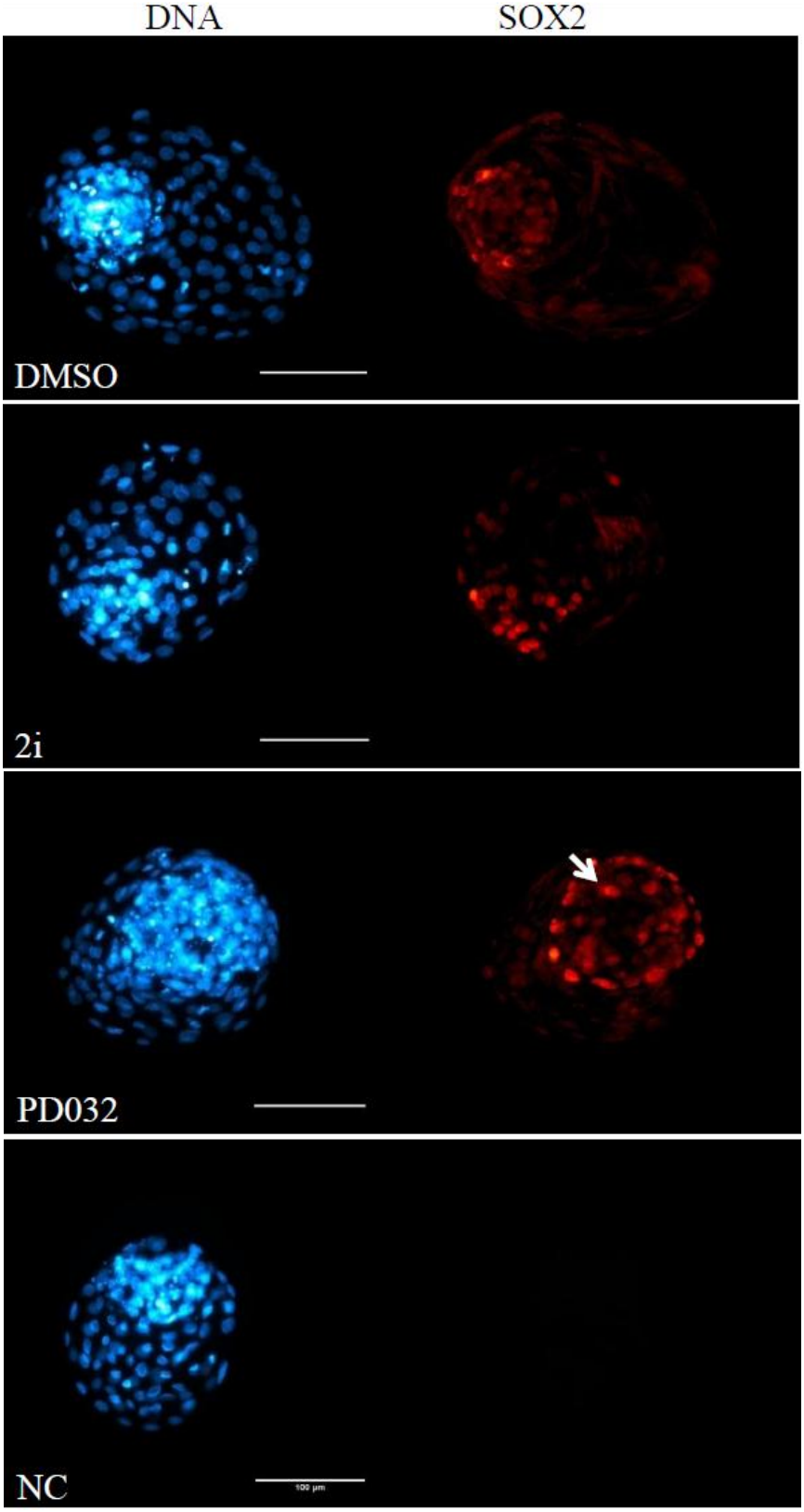

Figure 34. SOX2 protein localisation in D8 bovine blastocysts cultured in DMSO, 2i and PD0325901 treatments. White arrow indicates a cell positive within the 'outer ring' of SOX2-posiitive cells. NC = Negative control. Scale bar = 100 µm.

5.5 Protein Localisation Summary

Following thorough testing of NANOG antibodies we were unable to convincingly detect the NANOG protein in D7 and D8 bovine blastocysts. This was regardless of whether embryos were cultured in 2i or without the inhibitors. We did observe a strange nucleoli staining in the TE of most blastocysts at D7 and D8 however, these results are puzzling as to why this does not extend to the ICM. We propose that the sensitivity limit of the ICC technique is such that it might be incapable of detecting the NANOG protein. Nonetheless, we have now established antibodies that will detect the bovine NANOG protein for future work. These results are not able to reinforce our findings that *NANOG* is specific to the ICM and up-regulated in 2i.

In contrast, we observed convincing SOX2 stainings that were nuclear and specific to the ICM in D7 blastocysts. These results support our mRNA data that *SOX2* is an ICM-specific marker in cattle. We also obtained good staining from D8 blastocysts cultured with 2i, PD0325901 and DMSO controls and no difference could be observed between treatments. Alternatively, we hypothesise that the D8 blastocysts on the whole show a restriction of SOX2 expression to a subset of cells within the ICM, which could potentially be the foundation of epiblast tissue and/or the extra-embryonic ectoderm.

