Using putative bovine embryonic stem cell-like cells for nuclear transfer cloning to determine their functional potential

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Abstract

The potential for cloning to improve dissemination of elite genetics has been a goal of livestock scientists for many years. However, efficiency of this technique has remained low compared to other *in vitro* embryo production methods. One hypothesised method to improve cloning efficiency is to use donor cells that require less reprogramming, as donor cells must be reprogrammed to an embryonic state in order to generate a whole embryo. Somatic cells are the most readily available cell type for donor cells, making them the most often used, but also the most differentiated. Embryonic stem cells (ESCs) are pluripotent cells, capable of generating all cell types. As ESCs are at a much lower differentiated state, they would be hypothesised to improve cloning efficiency. However, ESCs have only been derived from mice and rats. Using a novel culture method, bovine ESC-like cells (ePSCs) can be maintained for several weeks *in vitro*. The aim of this research was to evaluate the efficiency of using these bovine ePSCs in nuclear transfer (NT) cloning.

ePSC colonies were generated from *in vitro* produced blastocysts and arrested into mitosis with an overnight incubation with 500 nM nocodazole. A method was devised to isolate single cells, using serial treatment in dispase, pronase, and trituration in Ca-Mg-free dissociation media containing cytochalasin B. Arrested colonies were characterised for the degree of mitotic arrest, showing approximately 40% of the colony was arrested in mitosis, and the proportion of cells in DNA synthesis phase had significantly decreased. Cells expressing pluripotency genes *SOX2* and *NANOG* were enriched in the central dome-shaped colony. Analysis of mitotic cells showed that 63% were also pluripotent. NT cloning was optimised for use with donor cells synchronised into mitosis. Pronase treated donor cells fused to metaphase II arrested cytoplasts in isoosmolar fusion buffer resulted in highest fusion rate. Comparison of artificial activation with ionomycin followed by DMAP, CHX, or anisomycin showed that ionomycin/DMAP activation produced the highest blastocyst development rate. Using these optimised conditions, cloning with ePSC donor cells resulted in a three-fold higher blastocyst development than with somatic cells. As donor cells were in metaphase, they contained double the normal amount of DNA. Therefore,

it was important to assess the karyotype of blastocysts for normal ploidy. A bimodal distribution of chromosome spreads of around 60 and 120 chromosomes was observed, with higher chromosome numbers apparently restricted to trophoblast cells.

Further validation of blastocyst ploidy, characterisation of donor cells used for NT, and continued optimisation of protocols, through double cytoplast cloning or timing of gap between fusion and activation, is required before completing *in vivo* cloning trials. Embryo transfer trials will be the final step in addressing the long-term aim of this project: to demonstrate that elite bovine embryos can be converted into ePSCs and then ePSC derived animals.

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List of Abbreviations

AC	Alternating current
BEF14	Bovine embryonic fibroblast line 14
BSA	Bovine serum albumin
CB	Cytochalasin B
Cdk1	Cyclin-dependent kinase 1
CHX	Cycloheximide
DC	Direct current
DMAP	6-dimethyl aminopurine
DMSO	Dimethyl sulfoxide
EdU	5-ethynyl-2'-deoxyuridine
EF5	Embryonic fibroblast line 5
ePSC	Embryo-derived pluripotent stem cells (Bovine ESC-like cells)
ERK	Extracellular signal-related kinase
ESC	Embryonic stem cells
FCS	Fetal calf serum
FGF	Fibroblast growth factor
G_0	Quiescent
G_1	First growth phase
G_2	Second growth phase
GP	Guinea pig complement serum
GSK3	Glycogen synthase kinase 3
H3	Histone 3
ICM	Inner cell mass
IF	Immunofluorescence
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
IVP	*In vitro* production
M	Mitosis
MII	Metaphase II
MEK	Mitogen-activated protein kinase

mOsm	Milli-osmoles per kilogram
mRNA	Messenger RNA
N	Number of biological replicates
n	Number of technical replicates
Nanog2	Transgenic embryonic fibroblast cell line
Nocodazole	Methyl N-[6-(thiophene-2-carbonyl)-1H-benzimidazol-2-yl]carbamate)
NPB	No pseudo-polar body
NT	Nuclear transfer
Oct4	Octamer-binding transcription factor 4
P	Statistical P-value
PBS	Phosphate buffered saline
PBS/PVA	Polyvinyl alcohol in PBS
PFA	Paraformaldehyde
PG	Parthenogenote
PLCζ	Phospholipase C isoform zeta
PPB	Pseudo-polar body
RαB	Anti-bovine serum developed in rabbit
S	DNA synthesis
SEM	Standard error of the mean
Sox2	Sex determining region y-box 2
TE	Trophoblast
WNT	Wingless-type MMTV integration site family
1° antibody	Primary antibody
1C/2C/4C	DNA content of cell
1N/2N/4N	Chromosomal content of cell
2i	Double inhibitor combination (PD0325901 and CHIR99021)

Chapter 1: Introduction

With the world population expected to reach 9.6 billion people by 2050, there is an ever increasing demand for food [1]. Moreover, this food needs to be produced in a sustainable and efficient manner, allowing additional food to be generated from the same area of land while reducing environmental impacts. In order to achieve this goal, new agricultural practices that incorporate biotechnological advances need to be developed. This is especially important in a country such as New Zealand that relies heavily on its agricultural sector, with exports from livestock animals reaching $19.5 billion in 2013 [2].

One such biotechnology-based agricultural practice is the use of embryonic stem cells (ESCs) to generate clones. Cloning has the potential to increase reproductive efficiency as it removes the element of chance that arises in natural mating, allowing the multiplication of animals with beneficial traits. Current methods use selective breeding programs based on the genotype of the parents, identifying individuals with elite genetics that can be paired to breed elite offspring [3; 4]. However, due to independent assortment of alleles during meiosis, the elite genetics of the parent may not be transferred to their offspring. An alternative would be to use genomic approaches to identify an individual with the traits of interest and 'copy' this individual through cloning. This approach would remove the element of chance, as the offspring will have the same genetics as the parent [4]. Unfortunately, in its current state, the efficiency of cloning is too low to be a commercially viable technique. The use of ESCs for cloning has the ability to improve cloning efficiency. ESCs can generate all somatic (body) cell types of an adult animal, as well as germline (egg and sperm) cells. This property is termed pluripotency. It is hypothesised that using ESCs will increase the efficiency of cloning because their genome requires less reprogramming to reach an embryonic state. Using ESCs would also be beneficial for agricultural and biomedical applications as they are more amenable to genetic modifications for generating modified clones [5].

However, this is currently not possible in livestock as ESCs only exist in mice and rats. Despite research efforts over two decades, all attempts to derive these cells in farm animals have failed [6]. This failure is significant as it delays important farming applications. For breeding, ESCs could be derived from embryos with outstanding genotypes, effectively capturing and multiplying their elite genetics. This would take genomic selection from the animal to the cellular level, accelerating genetic gain through the generation of animals with beneficial traits, such as increased milk yield or muscle mass that improve the productivity of the agricultural sector.

Using a novel culture method, bovine ESC-like cells (or embryo-derived pluripotent stem cells; ePSCs) can be maintained for several weeks [7]. These cells are referred to as 'ESC-like' because their capacity to fulfil all the standards that define ESCs has yet to be demonstrated. The purpose of this research project is to overcome the knowledge gaps in bovine ePSC biology by measuring the efficacy of using these cells for cloning. Ultimately, the long-term aim is to demonstrate that livestock embryos can be efficiently converted into ePSCs and then ePSC-derived animals, capturing their unique genetics for accelerated animal breeding and biomedical applications.

Chapter 2: Literature Review

2.1 Embryo development

Before the 19th century, it was widely believed that embryos developed from a miniature version of the adult, a homunculus, found in semen [8-11]. This idea was replaced by epigenesis, where the adult form develops from an egg with no pre-existing form [8; 10]. With the advancement of microscopy, the development of increasing complexity of form from a single-celled egg could be visualised. This aided the understanding of embryo development to the point where it could be replicated *in vitro*. For more than 30 years, embryos have been able to be developed *in vitro* from mice (*Mus musculus)* and other mammalian species, including humans [12].

Before fertilisation, an egg (oocyte) must resume the cell cycle and mature its nuclear and cytoplasmic contents. Premature oocytes are arrested at metaphase I. A cell in interphase has a single copy of two homologous chromosomes of paternal and maternal origin. This cell's chromosomal content is diploid (2N) and its genomic content is two chromatids (2C; Figure 2.1). In metaphase I, the oocyte has replicated its DNA, resulting in twice the normal amount of DNA (4C), but with still 2 homologous chromosomes (2N). Upon maturation, the oocyte resumes the first meiotic division of metaphase I and progresses to metaphase II (MII) of the cell cycle. At MII, one set of sister chromatids have been expelled to form a polar body, leaving a haploid cell with one homolog (1N) that still has its sister chromatid attached (2C). The polar body is located in the perivitelline space between the oocyte cell membrane and zona pellucida, the protective outer layer of the oocyte. During maturation of the nuclear material to MII, the cytoplasm must also prepare for fertilisation. Cytoplasmic maturation includes functions such as increasing sensitivity to intracellular calcium release and production of key proteins [12].

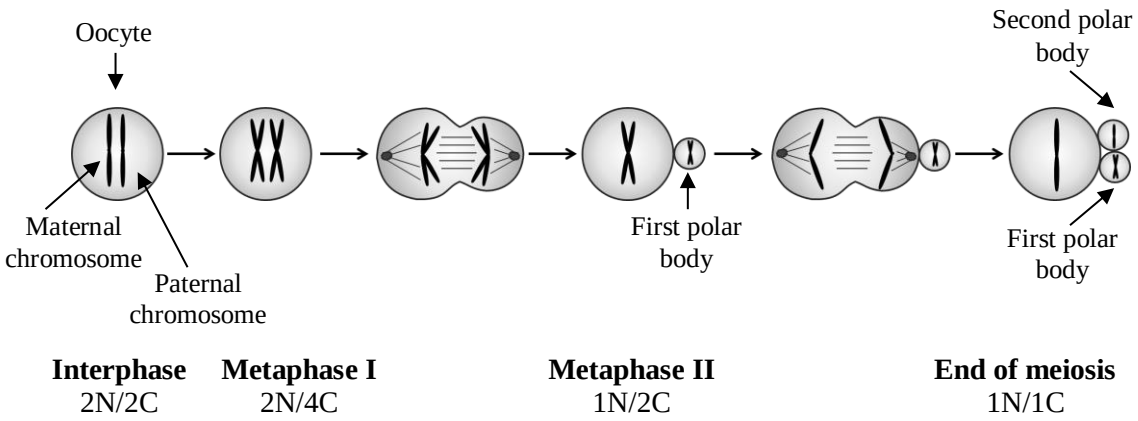

Figure 2.1: Meiosis in the developing oocyte. During interphase the oocyte has a single copy of each homologous chromosome (2N/2C). At metaphase I the chromosomes have been replicated (2N/4C). Between metaphase I and metaphase II the homologous chromosomes are separated, leaving the oocyte with one homolog with sister chromatids still attached (1N/2C). The other homolog is expelled as the first polar body (1N/2C). Following metaphase II, the sister chromatids separate and one is expelled as the second polar body. This leaves the oocyte with a single copy of the homolog (1N/1C). Modified from (Björn Oback, unpublished data).

The start of embryo development is triggered by the fusing of sperm (1N/1C) to the oocyte. This process causes an intracellular rise in calcium that then oscillates a number of times. It is widely believed that these calcium oscillations are stimulated by the introduction of phospholipase C isoform zeta (PLCζ) from sperm [13; 14]. Changes in intracellular calcium levels are important for preventing polyspermy and the resumption of the cell cycle from MII. Polyspermy, the fusing of multiple sperm onto the oocyte, is prevented by an increase in calcium causing the release of the contents of cortical granules into the perivitelline space. This causes the zona pellucida to harden, preventing sperm from penetrating it. The resumption of the cell cycle is necessary for the extrusion of the second polar body, resulting in an oocyte that is 1N/1C and ending meiosis (Figure 2.1). Both sets of DNA are then aligned to generate a 2N/2C zygote, where DNA can be replicated and mitotic divisions begin [14].

The growth of an embryo follows defined stages in all mammals (Figure 2.2). This development occurs in a hypoxic environment; *in vivo* it is caused by the embryo being enclosed in more layers of granulosa cells during development.

This is reproduced artificially *in vitro* by controlling oxygen levels [12]. Following fertilisation, the zygote divides to form two cells (blastomeres). These blastomeres are totipotent cells that are able to form the embryo and extra-embryonic tissue, such as the placenta. Cells continue to divide through to a compacted ball of blastomeres (a morula), and then a blastocyst. At the blastocyst stage, the blastomeres differentiate into two different cell types. The trophoblast (TE) is the outer layer of cells that contributes to the extra-embryonic tissue. Inside the TE is a collection of cells that compose the inner cell mass (ICM). As the embryo develops, the ICM is separated into a thin layer of hypoblast cells that are in contact with the blastocoele and will develop into extraembryonic tissue, and epiblast cells that will develop into the fetus. Continued growth of the blastocyst causes it to 'hatch' from the zona pellucida, freeing it to develop further [6; 12; 15; 16].

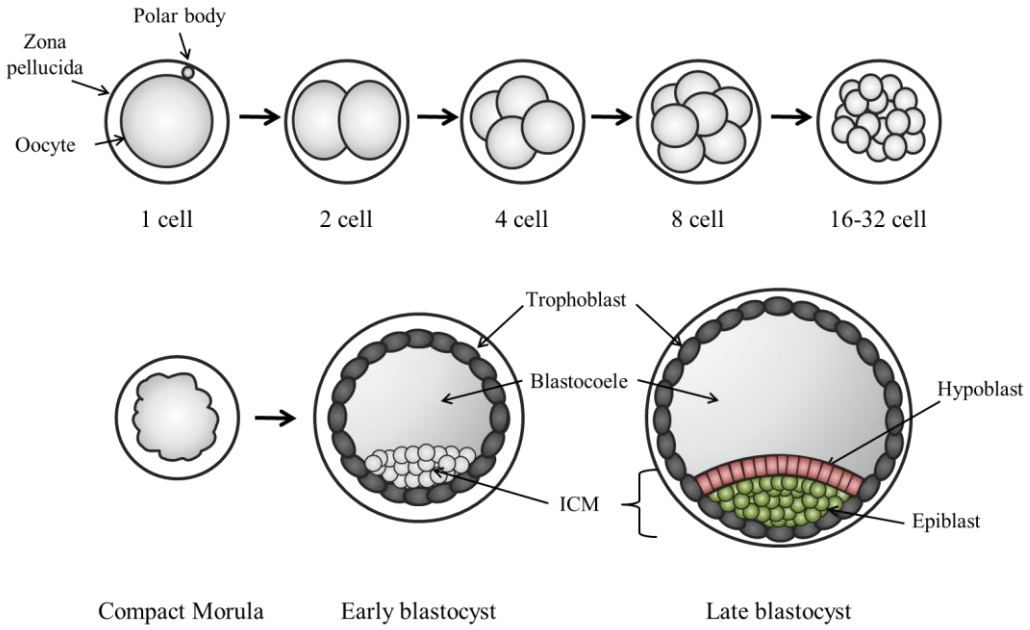

Figure 2.2: Stages of bovine embryo development from matured oocyte to blastocyst. The 1-cell stage is a matured oocyte with expelled polar body before fertilisation. Following fertilisation, oocyte cleaves forming a 2-cell embryo. Embryos continue cleaving, forming a compacted morula. A blastocoele develops within the morula, forming a blastocyst. In the early blastocyst, trophoblast (TE) and ICM cells become clearly distinguishable. ICM cells further segregate into epiblast and hypoblast cells in the late blastocyst. Modified from [17].

In bovine (*Bos taurus*), development from fertilisation to blastocyst stage takes one week. *In vivo* the blastocyst gains nutrients from uterine secretions and can continue to grow and develop into the fetus. *In vitro* development can be maintained until hatched blastocyst, a maximum of nine days after fertilisation. At this point the support for development cannot be simulated, requiring embryos to be transferred into a uterus for continued development *in vivo* [6].

2.2 Nuclear transfer cloning

Once development of an embryo was understood, methods to manipulate it were developed. Cloning captured attention by being able to 'copy' a desirable individual. There are a number of techniques for generating clones. Embryos can be split early in development into two to four blastomeres to generate twins, triplets, or quadruplets [18]. Alternatively, ESCs can be cloned by being added to a tetraploid embryo. The tetraploid cells contribute solely to the extra-embryonic tissue, while the ESCs form the embryo proper [19]. However, the most common technique is nuclear transfer (NT) cloning [18].

2.2.1 History of nuclear transfer cloning

The potential to replicate an individual through NT cloning was first investigated by studies in amphibians. In the 1950s, it was shown in two frog species, *Rana pipiens* and *Xenopus laevis*, that the nucleus of an embryonic cell could be introduced into an enucleated oocyte and successfully develop into an adult [20; 21]. Proof that a fully differentiated adult cell was capable of cloning was not demonstrated for another 25 years, until the nucleus from a functioning lymphocyte was used to clone *X. laevis* tadpoles [22]. However, clones from differentiated adult donor cells did not survive to adulthood. Nevertheless, the proof that a differentiated cell could be used for cloning confirmed that the cell's genome retains the ability to create all cell types from all germ layers, regardless of what cell it was originally [18].

Studies in mammals proved more difficult, with many years passing before successful cloning was achieved. In the 1980s, clones were generated through the use of blastomeres as donor cells. The lower success of cloning from

differentiated cells in amphibians discouraged their use in mammals. This was changed with the generation of clones from more differentiated embryonic cells in 1996 [23] and the birth of 'Dolly' the sheep (*Ovis aries*) from a fully differentiated mammary cell [24]. The success of Dolly lead to the generation of clones from differentiated somatic cells in cattle (*B. taurus)* [25; 26], goats (*Capra aegagrus hircus)* [27], pigs (*Sus scrofa domesticus)* [28], deer (*Cervus elaphus)* [29], rats (*Rattus norvegicus)*, mice (*M. musculus)* [30], horses (*Equus ferus caballus)* [31], dogs (*Canis lupus familiaris)* [32], and cats (*Felis catus)* [33].

2.2.2 Nuclear transfer cloning methodology

NT cloning is comprised of three main stages (Figure 2.3). The first is the collection of recipient oocytes and the removal of their nucleus. This process of enucleation produces an oocyte devoid of DNA in preparation for the entry of a new nucleus. Secondly, the nucleus from a selected donor cell is transferred to the enucleated oocyte. The cytoplasm of the oocyte is able to reprogram the donor nucleus to an embryonic state, capable of producing an embryo. Finally, reconstructed oocytes containing a transferred nucleus are artificially activated to resume meiosis. Once meiosis has completed, the cloned embryo undergoes mitosis, following the same developmental stages as a sperm-fertilised oocyte, reaching blastocyst stage *in vitro*. Further detail with respect to NT methodology is described below.

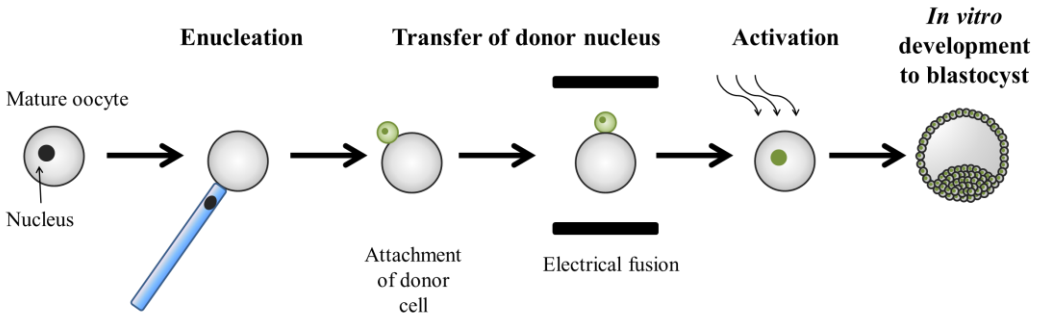

Figure 2.3: Outline of nuclear transfer protocol. *In vitro* matured oocytes are produced and enucleated using a finely pulled pipette. Donor cells are then attached to enucleated cytoplasts and electrically stimulated to fuse, transferring the donor cell nucleus into the cytoplast. Reconstructs are then artificially activated to resume mitosis and allowed to develop *in vitro* until late blastocyst stage. Modified from [5].

2.2.2.1 Enucleation of donor oocytes

The first step in NT cloning is the removal of the DNA from *in vitro* matured oocytes. Enucleation of donor oocytes can be achieved in three different ways: zona-intact, zona-free, or 'hand-made cloning'. Zona-intact enucleating involves aspirating the DNA and a small amount of cytoplasm using a finely pulled pipette [34]. This is the most common method, being first developed in mouse [35], and then adapted for sheep [36] and cattle [34; 37]. Zona-free enucleation uses the same process as zona-intact, only differing by the removal of the zona pellucida. This method is faster and simpler than zona-intact, allowing greater reproducibility of results [34; 38]. Use of zona-free enucleation changes the culture conditions of the embryos from standard group culture. Normal culture conditions use group culture, with up to ten embryos in a single drop of culture media. Without a zona pellucida to create a barrier in zona-free NT, embryos must be cultured singularly or using a well-of-well method where dimples are created in the base of the culture dish that embryos rest in [39]. Culturing embryos in a group improves development as embryos produce and excrete factors that aid growth [40]. However, studies have shown that the group effect is quite small on overall development [41]. Hand-made cloning uses two donor oocytes to generate a single enucleated oocyte. Donor oocytes are bisected so one half contains the nucleus, which is discarded, and the other half is only cytoplasm. Two cytoplasmic oocyte halves are then fused to generate an oocyte of the same original volume. This technique is the least common NT method as it halves the number of donor oocytes available for cloning [34; 42]. Once the nuclear material is removed from the oocyte, they are referred to as cytoplasts as they are only composed of cytoplasm.

2.2.2.2 Transfer of nucleus from donor cell to cytoplast

Following enucleation, cytoplasts are fused with the nucleus from a cell of the individual to be cloned. The most common method of achieving this is to attach a donor cell to the cytoplast and electrically fuse them. Attachment of the two membranes is achieved by the addition of lectin, a carbohydrate-binding protein, to the culture media, causing the cell to stick to the cytoplast. Electrical fusion is

stimulated by two direct current (DC) pulses. These pulses increase the permeability of the cell membranes, allowing them to fuse. The placement of the cell on the cytoplast must be parallel to the direction of the current for fusion to occur. Thus, cell and cytoplast couplets must be aligned prior to DC pulse. Automatic alignment can be achieved using an alternating current (AC) depending on cell size; AC alignment is more successful with larger cells. As the membranes of the cells fuse together the nucleus of a donor cell is transferred to the cytoplast [18; 43]. An alternative to fusing a whole cell is to remove the nucleus from the donor cell and microinject it into the oocyte [30]. This is less common as it requires a greater amount of time and skill to perform. Cytoplasts that have successfully fused the donor cell are termed reconstructs, as the oocyte has DNA again.

Two factors have been shown to aid fusion of a cell to a cytoplast. Pronase treatment of donor cells before sticking to cytoplasts has been shown to increase fusion. This is thought to be due to the removal of surface charges or by smoothening the surface of the cell by removing proteins, improving adhesion of the cell to the cytoplast for fusion [42-44]. The osmolality of the buffer used for fusion can also affect fusion efficiency. Osmolality is the concentration of solutes in a solution, with normal mammalian cell osmolality around 280 milli-osmoles per kilogram (mOsm). A hypoosmolar fusion buffer of 165 mOsm has been shown to increase alignment and fusion. Moving to a solution of lower osmolality is thought to improve fusion by causing cells to swell, making the membranes more permeable [43; 44].

Once DNA is received by the cytoplast, it interacts with the cytoplasm and is reprogrammed to an embryonic state able to generate all cell types, eventually developing into an adult animal [18]. Reprogramming is driven by factors within the cytoplasm of the oocyte that are able to override the current state of differentiation of the DNA [45]. This step is essential for reactivating genes that have been silenced throughout development, through epigenetic modification. This entails the modification of DNA or proteins associated with DNA that affect the expression of genes. The modifications do not affect the DNA sequence, only how the sequence is accessed for transcription by preventing factors from binding

to DNA or affecting how it is packaged. If the genome was not reset only a single cell type, that of the donor cell, would be able to be generated. Removal of epigenetic marks allows the generation of all cell types [18].

2.2.2.3 Activation of reconstruct to resume meiosis

After fusion, reconstructs are electrically or chemically stimulated to activate. Artificial activation stimulates the release of calcium in the cytoplasm of the reconstruct, releasing the oocyte from its meiotic block. This response mimics the natural activity of the oocyte after sperm fuses to its membrane [14; 42; 46]. Electrical activation stimulates the mobilisation of calcium into the cell, which triggers the release of internal stores of calcium. Alternatively, calcium ionophores, such as ionomycin, are used to chemically induce the release of calcium from internal stores, greatly increasing intracellular levels of calcium, while also mobilising external calcium into the cell [14; 47]. Artificial activation differs from activation following sperm fusion in that only a single increase in calcium levels is generated, instead of oscillating waves [14; 42; 47].

Along with increase in calcium, artificial activation causes the degradation of cyclin B and cyclin-dependent kinase 1 (Cdk1) complexes [48]. Cyclins are proteins that act as indicators of cell cycle stage [49], with cyclin B and Cdk1 complexes maintaining the oocyte in MII. Following chemical or electrical artificial activation, reconstructs are incubated in an inhibitor of protein phosphorylation or synthesis. This supresses the reformation cyclin B and Cdk1 complexes, allowing the oocyte to progress through the cell cycle [18; 48], reprogram the genome of the donor cell, and induce pronuclear formation. The development of a pronucleus with the donor genome is important for the successful development of the embryo [46; 50; 51]. There are a range of chemicals used to inhibit protein synthesis after ionomycin activation. The most common is the phosphorylation inhibitor 6-dimethyl aminopurine (DMAP). An alternative option is the general protein synthesis inhibitor cycloheximide (CHX), which prevents cyclin synthesis [14; 51; 52]. A less common general protein synthesis inhibitor is anisomycin [53; 54]. A benefit of using protein synthesis

inhibitors such as CHX and anisomycin is they allow the extrusion of a second pseudo-polar body, necessary for cloning with mitotic donor cells.

If a non-enucleated oocyte is artificially activated a parthenogenote (PG) is produced. Parthenogenesis is the generation of an embryo from an unfertilised oocyte, with the resulting offspring being generated solely from maternal DNA [47]. This is an asexual reproduction strategy used by many species including insects [55; 56], reptiles [57; 58], and amphibians [59]. In cloning, parthenogenetic activation can be used as an important tool for investigating artificial activation efficiency. This technique isolates the effect of the activation protocol and whether it is sufficient to stimulate normal development, removing variation caused by donor cell introduction [14; 47; 52; 60]. Although the PG only contains a single homolog (1C), following activation it maintains the correct DNA content (2C) permitting normal embryonic development. Polar body extrusion, using protein synthesis inhibitors such as CHX and anisomycin, is unwanted in this case; ejection of a second pseudo-polar body results in a haploid embryo (1N/1C) that has poor development due to having only half the normal amount of DNA (Figure 2.4). An inhibitor of actin network formation, cytochalasin B (CB), is used to maintain the correct ploidy. The disruption of actin filaments within the cellular membrane prevents expulsion of a polar body [61].

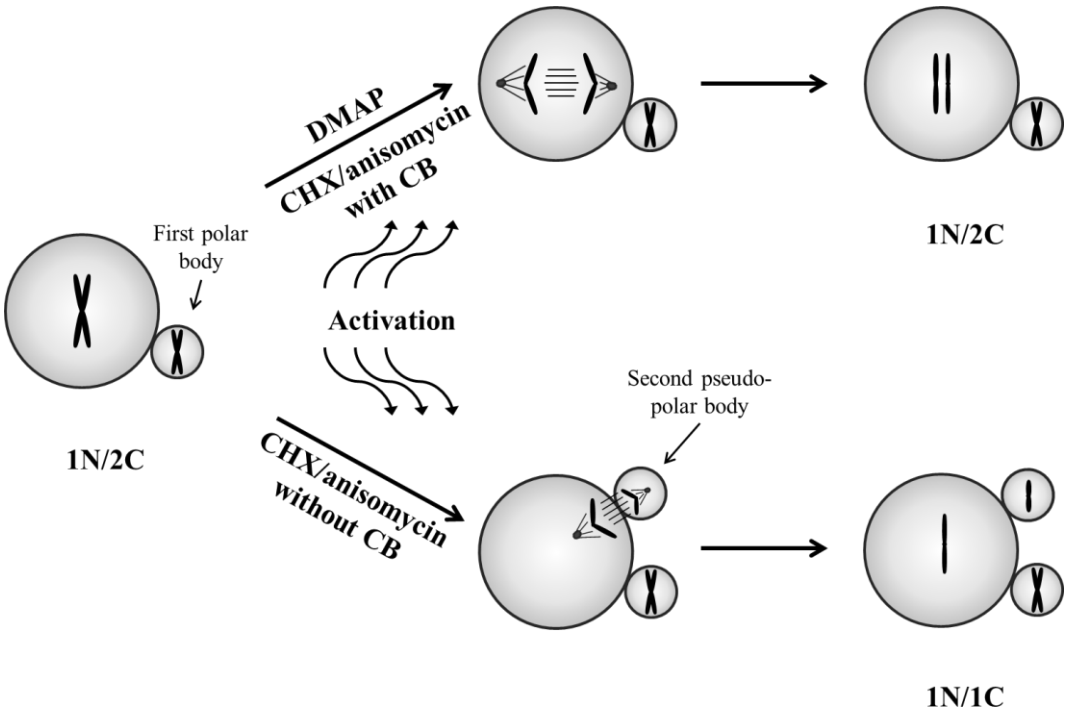

Figure 2.4: Chromosome and DNA content of PGs following activation. Incubation with DMAP or CHX/anisomycin with CB prevents a second pseudo-polar body being expelled. This results in a PG that still has only one homologous chromosome (1N), but two copies of it (2C). Having the correct DNA content allows normal embryonic development. Incubation with CHX or anisomycin without CB permits a second pseudo-polar body being expelled. This results in a PG that has one homologous chromosome (1N), and only one copy of it (1C). Having the incorrect DNA content affects embryonic development. Modified from (Björn Oback, unpublished data).

2.3 Factors that affect cloning efficiency

Although it has been nearly two decades since the cloning of Dolly and subsequent widespread increase in somatic cell NT cloning, the efficiency of using this technique is still considered low. Cloning efficiency is defined as the proportion of embryos that develop into a healthy animal after being transferred to a surrogate [62]. The efficiency of cloning is much lower than other *in vitro* reproductive techniques; NT cloning has a cloning efficiency of 1-9% in cattle compared with 40% with *in vitro* fertilisation (IVF) [38; 62; 63]. The main genetic factor affecting cloning efficiency is chromosomal abnormalities caused by the NT procedure, through abnormal chromosome segregation and inefficient reprogramming. These abnormalities affect development and can interfere with

pregnancy, causing miscarriage and other animal welfare issues for both fetus and mother [62; 63].

2.3.1 Abnormal chromosomal segregation

Incorrect chromosome number and structure is observed in a high number of NT embryos, and is attributed to having a larger effect on embryo viability than reprogramming errors [64]. Changes in chromosomal constitution of the cell occur via incorrect segregation of chromosomes during cleavage early in embryonic development. This may be caused by issues with spindle formation and distribution of chromosomes on the metaphase plate [60; 64]. Correct spindle formation is aided by the centrosome. In mice, there are a number of maternal centrosomes still present in the cytoplast following enucleation. In bovine, the centrosome is removed with the nucleus during enucleation. This means the only centrosomes present for chromosomal segregation following activation are those transferred from the donor cell [60].

Abnormal chromosomal compliment may not affect early morphology, requiring it to be screened for. The methods for screening chromosome number usually require fixation or denaturation, preventing further development. However, live imaging techniques have shown that in mice, abnormal chromosomal segregation after the 8-cell stage produced viable offspring. This suggests that changes in chromosome number that occur later in development do not have as strong of an impact on development to term, possibly because there are enough cells with correct chromosomal constitution to generate the embryo proper [64].

2.3.1.1 Donor cell cycle phase

For DNA to be correctly received by the cytoplast, chromosomes segregate, and develop into an embryo, the donor cell needs to be at a stage of the cell cycle compatible with the cytoplast. The cell cycle is split into four phases (Figure 2.5). Immediately following mitosis is the first growth phase (G_1) where cells increase in size and perform their normal metabolic functions, transcribing RNA and translating proteins for cell growth. If cells arrest in this phase, as they are no longer dividing or preparing to divide, they are said to be quiescent (G_0).

Progression from G_1, initiated by cyclins and cyclin dependent kinases, enters the cell into DNA synthesis phase (S). During this phase each chromosome is replicated in preparation for cell division. At the completion of DNA synthesis, cells continue to grow until ready to divide (G_2-phase). G_2 cyclin checkpoints maintain the cell in G_2 until all processes necessary for cell division have been completed, including generation of spindle proteins. The cell then enters mitosis (M) where chromosomes line up and are segregated by mitotic spindles. Correct segregation progresses the cell into generating two nuclei around the separated DNA, division of cellular components, and cleavage of the cell in half forming two daughter cells [18].

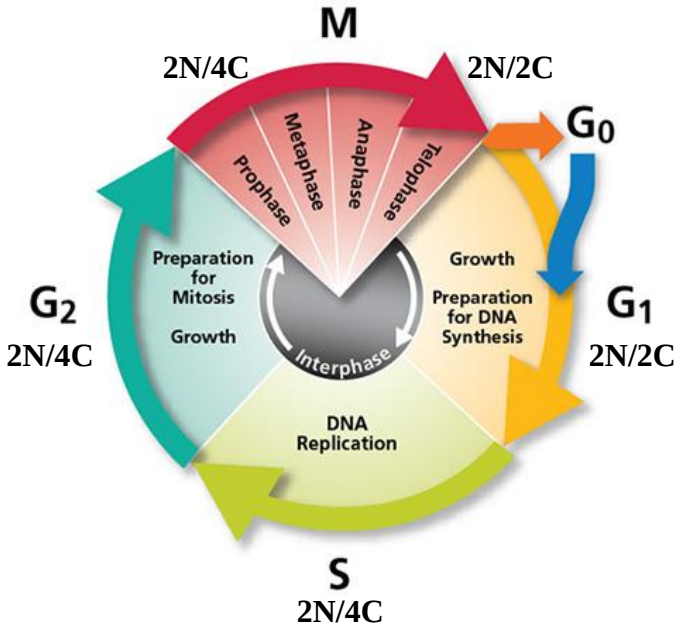

Figure 2.5: Cell cycle stages. Interphase is separated into three stages. 1) During first growth phase (G_1) cells grow and prepare for DNA synthesis. If cells arrest in this stage they are said to be quiescent or in G_0. 2) DNA is synthesised in S-phase in preparation for cell division. 3) Second growth phase (G_2) prepares cells for cell division. Mitosis (M) follows immediately after G_2. Image from [65].

2.3.1.2 Cell cycle compatibility with metaphase II cytoplasts

During standard NT cloning, a donor cell is fused to a non-activated, MII cytoplast. This presents a high cyclin B/Cdk1 environment for the donor cell to enter, compatible with donor cells in G_1/G_0- or G_2/M-phase. Donor cells in G_1- or G_0-phase entering a high cyclin B/Cdk1 environment have their nuclear

membrane degraded and chromosomes condensed in preparation for replication of chromosomes in the first mitotic division and enabling reprogramming. As G_1/G_0 cells have not replicated their genome, they have the correct ploidy (2N/2C) [18]. DNA from donor cells in G_2 or the early stages of mitosis are also compatible. G_2/M-phase cells match the state of the original oocyte genome; they have a high cyclin B/Cdk1 environment, have already replicated their DNA (2N/4C), and nuclear membrane is disassembled. While these cells have double the normal amount of DNA, a pseudo-polar body can be extruded to maintain correct ploidy; chromatids align on the spindle, originating from the metaphase donor cell, and segregate with half DNA content remaining in the cytoplast (2N/2C) and the other half being expelled as a pseudo-polar body [18; 66]. Cells in S-phase cannot be used for cloning. This is because their DNA is only partially replicated, with DNA content anywhere between 2-4C. If an S-phase cell fuses to a MII-phase cytoplast, the high cyclin B/Cdk1 environment will cause the nuclear membrane to break down, interrupting DNA replication which will not resume correctly, resulting in an embryo of unknown ploidy [18; 46].

2.3.1.3 Cell cycle compatibility with preactivated cytoplasts

An alternative to fusing into a MII-phase oocyte is preactivation. In this procedure, cytoplasts are activated with ionomycin and kept in an inhibitor of protein phosphorylation or synthesis while donor cells are fused. This means the donor cell is entering a low cyclin B/Cdk1 environment. This is compatible with donor cells in G_2/M- or S-phase. Donor cells in G_2/M-phase do not need high cyclin B/Cdk1 levels to initiate nuclear membrane degradation. Entry into a preactivated cytoplast commences chromosome segregation and progression out of metaphase. Low cyclin B/Cdk1 levels are compatible with S-phase donor cells as their nuclear envelope is left intact and chromosomes can continue to be replicated. G_1/G_0-phase donor cells are not compatible as their nuclear membrane remains intact, preventing chromatin remodelling necessary for cell division [18]. NT using preactivated cytoplasts has been shown to be beneficial with donor cells that do not require a large amount of reprogramming, such as blastomeres [67].

2.3.1.4 Synchronisation of cell population into a single cell cycle phase

Cells are normally synchronised to generate a large population of donor cells in the correct cell cycle for NT. The most widely used synchronisation method is serum starving, which produces cells in G_1/G_0-phase. Serum starving, culturing cells in growth media with low serum, causes cells to stop dividing. This quiescent state creates a large population of cells in G_0-phase that can be harvested for cloning. G_1/G_0-phase cells have a higher cloning efficiency than G_2/M, making it the more popular option [62]. However, some cell types cannot be easily synchronised into G_1/G_0, so require the use of cells in G_2/M-phase. Synchronisation into G_2/M can be achieved by using a drug that halts the cell cycle at mitosis, such as nocodazole (methyl N-[6-(thiophene-2-carbonyl)-1H-benzimidazol-2-yl]carbamate) [68; 69]. Nocodazole interferes with the microtubule spindles that are required for chromosome separation during mitosis. Cell cycle machinery detects abnormalities in spindle structure, halting the cell cycle. The disadvantage of using cells in G2/M-phase is that the extra set of sister chromatids must be expelled, as a second pseudo-polar body, before mitotic division can occur (Figure 2.6) [70].

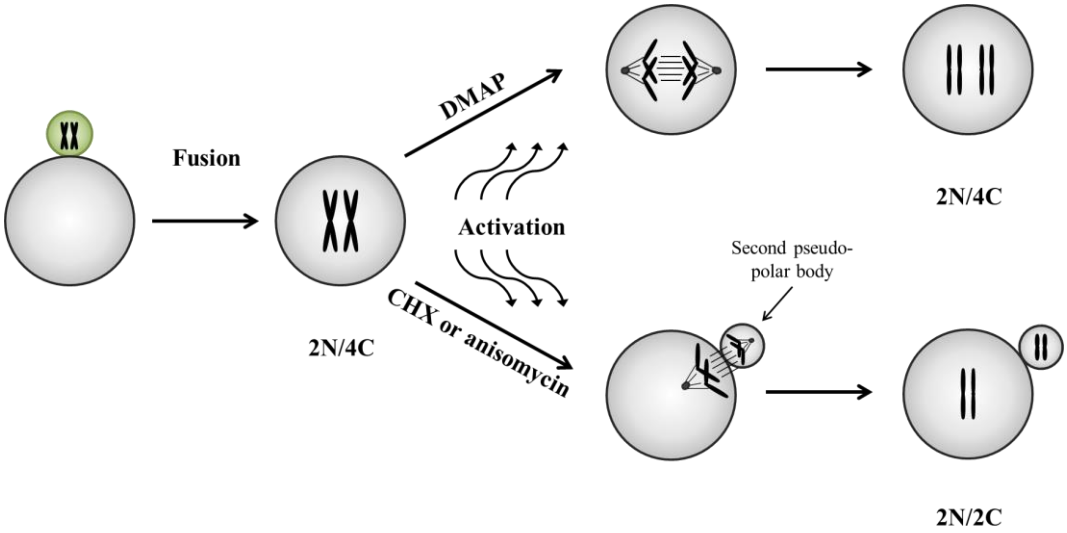

Figure 2.6: Chromosome and DNA content of M-phase NT reconstructs following activation. Incubation with DMAP prevents a second pseudo-polar body being expelled. This results in a reconstruct that still has two copies of each homologous chromosome (2N/4C). Incubation with CHX or anisomycin permits a second pseudo-polar body being expelled. This results in a reconstruct that has one copy of each homologous chromosome (2N/2C). Modified from (Björn Oback, unpublished data).

2.3.2 Epigenetic reprogramming

Incomplete reprogramming of the donor nucleus through epigenetic changes also affects cloning efficiency. The epigenetic state of the donor cell must be rapidly reversed to an embryonic state following NT; current epigenetic state of the nucleus must be wiped to permit expression of early embryonic genes to allow the correct development of an embryo. If the donor nucleus is not correctly reprogrammed, abnormal epigenetic histone and DNA patterns may be expressed, affecting development. Chemical modifications can be used to aid reprogramming of the genome such as incubation with histone deacetylase inhibitors [61; 64]. Reprogramming efficiency can also be affected by the differentiation status of the donor cell, making the choice of donor cell type an important consideration [62].

2.3.2.1 Donor cell type

The choice of donor cell requires a balance between cell availability and amount of genome reprograming required. Somatic cells are the most differentiated cell type, requiring the most reprogramming by the oocyte cytoplasm, but are the most abundant and easily accessible cell type. By comparison, the genome of blastomeres is at an embryonic state, needing less reprogramming to generate an embryo. This makes them an ideal cell type for use in cloning [5; 18]. Blastomeres are considered superior to somatic cells, having a higher cloning efficiency of 36% in mice and 28% in cattle compared to 0.6% and 5% with somatic cells, respectively [71-73]. Blastomeres also have a lower incidence of chromosomal abnormalities than their more differentiated counterpart. The problem with using blastomeres is there are a limited number of cells that can be harvested from an early stage embryo. This hindrance has caused somatic cells to be the most common donor cell type used in cloning [5; 18].

Even with improvements to NT techniques, cloning efficiency with somatic cells is still low compared to IVF (9% vs 40% in cattle) [38; 63]. To overcome this problem, it was hypothesised that ESCs would be a good middle ground, with a lower degree of differentiation than somatic cells. ESCs can also be grown in culture to increase the number of cells available. One drawback to using ESCs is that they cannot be serum starved into G_0, therefore they must be used in M-phase

for cloning with MII-phase cytoplasts, or M- or S-phase with preactivated cytoplasts [5; 18].

2.4 Embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent cells, capable of differentiating into any cell type, somatic or germ cells, and regenerating indefinitely [5; 6; 74]. Interest into ESCs started with their ability to be used in cloning and biomedical applications. In cloning, the ability of ESCs to multiply elite embryos or to preserve the genetics of rare breeds makes them an attractive cell type. ESCs are ideal for use in biomedical applications as they are more amenable to genetic modifications. ESCs have been used in biomedical applications such as understanding the function of mutations linked to skin disorders [75], Parkinson's disease [76], and liver disease [77], tissue engineering [78; 79], investigating drug toxicity [80], and cell transplantation therapies [81].

ESCs were first isolated in mouse in 1981 from early blastocysts [16]. These were the first isolated cells able to maintain their pluripotency *in vitro*. Since this initial discovery over 30 years ago, ESCs have only been able to be isolated in mice and rats. All attempts to derive *bone fide* ESCs in any other species have failed. This is likely to be due to differences in developmental timing across species making it hard to pinpoint the equivalent stage as a day 4.5 mouse embryo [6].

2.4.1 Defining ESCs

Molecularly, ESCs are characterised by the formation of dome-shaped colonies and expression of key pluripotency genes octamer-binding transcription factor 4 (*Oct4*), Sex Determining Region Y-Box 2 (*Sox2*), and *Nanog* (Figure 2.7) [6; 7; 74; 82; 83]. *Oct4*, *Sox2*, and *Nanog* activate genes involved in pluripotency, preventing differentiation. *Oct4* and *Sox2* are expressed throughout the developing embryo, slowly being downregulated in the TE and becoming restricted to the ICM. *Nanog* is exclusively expressed in the pluripotent epiblast cells [6; 74; 84]. Putative ESCs are identified by expression of these key pluripotency genes before more stringent analysis.

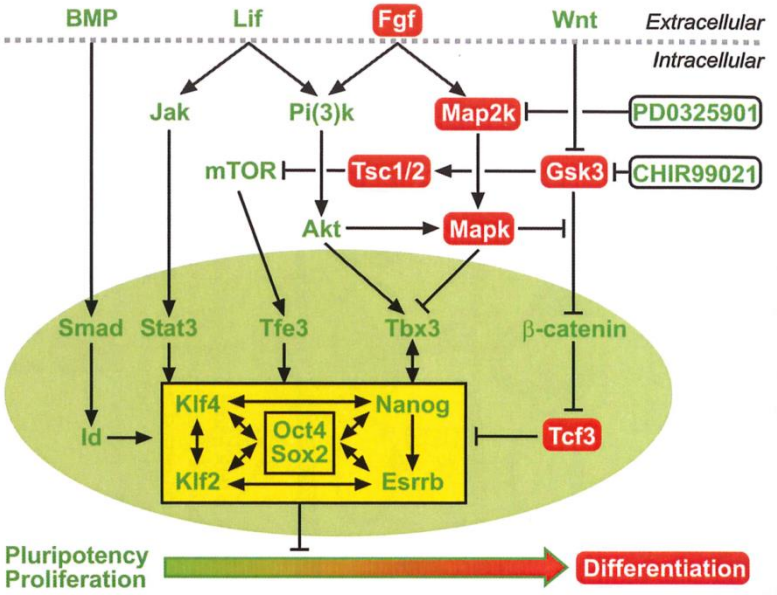

Figure 2.7: Molecular pathways involved in pluripotency. *Oct4, Sox2,* and *Nanog* are the three core pluripotency genes regulated by a number of signalling pathways. Two inhibitors used to promote pluripotency in double inhibition growth media (2i growth media), PD0325901 and CHIR99021, are outlined with the differentiation pathways they inhibit. Image from [6].

There are three functional criteria that must be demonstrated for pluripotent cells to be called true ESCs, which have not been demonstrated conclusively in any species other than mice. The first is the formation of teratomas. When injected into a recipient with a compromised immune system, ESCs should form a benign tumour containing cells representing the three germ layers of endoderm, mesoderm, and ectoderm. This is the least stringent hallmark of a true ESC, with representatives of all possible cell types are generated from one original cell type [6].

Secondly, true ESCs are able to generate germline chimeras. Animals partially derived from ESCs comprise two genetically distinct cell types and are referred to as somatic 'chimeras'. If ESCs also contribute to the formation of functional gametes, the resulting animals are referred to as germline chimeras. This proves the cells are pluripotent as they can still develop into germ cells. True ESCs are able to contribute to germline chimeras while cells derived later than day 4.5 in the mouse do not, indicating they have been biased towards certain lineages and are not truly pluripotent [7; 16; 82; 83].

The highest standard of pluripotency is tetraploid complementation. Embryos can be generated by combining cells of different lineages, creating an aggregate. In tetraploid complementation, diploid ESCs can be aggregated with tetraploid cells or injected into tetraploid embryos to form an ESC derived embryo. The presence of diploid cells restricts the distribution of tetraploid cells to the extra embryonic tissue. If an animal can be generated solely from the diploid cells then they were pluripotent as all cell types were able to be generated [6; 85].

2.4.2 Growth conditions to promote pluripotency

On the path to achieving ESCs from all species, pluripotency has been successfully maintained in culture through the use of specialised growth conditions. Along with standard media for growth in anoxic conditions, two small-molecule inhibitors are used to prevent cells from differentiating. The first inhibitor (PD0325901) acts on the fibroblast growth factor (FGF)/mitogen-activated protein kinase (MEK)/extracellular signal-related kinase (ERK) pathway (Figure 2.7) [86]. This pathway is involved in the differentiation of cells into the ICM or the hypoblast [87]. The second is a glycogen synthase kinase 3 (GSK3) inhibitor (CHIR99021), which allows the activation of the wingless-type MMTV integration site family (WNT) signal transduction pathway [86]. WNT pathway activation has been associated with promoting ESC self-renewal [83; 88]. This double inhibitor combination is termed ‘2i’ and it has been shown to promote epigenetic changes that prevent differentiation of ESCs, and bi-allelic expression of the key pluripotency markers such as *Nanog* [83; 84]. 2i has been successfully used to develop and maintain true ESCs in mice and rats *in vitro*, and prevent formation of the hypoblast *in vivo*. In non-rodent species, the focus has largely been on human ESCs for therapeutic use, however ethical restraints prevent verification of whether they are true ESCs through chimera generation [82; 83]. There has also been considerable attention on ruminant ESCs, with studies showing that 2i growth conditions promote pluripotency through increased expression of *SOX2* and *NANOG*, and limited self-renewal in cattle [7]. The cells produced are termed ESC-like cells due to their increased pluripotency, but not

achieving full true ESC status. True ESCs are yet to be achieved for any ruminant species [6].

2.4.3 ESCs in cloning

Previous studies have successfully used ESCs in cloning with mice [89-92]. The large focus on murine studies is due to true ESCs being isolated from only mice and rats. However, some bovine studies have been successful in using ePSCs in cloning [93; 94]. To date, no overall improvement in cloning efficiency has been achieved by using ESCs compared with somatic cells. Although overall efficiency is similar, NT with somatic cells is more efficient to *in vitro* developmental stage (hatched blastocyst), whereas the efficiency of blastocysts transferred to surrogates developing to term was greater with ES donor cells [95]. Thus, if development to blastocyst stage *in vitro* can be improved with NT reconstructs generated from ESCs, then overall cloning efficiency would increase.

While serum starvation into G_0/G_1 phase is routinely done with somatic cell NT, this is difficult to achieve with ESCs. Thus, synchronising into M-phase is the preferred method of cell synchronisation for NT [89]. Ionomycin activation followed by incubation in DMAP is the standard protocol used to generate NT blastocysts. However, when using donor cells arrested in M-phase this is not ideal; DMAP prevents pseudo-polar body expulsion, resulting in a NT reconstruct with double the normal amount of DNA (2N/4C), affecting development (Figure 2.6). The main activation combination used to artificially activate reconstructs generated from mitotically-arrested donor cells is ionomycin and 10 µg/ml (35.5 µM) CHX [14; 46; 52; 68; 96]. Using bovine somatic cells, activation with CHX for six hours in MII-phase cytoplasts resulted in 21% blastocyst development [66]. Alternatively, CHX preactivated cytoplasts had similar development rates up until six hours of preactivation [46].

An alternative option is activation with ionomycin followed by anisomycin incubation [54; 96]. A five hour incubation of MII-phase cytoplasts in 10 µM anisomycin is the most common activation procedure. No literature could be

found using anisomycin with M-phase donor cells, however, G_0-phase cells activated with anisomycin had a blastocyst development rate of 32% [96].

2.4.3.1 Problems with ePSC cloning

The first issue with attempting to clone with ePSCs is the isolation of single donor cells. Mouse ICM derived colonies are easily dissociated into single cells for passaging and further growth. In contrast, livestock ePSCs are tightly joined and depend on interactions between cells, preventing isolation of single cells. Attempts to dissociate cells using chemicals or enzymes standardly used to dissociate cells in passaging, such as trypsin, collagenase, pronase, or EDTA, have been unsuccessful. Not only is this an issue for NT cloning as single donor cells cannot be isolated, being unable to dissociated colonies means they cannot be used to expand cell lines or generate chimeras through cell injection or aggregation [6].

The second issue with cloning with ePSCs is that the cell cycle synchronisation method commonly used (G_2/M-phase) requires the use of a drug to arrest cells in mitosis. The most common drug is nocodazole [94] which has been shown to be the most reversible of the drugs used to synchronise cells into mitosis, dependant on concentration and length of exposure [97]. Incubation with nocodazole at high concentrations or for extended periods of time affects the ability of the cell to cleave once the drug is removed [94]. Nocodazole concentrations above 1 µM have been shown to cause complete depolymerisation of microtubules, whereas 100 nM still achieves maximal number of cells arrested while only mildly altering microtubule structure. Changes to centrosome structure and location were observed with exposure to 100 nM nocodazole, however, this was much less severe compared with higher concentrations; it is postulated that the survival of microtubules may be important to maintaining centrosome structure and function following removal of nocodazole [98]. The effect of nocodazole on microtubules and the centrosome is important in bovine NT cloning as the only centrosomes available to segregate chromosomes in the activated cytoplast are those associated with the donor cell, due to oocyte centrosomes being removed with the nucleus [60].

2.5 Research rationale and objectives

The aim of this thesis was to investigate and characterise the use of bovine ePS cells for NT cloning. The overall aim is to demonstrate that an elite livestock embryo can be converted into an ePSC colony and then into multiple ePSC-derived animals. This would enable the capture and multiplication of their genetics for accelerated animal breeding and biomedical applications.

To address the research aim, four research objectives were used for the purpose of this study. Objective 1 was to devise a method for single cell dissociation of ePSC colonies for use in NT cloning, using methodology used in mice and other bovine embryology techniques. Objective 2 was to characterise cells isolated for NT through DNA staining and immunocytochemistry for cell cycle stage and pluripotency gene expression. Objective 3 was to modify existing cloning protocols for use with ePSCs. Finally, objective 4 was to analyse cloning efficiency through blastocyst development rate and karyotyping for cell ploidy.

Chapter 3: Methods

3.1 Materials

Reagents and in-house recipes used for the purposes of this thesis are outlined in Appendix I. Solutions were prepared using Milli-Q water (18.2 MΩ·cm). Solutions containing non-sterile components were sterilised by passing through a polyethersulfone 0.2 µm pore filter membrane. Consumables were purchased sterile and all glassware autoclaved at 121°C for 15-20 min with high pressure saturated steam.

3.2 Ethics statement

All research was exempt from animal ethics approval as research was conducted on early stage bovine embryos or *in vitro* culture of bovine cell lines. This project received Environmental Protection Authority (EPA) approval (GM005/ARR003) under the New Zealand Hazardous Substances and New Organisms (HSNO) Act 1996 to develop genetically modified organisms.

3.3 Generation of ePSCs

3.3.1 *In vitro* production (IVP) of bovine blastocysts

Production of blastocysts was carried out under physical containment level 2 (PC2) conditions in the embryology laboratory at AgResearch Ltd, Ruakura, Hamilton, New Zealand. All embryo holding plates were prepared in a sterile laminar flow hood (Model CF 43/40, Gelman Sciences, Australia) at least two hours prior to use for gas levels to equilibrate in the appropriate gas conditions. All experimental research was completed on a warm stage of 35-37°C, unless otherwise stated. Wash plates were completed in 35 mm Petri dishes containing enough media to cover the base of the dish, approximately 3 ml. Information throughout IVP was recorded on an IVP sheet (Appendix II).

3.3.1.1 *In vitro* maturation (IVM) – Day -1

IVM plates were prepared on the morning of aspiration. To prepare the maturation media, cysteamine was added to a final concentration of 0.1 mM. Freshly made maturation media supplemented with cysteamine was then used to prepare IVM plates with 12x 40 µl drops of media in a 60 mm Petri dish (Appendix III, Figure III.1A). Drops were then overlaid with 8 ml of mineral oil using a stripette serological pipette and pipette aid. Plates were equilibrated in a 5% CO_2 incubator (Contherm biocell 1000 incubator, Contherm, New Zealand) at 38°C. Each IVM plate can hold up to 120 oocytes. Aspiration media, H199 + 10% FCS, and B199 + 10% FCS were placed in 5% CO_2, 38°C incubator to warm before use. The lid of B199 + 10% FCS was left loose to allow equilibration of gases to maintain pH.

Bovine ovaries were collected from abattoirs within the Waikato/Auckland regions and stored in a thermos flask with 0.9% saline at 29.5-30.5°C. Upon arrival at AgResearch, ovaries were washed three times with 30.5°C 0.9% saline to remove excess blood. Follicles between 3-8 mm were aspirated using an 18 gauge needle under vacuum of 48 mmHg (IVF Ultra Quiet VMAR-5100, Cook veterinary products, Switzerland). The needle was inserted into the follicle and moved around to dislodge the oocyte. Follicular contents were collected in 2 ml of aspiration media in a 15 ml conical tube.

Contents of aspiration tubes were searched for cumulus-oocyte complexes. Sediment from aspiration tubes was transferred to 90 mm Petri dish containing sufficient aspiration media to cover the bottom of the dish (approximately 20 ml). Using a grid, good quality oocytes were selected for evidence of a light, even colour, and were surrounded by cumulus cells. These oocytes were then transferred with a pipette in as little media as possible to a wash plate of H199 + 10% FCS. Oocytes were washed through a second H199 + 10% FCS wash plate, followed by a B199 + 10% FCS wash plate. Ten oocytes were transferred in 10 µl of B199 + 10% FCS to a single 40 µl drop of maturation media in the IVM plate. Once all oocytes were transferred to the IVM plate it was returned to 5% CO_2, 38°C incubator for 22-24 hrs to mature.

3.3.1.2 *In vitro* fertilisation (IVF) – Day 0

IVF plates were prepared on the morning prior to IVF. Freshly thawed additives were added to IVF media: 0.1 mM pyruvate, 10 mg/ml heparin, 0.2 mM penicillamine, and 0.1 mM hypotaurine in 10 ml of IVF medium. IVF plates consist of 12x 30 µl drops of IVF media in a 60 mm Petri dish, overlaid with 8 ml of mineral oil. Plates were equilibrated in a 5% CO_2 incubator at 38°C. Each IVF plate can hold up to 60 oocytes. Remaining IVF media and 10 ml HSOF were placed in 5% CO_2, 38°C incubator to warm before use. The lid of IVF media was left loose to allow equilibration.

Oocytes were removed from IVM plate using a 200 µl pipette and transferred into a wash plate of HSOF. A second HSOF wash plate was completed, and then a wash plate of IVF media. Five oocytes were transferred in 10 µl of IVF media to a single drop in the IVF plate. Once all oocytes were transferred to the IVF plate it was returned to 5% CO_2, 38°C incubator until sperm preparation was completed.

Sperm used was frozen stock of bull semen (Rotodale NHF Deacon) that requires preparation to induce capacitation and the use of a gradient that separates sperm from semen. Gradient was prepared by pipetting 1 ml of 45% percoll solution into a 15 ml conical centrifuge tube and gently underlaying this with 1 ml of 90% percoll. An interphase could be seen between percoll solutions if the gradient had been prepared correctly. A semen straw was removed from liquid nitrogen container and thawed by holding in air for 5-10 sec, followed by 30 sec in a 30-35°C water bath. The straw was dried and the ends sterilised with 70% ethanol. One end of the straw was cut with sterile scissors and then held against the side of the tube containing percoll gradient as the other end was cut, releasing the semen into the percoll gradient. The number of straws required had been previously determined as 1 straw per 100 oocytes. Sperm was centrifuged at 700 x g for 20 min at room temperature. During this time 1 ml of HSOF was added to a 15 ml centrifuge tube to warm to room temperature.

After centrifugation, supernatant was removed using a sterile Pasteur pipette. A small amount of HSOF was aspirated in a new Pasteur pipette and then used to gradually aspirate sperm pellet. Gradual mixing of sperm and HSOF prevented sperm death from dilution shock. Aspirated sperm was then gradually added to remaining HSOF and centrifuged at 200 x g for 5 min at room temperature. Supernatant was removed with a Pasteur pipette and sperm pellet gradually resuspended in 200 µl IVF media and mixed thoroughly. A 10 µl aliquot of sperm was transferred to 190 µl water for counting. Remaining sperm volume was measured using a Pasteur pipette on a 1 ml syringe and stored at 38°C.

Sperm were counted using a haemocytometer (Bürker Counting Chamber, Neubauer, Weber, UK). Preparation of haemocytometer involved cleaning with ethanol, moistening the two support shoulders, and carefully applying the coverslip with gentle pressure until neutons rings could be seen on the support shoulders. To each side of the chamber, 10 µl of sperm diluted in water was added and the 25 central squares enclosed by triple grid lines were counted under 400x magnification (Figure 3.1). Only sperm heads were counted as a single sperm can have multiple tails. Any sperm on the top and left edges of the grid were counted, whereas sperm on the bottom and right edges were discarded. Both sides of the chamber were counted and an average taken. The average sperm count was then applied to Equation 3.1 to calculate total volume of IVF media required.

Equation 3.1: Calculation of sperm dilution to 1.5 million per ml.

$$\frac{Volume\ of\ sperm\ measured\ x\ Average\ sperm\ count}{37.5} = Total\ volume\ of\ IVF\ media$$

$$Total\ volume\ of\ IVF\ media - volume\ of\ sperm\ measured = Volume\ of\ IVF\ to\ be\ added$$

Sperm was diluted by the amount calculated to a final concentration of 1.5 million sperm per ml and 10 µl of diluted sperm was added to each IVF drop containing oocytes. Motility of sperm was checked in IVF drop before plates were returned to 5% CO_2, 38°C incubator for 18-24 hrs to fertilise.

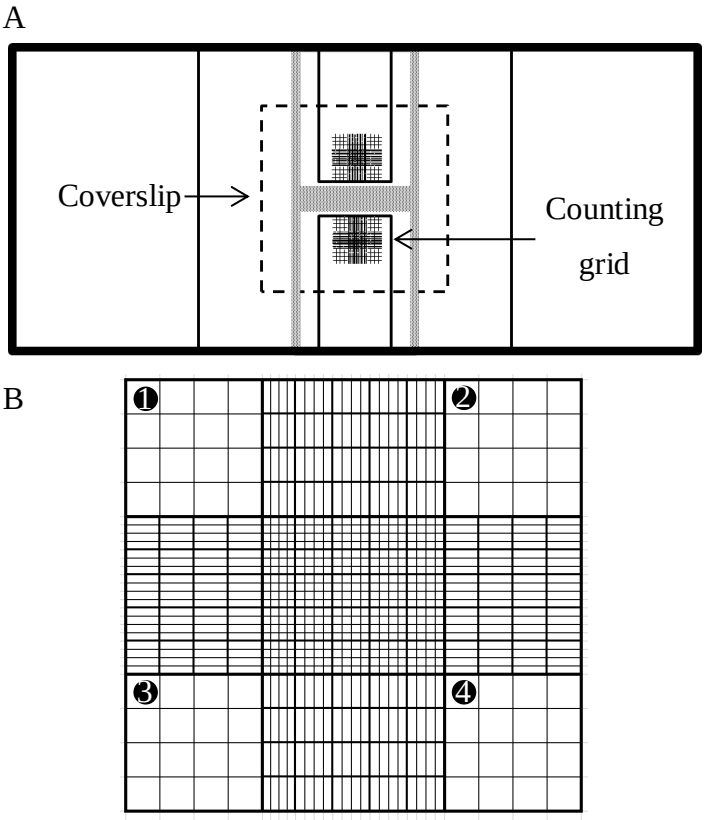

Figure 3.1: Haemocytometer layout. A) Aerial view of haemocytometer, showing counting grid and coverslip placement. B) Enlarged view of counting grid. Central grid with triple lines was used to count sperm. Squares 1-4 and central grid were used in cell culture counting. Modified from [99].

3.3.1.3 *In vitro* culture (IVC) – Day 1

IVC plates were prepared on the morning of IVC, or the evening prior. IVC plates consisted of 2x 40 µl wash drops and 6x 20 µl drops of ESOF in a 35 mm Petri dish, overlaid with 2-3 ml of mineral oil (Appendix III, Figure III.1B). Plates were equilibrated in a modular incubator chamber (QNA International Pty Ltd., Australia) within a 38°C incubator. Each IVC plate can hold up to 60 oocytes. Culturing of embryos occurs within a modular incubator chamber in order to generate anoxic conditions, similar to conditions *in vivo*. Modular incubator chambers contained a 90 mm Petri dish of Milli-Q water to provide humidity and a 35 mm Petri dish of B199 + 10% FCS to act as an indicator of pH of culture dishes. Anoxic conditions were generated using a gas mixture of 5% CO_2, 7% O_2,

and 88% N_2. Modular incubator chambers were gassed with this gas mix for 5 min, then sealed and stored in a 38°C incubator. HSOF (approximately 10 ml) was placed in 5% CO_2, 38°C incubator to warm before use.