Chapter 6: Discussion

6.1 The Effect of 3i/2i in Bovine Pre-implantation Embryo Development

6.1.1 Morphological assessment of culture in 2i

In 2008, the first completely chemically defined conditions for ESC culture were described (Ying et al, 2008). The authors used small molecular weight inhibitors (3i/2i) to block specific pathways associated with differentiation and subsequently maintain an undifferentiated, proliferative population of pluripotent cells. Nichols et al (2009) assessed the effect of these inhibitors on mouse embryos with convincing results showing a benefit of 2i culture for ESC derivation. Here we reported the findings of using these inhibitors during embryo culture in bovine, a species currently considered unpermissive for ESC derivation.

Embryo development was assessed by the total number of blastocysts and the proportion of embryos that were of a transferable grade (grade 1 and 2). Additionally, the stage of blastocyst development was recorded with embryos on a progressive scale from early to late (or hatched) blastocysts. Overall, we found that culturing embryos in 3i had no significant effect on embryo development to blastocyst. Following exclusion of the PD173074 inhibitor, we found no significant difference for overall development to blastocyst in 2i culture. Aparicio et al (2010) found a significant increase in the portion of bovine embryos reaching the blastocyst stage and cultured from D1 with only the inhibitor CHIR99021 (31% vs. 23% in the control). DMSO in this instance was also used as the solvent control. Our findings do not agree with the results of this study as we did not see a difference in 2i culture. However, we cannot exclude the possibility that the second inhibitor (PD0325901) in 2i could have the conflicting effect on development, which results in a balance between the two. Ying et al (2008) included the CHIR99021 inhibitor for ESC culture with the intention of relieving the GSK3-mediated negative regulation of biosynthetic pathways to restore

growth in ESCs. Apoptosis was relatively high in cells cultured with the other inhibitors (PDs), but the addition of CHIR99021 enhanced survival at low densities.

Furthermore, in a study by Madan et al (2005) the effect of the single inhibitor U0126 (a predecessor of PD0325901) on bovine embryo culture was reported. The authors found no significant difference in the proportion of embryos that progressed to the blastocyst stage compared to controls. Moreover, embryos cultured in U0126 were observed to blastulate at the same time as controls. These results resemble our findings in that we observed no difference in embryo development, however we did observe an increase in the rate of development in cumulus-free cultured embryos in 2i. We are aware that U0126 is less specific than the PD0325901 inhibitor and therefore the effect may be less pronounced. Interestingly, the authors key finding was that the combined blockade of two MAPK pathways; p38 and ERK (with the inhibitors SB220025 and U0126, respectively), during bovine embryo culture resulted in the complete failure of embryos to reach the blastocyst stage. They postulated that inhibition of one pathway, found to have no effect, could potentially compensate for the other (Madan et al. 2005). This could be important as inhibition of ERK could cause downstream regulatory pathways to be blocked, but embryogenesis may still persist through the action of p38. These results imply that bovine embryo development may not rely exclusively on one pathway, and therefore the inhibition of ERK may be recovered to some extent by the MAPK pathway p38 in cattle.

Within our results a small difference in total blastocysts was observed with and without the presence of cumulus cells. Cumulus cells are present within mammalian follicles and are vital for maturation and ovulation of the oocyte in vitro (Tanghe et al. 2002). In vitro, the removal of these cells prior to maturation in cattle is detrimental to the developmental competence of the embryo (Fukui & Sakuma 1980). Additionally, removal prior to fertilisation resulted in decreased sperm penetration and/or male pronucleus formation, however embryo culture without cumulus cells resulted in no significant difference in blastocyst rates compared to embryos grown in the presence of cumulus cells (Zhang et al. 1995).

Unexpectedly, in our experiments cumulus cells were abundant at the day five changeover in 2i and were often physically distinct to what was seen in the control group. Generally, cumulus cells form into a tidy ball of cells centred in the middle of a group of embryos. However, embryos in 2i were often completely engulfed in these cells, which could not be mechanically removed in some instances, and often embryos were stuck to one another or to the bottom of the petri dish. Production notes were documented indicating a greater number of degenerate embryos that were observed in 2i. This observation led us to speculate that 2i was eliciting an effect on these cumulus cells and consequently being a detriment to normal development. We decided to remove the cumulus cells prior to culture to: 1) remove any bias that may be caused by the cumulus cells and 2) to see if removing the cumulus cells would have any effect on development between the two groups. We used an exploratory approach to investigate the cumulus cell effect in 2i, but with no success as cumulus cells grown independent of embryos did not show the same effect. The explanation for this observation is therefore still unknown.