Oocytes were removed from IVF plate using a 200 µl pipette and transferred into a wash plate of HSOF. In as little media as possible, 180 oocytes were collected and transferred to a 1.5 ml Eppendorf tube of 500 µl hyaluronidase (in HSOF) to strip the cumulus cells from the oocyte. Tube was vortexed for 2 min at 2000 rpm on minishaker (SM1 minishaker IKA®, Germany), briefly spun on mini centrifuge (Spectrafuge mini C1301, Labnet International INC., USA) for less than 10 sec, and contents returned to HSOF wash plate. Lid and sides of tube were rinsed with HSOF to collect any oocytes remaining. Oocytes were washed through two more HSOF wash plates to stop hyaluronidase activity and remove from solution. Using a finely pulled Pasteur pipette, oocytes were transferred to the first IVC plate wash drop. Oocytes were washed through the second wash drop and 10 oocytes transferred to each 20 µl culture drop. Once all oocytes were in culture drops, plates were returned to the modular incubation chamber, gassed for 5 min with 5% CO_2, 7% O_2, and 88% N_2, valves sealed, and stored in a 38°C incubator.

3.3.1.4 Change of culture medium (Change-over) – Day 5

Five days after IVF, the culture medium was changed from ESOF to LSOF. This change in media was required to maintain development, with each medium aimed to simulate *in vivo* conditions during different stages of development.

Change-over plates were prepared in the morning or evening prior. Plates consisted of 2x 40 µl wash drops and 6x 20 µl drops of LSOF in a 35 mm Petri dish, overlaid with 2-3 ml of mineral oil. Plates were equilibrated in a modular incubator chamber, gassing with 5% CO_2, 7% O_2, and 88% N_2 gas mix for 5 min, then sealed and stored in a 38°C incubator. Each plate can hold up to 60 oocytes.

Using a finely pulled Pasteur pipette, embryos were transferred to the first LSOF wash drop and washed through the second. Within the second wash drop the embryos were grouped according to development and recorded; groups were

determined by embryo development reaching compact morula, greater than 8-cell stage, less than 8 cells, 1-cell, or degraded (Appendix IV). Grouped embryos were transferred to 20 µl culture drops, 10 embryos per drop. Uncleaved embryos (degraded and 1-cell) were left in the second wash drop. Culturing embryos that have reached the same developmental stage together was performed to avoid any detrimental effects of poor developing embryos in group culture. Once all embryos were in culture drops, plates were returned to the modular incubation chamber, gassed for 5 min with 5% CO_2, 7% O_2, and 88% N_2, valves sealed, and stored in a 38°C incubator.

3.3.1.5 Grading – Day 8

Embryos were graded for development and quality (grade) eight days after IVF. Total number, developmental stage and grade of blastocysts were recorded. Grade was assessed by the criteria shown in Appendix IV. Following grading, blastocysts were processed for immunosurgery or karyotype analysis.

3.3.2 ICM cell isolation

Immunosurgery was used to isolate the ICM of high quality embryos for the generation of ePSC colonies. The activity of complement protein is used to lyse the outer TE cells, leaving the ICM cells intact. Anti-bovine antibodies bind to the outer TE layer, while being blocked from accessing the ICM by zonular tight junctions between TE cells. Complement then binds to the antibodies and causes cell lysis. Embryos were handled with pulled Pasteur pipettes with the diameter of an expanded blastocyst or the ICM. As reagents were light sensitive, all incubations were performed under tin foil to protect from light.

3.3.2.1 Preparing culture dish for ICM cells

ICM colonies were cultured on an 8-chamber glass plate that was gelatin and laminin coated. Each chamber could hold up to two colonies. On the day prior to immunosurgery, enough gelatin to cover the base of each chamber (approximately 200 µl) was added to 8-chamber glass plates as required. On the day of

immunosurgery, gelatin was aspirated and each chamber washed with 200 µl PBS. Working solution of laminin was prepared by adding 950 µl PBS to 50 µl stock 1 mg/ml laminin. To each chamber, 125 µl 50 µg/ml laminin was added and incubated for 40 min at room temperature. Laminin was aspirated, chambers washed with 200 µl PBS, and 300 µl $2i^+$ culture media added. Prepared chambers were stored in 5% CO_2, 38°C incubator until required.

3.3.2.2 Immunosurgery

Stock solutions of anti-bovine serum developed in rabbit (RαB) and guinea pig complement serum (GP) were prepared previously (Pavla Turner, AgResearch). Working solutions were generated by diluting a 50 µl aliquot of each stock solution with Embryo Hold – BSA; RαB was diluted 1:4 and GP was diluted 1:3.7. Working solutions were centrifuged at room temperature for 10 min at 268 x g and plates prepared in a 60 mm Petri dish for both RαB and GP. Plate layout consisted of a central wash drop of 40 µl with a 20 µl wash drop either side, and underneath the wash drops were 6-8x 20 µl culture drops (Appendix III, Figure III.1C). Drops were covered with 8 ml mineral oil and placed on warm stage of 35-37°C under tin foil. A universal containing 20 ml Embryo Hold – BSA with 200 µl 1 mg/ml PBS/PVA was placed in the incubator to warm before use.

Grade 1-2 expanded blastocyst selected for immunosurgery were transferred into a wash plate of Embryo Hold – BSA + PVA. Zona pellucida was removed from embryos using a 30-50 µl drop of pronase. Once the zona pellucida appeared expanded, embryos were transferred back to Embryo Hold wash plate to stop pronase digestion. Zona-free embryos were washed three times in a fresh Embryo Hold wash plate. Transferred 5-10 embryos at a time into the central 40 µl wash drop of the RαB plate in as little media as possible. Embryos were washed three times within central wash drop and again in one 20 µl wash drop. Extensive washing was necessary to remove all protein from the solution. Washed embryos were spread in a single culture drop. This was repeated until all embryos were processed, alternating between 20 µl wash drops. Embryos were incubated in RαB for 40 min on warm stage under foil.

Following RαB incubation, embryos were washed three times in a fresh Embryo Hold wash plate and again in a second wash plate. In as little media as possible, 5-10 embryos at a time were transferred into the central 40 µl wash drop of the GP plate. Embryos were washed three times within central wash drop and again in one 20 µl wash drop. Extensive washing was necessary to remove any remaining RαB or other proteins from the solution. Washed embryos were spread in a single culture drop. This was repeated until all embryos were processed, alternating between 20 µl wash drops. Embryos were incubated in GP for 15-20 min on warm stage under foil.

Between 15-20 min the lysed TE cells started to dislodge from the ICM, allowing for separation of the two cell types. Embryos were removed from GP drops and transferred to a fresh Embryo Hold wash plate. Using a finely drawn Pasteur pipette with an internal diameter of an ICM, embryos were aspirated and expelled until all TE cells were removed. A maximum of two isolated ICM colonies were added to each prepared chamber. Plates were transferred to a modular incubation chamber, gassed for 5 min with 5% CO_2, 7% O_2, and 88% N_2, valves sealed, and stored in a 38°C incubator. ICM colonies were cultured in glass chamber for four to eight days, after which they were assessed for plating efficiency and morphology before use in nuclear transfer or immunofluorescence (IF).

3.4 Cell culture

Cell culture was carried out under PC2 conditions in the tissue culture laboratory at AgResearch Ltd, Ruakura, Hamilton, New Zealand. All work was conducted in a sterile laminar flow hood (Hera guard, Heraeus, Germany) using aseptic technique. Cells were cultured in a 5% CO_2, 38°C incubator (Series II water jacketed CO_2 incubator, Therma Forma, Thermo Scientific, USA). Personal protective equipment was worn at all times when using liquid nitrogen.

Three different bovine embryonic fibroblast cell lines were used for the purpose of this research study. Initial experiments were performed using a transgenic embryonic fibroblast line used for overexpression of Nanog (Nanog2) and

embryonic fibroblast line 5 (EF5). After these tests, bovine embryonic fibroblast line 14 (BEF14) was chosen for all remaining experiments.

3.4.1 Thawing cells

Cryovials (Cryotube ™ Vials, Thermo Scientific, Denmark) containing cell stocks were stored in liquid nitrogen (Chart MVE Biological systems, USA). To thaw cells, enough liquid nitrogen was poured into a small polystyrene box to cover the base ($1/5^{th}$ full). A cryovial of desired cells was quickly collected from the box in liquid nitrogen storage with forceps, placed in polystyrene box, and transported to tissue culture lab. Cells were incubated in 38°C water bath until it was nearly thawed; only a small ice crystal remained in the cryovial. Thawed cells were added to 9 ml pre-warmed standard culture media in a 15 ml centrifuge tube and centrifuged (Biofage Primo Centrifuge, Heraeus, Germany) for 3 min at 161 x g at room temperature. Supernatant was aspirated (Air Cadet, Thermo Scientific, USA) and cells resuspended in appropriate volume of media for culture dish (Table 3.1). Cells were added to tissue culture plates that were labelled with cell name, passage number and the date, and placed in incubator.

Table 3.1: Media requirements for different sizes of tissue culture plates.

Tissue culture dish size	Seeding density (number of cells)	Volume of media (ml)
4 well plate	50,000	0.8
35 mm dish	200,000	2
60 mm dish	500,000	5
90 mm dish	1,000,000	10

3.4.2 Passaging cells

When cells were 70-90% confluent they were passaged and reseeded onto a new plate to provide space for continued growth. Culture media was aspirated and cells were washed with pre-warmed PBS. After aspiration of PBS, enough warm TrypLE to cover the bottom of the dish (approximately 1-2 ml) was added and

incubated at 38°C for 3-5 min. Cells were visualised under phase contrast microscope (Nikon TMS, Nikon, Japan) at this time to check that cells were dislodged from the surface of the dish. If some cells were still attached, the plate was tapped gently. Cells were gently transferred to a 15 ml centrifuge tube and diluted with enough pre-warmed standard culture media to a final volume of 10 ml. Tube was centrifuged at room temperature for 3 min at 161 x g, supernatant aspirated and cells resuspended in appropriate volume of media for culture dishes (Table 3.1). Cells were added to tissue culture plates that were labelled with cell name, increased passage number and the date, and then placed in the CO_2 incubator.

3.4.3 Freezing cells

The bovine cells from the isolated fibroblast lines are not immortal and, therefore, could not be cultured indefinitely. When cells were no longer needed they were frozen for future use. Before cells were processed for freezing, fresh cryoprotectant was prepared. Cryoprotectant was used to protect cells from damage when freezing and thawing. To prepare cryoprotectant, dimethyl sulfoxide (DMSO) was slowly added to FCS and mixed to make a final concentration of 20% DMSO. Cryoprotectant was used within 20 min of making. Cells were grown to 70-90% confluency, ensuring cells were in the log phase of growth which allowed them to recover quickly from freezing and continue growing. Cells were passaged and a cell count was performed before tube was centrifuged for 3 min at 161 x g at room temperature. Supernatant was aspirated and pellet resuspended in enough standard culture media to have a concentration of 2 million cells per ml. An equal volume of cryoprotectant solution was slowly added to make a final concentration of 1 million cells per ml. To each cryovial, 1 ml of cells was added and labelled with cell line, current passage number, date, and number of cells. Cryovials were transferred to a Mr Frosty^{TM} freezing container (Cryo 1°C Freezing container, Nalgen, Thermo Scientific, USA) and stored at -80°C (Forma 900 series, Thermo Fisher Scientific Inc, USA). Mr Frosty^{TM} freezing containers control the decrease in temperature to one degree per minute. This slow freezing was the optimal rate to ensure survival of cells. After 24 hrs, cryovials were stored in a box in the liquid nitrogen storage tank.

3.4.4 Cell counting

Cell counts were performed using a haemocytometer when the number of cells was needed to be known for passaging or freezing. The haemocytometer was prepared as in 3.3.1.2. Using a phase contrast microscope at 100x magnification, cells in four corners and one central square of the nine large squares were counted (Figure 3.1). Cells touching the bottom and right edge of the grid were discarded. Total number of cells counted in the five squares was used in Equation 3.2 to calculate total number of cells collected. Cell solution was then diluted as needed to produce the correct concentration.

Equation 3.2: Calculation of total number of cells present.

$$\frac{Cells\ counted}{Number\ of\ squares\ counted} \times Volume\ of\ media \times 10^4 = Number\ of\ cells$$

3.5 Nuclear transfer cloning

Production of NT cloned blastocysts was carried out under the same conditions as production of IVP blastocysts (3.3.1). All plates were prepared in a sterile laminar flow hood at least two hours prior to use for gas levels to equilibrate in the appropriate gas conditions. All manipulation was completed on a warm stage of 35-37°C. Wash plates were completed in 35 mm Petri dishes containing enough media to cover the base of the dish, approximately 3 ml. All work on the day of NT cloning was carried out using finely pulled Pasteur pipettes, unless otherwise stated. Information on the day of NT was recorded on a cloning record sheet (Appendix V) and information throughout culture was recorded on a modified IVP sheet (Appendix VI).

Abattoir-derived oocytes were aspirated and cultured in IVM plates according to the method outlined in 3.3.1.1. Oocytes needed to be matured for 22-26 hrs to be used for cloning. At this stage the oocyte genome has matured and arrested at MII, and the cytoplasm has matured to contain all factors required to reprogram the donor genome and initiate the resumption of the cell cycle.

The day prior to NT, plates were prepared for holding oocytes during the stages of NT cloning (Table 3.2). The use of mitotic cells required nocodazole to be added to solutions holding cells or couplets before fusion to ensure cells remained in mitosis. Plates were stored at 4°C overnight.

Table 3.2: Cloning plates prepared the day before NT.

Plate name	Solution	Drop configuration	Number of plates
Oocyte	H199 + 3 mg/ml BSA	12 x 30 µl	1
Cytoplast	H199 + 3 mg/ml BSA	12 x 30 µl	1
Stain	5 µg/ml Hoechst in 1 ml H199 + 3 mg/ml BSA	12 x 30 µl	1
Couplet	H199 + 3 mg/ml BSA	12 x 30 µl	1 per treatment
Couplet + nocodazole	100-1670 nM nocodazole in 2 ml H199 + 3 mg/ml BSA	12 x 30 µl	1 per treatment
Post Fusion	H199 + 3 mg/ml BSA	12 x 30 µl	1 per treatment
ESOF – Ca	ESOF – Ca + 10% FCS	12 x 30 µl	1 per treatment
ESOF Hold	ESOF	12 x 30 µl	1
ESOF	ESOF	3 x 40 µl, 30 x 5 µl	1 per 30 oocytes, per treatment

On the morning of NT, final plates were prepared (Table 3.3) and all solutions stored in incubator to warm. Fusion buffer was stored on bench to warm to room temperature. Solutions containing lectin (40 µg/ml), DMAP (2 mM), CHX (17.8 – 35.5 µM), anisomycin (10 – 100 µM), and CB (5 µg/ml) were prepared on the day of NT. ESOF plates were equilibrated in modular incubator chamber. ESOF Hold, ESOF – Ca, DMAP, CHX (+/- CB), and anisomycin (+/- CB) plates were equilibrated in 38°C 5% CO_2 incubator. All remaining plates were stored on warm stage.

Table 3.3: Plates to be made on the day of NT.

Plate name	Solution	Drop configuration	Number of plates
Lectin	40 µg/ml lectin in 2 ml H199 + 3 mg/ml BSA	12 x 30 µl	1 per treatment
Lectin + nocodazole	40 µg/ml lectin, 100-1670 nM nocodazole in 2 ml H199 + 3 mg/ml BSA	12 x 30 µl	1 per treatment
Pronase	0.125% pronase in 100 µl H199 + 0.5% FCS + 100-1670 nM nocodazole	4 x 20 µl	1
Dissociation	Dissociation media	12 x 30 µl	1 per treatment
Dissociation + nocodazole	100-1670 nM nocodazole in 1 ml Dissociation media	12 x 30 µl	1 per treatment
DMAP	2 mM DMAP in 2 ml ESOF	3 x 40 µl, 30 x 5 µl	1 per 30 oocytes, per treatment
CHX	17.8-35.5 µM CHX in 2 ml ESOF	3 x 40 µl, 30 x 5 µl	1 per 30 oocytes, per treatment
Anisomycin	10-100 µM Anisomycin in 2 ml ESOF	3 x 40 µl, 30 x 5 µl	1 per 30 oocytes, per treatment
For parthenogenote controls			
DMAP	2 mM DMAP in 2 ml ESOF	3 x 40 µl, 30 x 5 µl	1 per 30 oocytes
CHX + CB	17.8-35.5 µM CHX, 5 µg/ml CB in 2 ml ESOF	3 x 40 µl, 30 x 5 µl	1 per 30 oocytes
Anisomycin + CB	10-100 µM Anisomycin, 5 µg/ml CB in 2 ml ESOF	3 x 40 µl, 30 x 5 µl	1 per 30 oocytes

3.5.1 Oocyte preparation

Matured oocytes (18-20 hrs post IVM) were removed from IVM plate using a 200 µl pipette and transferred into a wash plate of H199 + 10% FCS. In as little media as possible, 180 oocytes were collected and transferred to a 1.5 ml Eppendorf tube of 500 µl 0.1% hyaluronidase (in H199) to strip the cumulus cells from the oocyte. Tube was vortexed for 2 min at 1800-2000 rpm on minishaker, briefly spun on mini centrifuge for less than 10 sec, and contents returned to H199 + 10% FCS wash plate. Lid and sides of tube were rinsed with H199 + 10% FCS to collect any oocytes remaining. Oocytes were washed through another H199 + 10% FCS wash plate and one H199 + 3 mg/ml BSA to stop hyaluronidase activity and remove from solution.

Oocytes were then examined for the presence of a polar body, indicating they had matured through to MII. This involved rotating the oocyte with a finely pulled Pasteur pipette, to enable all sides of the oocyte to be examined for presence of a polar body. Oocytes with a polar body were pronased for 1-2 min, 50 oocytes at a time, to remove the zona pellucida. After washing in H199 + 3 mg/ml BSA to remove residual pronase, oocytes were stored in 'Oocyte' plate to return to their normal shape before enucleation. A number of oocytes were set aside to be activated as PGs, as a positive control for activation.

3.5.2 Oocyte enucleation

3.5.2.1 Production of enucleation tools

Two specialised tools were required for enucleation: a blunt aspiration pipette and a separation needle. Tools were made prior to the day of NT by pulling borosilicate capillaries (GC100T-15, Harvard Apparatus Ltd, UK) with a horizontal puller (P-87, Sutter Instruments, USA). Blunt aspiration pipette was formed by the pulled capillary being cut at an outer diameter of approximately 24 µm using a microforge (MP-9, Narishige, Japan) and bent at a 30° angle at the point where the capillaries diameter was 80 µm thick. Separation needle had a wider outer diameter of 100-150 µm. The end of the glass piece was melted closed and a 30° angle was introduced.

3.5.2.2 Enucleation

Oocytes were transferred 40 at a time to 'Stain' plate and incubated for 5 min. 'Stain' plate contained Hoechst which stained the DNA, enabling it to be visualised under fluorescent light so it could be removed. Stained oocytes were then washed briefly in H199 + 3 mg/ml BSA before transferring to the lid of a 10 cm Petri dish containing a drop of H199 + 10% FCS under mineral oil. This plate was kept on the warm stage of the micromanipulation microscope (MO-188, Nikon Narishige, Japan). Enucleation was performed using three-axis oil hydraulic hanging joystick micromanipulators and a 0.2 mL Gilmont® micrometer syringe (Cole-Parmer Instruments, USA) to control the aspiration pipette and separation needle. Metaphase plate was visualised using UV-light exposure with the diaphragm closed as much as possible to minimise UV exposure to the oocyte. Under 100x magnification, metaphase plate was aspirated into the separation needle and remaining cytoplast pushed out of UV-light. Enucleated cytoplasts were stored in 'Cytoplast' plate.

3.5.3 Cell preparation

3.5.3.1 Somatic cells

A mitotic shake-off method was used to isolate mitotic somatic cells for nuclear transfer. Cells were passaged 1-2 days before NT at a seeding density outlined in Table 3.1. On the day of NT, cells were washed once with pre-warmed PBS and cultured for 1 hr in standard culture media with 100 or 1670 nM nocodazole. Presence of mitotic cells was assessed using phase contrast microscope before shake-off was performed.

Media was gently removed and replaced with H199 + 0.5% FCS with 100-1670 nM nocodazole. The volume of H199 media added was half the volume used for culturing (outlined in Table 3.1) to ensure no media was spilled during shake-off. Mitotic cells were dislodged by gently tapping the culture dish two times, and success of shake-off was determined by visualising under phase contrast microscope. Media containing dislodged mitotic cells was transferred into a 15 ml

centrifuge tube and centrifuged for 3 min at 161 x g at room temperature. Supernatant was removed and cells resuspended in 1-2 ml H199 + 0.5% FCS with 100-1670 nM nocodazole. Into a 60 mm Petri dish, 6 x 30 µl drops of mitotic cells were overlaid with warm mineral oil.

3.5.3.2 ePS cells

ePSCs were cultured for four to eight days before use in NT. On the day prior to NT, colonies were graded and ones to be used in NT were chosen. Media from these colonies was aspirated and replaced with $2i^+$ culture media containing 100, 500, 1000 or 1670 nM nocodazole. Colonies were incubated for 4 hrs or overnight (18-22 hrs) in modular incubator chamber to arrest cells in mitosis. On the day of NT, cells were isolated into single cells using mechanical or chemical dissociation.

Mechanical dissociation

Cells were isolated by either shake-off or manual separation. Shake-off used a similar technique as somatic cells, where the chamber was gently tapped to dislodge only mitotic cells. Manual separation was performed using a finely pulled Pasteur pipette and triturating colonies until cells had dislodged.

Chemical and enzymatic dissociation

Media was aspirated from chamber and replaced with 200 µl of pre-warmed 0.5 mg/ml dispase. After 1-2 min colonies dislodged from chamber and were transferred to 'Dissociation' plate containing dissociation media with or without CB, or into drops of culture media. Alternatively, no dispase was used, and instead dissociation media was added straight to aspirated chamber. Cells were dissociated into single cell suspension by triturating colonies through a mouth pipette with increasingly smaller diameters.

3.5.4 Attachment of donor cell to cytoplast

Lectin was used to attach donor cells to the outside of the cytoplast. This kept the cells attached until ready for fusion. Before sticking, one group of cells were pronase treated to test fusion efficiency. Cells were transferred to a single drop in 'Pronase' plate and incubated for 5 min.

Prepared donor cells were transferred to ‘Lectin’ plate. Into an empty lectin drop, 5-10 cytoplasts were transferred and spread to avoid cytoplasts sticking. A single cytoplast was moved into a lectin drop containing donor cells and pushed against an appropriate donor cell to attach. Mitotic cells were chosen based on morphology; the largest cells were used as donor cells as they were most likely to be in mitosis. Cytoplast/donor cell couplets were incubated in original lectin drop for approximately 5 min. After washing in H199 + 3 mg/ml BSA to remove residual lectin, 10 couplets were spread in a single drop in the ‘Couplet’ plate, ensuring couplets were not touching.

3.5.5 Fusion of couplets

Donor cells were electrically stimulated to fuse to cytoplasts. The use of a custom-made parallel-plate fusion chamber attached to an electro cell manipulator (BTX ECM 200, Biotechnologies and Experimental Research Inc, USA) allowed simultaneous fusion of many couplets. From the ‘Couplet’ plate, 10-20 couplets were transferred into a wash plate of fusion buffer (either hypoosmolar or isoosmolar) to equilibrate. Couplets were checked that a single donor cell was still attached and had not lysed. Up to 10 couplets were transferred into the 3mm gap between electrodes in the fusion chamber, which contained the same fusion buffer as the wash plate. An alternating current (AC) of amplitude 21.2 was used to automatically align couplets to be perpendicular to electrodes (Figure 3.2). Any couplets not automatically aligned were removed and manually aligned in the following fusion. Aligned couplets were fused using two direct current (DC) pulses for 10 µsec at an amplitude of 1.5 kV/cm for small ePSC donors or 2.0 kV/cm for large ePSC and somatic donor cells. Fused couplets were transferred back into fusion buffer wash plate until all 10-20 couplets had been processed, and were then transferred to the ‘Post Fusion’ plate. These steps were repeated until all couplets had been exposed to the fusion pulse.

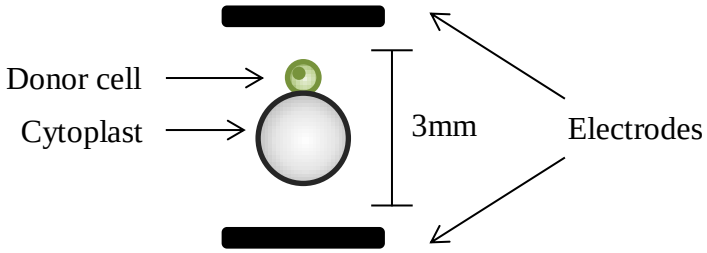

Figure 3.2: Alignment of couplet with fusion plates. Donor cell needed to lie between cytoplast and electrode for fusion to occur. Modified from [5].

Between 30-60 min post-fusion, couplets were scored for fusion by detecting cell lysis, detachment of the donor cell, or presence of an unfused donor cell still attached to the cytoplast. Fused couplets (reconstructs) were washed through HSOF – Ca + 10% FCS and transferred into the first row of drops in 'ESOF – Ca' plate, 10-20 reconstructs per drop. Reconstructs were washed through one drop in the top row, one in the middle row, and finally spread in a drop in the final row. 'ESOF – Ca' plates were incubated in a 5% CO_2 incubator at 38°C for maximum of 1 hr post-fusion.

3.5.6 Activation of fused reconstructs

Artificial activation of the cytoplast to resume mitosis was achieved by using the calcium ionophore ionomycin, approximately one hour post-fusion. Reconstructs and PGs were incubated in HSOF + 1 mg/ml BSA for 5-30 min before activation, one 35 mm dish per treatment. Using a 20 µl pipette, reconstructs and PGs were then transferred to fresh 5 µM ionomycin solution (5 µl ionomycin in 5 ml HSOF + 1 mg/ml BSA) in a 35 mm dish and incubated for 4.5 min. Ionomycin solution was prepared and used within 10 min. Reconstructs and PGs were washed for 3 min in HSOF + 30 mg/ml BSA in a 35 mm dish to remove ionomycin.

After washing, reconstructs were incubated in an inhibitor of phosphorylation or protein synthesis. This was to prevent reformation of cytoplasmic factors that would inhibit the resumption of mitosis. Using a finely pulled mouth pipette, reconstructs and PGs were transferred to plates as outlined below.

Standard process

Nocodazole-arrested reconstructs were cultured in 6-DMAP, anisomycin, or CHX for 4, 5, or 6 hrs, respectively. PGs were cultured in 6-DMAP, CHX + CB, or anisomycin + CB. The addition of CB is necessary to prevent the expulsion of a second polar body, which would result in the embryo being haploid. After washing thoroughly through the 3x 40 µl wash drops, reconstructs and PGs were cultured singularly, one per 5 µl drop. After incubation period, reconstructs cultured in CHX and anisomycin were scored for pseudo-polar body extrusion. DMAP prevents polar body extrusion, so reconstructs were not assessed.

Preactivation

Cytoplasts were activated with ionomycin and cultured in DMAP for 1 hr before attachment of donor cells. In this situation, cytoplasts were kept in the presence of DMAP throughout cell attachment and fusion. After fusion was scored, reconstructs were moved to standard DMAP culture dishes. Reconstructs were cultured for a total of 4 hrs in DMAP.

Pseudo-polar body extrusion

After washing in HSOF + 30 mg/ml BSA, reconstructs and PGs were transferred to a 30 µl drop in 'ESOF Hold' plate. A control group of reconstructs and PGs was then transferred straight to a 'DMAP' plate and handled according to the standard process. For remaining reconstructs and PGs, the presence of a second pseudo-polar body was assessed every 15 min in initial trials, every 30 min after expected time frame had been established. After 1, 1.5, or 2 hrs, reconstructs and PGs with a pseudo-polar body were transferred to DMAP, washed thoroughly through the 3x 40 µl wash drops, and cultured singularly in 5 µl drops. After 2 hrs all remaining reconstructs and PGs were transferred to DMAP culture. Total time cultured in DMAP was 4 hrs.

3.5.7 Embryo culture

After activation was complete, reconstructs and PGs were cultured in the same manner as in 3.3.1.3 and 3.3.1.4. However, single culture was used instead of group culture (Appendix III, Figure III.1D). Reconstructs and PGs were washed through two dishes of HSOF, moving around three times within each dish. Reconstructs and PGs were transferred to the first 40 µl wash drop of ESOF, and washed through the next two before being transferred to individual culture drops. Once all reconstructs and PGs were in culture drops, plates were returned to the modular incubation chamber, gassed for 5 min with 5% CO_2, 7% O_2, and 88% N_2, valves sealed, and stored in a 38°C incubator.

After 5 days in ESOF, embryos were transferred to LSOF culture. To prevent precipitation of media components, LSOF media was gassed for 5-10 min with 5% CO_2, 7% O_2, and 88% N_2 before preparing single culture LSOF plates. Using a finely pulled Pasteur pipette, embryos were transferred to the first LSOF wash drop and washed through the next two. Within the last wash drop the embryos were grouped according to development and recorded; groups were based on embryo development reaching compact morula, greater than 8-cell stage, less than 8 cells, 1-cell, or degraded (Appendix IV). Embryos were transferred to single culture drops in decreasing developmental grade. Once all embryos were in culture drops, plates were returned to the modular incubation chamber, gassed for 5 min with 5% CO_2, 7% O_2, and 88% N_2, valves sealed, and stored in a 38°C incubator.

Embryos were assessed for development and grade on day seven and eight after NT. Total number, developmental stage and grade of blastocysts were recorded. Grade was assessed by the criteria shown in Appendix IV. Following grading, blastocysts were processed for karyotype analysis.

3.6 Analysis of cells and embryos

3.6.1 DNA Staining

Nuclear DNA was stained with Hoechst to assess the degree of condensation as a marker of cells being in metaphase of mitosis. Cells and embryos were incubated in their current media with 5 µg/ml Hoechst 33342 for 5 min. Cells and embryos were analysed on EVOS microscope (Advanced microscopy group, Millenium Science, Australia). Alternatively, resuspended cells were deposited onto a microscope slide using the Cytospin®3 (Thermo Shandon, USA). Briefly, a Cytoclip was loaded with a 25 x 75 mm microscope slide (LabServ, Thermo Scientific, USA) pre-cleaned with ethanol, followed by a Cytofunnel with filter card attached, and spring clipped into place. The Cytofunnel was loaded with 100-500 µl of a suspended cell sample at a concentration of 1 million cells per ml. The Cytoclip was loaded into the Cytospin, centrifuged at room temperature for 3 min at 112.9 x g, and the Cytoclip was then unclipped and Cytofunnel was carefully removed as not to disturb cells on the slide. Finally, the cells were stored at room temperature in slide folder to protect from light until imaged.

3.6.2 Immunofluorescence

3.6.2.1 Standard protocol

Immunofluorescence (IF) was used to visualise the localisation of a protein within a cell. This is used by attaching fluorescent probes to antibodies that have been bound to the protein of interest. Analysis of ePSCs was performed on colonies within the 8-chamber glass plate. Volumes used in all steps were enough to cover the base, approximately 100-150 µl per chamber. Incubations were performed at room temperature unless otherwise stated. Antibodies used are outlined in Table 3.4 and Table 3.5. Target species of primary antibodies varied, however, all had been validated for use with bovine proteins.

Table 3.4: Primary antibodies used in IF.

Protein antibody	Host	Cloniality	Dilution required	Manufacturer
Ki67 (mitosis marker)	Rabbit	Polyclonal	1/200	Abcam #15580
Nanog (pluripotency marker)	Mouse	Monoclonal	1/100	eBiosciences #14-5768
Phosphorylated Histone 3 (H3) (mitosis marker)	Rabbit	Polyclonal	1/100	Upstate #06-570
Sox2 (pluripotency marker)	Goat	Polyclonal	1/30	R & D Systems #AF2018

Table 3.5: Secondary antibodies used in IF.

Antibody	Host	Reactivity	Fluorescent emission (nm)	Dilution required	Life Technologies number
DαG 568	Donkey	Goat	568	1/1000	A-11057
DαM 568	Donkey	Mouse	568	1/1000	A-10037
DαRab 488	Donkey	Rabbit	488	1/1000	A-21206
GαRab 568	Goat	Rabbit	568	1/1000	A-11036

ePSCs were cultured for four to six days before use in IF. On the day prior to IF, half the colonies to be analysed were treated with 500 nM or 1.67 µM nocodazole. The remaining samples were treated with DMSO, at the same concentration and length of time, to act as a vehicle control. Media from these colonies was aspirated and replaced with $2i^+$ culture media containing nocodazole or DMSO. Colonies were incubated overnight in modular incubator chamber at 38°C.

Approximately 18 hrs after initiation of nocodazole arrest, media was aspirated from chamber and replaced with 4% paraformaldehyde (PFA) that had been freshly depolymerised by incubating at 65°C for 5 min. PFA was incubated on

cells for 15 min to fix cells, preventing degradation of proteins. Cells were washed twice with PBS, followed by quenching with 50 mM NH_4Cl for 10 min. These washing steps removed any remaining PFA so it would not affect following reactions. NH_4Cl was removed and cells washed once with PBS. Permeability of cells was increased by incubation in 0.1% Triton X-100 for 10 min. Cells were washed twice with PBS. To prevent false positive stains, cells were blocked in a 5% serum of either donkey or goat. The serum used was determined by the species that the secondary antibody was raised (Table 3.5). Blocking was completed for 60 min at room temperature.

Cells were incubated with the primary antibody for the protein of interest (single stain) or for two proteins of interest (double stain). If a double stain was being used, each primary antibody was raised in a different species to allow for specific binding of secondary antibodies. Primary antibodies were diluted according to Table 3.4 in the serum used for blocking. One ePSC colony was incubated in blocking solution alone (No primary antibody; no 1° antibody). This acted as a control for unspecific binding of secondary antibodies, as no primary antibody was present, enabling the determination of background fluorescence levels. Cells were incubated with primary antibodies overnight at 4°C, or 60 min at room temperature.

On the following day, cells were washed three times with PBS to remove unbound primary antibody. Secondary antibodies were diluted in blocking solution (Table 3.5), along with 5 µg/ml Hoechst to stain DNA, and applied to cells. All subsequent steps were completed under tin foil to protect light sensitive fluorescent probes. Cells were incubated with secondary antibodies for 30 min at 37°C. Three PBS washes were used to remove any unbound secondary antibody, followed by a short wash in distilled water.

Excess water was aspirated before mounting cells. Using the tool supplied with chambers, the plastic walls were dislodged from the glass plate. A 5-10 µl drop of diamond antifade mounting media was added to each well of the chamber and a 22 x 50 mm glass coverslip (LabServ, Thermo Scientific, USA) applied. Air

bubbles were pushed out to the edges of the coverslip. Slides were stored at 4°C in a slide folder to protect from light.

3.6.2.2 5-ethynyl-2'-deoxyuridine (EdU) staining

Analysis of the proliferating fraction of a cell population was achieved by using 5-ethynyl-2'-deoxyuridine (EdU) staining. Cells that are actively synthesising DNA will incorporate the thymidine analogue, EdU, and can be visualised by binding a fluorescent probe. The Click-iT® EdU Imaging Kit (Invitrogen, Life Technologies, USA) was used to stain cells for DNA synthesis. Some components of the kit were missing, so were substituted for house-made versions (Appendix I). Incubations were completed at room temperature and volume of 100 µl, unless otherwise stated.

ePSCs were prepared as per standard IF protocol (3.6.2.1) by nocodazole arrest or DMSO control. Approximately 18 hrs post nocodazole arrest, 20 µM EdU (supplied in kit at stock solution 10 mM) was added to each well and incubated in modular incubator chamber at 38°C for 30 min. Cells were then fixed with freshly depolymerised 4% PFA for 15 min. Cells were washed three times with PBS, followed by quenching with 50 mM NH_4Cl for 10 min. Cells were then washed three times with PBS followed by incubation in 0.5% Triton X-100 for 20 min.

During Triton incubation, the Click-iT® reaction cocktail was prepared. Additives required to produce 1 ml of cocktail are listed in the order added (Table 3.6). Addition of components in the correct order was important for optimal reaction efficiency. Cocktail was used within 15 min of preparing.

Table 3.6: Click-iT® reaction cocktail components.

Reaction Component	Volume
100 mM Tris buffered saline pH 7.4	860 µl
100 mM $CuSO_4$	40 µl
Alexa Fluor® azide (diluted in DMSO)	2.5 µl
10X Click-iT® reaction buffer additive	10 µl
Milli-Q water	90 µl
Total	**1 ml**

Cells were washed three times with PBS before addition of Click-iT® reaction cocktail to each well. Reaction cocktail was incubated for 30 min under tin foil to protect light sensitive fluorescent probe. All subsequent steps were completed under tin foil. Cells were washed once with PBS. Cells were either processed through standard IF (for double stain) or DNA was stained using Hoechst diluted in blocking solution for 30 min. Three PBS washes were used to remove excess Hoechst, followed by a short wash in distilled water. Slide was mounted as per standard IF method as outline in 3.6.2.1.

3.6.3 Karyotyping

On day 7 or 8 after IVF or NT, embryos were processed for karyotyping. Mitotic chromosomal spreads were analysed to assess ploidy of the embryo. Embryos were handled with pulled Pasteur pipettes with the diameter of an expanded blastocyst.

Embryos were synchronised into mitosis using 1.67 µM nocodazole in LSOF for 3-4 hrs in modular incubator chamber at 38°C. After nocodazole arrest, PG blastocysts were manipulated to compare ploidy of TE and ICM cells. Blastocysts were bisected to isolate TE cells or processed through immunosurgery to isolate ICM cells. These samples were then handled the same as whole embryos. Arrested embryos were transferred to 500 µl 0.9% sodium citrate with 0.1 mg/ml PBS/PVA in a 4 well plate. Sodium citrate is hypotonic, which caused the cells to swell and burst upon spreading. Embryos were incubated at 4°C for a minimum of

20 min. Swollen embryos were prone to sticking to the inside of Pasteur pipette. To prevent this, pipettes were coated with dimethylpolysiloxane by drawing enough solution into the pipette to coat the finely pulled barrel and incubating at 60°C for 1 hr. Solution was expelled before use.

Fresh 3:2:1 fixative was prepared into a 15 ml conical tube and stored at -20°C for at least 1 hr. After sodium citrate incubation, fixative was dispensed into three 35 mm dishes and kept on ice. Using a microscope with a cold stage, an embryo was transferred to fixative for 2-3 min with coated pipette. Adequate time in fixative was assessed by embryos becoming more transparent and moving out of the field of view. Pipette barrel was filled with fixative and embryo was transferred into pipette with as small amount of fixative as possible. Pipette was touched onto microscope slide pre-cleaned with ethanol; the embryo and small amount of fixative transferred to slide due to capillary action. This was repeated until all embryos were spread. Dishes were rotated periodically to keep fixative ice-cold.

Slides were allowed to dry for 2 hrs on warm stage, or in a dust-free environment until staining. Chromosomes were stained with the DNA dye Giemsa. A fresh 5% Giemsa stain was prepared in Gurr buffer (pH 6.8). Slides were stained for 15 min then rinsed gently with tap water until water ran clear. Slides were allowed to air dry before analysing.

3.6.4 Imaging of IF and karyotypes

DNA staining of colonies and reconstructs was viewed on EVOS microscope. Images were captured at 100-200x magnification under phase contrast or brightfield and 405nm wavelength for Hoechst staining. The exposure time required for image capture was determined upon observation of first positive staining, comparing to the negative control, and kept constant for all images in that run.

IF staining and karyotypes were visualised on the Olympus fluorescent microscope (BX50 Olympus, Japan). Images were captured with Spot RT3-slider

camera and processed with SpotBasic software (Spot imaging solutions, USA). Standard IF stains were visualised at 100-400x magnification. Images were taken in black and white, while EdU stain was captured with colour images due to high background fluorescence. Images were taken at the wavelength specific to the secondary antibody: 405, 488, or 564. The exposure time required for each channel was determined upon observation of first positive staining, in comparison to the negative control. Exposure times were then kept constant for all images in that run. Black and white images were pseudo-coloured using ImageJ and background subtracted to remove background fluorescence. Karyotypes were visualised under brightfield light at 1000x magnification under oil immersion. Images were captured in black and white and processed using SmartType 3 (Digital Scientific UK).

3.7 Statistical analysis

Microsoft Excel (2010) was used to analyse data. Minimal time required for maximal pseudo-polar body extrusion data was fit with a polynomial trendline. Development on day 7 and 8 was expressed as a percentage of embryos placed into IVC. Dot plots had a 'jitter' function (± 0 - 0.15) added to prevent data points overlapping. Error was calculated using standard error of the mean (SEM). Significance was determined using Fisher's exact test of independence or Student's t-test. Fisher's exact test is used to compare proportions and is more accurate than other statistical tests when observations are small. Student's t-test is used to compare the means of a nominal variable. The null hypothesis for both tests is that the means are independent, and therefore equal, for the two treatments [100]. Fisher's exact test of independence was used to determine significance of blastocyst development as overall development numbers were expected to be low. Student's t-test was used to determine significance of ePSC colony characterisation (DNA staining and IF) as it was more appropriate due to the large number of cells in a colony. Significance was defined as *P* value less than 0.05 and highly significant as less than 0.01.

Chapter 4: Results

4.1 Objective 1: Isolation of single ePSCs

4.1.1 Generation of ePSC colonies

4.1.1.1 IVP development

IVP was used to generate blastocysts for ICM isolation (*n*=16; Figure 4.1). Development greater than 1-cell stage (cleavage rate) of *in vitro* fertilised oocytes was 78 ± 3%. On day 8, the total number of blastocysts developed from oocytes that were selected at IVC was 38 ± 2%. Blastocyst quality was further investigated by identifying high grade blastocysts (grade 1-2) as these are of transferable quality. Development of high grade blastocysts was 16 ± 1%.

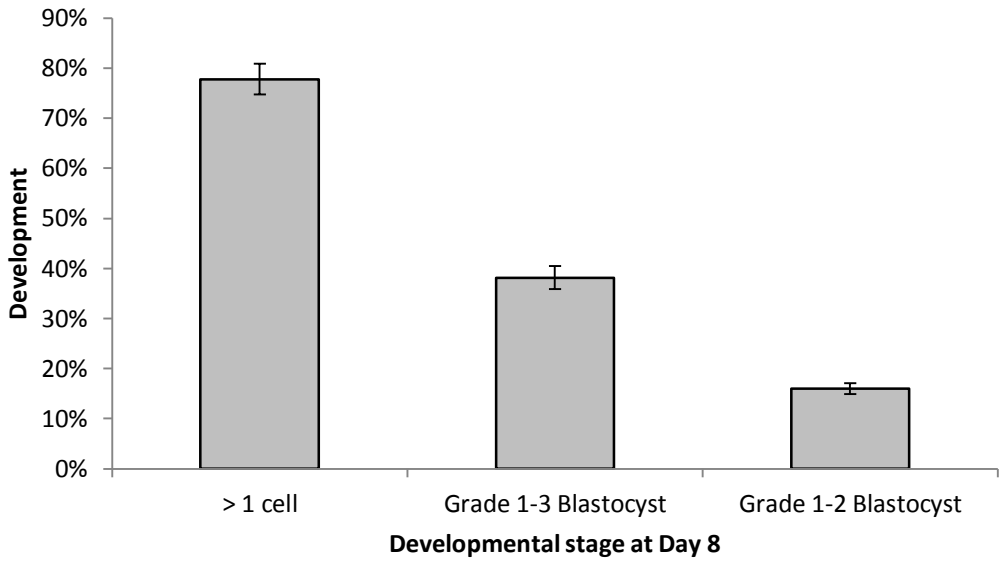

Figure 4.1: Efficiency of IVP. Development of >1 cell indicates number of IVF embryos that cleaved. Grade 1-3 development captures all blastocysts that had developed, whereas grade 1-2 isolates high grade blastocysts that are of transferrable quality. Error bars = SEM, replicates *n*=16, oocytes into IVC *N*=2331.

4.1.1.2 Isolating ICM colonies for culture

The efficiency of immunosurgery at isolating ICM cells and their attachment to the substrate was measured. Variability of immunosurgery efficiency was greatest at the start, becoming more consistent as technique was improved. Analysis was performed on the final five experiments as the technique was fully proficient (*N*=105 blastocysts). On average, 93 ± 5% of ICMs were isolated from the blastocyst and transferred to culture (Figure 4.2). Once in culture, 94 ± 2% of ICMs attached to the substrate (plating efficiency). Overall efficiency of using immunosurgery to generate ePSC colonies was 86 ± 6%.

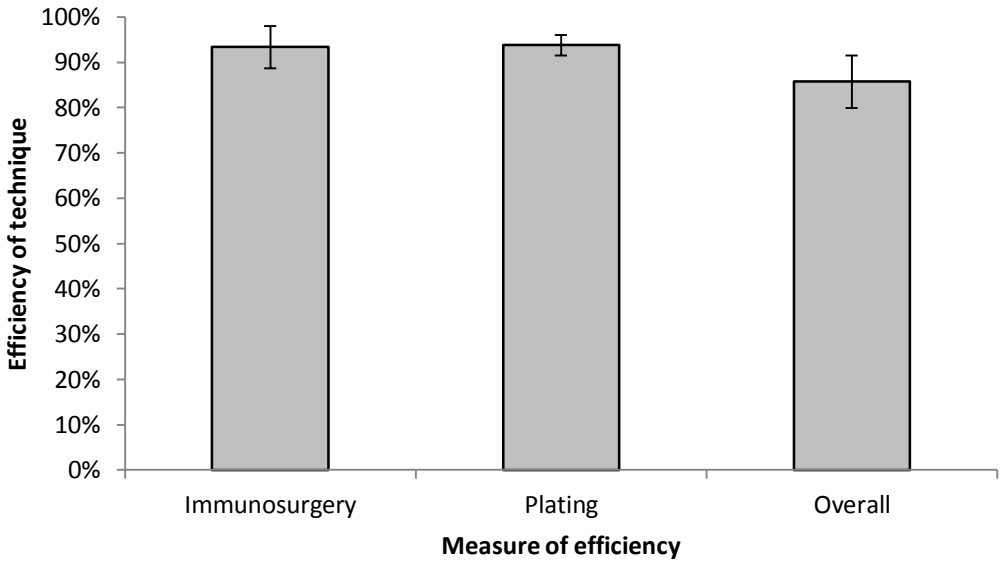

Figure 4.2: Efficiency of using immunosurgery to generate ePSC colonies. Efficiency was measured of isolating ICM cells from grade 1-2 blastocysts, plating efficiency of ICM cells to substrate of chambers in $2i^+$ media, and net yield of ePSC colonies from blastocysts. Error bars = SEM, replicates *n*=5, blastocysts processed *N*=105.

4.1.2 Dissociation of single ePSCs

Mechanical, chemical, and enzymatic methods were trialled to isolate single cells from ePSC colonies. Mechanical methods proved to be unsuccessful at separating single cells from the colony. Using manual shake-off, no single cells were dislodged from the colony and colonies remained attached to the substrate. Manual separation by trituration with a small mouth pipette detached a small number of whole colonies from the substrate, however, no single cells were isolated.

Chemical assisted dissociation methods allowed the separation of single cells. Addition of Ca-Mg-free dissociation media containing EDTA permitted the separation of few cells from the outside of the colony, with the majority of the colony remaining attached. Incubation in dispase for five minutes detached all colonies from the substrate. Single cells were not able to be separated when colonies were returned to $2i^+$ culture media. However, if detached colonies were transferred into pronase, followed by dissociation media, single cell isolation was achieved with a considerable amount of mechanical trituration of the colony. A reduction in the amount of manipulation required was achieved by addition of CB to dissociation media. Therefore, optimised conditions for generating single ePSCs was incubation with dispase for five minutes, pronase for five minutes, and triturated in dissociation media with CB.

4.2 Objective 2: Characterisation of ePSC donor cells

4.2.1 Length of nocodazole arrest for synchronisation into M-phase

ePSC colonies were exposed to 1.67 µM nocodazole for 4 and 21 hrs to optimise the length of nocodazole arrest to generate sufficient mitotic donor cells for NT. Cells in mitosis were assessed by Hoechst stain to identify condensed nuclei (Figure 4.3). Nocodazole-arrested colonies were compared with non-arrested colonies incubated with DMSO (vehicle control). Two colonies were assessed per treatment, with an average of 114 nuclei counted per colony (44-193 nuclei per colony, total cells counted N=681). No difference was observed between DMSO control and colonies arrested with nocodazole for 4 hrs (8 ± 9% and 12 ± 5%, respectively; Figure 4.4). By comparison, incubation with nocodazole for 21 hrs arrested nearly half the colony in mitosis (49 ± 1%; $P < 0.05$).

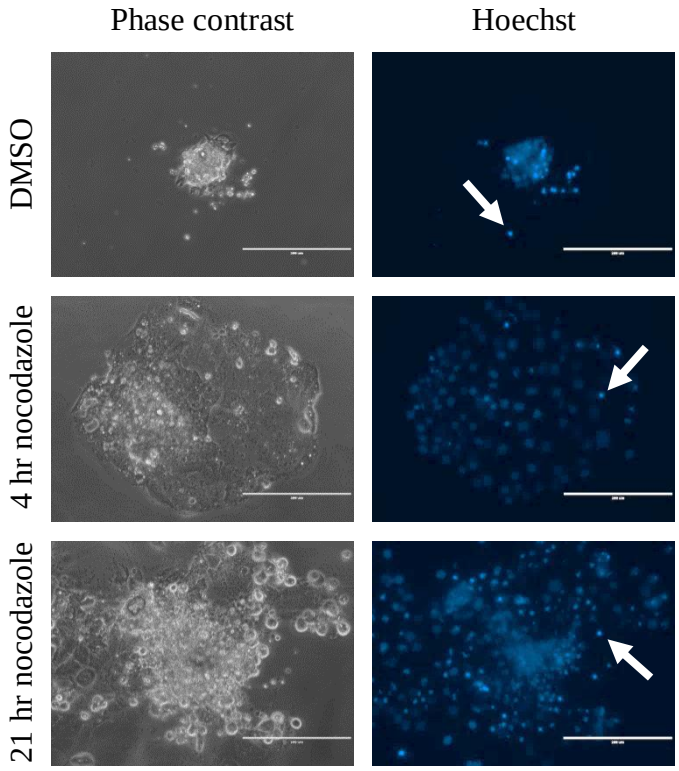

Figure 4.3: ePSC colonies treated with DMSO for 21 hrs, or 1.67 µM nocodazole for 4 hrs or 21 hrs. Arrows indicate condensed nuclei. Scale bar = 200 µm. Images taken on EVOS microscope under phase contrast and Hoechst stain visualised with 405 nm UV light.

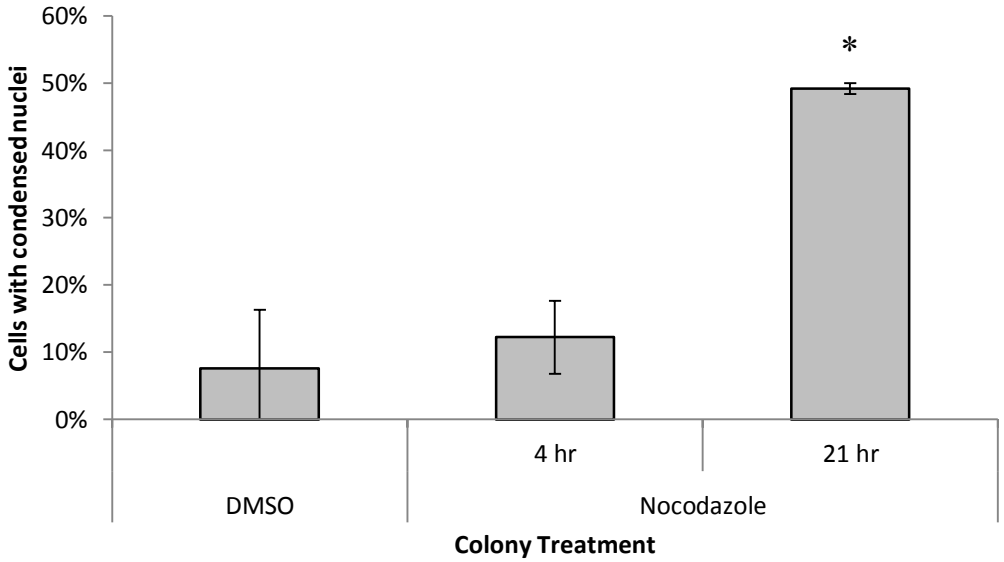

Figure 4.4: Average condensed nuclei in ePSC colonies after DMSO and nocodazole treatment. Significance * = $P < 0.05$ compared with DMSO. Error bars = SEM, colonies counted per treatment N=2, total nuclei counted N=681.

4.2.2 Nocodazole dose-response curve for M-phase arrest

To find the minimum effective concentration of nocodazole, four concentrations were tested over a range of 100-1670 nM. Colonies were arrested for 24 hrs prior to being stained with Hoechst (Figure 4.5). A minimum of two colonies were counted per treatment, with an average of 144 nuclei counted per colony (56-305 nuclei per colony, total cells counted N=1724). Compared with 100 nM (28 ± 3%; colonies N=4), there was a higher number of cells arrested with 500 nM nocodazole (41 ± 6%, $P < 0.05$; colonies N=2) and 1 µM nocodazole (45 ± 2%, $P < 0.01$; colonies N=3; Figure 4.6). Incubation with the standard concentration used within the laboratory of 1670 nM nocodazole produced a higher proportion of cells arrested (37 ± 5%; colonies N=3), but this was not significant compared with 100nM ($P = 0.08$). No significant difference was observed between 500, 1000, and 1670 nM nocodazole arrest.

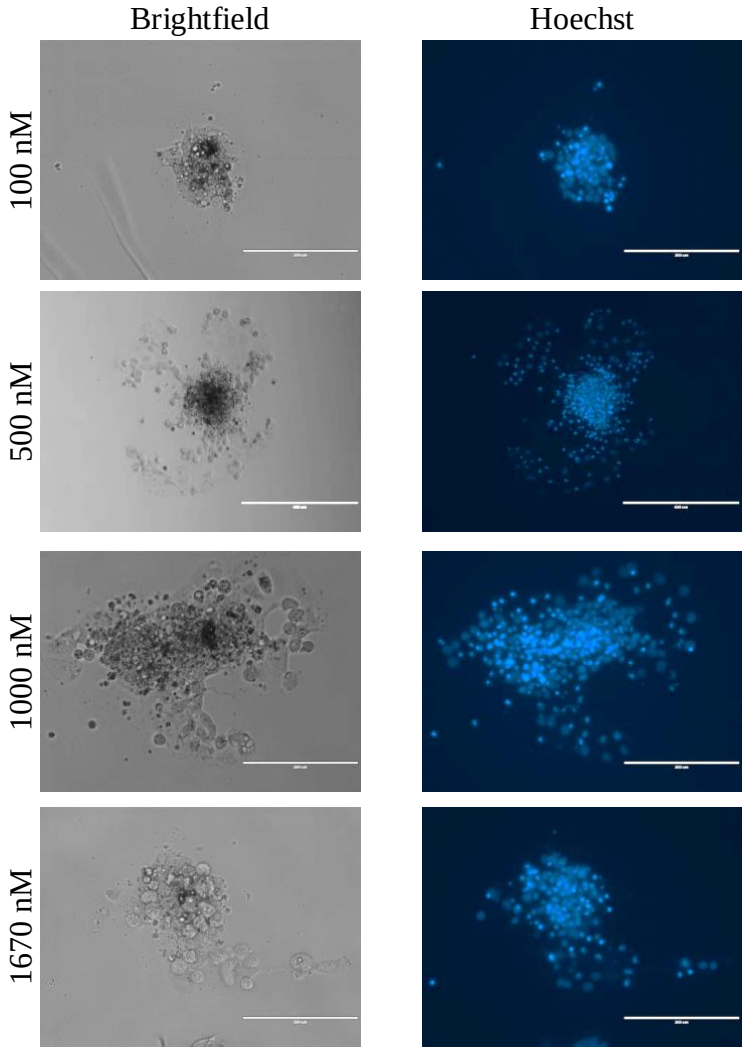

Figure 4.5: ePSC colonies treated with 100 nM, 500 nM, 1000 nM, or 1670 nM nocodazole for 24 hrs. Scale bar = 400 µm for 500 nM, and 200 µm for 100, 1000, and 1670 nM. Images taken on EVOS microscope under brightfield and Hoechst stain visualised with 405 nm UV light.

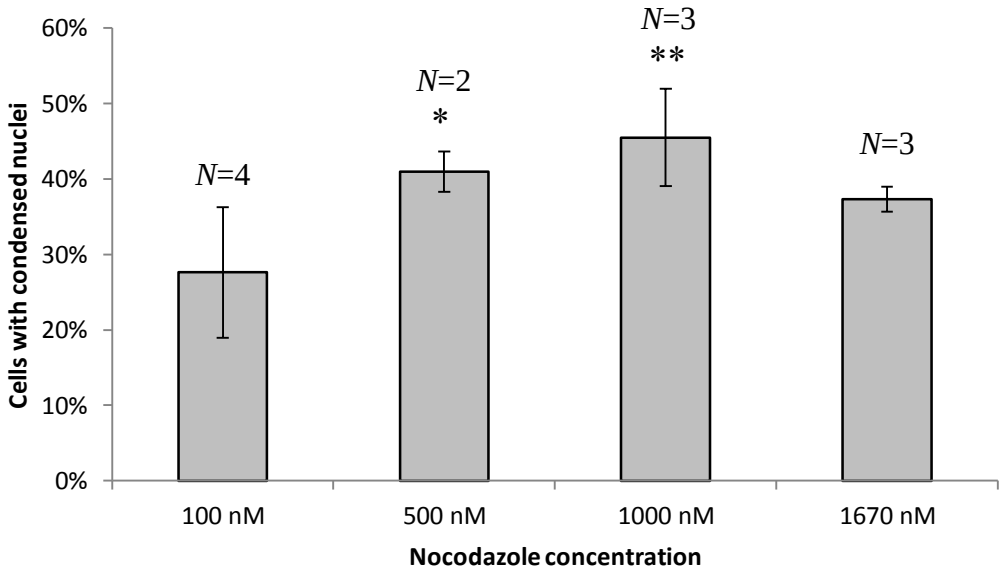

Figure 4.6: Average condensed nuclei in ePSC colonies after 24 hr nocodazole treatment. *N* value on graph = number of colonies counted. Significance * = $P < 0.05$, ** = $P < 0.01$ compared with 100 nM nocodazole. Error bars = SEM, total nuclei counted *N*=1724.

4.2.3 Immunofluorescence characterisation of cell cycle markers

4.2.3.1 Phosphorylated Histone 3

The accuracy of evaluating mitotic arrest via nuclei condensation was further addressed by staining cells for presence of phosphorylated histone 3 (H3), an M-phase arrest marker. Colonies were incubated with nocodazole (500 nM or 1.67 µM) or DMSO for 18-22 hrs prior to staining (Figure 4.7). Per treatment, 16-18 colonies (*n*=4) were counted with an average of 444 cells per colony (24-1115 cells per colony, total cells *N*=15346). Nocodazole-arrested colonies had a higher number of cells positively stained for phosphorylated H3 compared with DMSO control (40 ± 2% and 11 ± 3%, respectively; $P < 0.01$; Figure 4.8).

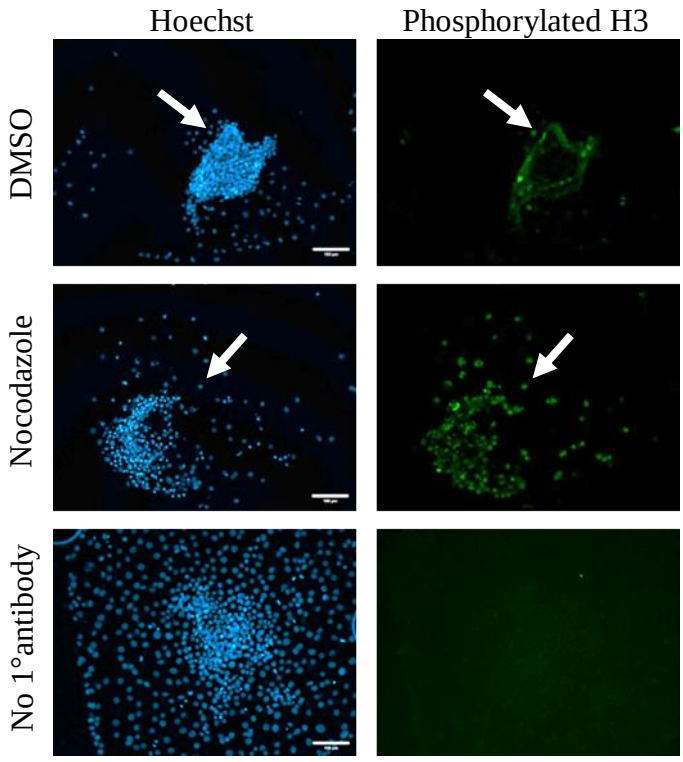

Figure 4.7: ePSC colonies stained for phosphorylated H3. Colonies were treated with DMSO or nocodazole (500 nM or 1.67 µM) for 18-22 hrs. Negative control colony was stained without binding of Phosphorylated H3 primary antibody. Arrow indicates nuclei positive for phosphorylated H3. Scale bar = 100 µm. Images taken on Olympus microscope, Hoechst stain visualised with 405 nm UV light and phosphorylated H3 with 488 nm.

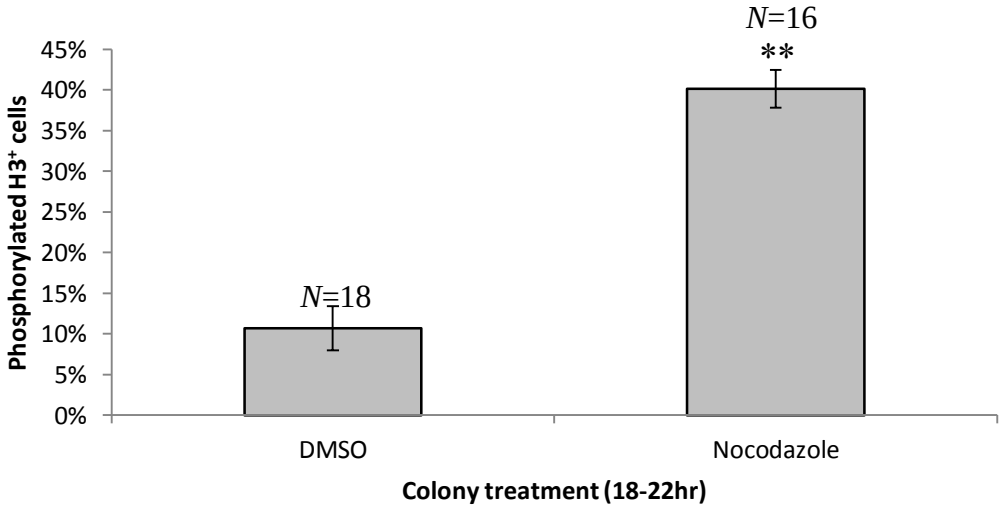

Figure 4.8: Average cells positively stained for phosphorylated H3. ePSC colonies stained after 18-22 hr DMSO or nocodazole treatment. *N* value on graph = number of colonies counted. Significance ** = $P < 0.01$ compared with DMSO. Error bars = SEM, replicates $n=4$, total nuclei counted $N=15346$.

4.2.3.2 EdU

Proportion of colony in S phase after nocodazole arrest was evaluated by incorporation of EdU. Colonies were incubated with nocodazole (500 nM or 1.67 µM) or DMSO for 18-22 hrs prior to incubation with EdU for 30 min (Figure 4.9). Per treatment, 4-5 colonies (n=2) were counted with an average of 530 cells per colony (73-1196 cells per colony, total cells counted N=4766). Nocodazole-arrested colonies had a lower proportion of cells incorporating EdU than DMSO control (11 ± 2% and 28 ± 2%, respectively; $P < 0.01$; Figure 4.10).