As mentioned previously, an increase in the rate of development was observed in cumulus-free cultured blastocysts as there were a significantly greater proportion of embryos that had hatched and significantly fewer early blastocysts in 2i. This is in contrast to what was observed in mouse embryos. Lu et al (2008) found a marked delay in development of embryos cultured in PD908059 (the less specific, first generation inhibitor of ERK). At the time of grading only 6.35% of embryos cultured in the inhibitor were at the blastocyst stage, compared with 71.12% in the control. In this study, embryos were cultured from the 8-cell stage in the inhibitor. Similarly, Nichols et al (2009) cultured embryos from the 8-cell stage in 3i/2i, yet no difference in developmental rate was reported. Our finding does not agree with either of the previous studies and indicates a difference that might exist in either the ERK or GSK pathway between mice and cattle.

In all treatments expanded and hatched stage blastocysts were present at D7, which indicated that the inhibitors did not compromise the integrity of trophectoderm cells. A loss in trophectoderm function could have resulted in embryos that failed to progress through to blastulation. Fortunately, this was not

the case and in contrast we observed larger blastocysts in 2i to their counterparts in the DMSO control. In accordance with our morphological observations we identified a significant increase in the total number of cells, which was equally contributed to both the ICM and TE lineages. If 2i enhances pluripotent characteristics, especially in the ICM, a larger number of cells is certainly not a disadvantage.

The increase in blastocyst cell nuclei was similarly found to increase in a study carried out by Aparicio et al (2010), which used the inhibitor CHIR99021 in bovine embryo culture. This study analysed the total number of cells in D8 blastocysts and found that hatched blastocysts cultured in CHIR99021 had significantly more cell nuclei. Unfortunately, this study did not look into the individual lineages. Taken together with these results, we conclude that the increase in cell number is most likely attributed to CHIR99021 inhibition. Cell numbers can be influenced by either proliferation or conversely cell death by the process of apoptosis. Byrne et al (1999) found that the total number of cells was negatively correlated with the extent of apoptosis in bovine blastocysts. The occurrence of apoptosis was observed mainly at the blastocyst stage in development and predominantly in the ICM. It has been suggested that apoptosis at this time may function as a cellular 'quality control' in the ICM to remove cells that are damaged or developmentally incompetent (Byrne et al. 1999). Perhaps a larger number of cells in 2i blastocysts fit the survival criteria in the ICM, and are therefore not destroyed. Whether the 2i blastocysts experience fewer apoptotic cells or an increase in cell proliferation would be an interesting investigation for the future.

If there is an increase in the proliferation of cells, or alternatively a decrease in cell death by apoptosis in 2i this would definitely be a factor effecting development. The combination of mechanical force and the production of regulatory molecules govern the process of zona hatching (Seshagiri et al. 2009). Therefore, embryo size driven by a greater number of cells could also be the reason we observe a greater proportion of hatched blastocysts in 2i due to an increased hydrostatic pressure on the zona pellucida at an earlier time point.

The grading system is a common laboratory practise, both in research and commercial in vitro embryo production. An obvious limitation of this method of evaluation is that it is subjective and is dependent on the individual researcher's experience. It can also lead to inconsistent results should researchers individual perceptions on what constitutes a certain type of blastocyst be different. However, grading provides a non-invasive and rapid evaluation based on visual assessment, that in conjunction with other parameters has been shown to reliably determine an embryo's potential viability (Overstrom 1996). Given that our embryo development results are all graded by the same researcher we can exclude that this is an extraneous variable within our data set.

6.1.2 Molecular assessment of culture in 2i

We proceeded to measure the effect of 2i by investigating the molecular aspects of the pluripotent state, namely mRNA and protein expression of key transcription factors. We looked at four genes (*NANOG, OCT4, SOX2* and *DPPA3*) at the core of the pluripotent network in mice (Nichols et al. 1998; Mitsu et al. 2003; Payer et al. 2003; Masui et al. 2007) and assessed their relative abundance as a ratio to 18S in bovine blastocysts and within the TE and ICM of bisected blastocysts. *CDX2* as a known TE marker in mice (Strumpf et al. 2005) and bovine (Berg et al. 2011) was included in analyses to assess if its expression was altered by culture of embryos in 2i. Of the five genes, only *NANOG* and *SOX2* were significantly affected by the presence of the inhibitors or DMSO supplementation. Interestingly, these two genes were also the only transcripts shown to be ICM-specific in D7/D8 bisected blastocysts. If 2i enhances pluripotent characteristics as seen in mice, then we would expect to observe changes primarily associated with the establishment of the epiblast lineage within the ICM.

6.1.2.1 NANOG

Our mRNA analyses showed a reliable increase in *NANOG* transcripts, which was up-regulated in the ICM in 2i blastocysts. The authors of a recent study showed that ERK inhibition in bovine blastocysts by the FGF receptor inhibitor PD173074 (constituent of 3i) similarly increased *NANOG* expression ($P < 0.05$) in D8 blastocysts after 24 hours of culture (Yang et al. 2011). We reported a highly

significant ($P < 0.01$) increase in *NANOG* after culture from D1 to D7 in 2i. This proposes the idea that there may be a critical period for when culture with these inhibitors does elicit the observed increase in *NANOG* transcription and that the ERK inhibitor (PD0325901) is most likely responsible for this increase. As we observed no morphological detriment to culturing embryos in 3i from D1, D3 and D5 post-fertilisation, it may be of interest to define if the observed *NANOG* up-regulation is affected by length of exposure to these inhibitors.

The convincing increase in the abundance of *NANOG* transcripts has suggested that regulation may be somewhat conserved between species as up-regulation of this gene is also apparent in mouse blastocysts cultured in 2i (Nichols et al. 2009). The Nanog transcription factor in mice has been surmised as the main choreographer in the acquisition of naïve pluripotency, which is crucial for epiblast development and for the derivation of viable ES cells (Mitsu et al. 2003; Silva et al. 2009). Mitsu et al (2003) demonstrated that the deletion of *Nanog* resulted in a loss of pluripotency in both ICM and ESCs, and elevated *Nanog* expression was sufficient to maintain self-renewal of ESCs in the absence of the cytokine LIF. This report first demonstrated the importance of *Nanog* for the establishment of pluripotency in mice. Likewise, NANOG is considered a key player in the network of pluripotent regulators in human ESCs (Boyer et al. 2005), and is one of four factors that can reprogram human somatic cells back to a state of pluripotency (Yu et al. 2007), referring to the ability to obtain induced pluripotent stem cells or iPSCs (Takahashi & Yamanaka 2006).

In cattle, the role of NANOG is yet to be characterised, but it is progressively expressed in pre-implantation blastocysts, ESC-like cells and iPSCs that exhibit pluripotent characteristics (Kuijk et al. 2008; Gong et al. 2010; Huang et al. 2011; Sumer et al. 2011). A few of these studies have speculated that NANOG localisation has similarities between species and that there may be functional preservation in mammals for this particular protein. If this is true our finding that *NANOG* is increased in the presence of 2i could be a vital piece of information that links ERK signalling with lineage establishment, and subsequently the pluripotent cell population, in cattle.

Embryo culture in 2i dramatically expanded the Nanog-positive compartment (epiblast) in the ICM of mouse blastocysts which could then be clonally expanded in vitro in the form of ESCs. The resulting colonies were established at high efficiencies (54%) in the presence of 2i and LIF from single epiblast cells (Nichols et al. 2009). The process of obtaining embryo-derived stem cells from the ICM of 2i cultured bovine blastocysts will be the next step to assessing whether embryo culture in 2i has ensuing benefits for establishing colonies of potential pluripotent cells.

In mice, the dramatic increase in Nanog protein as a result of 2i culture was due to an expansion in the number of cells that were Nanog-positive (epiblast) at the expense of those that were Gata4-positive (hypoblast) in the ICM (Nichols et al. 2009). As we were unable to detect the NANOG protein in bovine ICM, we could not investigate whether we would find analogous results to those observed in mice. Kuijk et al (2008) illustrated, with the NANOG antibody Ab21603, that several cells of the ICM in bovine blastocysts stained positively for NANOG while others did not. They suggested that the restriction of NANOG to a subset of cells in the ICM would most likely correspond to epiblast tissue. However, we could not detect a specific stain with the identical antibody even after repeating the authors' exact ICC method. Additionally, we have found no other literature that has shown the convincing detection of NANOG in bovine blastocysts using the ICC method. The Ab21603 antibody was discontinued and unfortunately we were unable to make any further conclusions using this antibody.

We did however obtain a positive stain for NANOG that was located in TE cells. The staining displayed a 'spotted' pattern within the nucleus. Shuyang et al (2006) described a NANOG staining in goat blastocysts that was located in the nucleus of ICM cells, but additionally had a very intense staining in the nucleoli of TE cells. The nucleolus is a sub-structure of the nucleus that is not bound by a membrane. Its functions are numerous, including protein sequestration, but it is mainly regarded as an RNA processing centre (Shaw & Brown 2012). We were unable to detect a positive signal in the ICM of bovine blastocysts, but our stainings in the TE were similar to those seen in the previous study of goat blastocysts. Furthermore, we had used the same antibody (Peprotech, 500-P236) to detect the

NANOG protein. Shuyang et al (2006) believe this observation suggests that the NANOG protein is being inactivated and subsequently destroyed by a mechanism reported to occur with transcription factors isolated in the nucleoli (Song & Wu 2005). This idea is plausible as we have observed a very low abundance of *NANOG* transcripts present in TE compared with the ICM in bovine blastocysts. Proteins generally persevere longer in cells than mRNA transcripts and therefore, what we observe may be the clearance of NANOG protein following a down-regulation in *NANOG* message in the TE. Further studies would be needed to validate this idea. However, the reason we do not detect NANOG protein in ICM cells is still unclear.

6.1.2.2 SOX2

In addition to the change in NANOG expression, we also found a significant down-regulation of *SOX2* mRNA in the presence of the inhibitor solvent; DMSO. Intriguingly, *SOX2* levels were restored in the presence of 2i to values equivalent to the untreated control. This suggests that 2i may have a positive effect on *SOX2* expression, which counteracts the negative effect seen with DMSO. DMSO is a common organic solvent with acknowledged embryo-toxic effects. It is known to variably alter gene expression and have a differentiation-inducing effect on ESCs (Adler et al. 2006). This is an important reason why we included the solvent control in our experiments to ensure we were aware of any alteration DMSO would cause. We were also able to show that the abundance of *SOX2* transcripts was significantly greater in the ICM as opposed to the TE in D7/D8 blastocysts. Therefore, we can conclude that the changes in gene expression as a result of DMSO and 2i culture occur predominately in the ICM of bovine blastocysts. To our knowledge, the effect of 2i on *SOX2* expression has not been reported for any other species, including mouse.