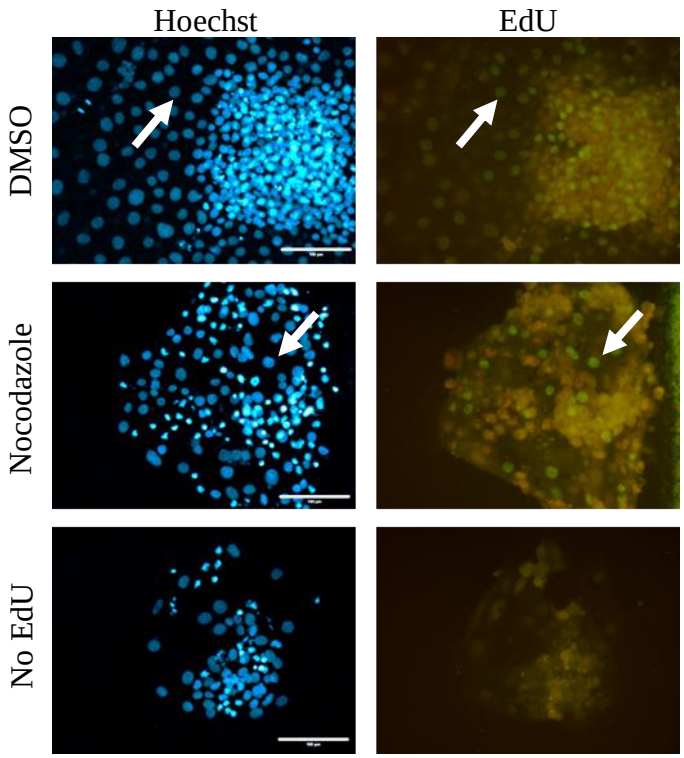

Figure 4.9: ePSC colonies stained for incorporation of EdU. Colonies were treated with DMSO or nocodazole (500 nM or 1.67 µM) for 18-22 hrs. Positive staining identified by bright green fluorescence (indicated by arrow), whereas background staining was a dull yellow. Negative control colony was stained without incubation with EdU. Scale bar = 100 µm. Images taken on Olympus microscope, Hoechst stain visualised with 405 nm UV light and EdU with 488 nm.

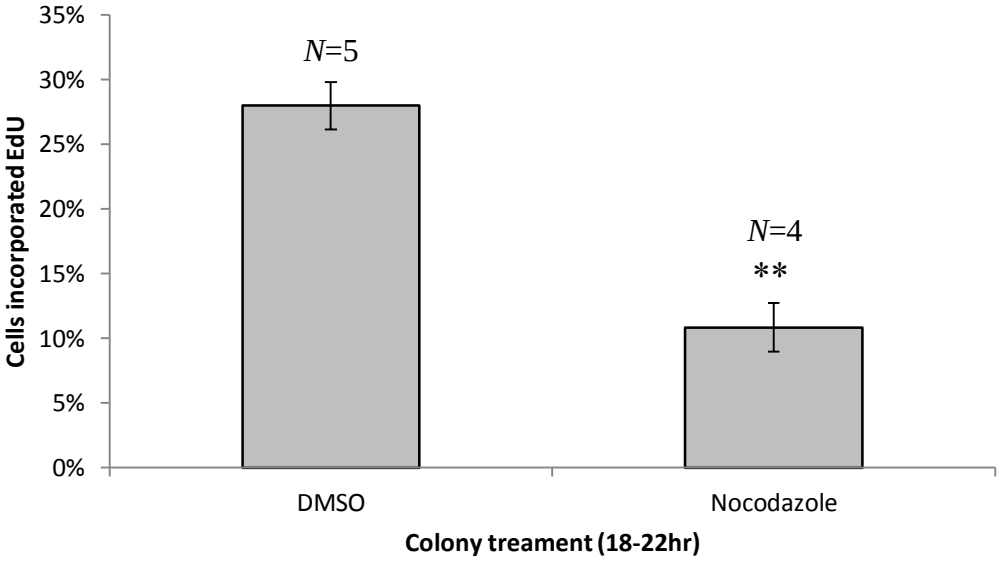

Figure 4.10: Average cells positively stained for EdU. ePSC colonies stained after 18-22 hr DMSO or nocodazole treatment with 30 min EdU incubation. *N* value on graph = number of colonies counted. Significance ** = $P < 0.01$ compared with DMSO. Error bars = SEM, replicates $n=2$, total nuclei counted $N=4766$.

4.2.3.3 Ki-67

The proliferating fraction of the colony was evaluated by staining for Ki-67 protein. Colonies were incubated with nocodazole (500 nM or 1.67 µM) or DMSO for 18-22 hrs prior to staining (Figure 4.11). Per treatment, 8-11 colonies ($n=3$) were counted with an average of 185 cells per nocodazole colony (71-354 cells per colony, colonies counted $N=11$, total cells counted $N=2037$), and 688 cells per DMSO colony (283-1191 cells per colony, colonies counted $N=8$, total cells counted $N=5505$). Nocodazole-arrested colonies had a higher number of cells staining positive for Ki-67 than DMSO control ($65 \pm 5\%$ and $29 \pm 8\%$, respectively; $P < 0.01$; Figure 4.12).

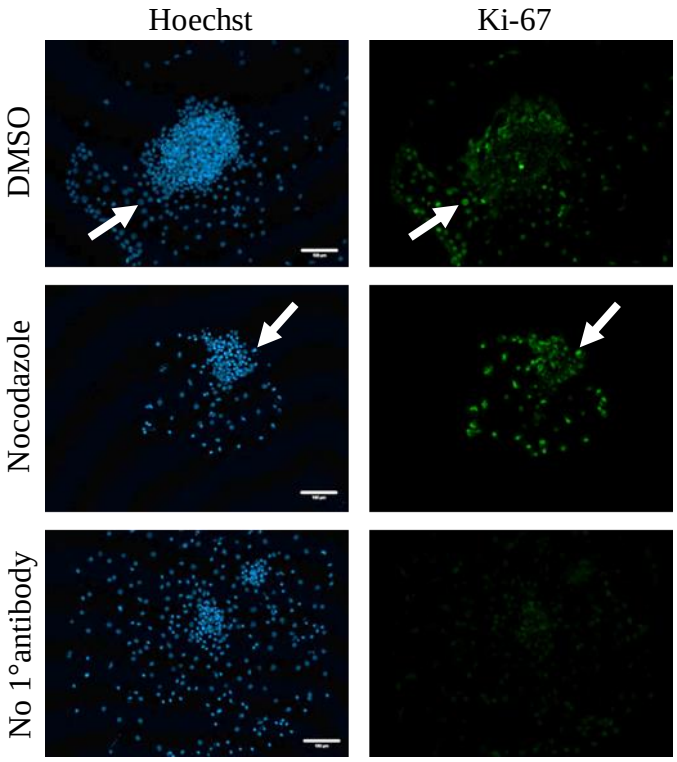

Figure 4.11: ePSC colonies stained for Ki-67. Colonies were treated with DMSO or nocodazole (500 nM or 1.67 µM) for 18-22 hrs. Negative control colony was stained without binding of Ki-67 primary antibody. Arrow indicates nuclei positive for Ki-67. Scale bar = 100 µm. Images taken on Olympus microscope, Hoechst stain visualised with 405 nm UV light and Ki-67 with 488 nm.

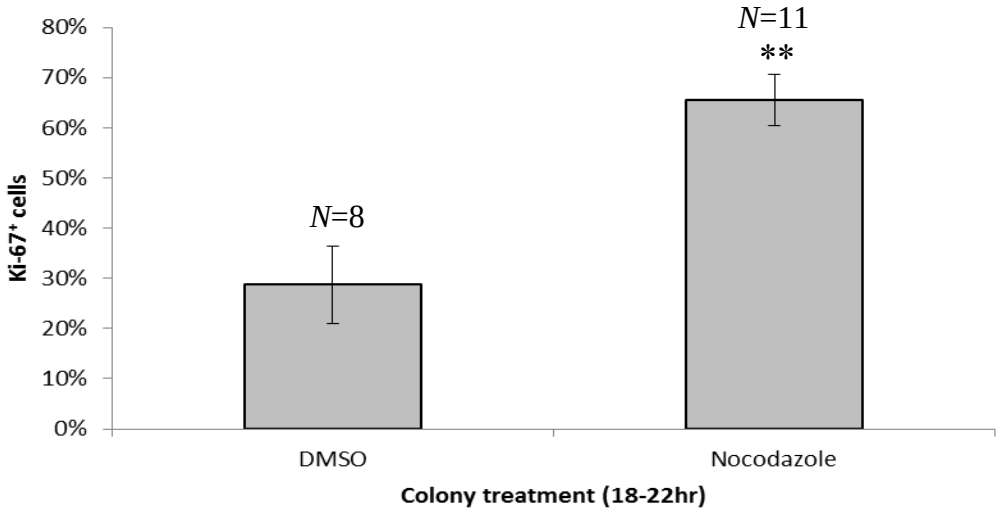

Figure 4.12: Average cells positively stained for Ki-67. ePSC colonies stained after 18-22 hr DMSO or nocodazole treatment. *N* value on graph = number of colonies counted. Significance ** = $P < 0.01$ compared with DMSO. Error bars = SEM, replicates $n=3$, total nuclei counted $N=7542$.

4.2.4 Immunofluorescence characterisation of pluripotency genes

4.2.4.1 NANOG

Expression of NANOG was assessed to ensure cells selected for NT were ePSCs. Colonies were incubated with nocodazole (500 nM or 1.67 µM) or DMSO for 18-22 hrs prior to staining (Figure 4.13). Per treatment, four colonies (*n*=2) were counted with an average of 169 cells per nocodazole colony (58-356 cells per colony, total cells counted *N*=675), and 351 cells per DMSO colony (34-639 cells per colony, total cells counted *N*=1404). Slightly higher expression of NANOG was observed between nocodazole-arrested colonies and DMSO control (48 ± 18% and 37 ± 13%, respectively), but this was not significant (*P*=0.63; Figure 4.14).

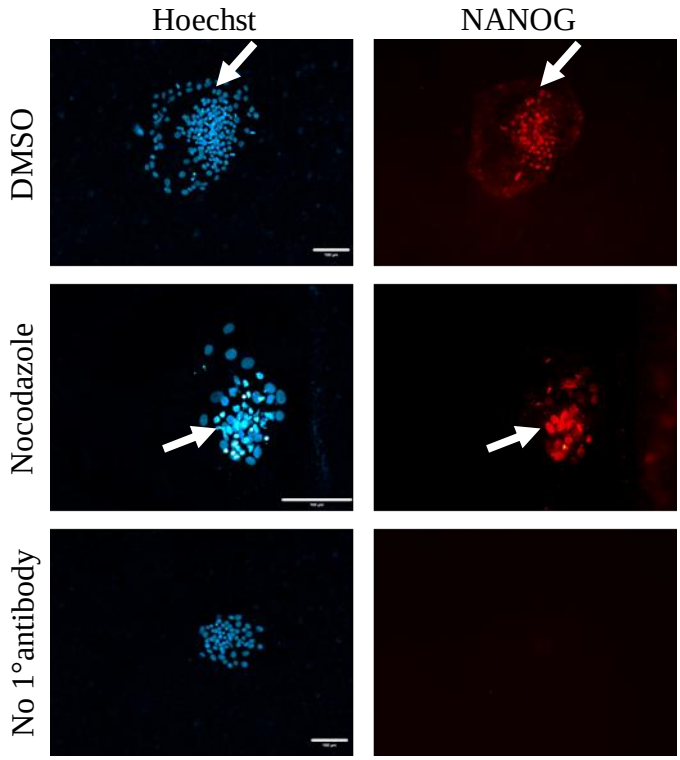

Figure 4.13: ePSC colonies stained for NANOG. Colonies were treated with DMSO or nocodazole (500 nM or 1.67 µM) for 18-22 hrs. Negative control colony was stained without binding of NANOG primary antibody. Arrow indicates nuclei positive for NANOG. Scale bar = 100 µm. Images taken on Olympus microscope, Hoechst stain visualised with 405 nm UV light and NANOG with 568 nm.

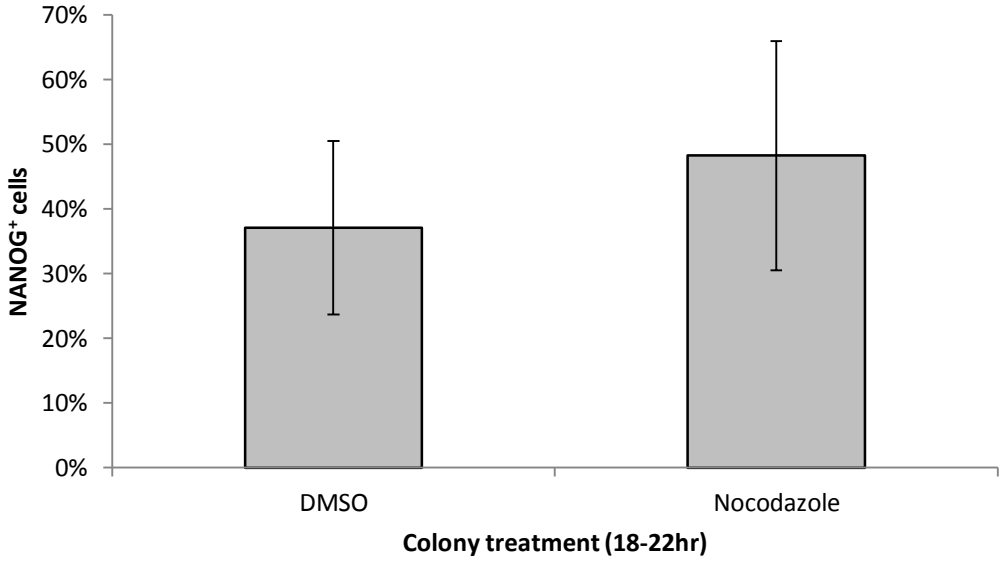

Figure 4.14: Average cells positively stained for NANOG. ePSC colonies stained after 18-22 hr DMSO or nocodazole treatment. Error bars = SEM, replicates *n*=2, colonies counted per treatment *N*=4, total nuclei counted *N*=2079.

4.2.4.2 SOX2

Expression of SOX2 was also examined to ensure NT donor cells were ePSCs. Colonies were incubated with nocodazole (500 nM or 1.67 µM) or DMSO for 18-22 hrs prior to staining (Figure 4.15). Per treatment, 7-10 colonies (*n*=2) were counted with an average of 218 cells per nocodazole colony (82-371 cells per colony, total colonies counted *N*= 7, total cells counted *N*=2357), and 330 cells per DMSO colony (54-669 cells per colony, total colonies counted *N*=10, total cells *N*=5878). Slightly higher expression of SOX2 was observed in DMSO control colonies compared with nocodazole arrest (47 ± 7% and 36 ± 4%, respectively), but this was not significant (*P*=0.27; Figure 4.16). The morphology of some cultures consisted of a central dome colony with a large outgrowth surrounding. In both DMSO and nocodazole-arrested cultures, there was a higher expression of SOX2 in the central colony (63 ± 6% and 48 ± 6%, respectively) than outgrowth cells (26 ± 10% and 29 ± 9%, respectively; Figure 4.17). However, this difference was only significant in DMSO ($P < 0.01$), not nocodazole-arrested cultures (*P*=0.11).

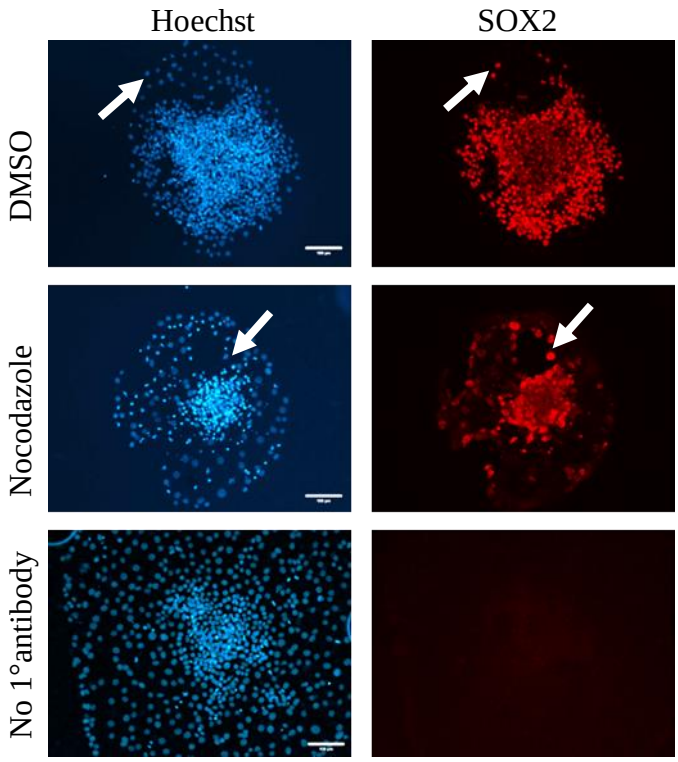

Figure 4.15: ePSC colonies stained for SOX2. Colonies were treated with DMSO or nocodazole (500 nM or 1.67 µM) for 18-22 hrs. Negative control colony was stained without binding of SOX2 primary antibody. Arrow indicates nuclei positive for SOX2. Scale bar = 100 µm. Images taken on Olympus microscope, Hoechst stain visualised with 405 nm UV light and SOX2 with 568 nm.

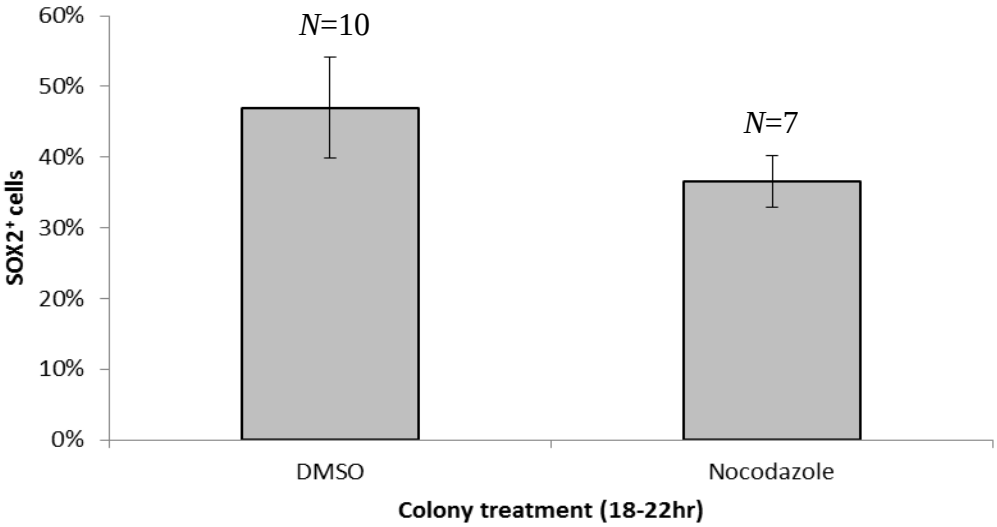

Figure 4.16: Average cells positively stained for SOX2. ePSC colonies stained after 18-22 hr DMSO or nocodazole treatment. *N* value on graph = number of colonies counted. Error bars = SEM, replicates *n*=2, total nuclei counted *N*=8235.

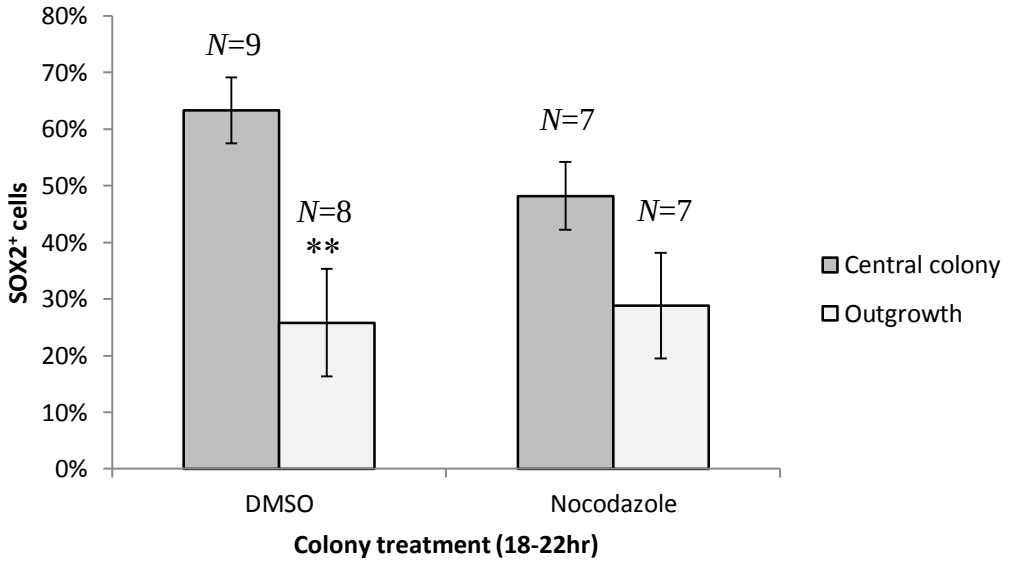

Figure 4.17: Average cells positively stained for SOX2 in central colony vs outgrowth. ePSC colonies stained after 18-22 hr DMSO or nocodazole treatment. *N* value on graph = number of colonies counted. Significance ** = $P < 0.01$ compared with central colony. Error bars = SEM, replicates $n=2$, total nuclei counted $N=8211$.

The mitotic fraction of the population was examined for SOX2 expression. Colonies were incubated with nocodazole (500 nM or 1.67 µM) or DMSO for 18-22 hrs prior to staining (Figure 4.18). Per treatment, 3-5 colonies were counted with an average of 134 phosphorylated H3 positive cells per nocodazole colony (121-157 cells per colony, total colonies counted $N=3$, total cells counted $N=401$), and 34 cells per DMSO colony (16-58 cells per colony, total colonies counted $N=5$, total cells counted $N=135$). Over half the cells positive for phosphorylated H3 were also positive for SOX2 in DMSO and nocodazole-arrested colonies (70 ± 15% and 53 ± 7%, respectively; $P = 0.45$; Figure 4.19).

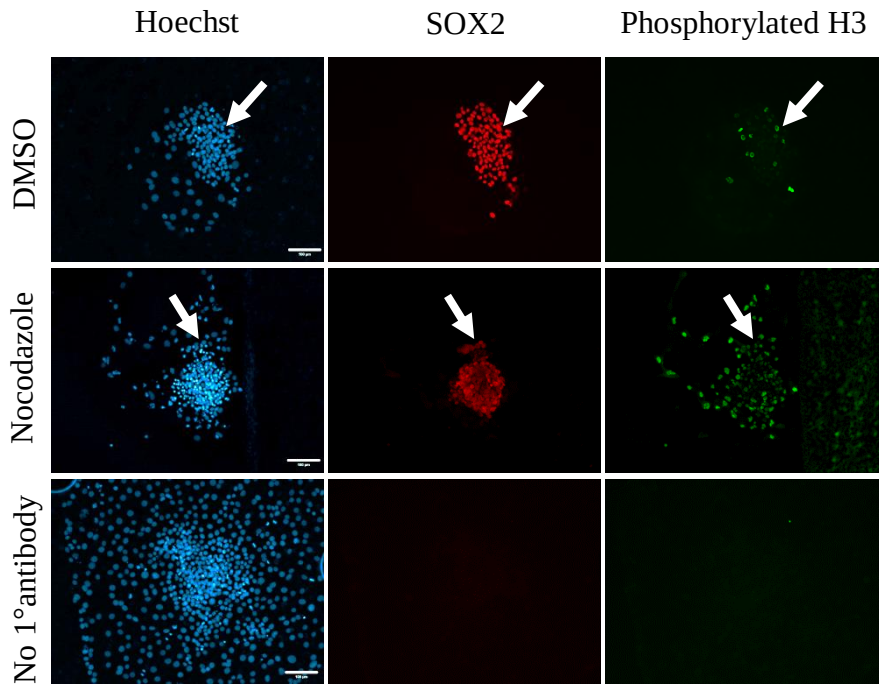

Figure 4.18: ePSC colonies stained for SOX2 and Phosphorylated H3. Colonies were treated with DMSO or nocodazole (500 nM or 1.67 µM) for 18-22 hrs. Negative control colony was stained without binding of SOX2 or Phosphorylated H3 primary antibodies. Arrow indicates nuclei positive for phosphorylated H3 and SOX2. Scale bar = 100 µm. Images taken on Olympus microscope, Hoechst stain visualised with 405 nm UV light, SOX2 with 568 nm, and Phosphorylated H3 with 488 nm.

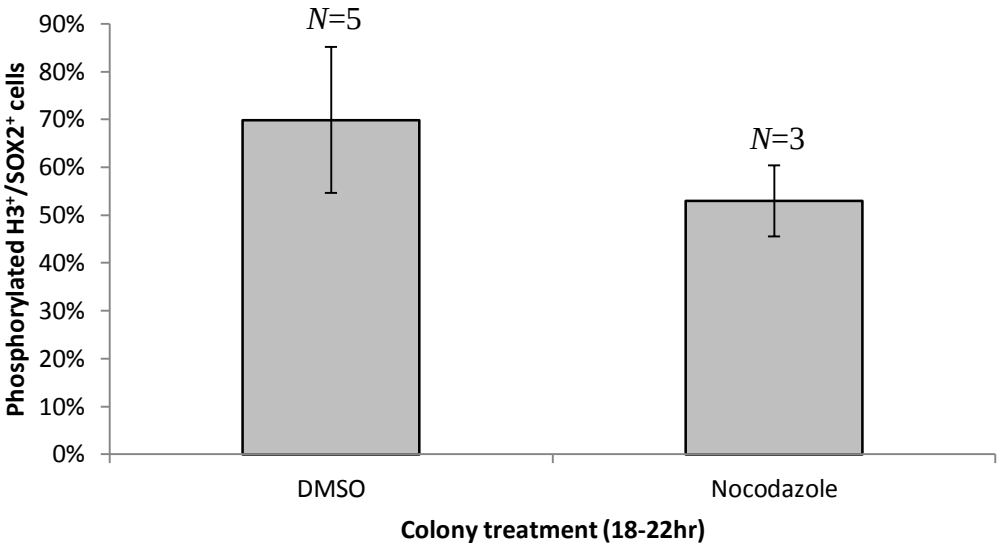

Figure 4.19: Cells positively stained for Phosphorylated H3 that also express SOX2. ePSC colonies stained after 18-22 hr DMSO or nocodazole treatment. *N* value on graph = number of colonies counted. Error bars = SEM, total nuclei counted *N*=536.

4.3 Objectives 3: Establishing a NT cloning protocol for M-phase bovine donor cells

4.3.1 Optimising artificial activation with PGs

4.3.1.1 Optimising CHX concentration

PGs were used as a proxy for optimising artificial activation of NT reconstructs and also served as positive controls. Two different concentrations were tested for CHX and anisomycin before comparison with DMAP. PGs were activated with ionomycin followed by 6 hr incubation in 17.8 µM (replicates n=9, total PGs N=196) or 35.5 µM (replicates n=13, total PGs N=387) CHX (Figure 4.20). Cleavage rate was higher with 35.5 µM CHX than 17.8 µM (94 ± 1% vs 88 ± 4%, respectively; $P < 0.05$). Development to day 7 was higher with 35.5 µM than 17.8 µM CHX for total blastocysts (22 ± 5% and 13 ± 7%, respectively; $P < 0.01$) and high grade blastocysts (14 ± 3% and 4 ± 3%, respectively; $P < 0.01$). No differences were observed in total development (26 ± 4% and 27 ± 5%, respectively) or high grade development (6 ± 3% and 8 ± 2%, respectively) to day 8 with 35.5 µM or 17.8 µM CHX.

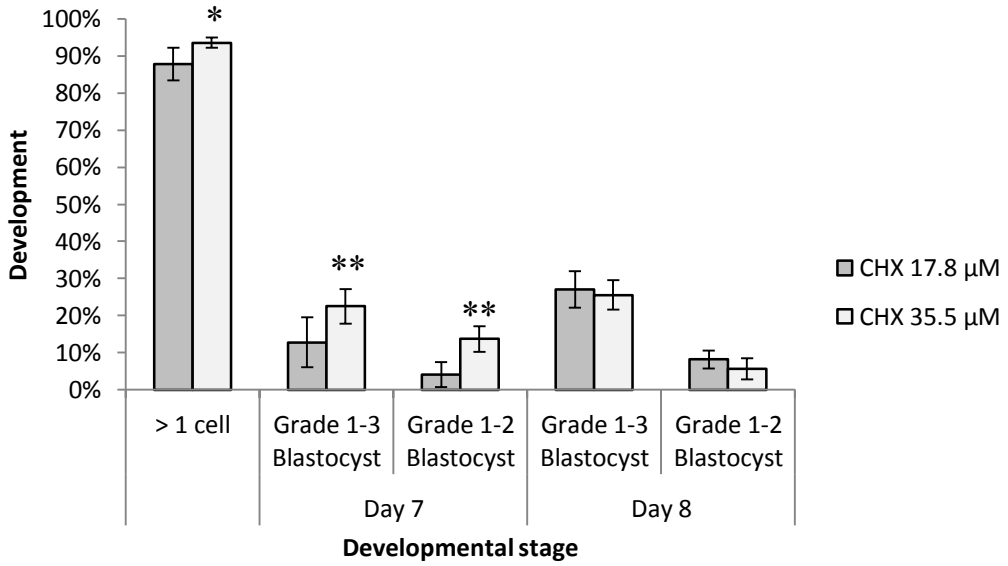

Figure 4.20: Development of PGs activated with ionomycin and 17.8 µM or 35.5 µM CHX. Significance * = $P < 0.05$, ** = $P < 0.01$ compared with 17.8 µM CHX. Error bars = SEM, replicates 17.8 µM CHX n=9, 35.5 µM n=13, total PGs N=583.

4.3.1.2 Optimising anisomycin concentration

Anisomycin incubation for 5 hrs at concentrations of 10 µM (replicates n=8, total PGs N=267) and 100 µM (replicates n=3, total PGs N=70) were used to complete activation of PGs following ionomycin (Figure 4.21). Cleavage of PGs incubated in 100 µM anisomycin was significantly decreased in comparison with 10 µM (3 ± 2% and 97 ± 1%, respectively; $P < 0.01$). No development was observed to blastocyst stage to day 7 or day 8 with 100 µM anisomycin. PGs incubated in 10 µM had greater total developed on day 7 and day 8 (9 ± 4% and 33 ± 8%, respectively; $P < 0.01$), and high grade blastocyst development on day 8 (7 ± 3%; $P < 0.05$). Difference in development of high grade blastocysts on day 7 was not significant (4 ± 2%).

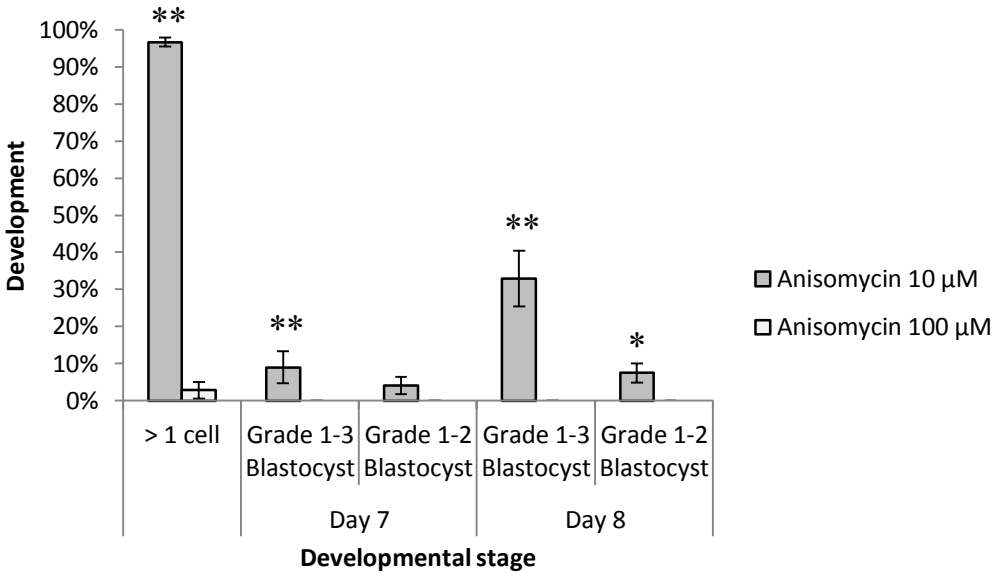

Figure 4.21: Development of PGs activated with ionomycin and 10 µM or 100 µM anisomycin. Significance * = $P < 0.05$, ** = $P < 0.01$ compared with 100 µM anisomycin. Error bars = SEM, replicates 10 µM anisomycin n=8, 100 µM n=3, total PGs N=337.