In addition to mRNA analyses, we were able to detect the localisation of the SOX2 protein in D7 and D8 bovine blastocysts using ICC. We did not observe any differences at D8 in protein abundance between 2i, PD0325901 and control blastocysts, yet as a limited number of blastocysts were stained in the control group we hold our conclusions until further repeats are undertaken. The stainings

on the whole showed SOX2 was conclusively expressed in the ICM of D7 embryos, which is complementary to our finding that mRNA transcripts were also detected primarily in the ICM. To our knowledge, these illustrations are the first to be published that show the SOX2 protein is specifically restricted to the ICM in D7 bovine blastocysts. Moreover, at D8 we observed a specific stain in the ICM, comparable with staining at D7, but the number of cells positive for SOX2 had reduced. The observed confinement of cells may reflect changes occurring in the ICM at this time. These findings would be consistent with studies that report the emergence of GATA4-producing primitive endoderm (or hypoblast) between days 8-10 in cattle (Yang et al. 2011). Additionally, Vejlsted et al (2005) had identified the existence of two cell populations in day 12 bovine epiblast, which suggested a certain level of differentiation had already occurred prior to this stage in development.

Sox2 is a known gene associated with pluripotency, and thus the epiblast lineage, in mice (Avilion et al. 2003) and humans (Boyer et al. 2005; Takahashi et al. 2007). However, Avilion et al (2003) stated that Sox2 remained highly expressed in extra-embryonic ectoderm, in a ring of cells adjacent to the epiblast, in mice blastocysts. Therefore, Sox2 has a secondary function associated with extra-embryonic progenitors, as well as the epiblast. Therefore, our D8 stainings were interesting in that if Sox2 function is conserved between species than the restriction of expression may represent both the epiblast and extra-embryonic ectoderm lineages. One particular blastocyst added support to this hypothesis as a very obvious ring of cells appeared to line the outer surfaces of the ICM. A closer look using a confocal microscope may provide answers to the localisation of these cells within the ICM. Regardless of SOX2s status as a core transcription factor associated with pluripotency, little attention has been paid to its role in other species. Here we show that SOX2 is specifically expressed in the ICM at D7 and that around D8 its expression becomes restricted to a subset of cells in the ICM. The exact identification of this cell population requires further investigation.

6.1.2.3 Other transcription factors

In mice, the transcription factor Oct4 has been found to be exclusively expressed in the ICM (Palmieri et al. 1994). In contrast, the distribution of OCT4 has been documented to occur in the TE and ICM of bovine blastocysts (Kirchhof et al. 2000; Kuijk et al. 2008). Similarly, goat blastocysts express OCT4 in both lineages, which together with the bovine expression profile, suggests a difference in *OCT4* regulation in ruminants compared with mice. It has been proposed that this could be related to the longer period before implantation and the requirement for OCT4 to maintain TE proliferation in these species (Shuyang et al. 2006). Our mRNA data is consistent with these observations. In mouse blastocysts, the number of Oct4-positive cells increased as the epiblast domain expanded in 2i. Additionally, the protein was also maintained in the hypoblast which is characteristic of embryos in diapause, the stage of arrested development from which ESCs are readily derived (Nichols et al. 2009). We did not see any difference in the expression of *OCT4* in bovine blastocysts that would suggest a change in transcription level of this gene. This does not exclude the possibility that there are more cells expressing OCT4 as we only measured *OCT4* relative to 18S. The next step would be to investigate the OCT4 protein in bovine blastocysts cultured in 2i.

There is currently insufficient information available on *DPPA3* expression in bovine pre-implantation embryos, therefore highlighting the requirement for further studies. We have shown that *DPPA3* was found to be expressed to a greater extent in the ICM of bovine blastocysts. However, this observation was not significant, but may indicate that this factor becomes gradually restricted to the ICM later in development. No significant difference in *DPPA3* expression was observed between blastocysts cultured with and without the presence of 2i.

As we were curious regarding the significant increase in TE cells, we decided to investigate the expression levels of *CDX2*, a lineage-specific marker for TE in mouse blastocysts (Strumpf et al. 2005). Kuijk et al (2008) reported CDX2 protein to be localised within the TE of bovine blastocysts. Additionally, *CDX2* transcripts have been shown to be enriched in the TE relative to the ICM by 2-fold

between D7-11 post-fertilisation (Berg et al. 2011). Our mRNA results did not show the same enrichment in the TE at D7/D8, but we did observe the same trend in untreated, DMSO and 2i cultured embryos. We acknowledge that our ICM samples do also contain some TE cells, and that this may interfere with the accuracy of the comparison for this particular TE-associated gene.

The relative analysis of real-time qPCR results has its limitation in that we were unable to quantify absolute abundance of gene transcripts. However, as our main aim was to assess the differences between the 2i treatment and controls, it served as a good tool for our comparative studies. The blastocysts in our experiments averaged between 100-150 cells (dependent on treatment) and therefore, samples of 15 blastocysts, for example, would only contain the RNA from between 1500-2250 cells. Large numbers of blastocysts were not always possible to obtain. This meant that the total cDNA generated from our starting material was low, which was reflected by the higher number of cycles required for the Lightcycler® to detect the amplified DNA product (CP). Unfortunately the higher the CP , the greater the variability in results. We were unable to efficiently increase the starting material without compromising the quality of embryos produced by IVP and encountering logistic issues. However, we tried to reduce this variability by repeating each sample a number of times and increasing the overall number of individual samples per treatment.

6.2 Future Work

The overall objective of the work carried out in this thesis was to assess if 2i enhances pluripotent characteristics in bovine blastocysts, for the purpose to improve derivation of embryo-derived stem cells in cattle. Therefore, future work would include the functional testing of whether cells from the ICM of bovine blastocysts cultured in 2i would establish and maintain colonies at greater efficiencies than untreated blastocysts.

Aside from the functional aspect, there are some findings which could be investigated further. It would be interesting to explore the mechanism behind why we observe an increase in cells with 2i. Is this due to fewer apoptotic cells or a greater proliferative effect of 2i? Fouladi-Nashta et al (2005) describes a unique method which incorporates a TUNEL labelling assay with a differential stain. This enabled the detection of apoptotic cells (TUNEL) and the concurrent visualisation of ICM and TE cells (differential stain). This method of detection may be especially applicable for us to assess the level of apoptosis, whilst still gaining valuable information on where the apoptosis is occurring, and subsequently the number of cells in both lineages in our control and 2i-treated embryos.

Additionally, experiments testing the use of individual inhibitors (CHIR99021 and PD0325901) as treatments during embryo culture would enable us to delineate which inhibitor is responsible for some of our key findings e.g. the increase in cell numbers in blastocysts, and *NANOG* and *SOX2* up-regulation. It would also be important to expand our gene expression analyses to other genes essential in early pre-implantation development. A gene of particular interest would be the hypoblast marker *GATA4.* This gene has already been implicated to be negatively effected by the inhibitor PD173074 in bovine blastocysts (Yang et al. 2011). It would also be an interesting combination to assess the protein localisation of both GATA4 and SOX2 simultaneously, as these are two proteins found to be specifically detected in the ICM of bovine blastocysts. This would help confirm when lineage segregation, and importantly the pluripotent population of cells, is established in cattle.

6.3 Conclusions

In conclusion, we have shown that bovine blastocysts cultured in 2i show accelerated blastocyst development. These blastocysts have more cell nuclei, which is attributed to a significant increase in both ICM and TE lineages compared with that of control embryos. The pluripotency transcription factor *NANOG* was significantly up-regulated in 2i blastocysts, which was due to an increase in transcription in the ICM. Another ICM-specific marker *SOX2* was negatively affected by DMSO and restored to normal levels in the presence of 2i. All other lineage-specific or pluripotency-related genes tested were unaffected. Table 11 summarises our observations of bovine embryo culture in 2i compared with observations in mice detailed by Nichols et al (2009). Overall, we conclude that bovine blastocysts differ from mouse in their response to 2i signal inhibition and molecular control of pluripotency.

Table 11. Embryo culture in 2i: Murine vs. Bovine observations

Murine Observations	Bovine Observations
Delayed development	Accelerated development
Reduced TE cell number	Increased TE and ICM cell numbers
Increased Nanog-positive cells in the ICM	Increased *NANOG* transcription. Protein undetectable
Oct4 expression maintained in entire ICM (epiblast/hypoblast).	*OCT4* expression not affected and detected in both ICM and TE
Sox2 expression not analysed	Increased *SOX2* transcription. ICM-specific protein.

TE= Trophectoderm, ICM= Inner cell mass.

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Appendix 1: Reagents, Solutions and Media Composition

All chemicals, unless otherwise stated, are sourced from Sigma-Aldrich, New Zealand Ltd.

TCM 199 (Base Medium)

Tissue Culture Medium 199 containing Earle's salts and L-glutamine is supplied by Life Technologies (# 31100-035).

H199 Media

Hepes-buffered TCM 199 contains: TCM 199 with 250 mM Stock H (Combination of Hepes-free acid, and Hepes Sodium Salt).

B199 Media

Bicarbonate-buffered TCM 199 contains: 0.2 mM C3H3O3Na, and 250 mM NaHCO3 with antibiotic: 50 mg/ml C18H36N4O11H2SO4 from Streptomyces (K1377).

Aspiration Media

Aspiration media contains: H199 medium + 925 IU/ml Heparin (#016128, CP Pharmaceuticals Ltd., UK) + 2% (w/v) foetal calf serum (FCS; Life Technologies).

IVM Media

IVM medium contains: B199 medium + 10% FCS (Life Technologies) with 10 μg/ml ovine follicle-stimulating hormone (FSH; ICP), 1 μg/ml ovine luteinising hormone (LH; ICP), 1 μg/ml β-estradiol in absolute ethanol and 0.1 mM cysteamine (2-Mercaptoethylamine) added prior to use.