4.3.1.3 Development following activation with CHX vs anisomycin vs DMAP

In order to determine the best activation drug following ionomycin activation, parthenogenesis was compared with DMAP (2 mM; *N*=186), CHX (35.5 µM; *N*=127) and anisomycin (10 µM; *N*=127) over three trials (Figure 4.22). Cleavage rates were similar across all three treatments (DMAP 96 ± 1%, CHX 92 ± 2%, and anisomycin 96 ± 2%). Total and high grade blastocyst development to day 7 was highest with DMAP (46 ± 8% and 18 ± 3%, respectively; $P < 0.01$ compared with CHX and anisomycin), followed by CHX (24 ± 7% and 11 ± 5%, respectively; total development $P < 0.01$, high grade development $P < 0.05$ compared with anisomycin), then anisomycin (9 ± 3% and 3 ± 1%, respectively). Day 8 total and high grade blastocyst development was again highest with DMAP (total 56 ± 9%, $P < 0.05$; high grade 24 ± 6%, $P < 0.05$ compared with CHX, $P < 0.01$ compared with anisomycin). However, no difference was observed between CHX and anisomycin (total development 43 ± 10% and 43 ± 10%, respectively; high grade 14 ± 6% and 12 ± 4%, respectively). A higher number of compact morulas were observed on day 7 with anisomycin (30 ± 2%) compared with DMAP (13 ± 1%, $P < 0.01$) and CHX (17 ± 4%, $P < 0.05$). No difference in compact morulas was observed between DMAP and CHX (data not shown). A significant net increase in high grade blastocysts from day 7 to day 8 was observed with DMAP (18 vs 24%; $P < 0.05$), and anisomycin (3 vs 12%; $P < 0.05$) activation. A small increase in development with CHX (11 vs 14%) was also observed, but was not significant ($P = 0.57$). Since DMAP resulted in higher development compared to CHX and anisomycin, it was used for subsequent experiments.

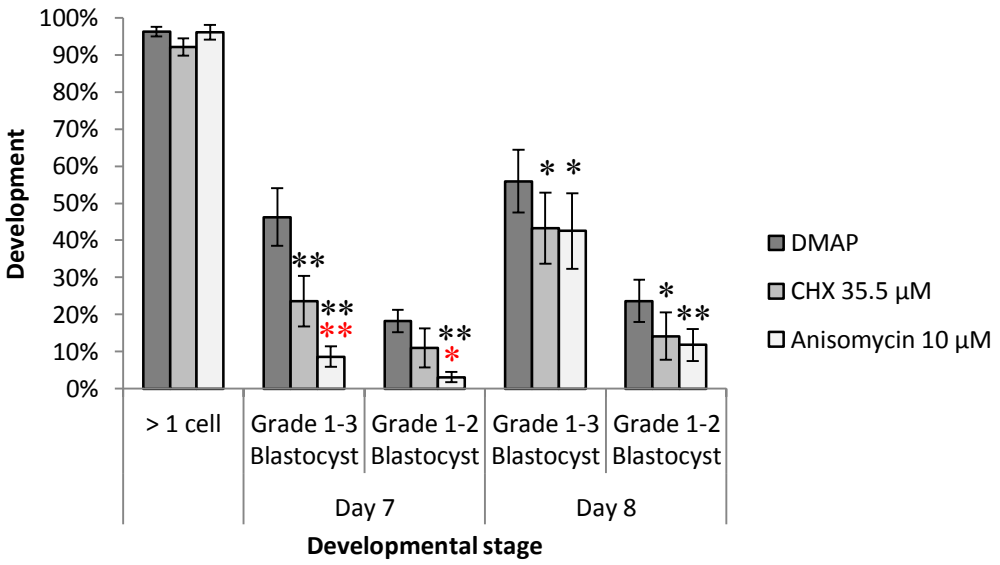

Figure 4.22: Development of PGs activated with ionomycin and 2 mM DMAP, 35.5 µM CHX, or 10 µM anisomycin. Significance * = $P < 0.05$, ** = $P < 0.01$ compared with DMAP (black) and CHX (red). Error bars = SEM, replicates n=3, total PGs N=440.

Development of individual blastocysts from day 7 and 8 was tracked to observe if net increase was caused by existing embryos increasing in grade or by new blastocysts developing, while day 7 blastocysts degrade (n=3, N=186; Figure 4.23). DMAP day 8 blastocysts were mostly those that were present on day 7, rather than new blastocysts (72 and 29%, respectively; $P < 0.01$). Whereas, CHX was evenly split between the two groups (54.5 and 45.5%, respectively; $P = 0.45$), and anisomycin had more new blastocysts (21 and 79%, respectively; $P < 0.01$). Of those that were blastocysts on day 7, the majority of DMAP- (N=56; Figure 4.23A) and CHX-activated blastocysts (N=30; Figure 4.23B) did not change in grade on day 8 (52 and 67%, respectively), with a trend to increase (29 and 20%, respectively) rather than decrease (20 and 13%, respectively). Anisomycin-activated blastocysts (N=11; Figure 4.23C) had an equal number blastocysts maintain their grade on day 8 and increase to high grade (45%). Again, a trend of number of blastocysts increasing in grade being higher than those decreasing (9%) was observed. Of the blastocysts that developed between day 7 and day 8, 23% DMAP (N=22), 8% CHX (N=25), and 14% anisomycin (N=42) were of high grade.

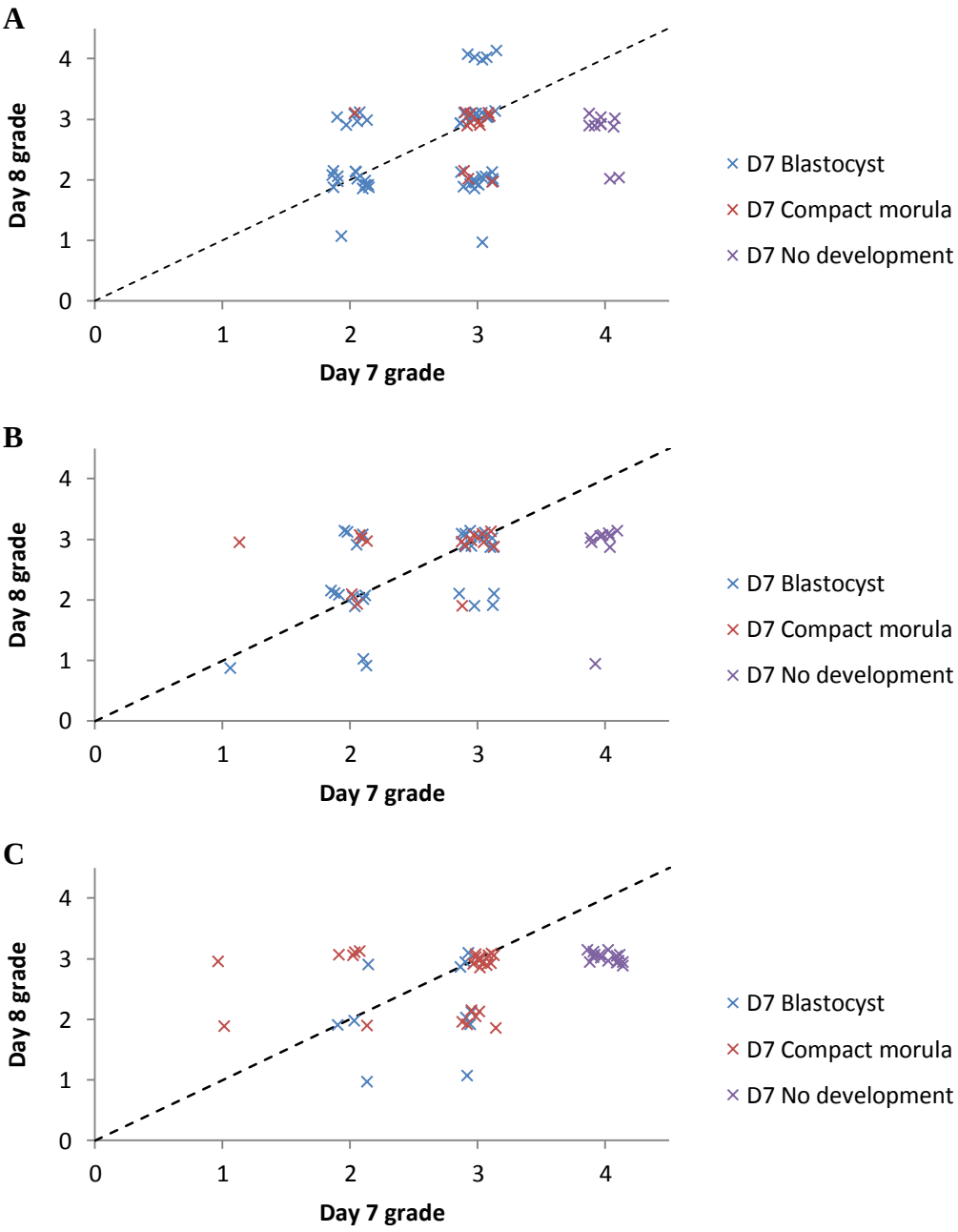

Figure 4.23: Developmental grade of individual PG blastocysts activated with A) DMAP, B) CHX, or C) anisomycin. Grade value 1-3 based on morphology of blastocysts or compact morulas. Grade of 4 given to embryos that were not at one of the defined developmental stages (had not developed to compact morula stage or had degraded and were no longer a blastocyst). Blue crosses = blastocysts that had developed on day 7, red = compact morula on day 7, purple = no defined development on day 7. Dashed line indicates no change in grade between day 7 and 8. Blastocysts above the dashed line decreased in grade from day 7-8. Replicates *n*=3, total embryos tracked DMAP *N*=78, CHX *N*=55, and anisomycin *N*=53.

4.3.1.4 Pseudo-polar body extrusion prior to DMAP incubation

The issue with using ionomycin/DMAP activation with NT reconstructs was that DMAP prevents the expulsion of a pseudo-polar body, necessary with M-phase donor cells to correct ploidy. In order to correct this, a delay between ionomycin activation and incubation in DMAP was trialled to assess the length of time required for a pseudo-polar body to be expelled. After activation with ionomycin, PGs were incubated in ESOF for up to 180 min to allow the extrusion of a pseudo-polar body containing half the original chromatin of the oocyte (Figure 4.24). Extrusion was assessed every 15 min to determine the minimum length of time to capture the maximum number of PGs with an extrusion (Figure 4.25). Pseudo-polar body extrusion plateaued around 90 min (n=8, total PGs N=557). PGs that extruded a pseudo-polar body were cultured in DMAP for 4 hrs, and development assessed on day 7 (Figure 4.26). Development was compared with PGs activated under standard conditions (ionomycin followed by culture in DMAP with a delay of less than 10 min) over 2-7 runs (N=517). Standard PG total and high grade blastocyst development (51 ± 6% and 28 ± 5%, respectively) was higher than development of pseudo-polar body extruded PGs ($P < 0.01$ compared with 90 and 120 min, $P < 0.05$ compared with 60 min). A trend of higher total and high grade development with pseudo-polar body PGs with a delay of 60 min (33 ± 8% and 12 ± 10%, respectively) was observed compared with 90 min delay (20 ± 3% and 11 ± 3%, respectively), and significantly higher development than 120 min delay PGs (17 ± 5% and 7 ± 3%, respectively; $P < 0.05$).

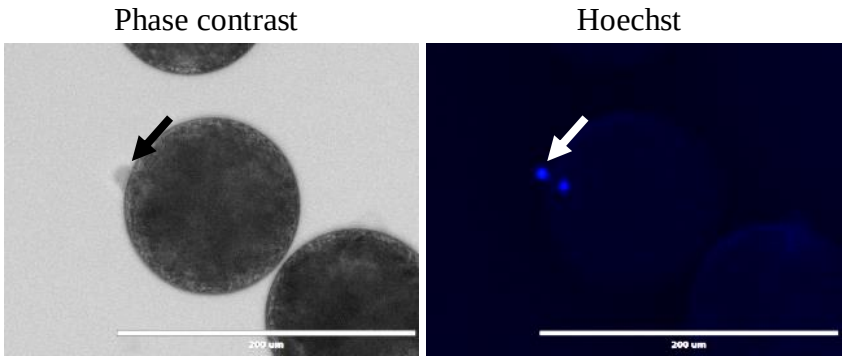

Figure 4.24: PG with pseudo-polar body extruded after 90 min. Arrows indicate single pseudo-polar body containing chromatin. Remainder of chromatin was located within oocyte. Scale bar = 200 µm. Images taken on EVOS microscope under phase contrast and Hoechst stain visualised with 405 nm UV light. Stained PG *N*=3.

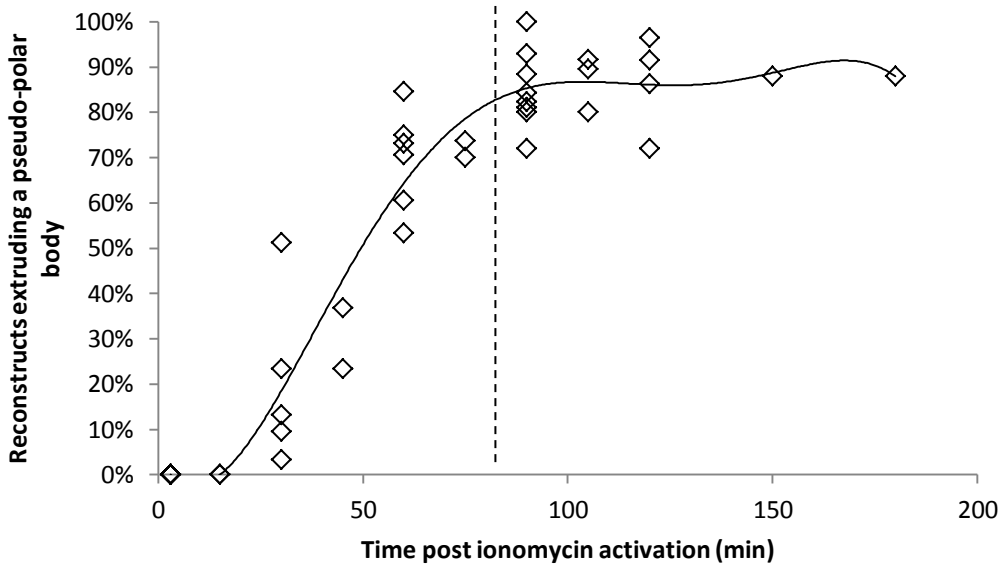

Figure 4.25: Extrusion rate of pseudo-polar body following ionomycin activation of PGs. Dashed line indicates plateau of pseudo-polar body extrusion around 90 min. Replicates *n*=8, total PGs *N*=557.

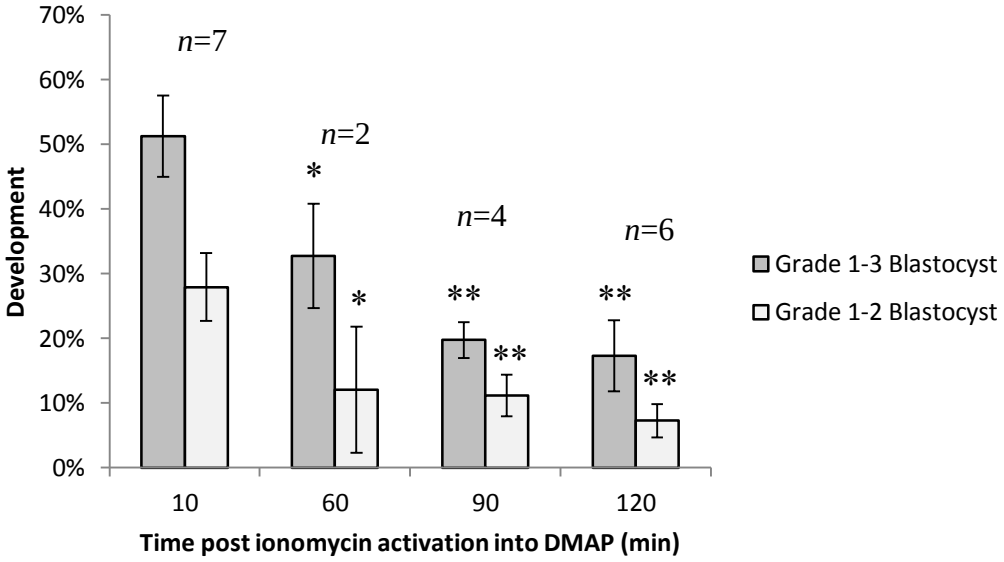

Figure 4.26: Development of PGs following pseudo-polar body extrusion. Values of *n* on graph = number of technical replicates. Significance * = $P < 0.05$, ** = $P < 0.01$ compared with standard activation (culture in DMAP with a delay of less than 10 min). Error bars = SEM, total PGs N=517.

4.3.2 Nuclear transfer trials

4.3.2.1 Optimising degree of cell lysis prior to fusion in hypoosmolar and isoosmolar fusion buffer

To determine the appropriate fusion buffer to use for M-phase donors, the quantity of cell lysis occurring in fusion buffer prior to administering the fusion pulse was measured using somatic (BEF14) and ePSC donor cells (couplets *N*=494). In hypoosmolar fusion buffer, 22 ± 2.5% of mitotic donor cells lysed pre-fusion (*n*=3; Figure 4.27). The use of isoosmolar fusion buffer decreased the rate of lysis to 9 ± 0.4% (*n*=2; $P < 0.01$).

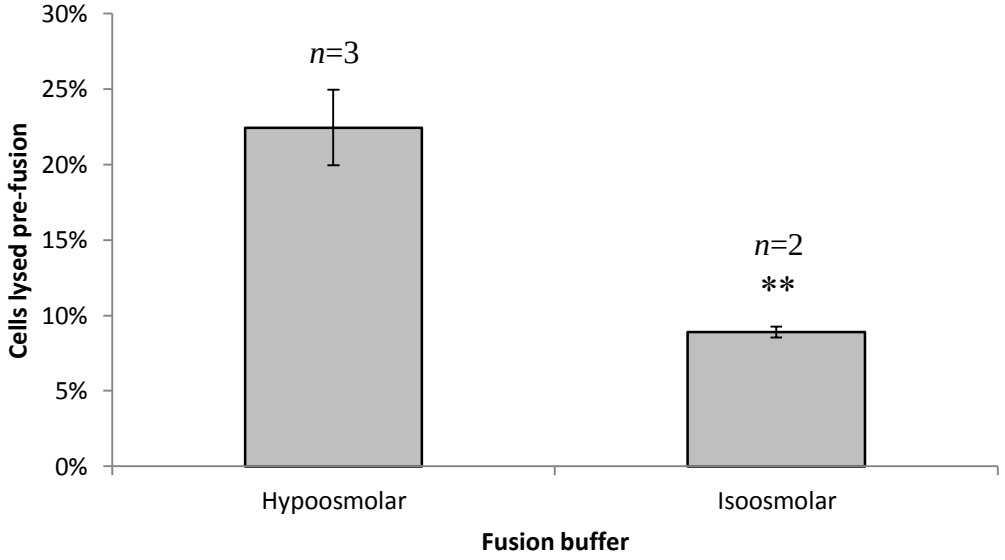

Figure 4.27: Lysed cells pre-fusion in hypoosmolar and isoosmolar fusion buffer. Values of *n* on graph = number of technical replicates. Significance ** = $P < 0.01$ compared with hypoosmolar fusion buffer. Error bars = SEM, total cytoplasts with attached donor cell *N*=494.

4.3.2.2 Optimising fusion rate with pronase treatment of donor cells

The treatment of somatic donor cells with pronase for five minutes prior to attachment to the cytoplast was evaluated for its effect on fusion rate. A direct comparison was made between BEF14 cells treated with pronase and untreated cells from the same mitotic shake-off. In hypoosmolar fusion buffer, no significant difference was observed in fusion rate between untreated and pronase-treated cells (65 ± 7% and 75 ± 10%, respectively; $P = 0.15$; *n*=3, *N*=260; Figure

4.28A). In isoosmolar fusion buffer, fusion rate of pronase treated cells was higher than untreated cells (72 ± 6% and 44 ± 5%, respectively; $P < 0.01$; n=7, N=351; Figure 4.28B).

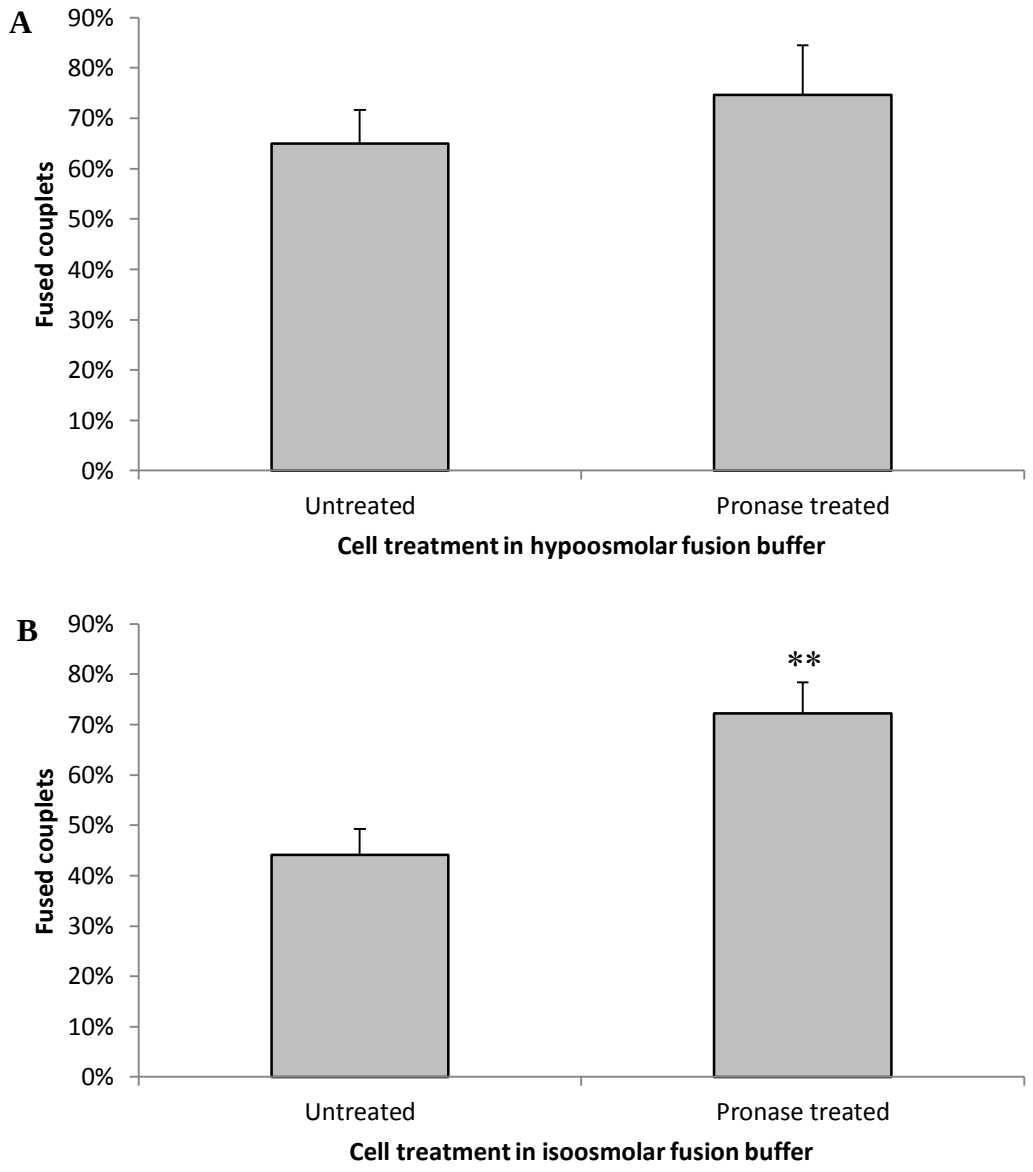

Figure 4.28: Fusion rate of cells in A) hypoosmolar and B) isoosmolar fusion buffer following treatment with pronase compared to untreated. Treated cells were incubated in pronase for 5 min. Significance ** = $P < 0.01$ compared with untreated cells. Error bars = SEM, hypoosmolar n=3, N=260, isoosmolar n=7, N=351.

4.3.2.3 Net efficiency gained from optimisations with hypoosmolar and isoosmolar fusion buffer

The results of cell lysis (4.3.2.1) and pronase treatment (4.3.2.2) optimisations were combined to compare the net efficiency of couplets generating reconstructs in hypoosmolar and isoosmolar fusion buffer (Figure 4.29). The net efficiency of using pronase treated donor cells in hypoosmolar fusion buffer was lower than in isoosmolar fusion buffer (58% vs 66%, respectively). Therefore, isoosmolar fusion buffer with pronase treated donor cells was the optimised condition for fusion.

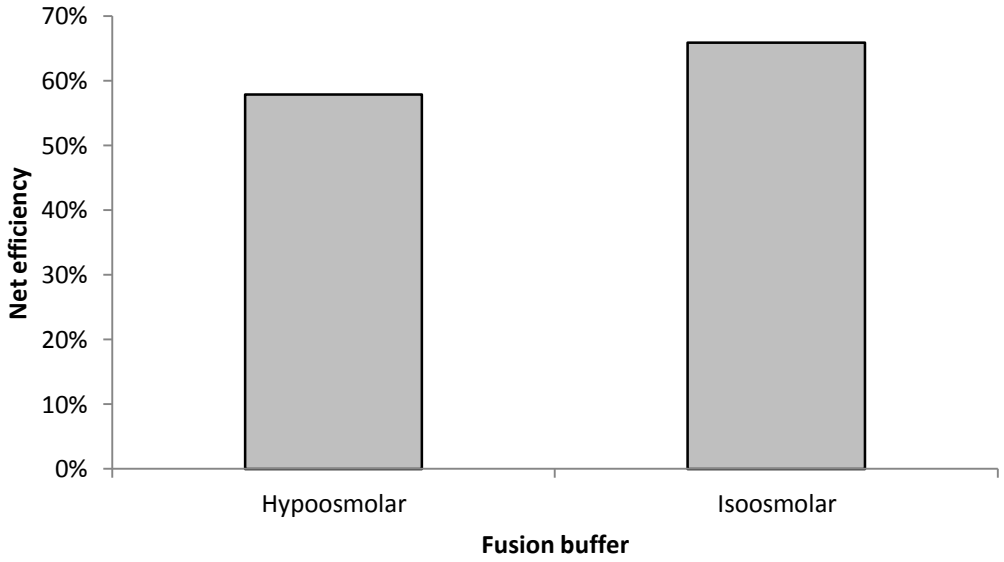

Figure 4.29: Net efficiency of using hypoosmolar and isoosmolar fusion buffer. Cell lysis and pronase treatment optimisations combined to generate net efficiency of fusion. Cells were incubated in pronase for 5 min and attempted to fuse in hypoosmolar or isoosmolar fusion buffer.

4.3.2.4 Comparison of somatic donor cell lines

Three different somatic cell lines, two non-transgenic (BEF14 and EF5) and one transgenic (Nanog2), were used in initial somatic cell NT trials. EF5 (N=25) and Nanog2 (N=17) were each used for a single run. BEF14 was the most readily available cell line and was assessed in over nine runs, three of which were comparable to EF5 and Nanog2 trials (N=166; Figure 4.30). These comparisons were made before optimisations, so couplets were fused in hypoosmolar fusion

buffer without pronase treatment. No difference was observed in cleavage (81 ± 13% vs 68% vs 65%), total blastocyst (5 ± 3% vs 4% vs 6%), or high grade blastocyst development (4 ± 3% vs 4% vs 6%) to day 7 for BEF14, EF5 and Nanog2, respectively.

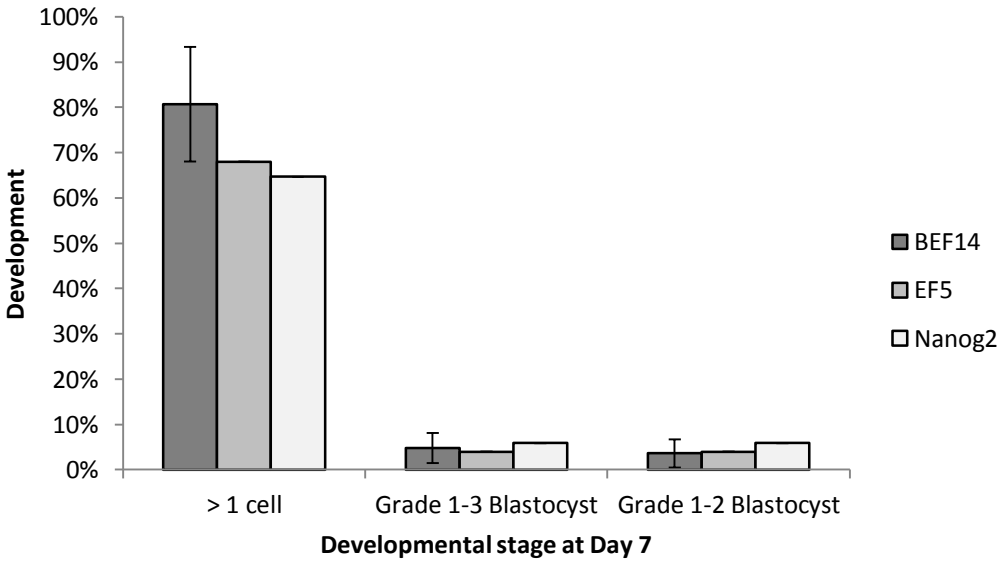

Figure 4.30: Development of NT reconstructs generated from different somatic cell lines. Cells arrested with nocodazole for 1 hr, couplets fused in hypoosmolar fusion buffer, and activated with ionomycin and CHX for 6 hrs. Error bars = SEM, replicates EF5 *n*=1, Nanog2 *n*=1, BEF14 *n*=3, total reconstructs *N*=208.

4.3.2.5 NT with CHX activation of ePSCs vs somatic cells

Development of ePSCs and somatic cells (BEF14, EF5, and Nanog2) were compared using ionomycin and CHX activation. ePSCs were arrested with 1.67 µM nocodazole for 18-22 hrs and reconstructs incubated in 17.8 µM CHX for 6 hrs following ionomycin activation (*n*=3, *N*=143). Somatic cells were arrested with 1.67 µM nocodazole for 1 hr and reconstructs incubated in 35.5 µM CHX for 6 hrs (*n*=3, *N*=105). Fusion occurred in hypoosmolar or isoosmolar buffer. No difference was observed between ePSCs and somatic cells in cleavage (80 ± 6% and 78 ± 16%, respectively), total blastocyst development (14 ± 12% and 7 ± 3%, respectively), or high grade development (7 ± 5% and 7 ± 3%, respectively) on day 7.

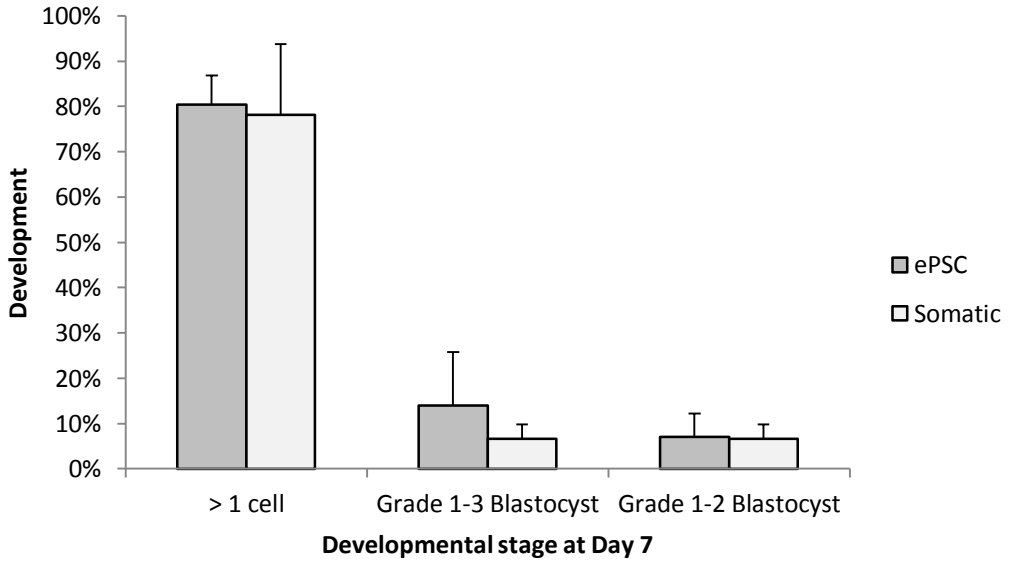

Figure 4.31: Development of ePSC and somatic cell NT reconstructs activated with CHX. ePSCs were arrested with 1.67 µM nocodazole for 18-22 hrs and activated with 17.8 µM CHX. Somatic cells were arrested with 1.67 µM nocodazole for 1 hr and activated with 35.5 µM CHX. Couplets fused in hypoosmolar or isoosmolar fusion buffer and activated with ionomycin and CHX for 6 hrs. Error bars = SEM, replicates *n*=3, total reconstructs *N*=248.