IVF Media

IVF medium contains: 107.7 mM NaCl, 7.15 mM KCl, 0.3 mM KH_2PO_4, 25 mM $NAHCO_3$, 0.33 mM $C_3H_3O_3Na$, 3.32 mM $C_3H_5O_3Na$, 1.71 mM $CaCl_2$:$2H_2O$, 8 mg/ml fatty-acid free bovine serum albumin (BSA; MP Biologicals, Auckland, NZ), with 0.04 mM $C_{18}H_{36}N_4O_{11}H_2SO_4$, and supplemented with 0.1 mM pyruvate, 10 mg/ml heparin, 0.2 mM penicillamine and 0.1 mM hypotaurine.

HSOF

Hepes-buffered synthetic oviduct fluid (HSOF) contains: 107.7 mM NaCl, 7.15 mM KCl, 0.3 mM KH_2PO_4, 5 mM $NaHCO_3$, 3.32 mM $C_3H_5O_3Na$, 0.04 mM $C_{18}H_{36}N_4O_{11}H_2SO_4$, 20 mM Hepes, 0.33 mM $C_3H_3O_3Na$, 1.71 mM $CaCl_2$:$2H_2O$, 3 mg/ml fatty-acid free BSA (MP Biologicals, Auckland, NZ).

90% Percoll Plus

4.5 ml Percoll Plus (GE Healthcare), 0.525 ml 10x SPTL, 0.05 ml 100x SPAD, 0.05 ml Stock B (250 mM $NaHCO_3$).

45% Percoll Plus

2 ml 90% Percoll Plus diluted with 2 ml 1x SPTL

Hyaluronidase

Hyaluronidase (Sigma H3506) dissolved at 0.1% concentration in H199 -BSA.

ESOF

Early Synthetic Oviduct Fluid medium contains: 107.7 mM NaCl, 7.15 mM KCl, 0.30 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.33mM $C_3H_3O_3Na$, 1.71 mM $CaCl_2$:$2H_20$, 0.15 mM $C_6H_{12}O_6$, 3.32 mM $C_3H_5O_3Na$, 0.04 mM $C_{18}H_{36}N_4O_{11}H_2SO_4$, and 0.081 g/L Non Essential Amino Acid, 1 mM Gluta-Max, and 8 mg/ml BSA.

LSOF

Late Synthetic Oviduct Fluid medium contains: 107.7 mM NaCl, 3.99 mM KCl, 1.20 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.33 mM $C_3H_3O_3Na$, 1.71 mM $CaCl_2$:$2H_20$, 0.49 mM $MgCl_2$:$6H_2O$, 3.32 mM $C_3H_5O_3Na$, 0.04 mM $C_{18}H_{36}N_4O_{11}H_2SO_4$, 1.5 mM $C_6H_{12}O_6$, 1 mM DNP (2-, 4-dinitrophenol) and 0.081 g/L Non Essential Amino Acid, 1 mM Gluta-Max, 0.22 g/L BM Essential Amino Acid and 8 mg/ml BSA.

Pronase

Protease from *Strep.Griseus* (Sigma P8811) diluted 0.5% in HSOF -Ca, -BSA (Osmolarity~ 305). Add 0.1 mg/ml $CaCl_2$:$2H_2O$, 0.1 mg/ml $MgCl_2$:$6H_2O$ and 1 mg/ml PVA.

Hoechst DNA stain

Bis-Benzimide (H33342; Sigma B2261) in 1 mg/ml MQ-H_2O. Stored at 4°C and used at working solution of 5 µl/ml.

THSOF

Transfer HSOF medium contains: 107.7 mM NaCl, 3.99 mM KCl, 1.20 mM KH_2PO_4, 5 mM $NaHCO_3$, 0.33 mM $C_3H_3O_3Na$, 1.71 mM $CaCl_2$:$2H_20$, 0.49 mM $MgCl_2$:$6H_2O$, 3.32 mM $C_3H_5O_3Na$, 0.04 mM $C_{18}H_{36}N_4O_{11}H_2SO_4$, 1.5 mM $C_6H_{12}O_6$, 20 mM Hepes, 0.081 g/L Non Essential Amino Acid, 1 mM Gluta-Max, 0.22 g/L BM Essential Amino Acid and 8 mg/ml BSA.

Fixative

Depolymerised 4% (w/v) paraformaldehyde (PFA), 4% (w/v) sucrose, 1 M NaOH in PBS with a few drops phenol red indicator.

TAE buffer

2 M Tris, 250 mM Glacial acetic acid, 50 mM EDTA + 1 L H_2O.

PBS

Phosphate buffered saline contains: 1.9 mM sodium dihydrogen orthophosphate 1-hydrate, 8.4 mM disodium hydrogen orthophosphate 2-hydrate, and 150 mM sodium chloride in MQ-H_2O.