4.3.2.6 Pseudo-polar body extrusion prior to DMAP incubation with somatic donor cells

The expulsion of a pseudo-polar body from reconstructs generated from mitotic somatic cells was examined in a similar manner to the PG trials. After activation with ionomycin, reconstructs were incubated in ESOF to allow the extrusion of a pseudo-polar body containing half the original chromatin of the cell. Unlike in PG trials, it was observed that pseudo-polar bodies contained either all of the chromatin leaving an empty cytoplast, or no chromatin (*n*=9; Figure 4.32). Extrusion was assessed every 30 min to determine the minimum length of time to capture the maximum number of reconstructs with an extrusion (Figure 4.33). Pseudo-polar body extrusion plateaued around 90 min (4 replicates, *N*=104). All reconstructs were cultured in DMAP for 4 hrs, and development assessed on day 7 (Figure 4.34). Development was compared with reconstructs activated under standard conditions (ionomycin followed by culture in DMAP with a delay of less

than 10 min) over 2-4 runs (*N*=153). Cleavage rates were similar across all groups (10 min 80 ± 3%, 90 min 73 ± 2%, and 120 min 56 ± 9%). A trend was observed with total (10 min 14 ± 4%, 90 min 4 ± 2%, and 120 min 0%) and high grade blastocyst development (10 min 8 ± 3%, 90 min 4 ± 2%, and 120 min 0%) decreasing as delay into DMAP was increased. Reconstructs that did not extrude a pseudo-polar body after 90 or 120 min (*n*=1, *N*=19) were also cultured to day 7 and had similar cleavage rates (90 min no PPB 69%, 120 min no PPB 100%). No blastocyst development was observed after 90 min or 120 min without a pseudo-polar body extruded.

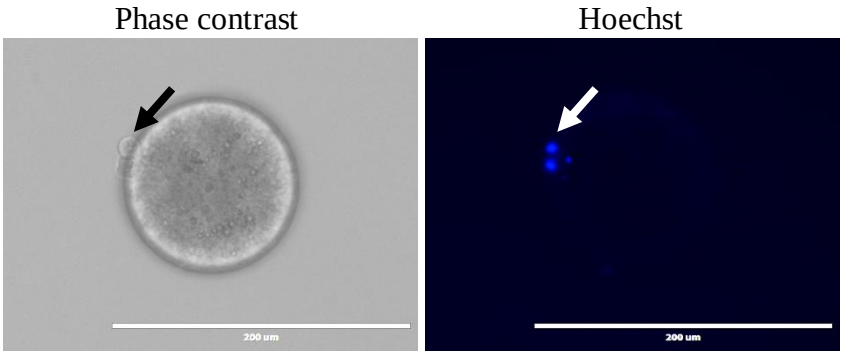

Figure 4.32: NT reconstruct with pseudo-polar body extruded after 90 min. Arrows indicate two pseudo-polar bodies each containing all chromatin from cell. Scale bar = 200 µm. Images taken on EVOS microscope under phase contrast and Hoechst stain visualised with 405 nm UV light. Stained reconstructs *N*=9.

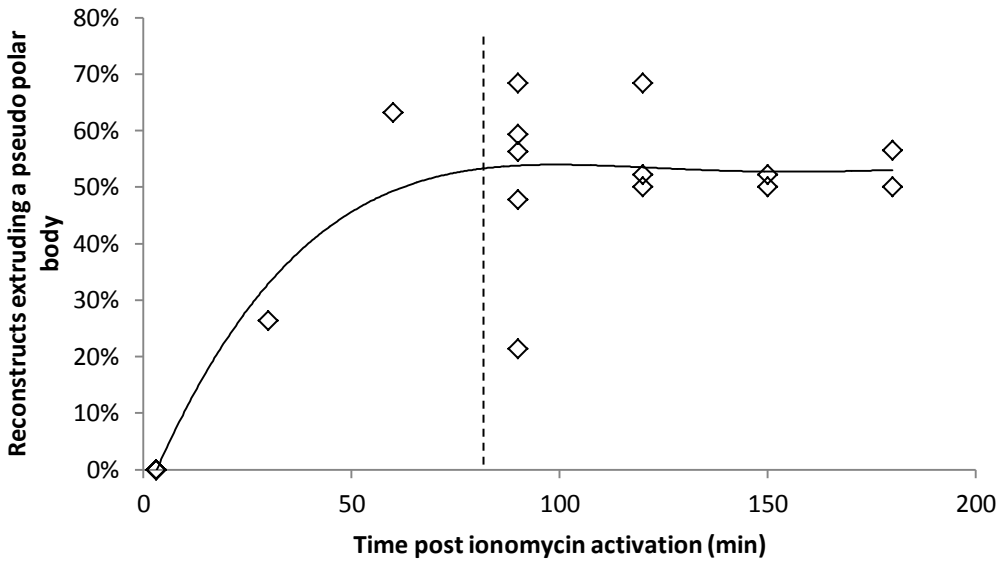

Figure 4.33: Extrusion rate of pseudo-polar body following ionomycin activation of NT reconstructs. Dashed line indicates plateau of pseudo-polar body extrusion around 90 min. Replicates *n*=4, total reconstructs *N*=104.

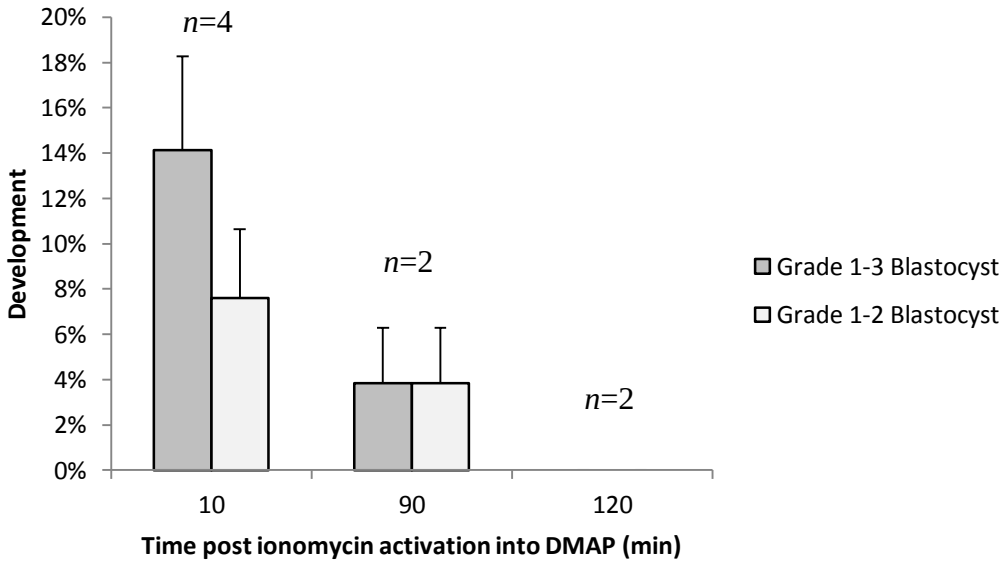

Figure 4.34: Development of NT reconstructs following pseudo-polar body extrusion. Pseudo-polar body extruded reconstructs were compared with standard activation (culture in DMAP with a delay of less than 10 min). Values of *n* on graph = number of technical replicates. Error bars = SEM, total NT reconstructs *N*=134.

4.3.2.7 NT with DMAP activation of ePSCs vs somatic cells

Development of reconstructs generated from ePSCs and somatic cells (BEF14) were compared using ionomycin and DMAP activation (Figure 4.35). ePSCs (n=3, N=125) were arrested with 500 nM nocodazole for 18-22 hrs. Somatic cells (n=4, N=173) were arrested with 100 nM nocodazole for 1 hr. Donor cells were treated with pronase prior to attachment, fusion occurred in hypoosmolar or isoosmolar buffer, and reconstructs incubated in DMAP for 4 hrs following ionomycin activation. No difference in cleavage was observed between ePSCs and somatic cells (82 ± 4% and 84 ± 4%, respectively). Development was higher with ePSCs than somatic cells in both total blastocyst development (24 ± 7% and 8 ± 5%; $P < 0.01$) and high grade development (12 ± 5% and 5 ± 3%; $P < 0.05$).

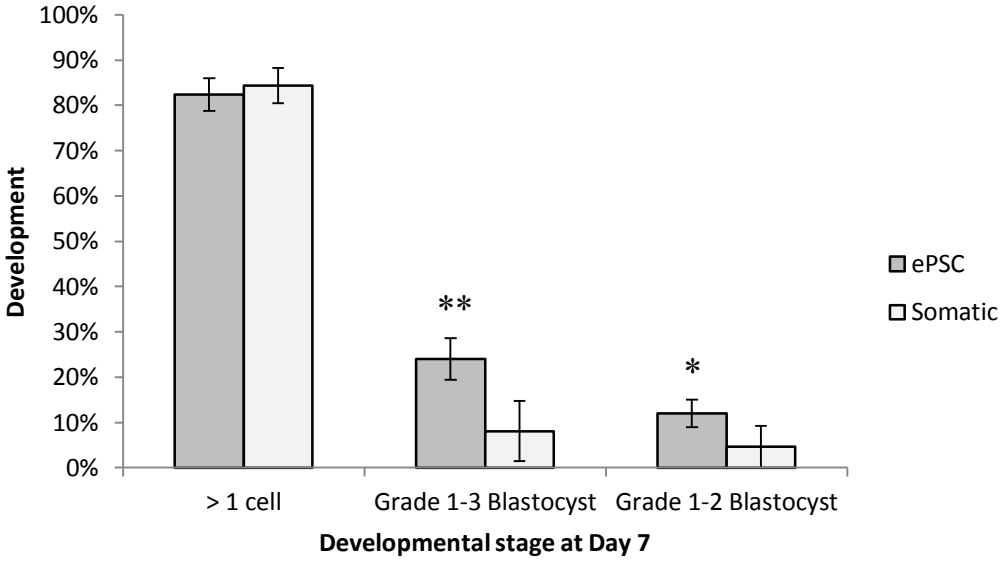

Figure 4.35: Development of NT reconstructs from ePSC and somatic donor cells. ePSCs were arrested with 500 nM nocodazole for 18-22 hrs and somatic cells were arrested with 100 nM nocodazole for 1 hr. Donor cells were treated with pronase, couplets fused in hypoosmolar or isoosmolar fusion buffer, and activated with ionomycin and DMAP for 4 hrs. Significance * = $P < 0.05$, ** = $P < 0.01$ compared with somatic cells. Error bars = SEM, replicates ePSC n=3, somatic n=4, total reconstructs N=298.

Development to day 7 vs day 8 of reconstructs generated from ePSCs and somatic cells (BEF14) were compared (Figure 4.36A). ePSCs (*n*=3, total into IVC *N*=81) were arrested with 500 nM nocodazole for 18-22 hrs. Somatic cells (*n*=6, total into IVC *N*=112) were arrested with 100 nM nocodazole for 1 hr. Donor cells were treated with pronase prior to attachment, fusion occurred in isoosmolar buffer, and reconstructs incubated in DMAP for 4 hrs following ionomycin activation. No difference was observed in total development of ePSC on day 7 vs day 8 (16 ± 2% and 16 ± 5%, respectively; $P = 0.1$), and a trend of decrease in total development from somatic donor cells (13 ± 4% and 9 ± 3%, respectively; $P = 0.52$). A trend of decrease in development to high grade blastocysts from day 7 to day 8 was observed in both ePSC (9 ± 5% and 1 ± 1%; $P = 0.06$) and somatic donors (7 ± 2% and 2 ± 1%; $P = 0.10$). Development of individual blastocysts on day 7 and 8 was tracked to assess whether any existing embryos were increasing in grade or if any new blastocysts were developing, while day 7 blastocysts degrade (Figure 4.36B). Of ePSC donor cell derived blastocysts (*n*=2, *N*=16), 44% decreased in grade from day 7 to day 8 and 6% increased. Of somatic donor cell derived blastocysts (*n*=4, *N*=15), 67% decreased from day 7 to day 8 and no blastocyst grade increased. The grade of the remaining blastocysts (50% and 33%, respectively) did not change. A single blastocyst developed from a day 7 compact morula, from a somatic donor cell (7%), which did not develop to a high grade embryo.

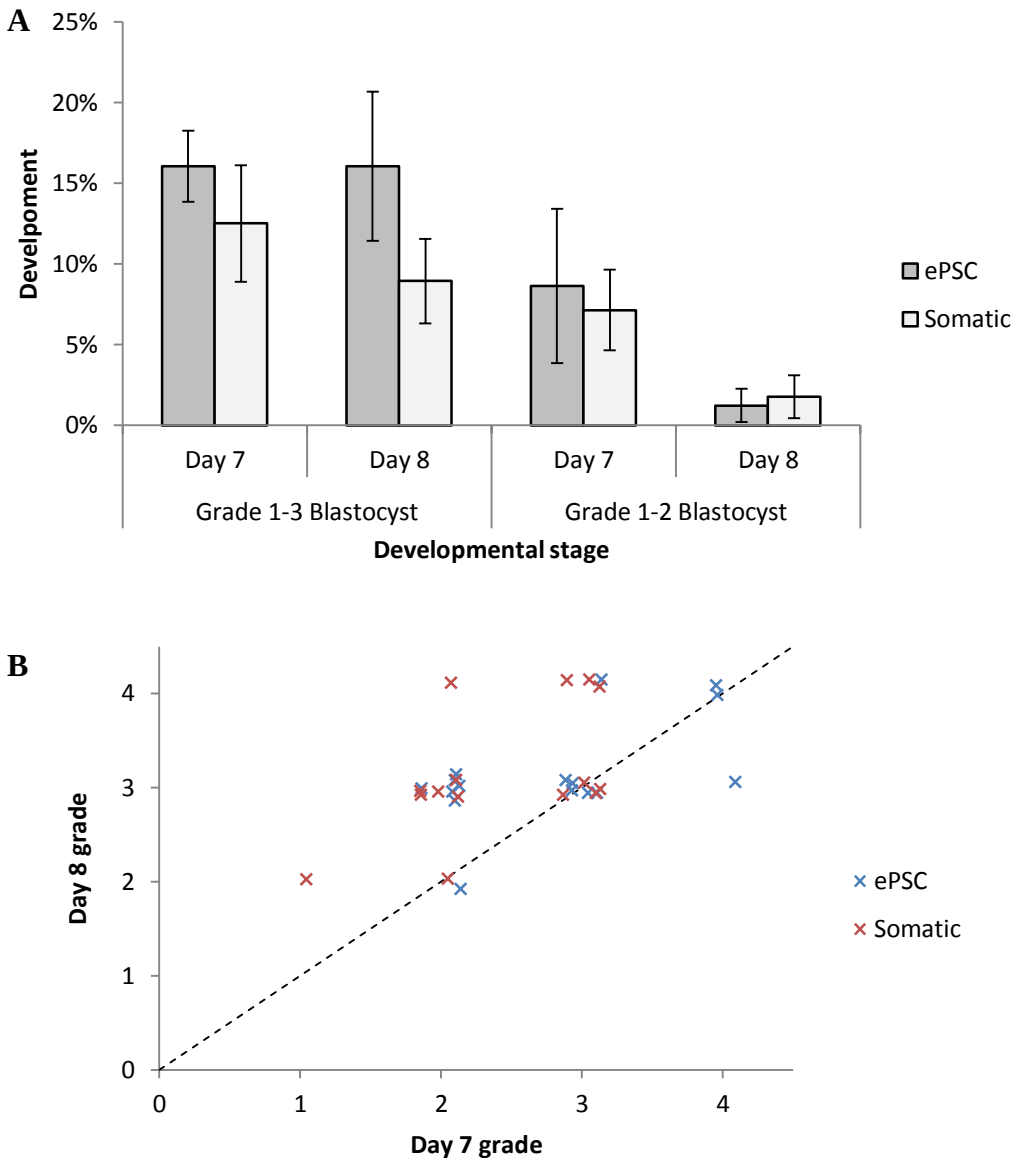

Figure 4.36: Comparison of development on Day 7 vs 8 of DMAP activated NT reconstructs. ePSCs were arrested with 500 nM nocodazole for 18-22 hrs and somatic cells were arrested with 100 nM nocodazole for 1 hr. Donor cells were treated with pronase, couplets fused in isoosmolar fusion buffer, and activated with ionomycin and DMAP for 4 hrs. A) Overall development of blastocysts separated into total development (Grade 1-3) and high grade development (Grade 1-2). Error bars = SEM, replicates ePSC *n*=3, somatic *n*=6, total reconstructs *N*=193. B) Developmental grade of individual blastocysts. Grade value of 4 given to embryos that were not at one of the defined developmental stages (had not developed to compact morula stage or had degraded and were no longer a blastocyst). Dashed line indicates no change in grade between day 7 and 8. Blastocysts above the dashed line decreased in grade from day 7-8. Replicates ePSC *n*=2, *N*=16; somatic *n*=4, *N*=15.

4.3.2.8 Preactivation of cytoplasts prior to cell fusion

Preactivation of cytoplasts before fusion with a donor cell was investigated using DMAP. Two trials were completed comparing preactivation of cytoplasts in DMAP (N=56) with standard activation (N=77). Cytoplasts were activated in ionomycin and cultured in DMAP for 1 hr before fusing an ePSC that had been arrested with 500 nM nocodazole for 18-22 hrs (Figure 4.37). Donor cells were treated with pronase prior to attachment, fusion occurred in isoosmolar buffer, and total incubation in DMAP was four hours. A trend was seen of higher cleavage (81 ± 2% and 68 ± 7%, respectively) and total blastocyst development on day 7 (27 ± 9% and 14 ± 5%, respectively) with standard activation compared with preactivated cytoplasts. Development of high grade blastocysts on day 7 was significantly higher with standard activation compared with preactivation (10 ± 6% and 0%, respectively; $P < 0.05$).

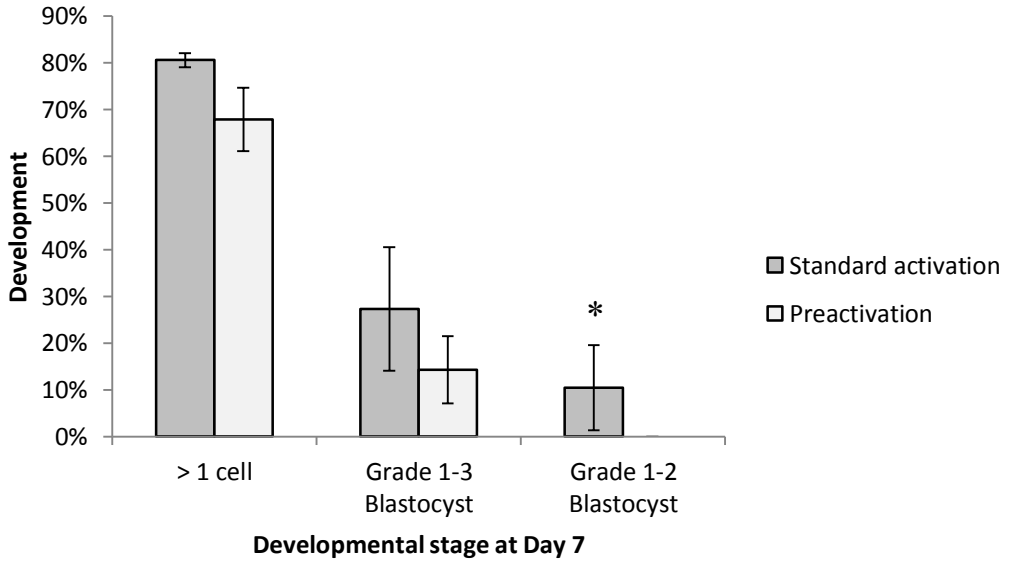

Figure 4.37: Development of 1 hr preactivation with DMAP and standard activation NT reconstructs from ePSCs. ePSCs were arrested with 500 nM nocodazole for 18-22 hrs, treated with pronase, couplets fused in isoosmolar fusion buffer. All reconstructs were activated with ionomycin and cultured in DMAP for a total of 4 hrs. Significance * = $P < 0.05$ compared with preactivated cytoplasts. Error bars = SEM, replicates n=2, total reconstructs N=133.

4.4 Objective 4: Validating different activation and NT cloning protocols by karyotype analysis

Chromosomes spreads were successfully produced for PGs activated with 17.8 µM CHX (*N*=6), DMAP (*N*=62), and 90 min delay before incubation in DMAP (*N*=17; Figure 4.38) and analysed using SmartType software (Figure 4.39). NT blastocysts produced from somatic cells activated with DMAP (*N*=7), and ePSCs activated with DMAP (*N*=21) and 17.8 µM CHX (*N*=2) were also successfully karyotyped (Figure 4.38). A small number of DMAP-activated PGs were processed through immunosurgery (*N*=6) to isolate ICM cells or bisected (*N*=8) to separate TE cells. Unfortunately, only four isolated ICMs and one bisected TE sample produced quality chromosome spreads.

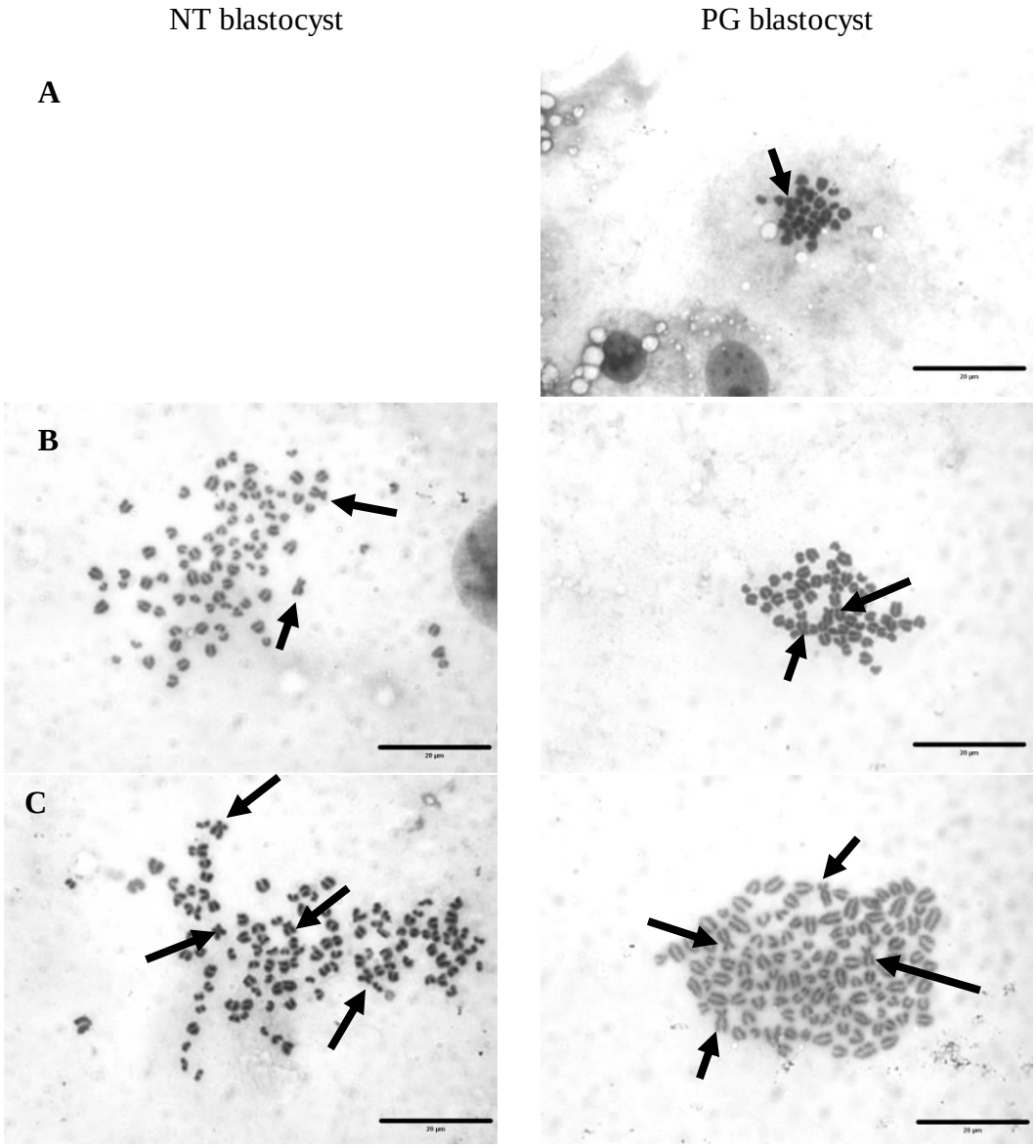

Figure 4.38: Chromosome spreads from PG- and NT-derived bovine blastocysts. Chromosome spreads contain approximately A) 30 chromosomes (1N), B) 60 chromosomes (2N), and C) 120 chromosomes (4N). Arrows indicate metacentric sex chromosomes. Scale bar = 20 µm.

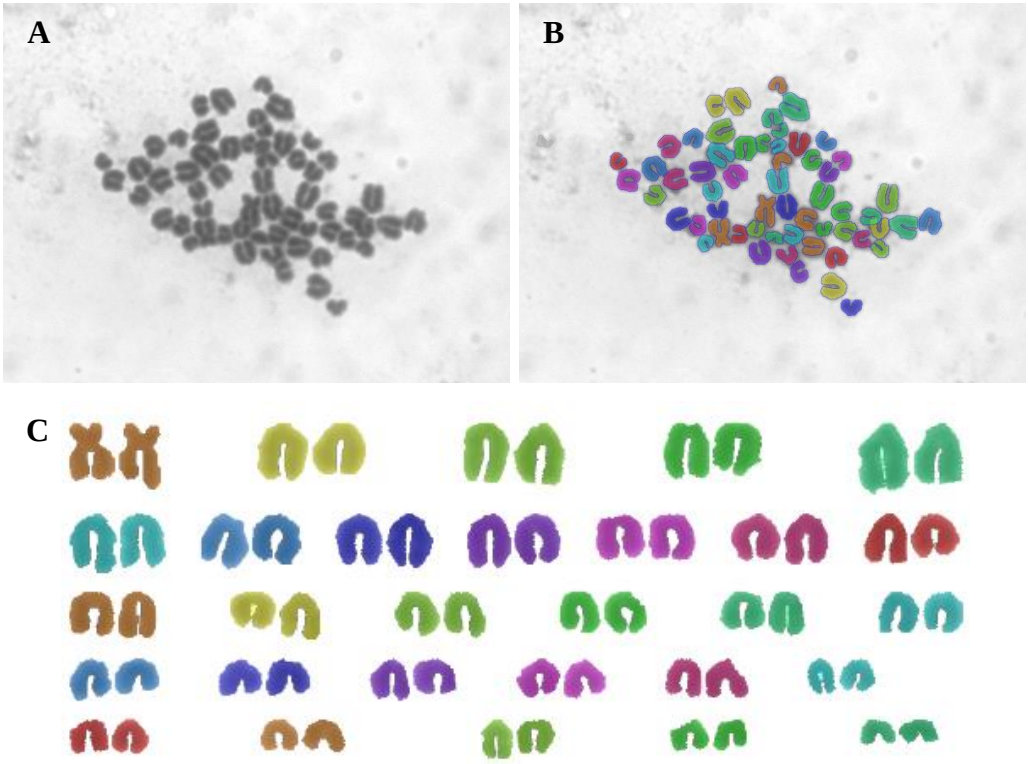

Figure 4.39: Example karyotype of a PG blastocysyt containing 58 chromosomes, analysed by SmartType. A) Original image taken under oil immersion at 1000x magnification. B) Individual chromosomes outlined using SmartType. Chromosomes falsely coloured for identification in karyotype. C) Chromosome karyotype separated from background and arranged according to size. Metacentric sex chromosomes (brown) paired at start.

Chromosomes were counted and ploidy determined ± 10% (Table 4.1). A bimodal distribution was evident in DMAP- and CHX-activated PG karyotypes (Figure 4.40), with chromosome spreads counted grouping around 2N/60 chromosomes (68 and 50% of total spreads counted, respectively) and 4N/120 chromosomes (16 and 50%). ICM spreads from DMAP-activated PGs were grouped closest to 60 chromosomes (75%) and the bisected TE spread was over 100 chromosomes. PG blastocysts developed from 90 min delay pseudo-polar body extruded DMAP protocol had chromosome spreads with a highly variable number of chromosomes.

Chromosome spreads from NT blastocysts had more varied chromosome numbers than their PG counterparts, with less clearly defined groups. DMAP-activated blastocysts from both somatic and ePSC donor cells had a small number of spreads around 60 chromosomes (14 and 10%, respectively), with the majority of

spreads containing over 100 chromosomes (71% in both groups). Only two quality spreads were achieved from CHX-activated blastocysts: one blastocyst that had expelled a pseudo-polar body (PPB) after six hours incubation in CHX, and one that did not (NPB). The blastocyst that had expelled a pseudo-polar body had around 60 chromosomes, whereas the one that did not had around 120 chromosomes.

Table 4.1: Proportion of chromosome spreads counted in each category.

Treatment		Number of chromosomes					
		30 (1N)	30-60	60 (2N)	60-120	120 (4N)	> 120
PG	CHX	-	-	50%	-	50%	-
	DMAP	-	11%	68%	5%	16%	-
	DMAP isolated ICM	-	25%	75%	-	-	-
	DMAP bisected TE	-	-	-	100%	-	-
	DMAP 90 min delay	12%	18%	29%	18%	24%	-
NT	CHX ePSC PPB	-	-	100%	-	-	-
	CHX ePSC NPB	-	-	-	-	100%	-
	DMAP ePSC	-	5%	10%	33%	52%	-
	DMAP somatic	-	-	14%	29%	29%	29%

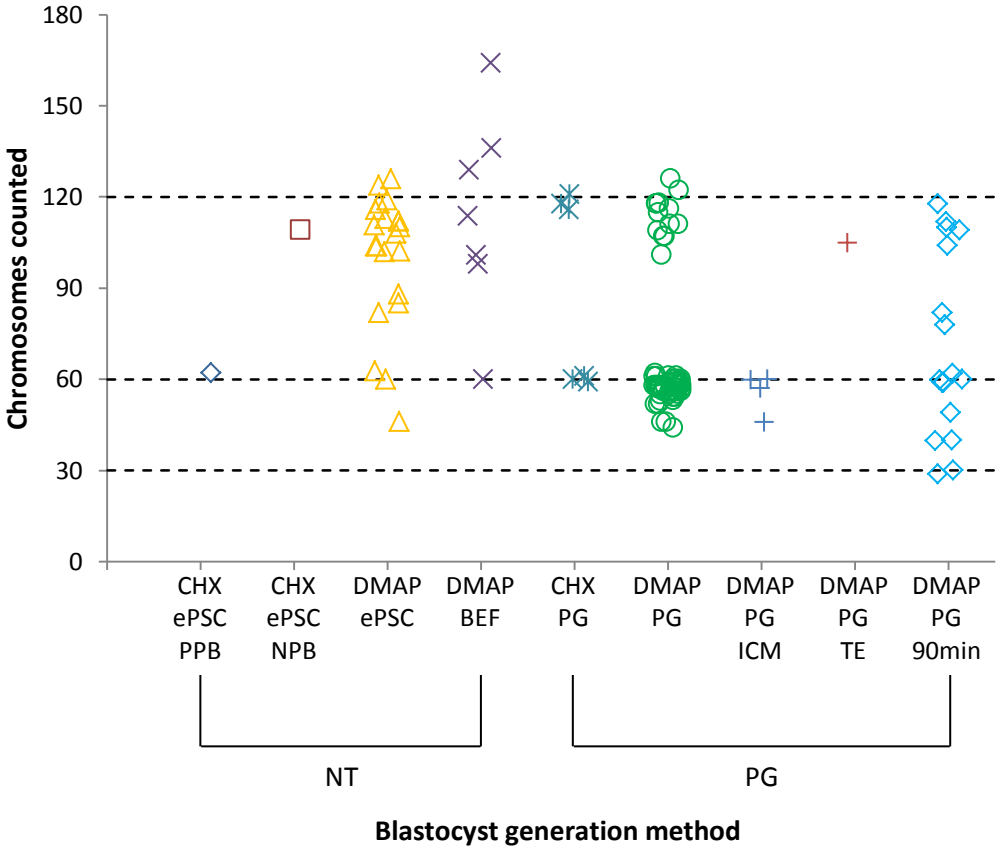

Figure 4.40: Distribution of chromosome spreads. Horizontal dashed lines indicate haploid (1N, 30 chromosomes), diploid (2N, 60 chromosomes), and tetraploid (4N, 120 chromosomes). NT reconstructs karyotyped were generated with ePSC or BEF14 donor cells, and activated with CHX or DMAP. Reconstructs activated with CHX were evaluated for presence (PPB) or absence (NPB) of a pseudo-polar body after 6 hr incubation. PGs were activated directly after ionomycin activation with CHX (CHX PG) or DMAP (DMAP PG, DMAP PG ICM, and DMAP PG TE), or after a delay following ionomycin activation of 90 min before incubation in DMAP to allow pseudo-polar body extrusion (DMAP PG 90 min). Immunosurgically isolated ICM (DMAP PG ICM) and bisected TE (DMAP PG TE) from DMAP PG blastocyst were also karyotyped.