DEPC-H_2O

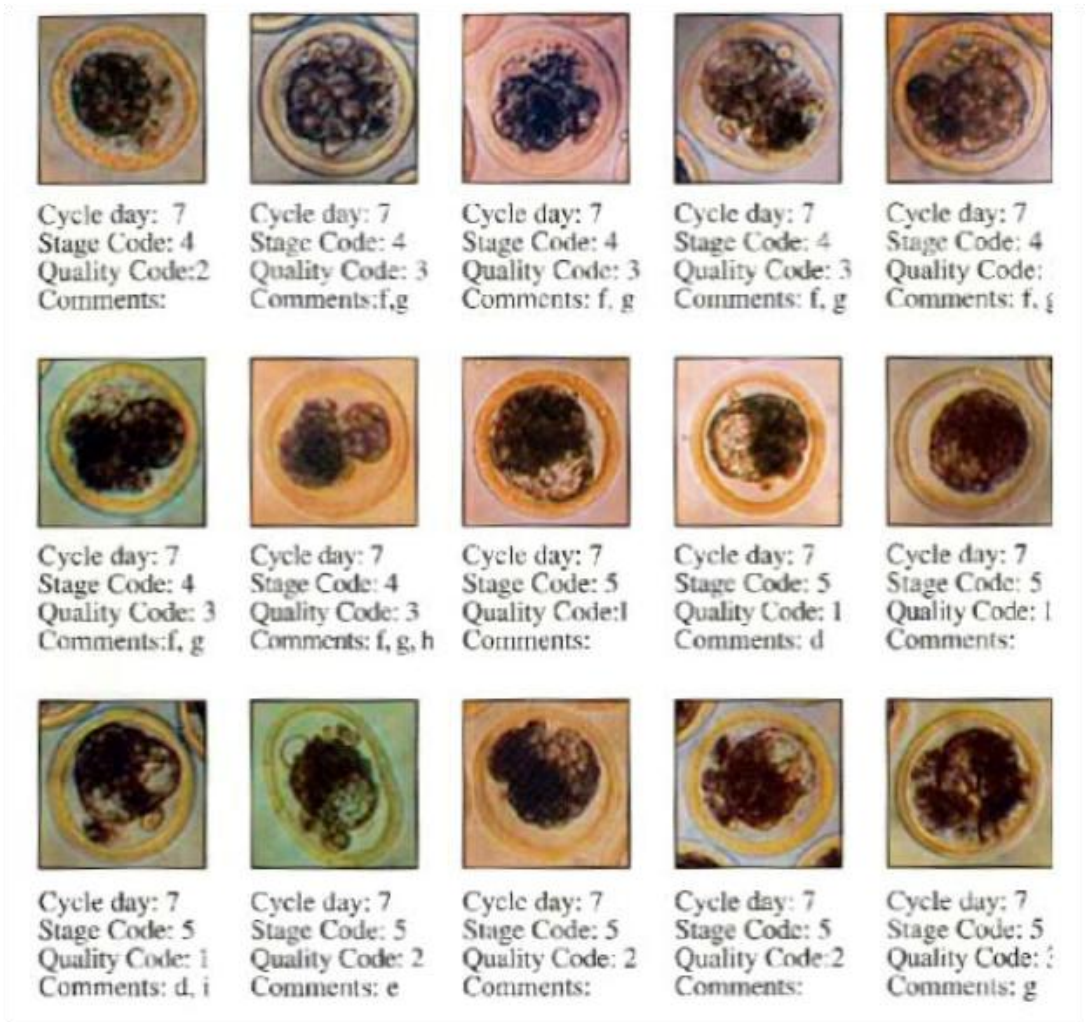
0.1% (v/v) diethyl procarbonate in MQ-H_2O. Mixed overnight, then autoclaved for 30 min at 121°C.

Appendix 2: Production Sheet

IVP Production Run sheet					Run Number:			
Purpose of run:								
Treatments:								
IVM								
Date:								
Time:								
Number of ovaries								
Number of oocytes into MATS								
Comments								
IVF								
Date:					Bull:			
Time:								
Number of oocytes:								
Sperm count & dil calc:								
Comments								
IVC								
Date:								
Time:								
Number of embryos:								
Comments								
C/O								
Date:								
Number of embryos:								
Comments								
Grading								
Date:								
Time:								
Number of embryos dev:								
Comments								

Appendix 3: Criteria for D7 Grading of Bovine Blastocysts

All images in Appendix 3 were obtained from the "Manual of the International Embryo Transfer Society" (3rd edition). Edited by David A. Stringfellow and Sarah M. Seidel. Published April 1988 by the International Embryo Transfer Society, 1111 North Dunlap Ave, Savoy, IL, USA.
Using Agresearch terms, stage code in the following pictures is equivalent to; 4= Tight Morula, 5= Early blastocyst, 6= Blastocyst, 7= expanded blastocyst and 8= hatched blastocyst. The cycle day is the corresponding day at which the stage code would appear post-fertilisation. See associated comments for more information on quality code or grade (1-3) of pictures below.



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 a code 4 (non-viable).

i) Irregular shape is a common variation in blastocoel development.

j) Collapsing of the blastocoel is considered a normal physiological process that does not lower the quality grade.
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 cryoprotective additive is introduced.
l) This embryo has a flat (even concave) surface of the zona pellucida that can cause the embryo to stick to the petri dish or straw. This defect alone keeps the embryo from being classified as quality grade 1 and should not be utilised 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 utilised in international commerce