Multiple spreads counted from a single DMAP PG blastocyst (N=18) were compared to evaluate whether blastocysts were mixoploid (Figure 4.41). A single blastocyst had spreads containing only 120 chromosomes, and 12 blastocysts containing spreads of only 60 chromosomes (6% 4N, 67% 2N). The remaining five blastocysts contained a mix of spreads around 60 and 120 chromosomes (28% mixoploid).

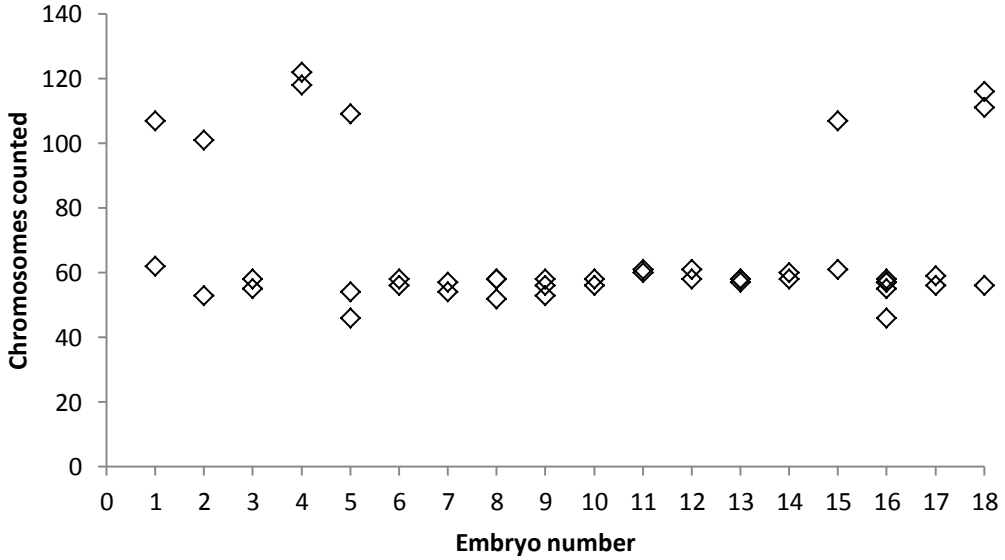

Figure 4.41: Distribution of multiple chromosome spreads counted from individual DMAP-activated PG blastocysts. Mixoploid embryos contain chromosome spreads of both 60 (2N) and 120 chromosomes (4N). Each data point represents one chromosome spread.

Chapter 5: Discussion

The main objective of this thesis was to investigate the use of bovine ePSCs in NT cloning, with the long-term goal of being able to convert superior bovine embryos into ePSCs and then ePSC-derived animals for accelerated animal breeding and biomedical applications. There were four main objectives achieved working towards this overall goal. Firstly, ePSC colonies were generated and single mitotic cells isolated for use as donor cells in NT cloning. Secondly, the duration and concentration of nocodazole arrest was established and colonies characterised for their cell cycle stage and expression of pluripotency genes. Thirdly, artificial activation and fusion parameters were optimised, followed by NT cloning trials comparing development with ePSC and somatic donor cells. Finally, blastocysts were assessed for chromosome number via karyotype analysis.

5.1 Objective 1: Isolation of single ePS cells

The first objective was to generate blastocysts and immunosurgically isolate the ICM to form ePSC cultures. Using this technique, on average 86% of blastocysts were converted to an ePSC colony. This finding means that an embryo identified as having desirable genetics has an 86% chance of being efficiently converted to an ePSC colony that can be used in NT cloning. This efficiency may improve with proficiency in the technique and by using smaller numbers of embryos. The effect of increase in technique proficiency was accounted for by analysing the final five immunosurgery trials. This set of experiments occurred after a break during which NT optimisations were performed. By only analysing these final trials, variation caused by different stock reagents and experimenter inexperience was excluded. Using a smaller number of embryos could improve efficiency as it would be more important to retrieve the ICM from every blastocyst. Immunosurgery completed for the purpose of this thesis was performed on large numbers of blastocysts at a time. When dealing with this number of blastocysts it was not crucial that all blastocysts had an ICM isolated and cultured. For practical applications, a greater emphasis would be placed on isolating ICMs from a relatively small number of

genetically identified blastocysts. This would increase the efficiency of immunosurgical isolation, resulting in the main determinant of net gain of ICM cultures being plating efficiency.

Once cultured, the isolation of single cells from ePSC colonies has been an issue in the past, hindering the use of these cells in cloning [6]. Studies using mice ESCs for NT cloning were able to culture cells and trypsinise them to separate into single cells [101]. This, however, is not possible in other species; cells have not survived chemical or enzymatic attempts to dissociate, and cells lysed in Ca-Mg-free media [6]. Thus a method that limited cell lysis was required. A combination of enzymatic and chemical dissociation, with mechanical manipulation of colonies, resulted in isolation of single cells from nocodazole-arrested colonies. The optimized conditions identified were the detachment of colonies from the substrate with dispase treatment for five minutes, followed by incubation in pronase for five minutes, and trituration in Ca-Mg-free dissociation media including CB. This allowed effective isolation of cells with minimal cell lysis by reducing the amount of trituration required. These techniques were less efficient with non-arrested colonies. This is likely due to the morphology of cells in the nocodazole-arrested colonies; cells in mitosis condense their shape to a more rounded morphology [102], which is associated with a loss of adhesion [103]. The reduced adhesion of the mitotic cells aids separation from the colony. As nocodazole-arrested colonies have a larger fraction of the population in mitosis than non-arrested colonies, single cells are easier to isolate. This is beneficial for use in NT cloning as M-phase cells are required for donor cells, and these are the cells that are more likely to separate from the colony.

5.2 Objective 2: Characterisation of ePSC donor cells

5.2.1 Duration and concentration of nocodazole arrest for synchronisation into M-phase

The microtubule-depolymerising action of nocodazole for long incubation times or at high concentrations is detrimental to centrosome structure [98]. Microtubule structure is required for functioning of the centrosome. High concentrations of nocodazole are associated with complete depolymerisation of microtubules, whereas lower concentrations have been shown to maintain structure similar to normal microtubules while still blocking function [98]. Maintenance of microtubule structure and centrosome functioning are important for accurate segregation of chromosomes once the drug is removed. For example, arresting with the microtubule depolymerising drug colcemid has been shown to cause aneuploidy, impacting further cell division [97; 98]. Loss of microtubules and centrosome structure is an issue for bovine cloning as the maternal centrosome is removed with the nucleus. This requires the centrosome introduced from the donor cell to remain intact for the correct segregation of chromosomes, allowing progression through the cell cycle [60]. Thus, minimisation of the length of arrest and concentration of nocodazole was important to reduce any negative effects of its use.

Two time points (4 hr and 18-22 hr) were compared with no nocodazole arrest (DMSO control). These time points were chosen as they were the most practical for use with the timings required for NT cloning. Short incubation of four hours was not sufficient to arrest a greater number of cells than what was observed in a non-arrested colony. By contrast, overnight (18-22 hr) incubation with nocodazole was sufficient in arresting around half the colony in mitosis. Due to the negative effects of long nocodazole exposure, a shorter incubation was preferred to overnight. However, practical incubations between 4-18 hr would have altered timing by only a few hours.

The inability to reduce the length of arrest resulted in an emphasis on reducing the concentration of nocodazole. Four nocodazole concentrations were tested to determine the minimal effective concentration. The highest (1.67 µM) was the standard concentration used in the laboratory where experiments were completed (Björn Oback, personal communication, 2014). The lowest concentration was based on the study by Jordan et al. that showed 100 nM nocodazole successfully arrested cultured somatic cells without completely depolymerising microtubules [98]. While 100 nM did arrest more cells compared with a non-arrested colony, it arrested significantly less than all higher concentrations. As only mitotic cells were desired for NT cloning, the concentration that produced the highest number of these cells was required; 100 nM was too low to maximise the number of donor cells. Of the three higher concentrations, there was no difference in the number of cells arrested at 500 nM, 1 µM, or 1.67 µM. Therefore, 500 nM was the lowest effective dose that produced maximal numbers of donor cells. This is lower than the 1 µM that was identified as causing complete depolymerisation of microtubules [98].

5.2.2 Immunofluorescence characterisation of cell cycle markers

All IF was carried out on colonies that had been arrested with 500 nM or 1.67 µM nocodazole overnight, depending on when experiments were performed in relation to optimisation of nocodazole concentration. There was no difference in the proportion of cells with condensed chromatin between 500 nM and 1.67 µM observed during nocodazole optimisations, therefore, this difference in nocodazole concentration should not have affected the IF quantification, only the impact the drug had on live cells in cloning.

The number of M-phase donor cells produced from an ePSC colony for NT following nocodazole arrest was evaluated with chromatin condensation, phosphorylated histone 3 (H3), EdU, and Ki-67 staining. Chromatin condensation, visualised with Hoechst, was used as a proxy for mitosis as chromosomes condense during metaphase. Phosphorylated H3 was a direct measure of mitosis;

phosphorylation at serine 10 of H3 begins in late G_2-phase, completed in late prophase of mitosis and meiosis, and maintained throughout metaphase [104]. Analysis of chromatin condensation in nocodazole incubation trials revealed 42% of cells arrested in mitosis after overnight incubation with 1.67 µM nocodazole. This was reinforced with staining for phosphorylated H3 showing that around 40% of cells were arrested with nocodazole, a 3.6 fold increase compared with non-arrested colonies. This means that close to 40% of the ePSC colony, around 180 cells (average colony 446 cells), were synchronised into the correct donor cell stage for use in NT cloning. This is further increased by selecting cells with the correct morphology once isolated, as mitotic cells can be distinguished by their large, round morphology. Correct identification of cells in mitosis was established by staining isolated ePSCs and couplets: 66% (N=280) of isolated single ePSCs were mitotic, while 94% (N=18) of selected donor cells attached to a cytoplast were mitotic (data not shown).

Cells in S-phase are incompatible with NT using MII-arrested cytoplasts, so EdU staining was used to evaluate the proportion of the colony that would be unsuitable for NT. EdU is a thymidine analogue that is incorporated into DNA during synthesis. This can then be detected by reaction with fluorescent azides, identifying the number of cells in S-phase at a given time [105]. A short (30 min) EdU incubation was used to measure the steady-state distribution of cells in S-phase. An alternative use for EdU labelling is determining the total fraction of proliferative cells with a long (>24 hr) incubation. In a non-arrested colony, EdU incorporation revealed 28% of the colony was in S-phase. This was reduced to 11% with an overnight nocodazole arrest. This data supports the observation that around 40% of the colony is arrested in metaphase as such cells will no longer be reaching S-phase. If about 40% of the colony is no longer cycling, then it would be expected that 28% of the 60% of non-arrested cells would still be in S-phase. Provided that nocodazole does not change the relative length of other cell cycle stages, only about 16% of an arrested colony would be estimated to be in S-phase. The observed drop in cells in S-phase roughly matches this prediction, indicating

that the around 40% the colony is arrested, and the remaining cells are progressing normally through S-phase.

Ki-67 is an indicator of cells proliferating. The Ki-67 protein is present throughout G_1, S, G_2, and M phases at varying concentrations and locations, but absent from quiescent G_0 cells. An increase in Ki-67 expression has been identified progressing through the cell cycle, with lowest expression in G_1 and maximum expression in metaphase [49]. About 29% of cells in the non-arrested colonies were Ki-67 positive. This is similar to the proportion of S-phase cells as marked by EdU incorporation. This overlap indicates that in non-manipulated colonies i) all proliferative cells are in S-phase and ii) most cells (>70%) are non-proliferative. This is supported by studies by Burdon et al. that showed the G_1-phase in mouse ESCs is almost entirely absent, thus proliferating cells will either be in S- or M-phase [106]. Alternatively, cells in G_1 may express low levels of Ki-67 and were not counted as positive, underestimating the proliferative fraction. In nocodazole-arrested colonies, the proportion of Ki-67-positive cells increased to 65%. This again supports the observation that around 40% of the colony is arrested at M-phase following nocodazole arrest. If about 40% of the colony is arrested in mitosis, then it would be expected that these cells would also be Ki-67 positive. This would increase the number of Ki-67 positive cells from 29% to 69%. The observed increase in Ki-67 positive cells in nocodazole-arrested colonies roughly matches this prediction, indicating that the around 40% of the colony is arrested, and the remaining cells are progressing normally through S-phase.

5.2.3 Immunofluorescence characterisation of pluripotency genes

The expression of two key pluripotency genes, *NANOG* and *SOX2*, were examined to ensure the cells used for NT cloning were ePSCs. Cultures of ePSCs either had a flat morphology or a central dome-shaped colony with an outgrowth of cells. Pluripotency genes were examined to assess whether i) the whole culture was comprised of ePSCs and ii) if there was a difference in dome-shaped colonies

compared with their outgrowth. *NANOG* was chosen as it is expressed exclusively in pluripotent cells, and *SOX2* as it is ICM-specific [6; 74], allowing the identification of cells as being non-pluripotent or of epiblast origin.

NANOG expression was evaluated in both arrested and non-arrested colonies. Together, 43% cells within an ePSC culture were NANOG-positive. No significant difference between the two colony types was observed, indicating that arresting cells did not affect levels of NANOG expression. However, staining was inconsistent; only two technical replicates yielded positive staining results. This may have been due to variation between different primary antibody stocks or other technical failure. These difficulties prompted the use of the alternative pluripotency gene, *SOX2*.

SOX2 was not the primary gene used for evaluating pluripotency as not every SOX2 positive cell will be expressing NANOG, but all NANOG positive cells are SOX2 positive [74]. SOX2 expression was evaluated in both arrested and non-arrested colonies. Overall, ePSC cultures had 43% SOX2 positive cells. This was similar to observations with NANOG staining, supporting these observations, despite the difficulties in achieving a positive NANOG stain. As SOX2 staining was more robust, a comparison between dome-shaped central colonies and outgrowth cells could be made on cultures with this morphology. This revealed that SOX2 positive cells were enriched in the dome-shaped colony, with the outgrowth showing lower expression. Low SOX2 expression in outgrowth cells presents a different population of cells that could be used for NT. Outgrowth cells would be more differentiated than the inner colony, with identical genetic backgrounds. Therefore, comparison of outgrowth and inner colony cells could highlight any differences in cloning efficiency from this increase in differentiation. Alternatively, these two populations can be used together if there is no difference in reprogramming efficiency. It is unlikely that there will be any differences in cloning efficiency, as no difference between TE and ICM cells in mice [106], or between stem cells and their differentiated progeny in a number of species [107] has been observed to date.

Finally, the mitotic fraction of the population was examined for expression of SOX2. During NT cloning, donor cells were selected based on morphology, using only those cells that were large, round, mitotic cells. This meant that the population of donor cells was enriched for those in mitosis and in the outgrowth, as these cells were larger than the central colony cells. Hence, it was important to analyse this population of cells for its SOX2 expression, rather than the whole colony. Of those cells identified as mitotic through staining for phosphorylated H3, 64% were SOX2 positive. Therefore, choosing a cell based on its mitotic status results in a ~60% chance that it would be an ePSC. This could be further increased by avoiding outgrowth cells when transferring colonies through treatments used to isolate single donor cells.

5.2.4 Issues with immunofluorescence characterisation of ePSC colonies

The main issue with quantification with IF is the reliability of counts due to the morphology of the colonies. In cultures with a dome-shaped central colony there were a large number of cells clustered on top of each other, making counting individual nuclei difficult. This observation will have affected the quantification of proportions of colonies positive for a particular stain. Hence, IF analysis should only be viewed as semi-quantitative. The morphology of colonies also affected analysis; dome-shaped central colonies would occasionally show a decrease in signal in the centre compared to the edges of the colony and the outgrowth. This could be because the camera was saturated with information from the large number of cells, or that the morphology of the colony affected the efficiency of the staining protocol. The morphology did not affect Hoechst staining, resulting in nuclei being included that did not have efficient IF signal. Again, this will have affected quantification of colony proportions. This issue could be resolved by analysing colonies with a dome-shaped morphology with a confocal microscope.

A technical issue with the IF procedure was that round, mitotic cells were more likely to be dislodged. Mitotic cells were less strongly attached to the substrate, meaning they could be easily dislodged during the various aspirations in IF and removed from the well. This will have impacted the accurate quantification of the number of cells in mitosis in a colony by preferentially losing mitotic cells. This was reflected in the lower number of overall cell counts in nocodazole arrested colonies. The impact of this was attempted to be reduced by using a pump for aspiration that had a lower vacuum pressure, to remove the solutions as gently as possible, and by discarding colonies that had large sections of outgrowth cells missing. Even with these attempts to mitigate the effects, the characterisation of number of cells arrested is likely to be underestimated.

Finally, the discrimination of positive and negative stains in IF staining is highly subjective. While some antibodies produce well-defined stains, other have a large amount of background fluorescence or weak positive signal. This was a particular issue with the dome-shaped central colonies that had decreased signal in the centre. The subjective nature of IF analysis was reduced by having all analysis performed by the same operator and positive signals having a determined cut-off based on signal being higher than the controls with no primary antibody.

5.2.5 Summary of characterisation of ePSC donor cells

Characterisation of ePSC colonies showed that a nocodazole arrest for 18-22 hr with a concentration of 500 nM was required to arrest the maximal number of cells in mitosis practical for cloning. This incubation resulted in ~40% cells arrested in mitosis, confirmed by quantification with condensed chromatin and staining for phosphorylated H3. Nocodazole-arrested colonies reduced the proportion of cells in S-phase and increased the fraction stained as ‘proliferating’. Generated ePSC colonies had 43% cells positive for NANOG or SOX2 in the whole colony, with a greater proportion of SOX2 cells enriched in the central dome-shaped colony. Of those cells identified in mitosis by phosphorylated H3 staining, 64% were also positive for SOX2, the fraction of the population desired

for NT cloning. However, these numbers are confounded by the subjective nature of IF analysis and difficulty of analysing the central dome-shaped colony, resulting in this technique to be viewed as semi-quantitative.

5.3 Objective 3: Establishing an NT cloning protocol for M-phase bovine donor cells

5.3.1 Optimising artificial activation with PGs

Parthenogenesis was used to as a proxy for optimising artificial activation protocols. Using PGs removed variation in development introduced by compatibility of the donor cell, allowing examination of efficiency of activation protocol alone [47]. Firstly, two concentrations of CHX and anisomycin were assessed to select the concentration that produced the optimal development rate. The concentrations trialled were one previously used in the laboratory where experiments were conducted at AgResearch, and a concentration used frequently from the literature. Activation with 17.8 µM CHX was used previously at AgResearch (Björn Oback, personal communication, 2014), whereas 35.5 µM was used commonly in the literature [14; 42; 46; 52; 68; 96]. The higher concentration of CHX produced a higher blastocyst development rate on day 7, but no differences on day 8. It was hypothesised at this point that NT reconstructs do not develop well to day 8, making 35.5 µM CHX the more desirable concentration to maximise development on day 7.

Activation with anisomycin has been used at AgResearch previously at a concentration of 100 µM (Björn Oback, personal communication, 2014). This was ten times higher than the 10 µM used in the literature [54]. Use of 100 µM anisomycin resulted in very low cleavage and no blastocysts developed. This suggests that this concentration is too high, causing the action of the drug to be less reversible, preventing development. Therefore, 10 µM anisomycin was the concentration used in further trials. Recently, a study by Felmer et al. systematically addressed the optimal concentration of anisomycin for activation of bovine PGs [96]. This study concluded that 3.8 µM (1 µg/ml) produced the

highest blastocyst development rate of 25% by day 7. The closest comparison they performed to 10 µM was 9.4 µM (2.5 µg/ml). Development following incubation with 9.4 µM anisomycin was 17%, which was not significantly lower than 3.8 µM. By comparison, experimental work for this thesis using 10 µM anisomycin resulted in 9% total development on day 7. It is unclear why these differences in developmental rates have occurred. Incubations occurred for four hours, compared with five for this thesis, but Felmer et al. found no significant difference between these incubation lengths. Developmental rates similar to Felmer et al. could only be achieved by culturing to day 8.

Following optimisation of CHX and anisomycin concentrations, development of PGs was compared between DMAP, CHX, and anisomycin. Optimisation of DMAP was not required as concentration of 2 mM has been well established [34]. No difference in cleavage was observed, indicating that activation had occurred correctly in all three groups. The highest blastocyst development, in both total number of blastocysts and those of transferrable quality, was produced by incubation in DMAP. This was evident on both day 7 and day 8. By contrast, Felmer et al. found no difference in PG development between DMAP and anisomycin. While their CHX development was similar to experimental work for this thesis, they had lower DMAP and higher anisomycin development [96]. It is unknown why these differences have occurred, as concentrations and incubation lengths were either identical between the two protocols, or not significantly different. These differences may be caused by inherent variation between laboratories. Therefore, for this thesis, DMAP was the optimal activation agent.

Interestingly, the difference in development between CHX and anisomycin was only observed on day 7. By day 8, development of anisomycin blastocysts had increased to that of CHX. The difference was explained by developmental stage of embryos on day 7. Activation with anisomycin resulted in a large proportion of compact morulas on day 7. By day 8, these had developed to blastocysts, reaching the same level of development as CHX. This may reflect the potency of these two drugs, indicating that embryos take longer to recover from anisomycin incubation.

A significant net increase in transferrable grade embryos from day 7 to day 8 was observed with DMAP and anisomycin activation, and a trend of increased development with CHX. The increase in grade 1-2 blastocysts was investigated by tracking development of individual embryos. This was to examine whether the total increase was caused by i) new blastocysts developing or ii) blastocysts increasing in grade from day 7 to day 8. In all groups the majority of blastocysts remained the same grade, with blastocysts more likely to increase than decrease in grade. Of the blastocysts that newly developed between day 7 and day 8, less than 30% were of transferrable grade in all groups. Therefore, the net increase was due to both grade 3 compact morulas and existing blastocysts improving to grade 1-2. These results indicate that for PGs it is beneficial to wait until day 8 as there is a net increase in transferrable grade blastocysts, with minimal degradation below transferrable quality.

Overall, DMAP was shown to be the optimal activation agent. However, DMAP prevents extrusion of a pseudo-polar body, which was perceived to be necessary to correct ploidy when cloning with M-phase donor cells. Cloning with M-phase donor cells using DMAP activation should result in 4C blastocysts that would not develop further. In order to resolve this issue, a delay between ionomycin activation and DMAP incubation was introduced. During this delay PGs were allowed to extrude a pseudo-polar body before being transferred to DMAP. This would correct the ploidy of the cell, while enabling the use of the best activation combination. Monitoring pseudo-polar body extrusion showed that after 90 min there was maximal expulsion of pseudo-polar bodies. However, the development of blastocysts significantly decreased with a delay into DMAP of 60-120 min. This was thought to be due to the reduction in chromosome number in pseudo-polar body PGs; extrusion of a pseudo-polar body in PGs should generate a haploid cell (1N/2C to 1N/1C), reducing development. This would not be an issue with NT reconstructs as cells would be going from double the normal DNA content to diploid (2N/4C to 2N/2C). Therefore, the decrease in development was not considered a problem.

5.3.2 Optimising NT fusion parameters

Following optimisation of artificial activation, fusion parameters were examined. Firstly, hypoosmolar and isoosmolar fusion buffer were compared. Hypoosmolar is the standard fusion buffer used as it increases fusion rate with G_0 somatic cells. This is thought to be due to the increase in osmotic pressure causing the cells to swell, increasing contact between cell and cytoplast [43; 44]. However, with M-phase cells, hypoosmolar fusion buffer resulted in a greater amount of cell lysis prior to fusion. Mitotic cells are generally larger than G_0 cells, as the cells have increased in size in preparation for cell division. During growth, the volume of the cell increases by a power of three, whereas the surface area by a power of two. This means there is a disproportional increase in cell volume compared to plasma membrane of the cell. When these cells are introduced to hypoosmolar fusion buffer, the osmotic difference between the cell and buffer causes the cells volume to increase further. As this cell already has a proportionally smaller amount of plasma membrane, the increase in volume causes the cell to lyse. Isoosmolar fusion buffer, therefore, is more beneficial for the survival of M-phase cells prior to fusion.

Secondly, pronase treatment of donor cells was examined. In mice somatic cell NT cloning, pronase treatment of donor cells aids fusion. This is thought to be due to 'smoothening' the surface of the cell by removing surface proteins to aid contact between cell and cytoplast [42-44]. Pronase treatment was trialled in both hypoosmolar and isoosmolar fusion buffer. In hypoosmolar fusion buffer, pronase treating cells made no difference to the fusion rate. However, in isoosmolar fusion buffer, pronase treating cells was necessary to achieve fusion rates similar to those with hypoosmolar fusion buffer; without pronase treatment, fusion rate was decreased 1.6 fold in isoosmolar fusion buffer. The different effect of pronase treatment in the two fusion buffers may be due to the different ways of improving surface area of contact between donor cell and cytoplast. In hypoosmolar fusion buffer, the osmotic stress causes the cells to swell, increasing contact. The

increase in contact between cells is not further increased by removing surface proteins, suggesting a physiological limit has been reached at optimising the improvement in contact. However, isoosmolar fusion buffer does not cause the cell to swell, so pronase treatment is required to remove surface proteins from the donor cell to increase contact between the two cells, aiding fusion.

Analysing fusion rate alone, hypoosmolar fusion buffer produces optimal fusion rate. However, when these are taken in conjunction with the amount of cell lysis prior to fusion, hypoosmolar fusion buffer is no longer the optimal choice. The net efficiency of fusion with pronase treated cells in hypoosmolar fusion buffer is lower than that of isoosmolar. This is due to the large amount of cell lysis in hypoosmolar fusion buffer; although the fusion rate is better, this is offset by increased cell lysis. Overall, the most efficient option is isoosmolar fusion buffer as it only has slightly lower fusion efficiency than hypoosmolar, and a significantly lower degree of cell lysis.

5.3.3 Somatic cell lines used for NT trials

Three different somatic lines were used in NT trials, depending what was available at the time of cloning. BEF14 was selected as the main line as it was readily available at low passage numbers. This work occurred before fusion buffer and pronase treatment optimisations, so somatic cells were fused in hypoosmolar fusion buffer without pronase treatment. Comparing lines showed no significant difference in cleavage or development, therefore, these results were pooled for further analysis.

5.3.4 NT with CHX activation of ePSCs vs somatic cells

As CHX had better blastocyst development rates than anisomycin, it was used in NT cloning trials in an attempt to avoid the issue with 4C cells and DMAP activation. Both ePSC and somatic cells were used as donor cells with CHX activation to compare development. Different concentrations of CHX were used for ePSCs and somatic cells due to the timing of trials with activation

optimisations, however, concentrations were kept constant within cell type. Hypoosmolar and isoosmolar fusion buffers were used as fusion optimisation had not been completed. This data was pooled as the different fusion buffers only affect fusion rate, not blastocyst development. No difference in development was observed between ePSCs and somatic cells. Observations were lower than that of G_0- and M-phase somatic cells using CHX activation previously published by Tani et al. [46] and Felmer et al. [96]. There were minor differences in cloning protocols used by Tani et al. and Felmer et al. compared with work for this thesis. Somatic donor cells used by Tani et al. were cumulus cells, whereas Felmer et al. and work for this thesis used embryonic fibroblasts. Thus, donor cell type can only account for differences between this thesis and Tani et al. Genetic background and passage number of embryonic fibroblasts differed between this thesis and Felmer et al., potentially affecting developmental rates.

5.3.5 NT with DMAP activation of ePSCs vs somatic cells

5.3.5.1 Pseudo-polar body extrusion prior to DMAP incubation

Poor development using CHX prompted the search for an alternative activation protocol. PG activation trials suggested activation with DMAP would improve development to day 7 and day 8. However, it was perceived necessary to allow the extrusion of a pseudo-polar polar body before incubation with DMAP to correct chromosome content. From PG optimisation trials it was known that the number of pseudo-polar bodies expelled plateaued after 90 min and these developed to blastocysts, albeit at a lower rate. However, the decrease in blastocyst development was thought to be due to pseudo-polar body PGs being haploid. Timing of pseudo-polar body extrusion was evaluated with NT reconstructs, with plateau of pseudo-polar body extrusion occurring around 90 min. As with PGs, a decrease in development was observed in reconstructs that had extruded a pseudo-polar body compared with the standard DMAP protocol. The decrease in development was similar to that with PGs; over 2.5 times lower development observed following a 90 min delay between ionomycin and DMAP incubation. By contrast, no reconstructs developed after a 120 min delay, unlike their PG

counterparts, which developed only slightly lower than after a 90 min delay. The decrease in development of the 90 and 120 min delay reconstructs was unexpected as the extrusion of a pseudo-polar body was thought to correct the DNA content of the cell to the correct amount (2N/4C to 2N/2C). The poor development was caused by inaccurate expulsion of chromatin; Hoechst-stained reconstructs showed that all chromatin was expelled in the pseudo-polar body, leaving an empty cytoplast. Therefore, development only occurred in the few reconstructs that segregated chromosomes correctly. The proportion of reconstructs that expelled all chromatin in the pseudo-polar body was not quantified. This was because reconstructs could either be stained or cultured to observe development; no live staining protocols were available, such as the injection of messenger RNA (mRNA) for fluorescent proteins used by Mizutani et al. [64] that allowed the visualisation of chromosomal segregation patterns. Such live staining protocols would allow the analysis of chromosome segregation and continued development of the embryo.

A concerning observation was that reconstructs with delayed DMAP incubation (90 or 120 min) that did not expel a pseudo-polar body did not develop into blastocysts. These reconstructs should have had the same DNA content as reconstructs processed with the standard DMAP protocol, and therefore should develop at a similar rate. One reason for this observation may be that these reconstructs had been incorrectly scored as fused. However, earlier experiments that had been stained with Hoechst showed the rate of incorrect fusion scoring was very low (data not shown). Alternatively, the delay between ionomycin and DMAP may be sufficiently long that cyclin B/Cdk1 complexes are able to reform. This would prevent nuclear membrane reformation, interrupting the resumption of the cell cycle, affecting subsequent development. If reformation of cyclin B/Cdk1 was occurring, it would also be impacting the development of reconstructs that correctly expelled a pseudo-polar body. Work by Susko-Parrish et al. showed that within the first hour following activation with ionomycin oocytes had resumed meiosis, and without incubation in DMAP for more than an hour oocytes were observed to form multiple pronuclei or multiple polar bodies. However, if oocytes

were cultured in DMAP following ionomycin activation, correct pronucleus formation occurred and development to blastocysts [108]. Alternatively, delay into DMAP could be affecting calcium oscillations. Ionomycin generates a single large increase in intracellular calcium, whereas sperm-mediated activation initiates calcium waves. Incubation in protein phosphorylation or synthesis inhibitors, such as DMAP, is thought to play a role in inducing calcium oscillations [52]. Therefore, delay into DMAP culture may be affecting calcium oscillations and subsequent activation of the cytoplast.

5.3.5.2 Standard NT with DMAP activation

While development was poor from DMAP delayed reconstructs, the control group of standard DMAP development showed promising results. This prompted the comparison of NT reconstructs generated from both ePSCs and somatic cells using ionomycin and DMAP activation. As with CHX NT trials, hypoosmolar and isoosmolar fusion buffers were used as fusion optimisation had not been completed and the data was pooled as this only affected fusion rate, not blastocyst development. Three times higher blastocyst development rate was observed using ePSC donor cells. This showed that the increase in DNA content in DMAP-activated compared with CHX-activated reconstructs did not prevent blastocyst formation. In fact, development was greater using DMAP than CHX for ePSCs. This development rate is similar to what was achieved with G_0 embryonic fibroblast cells by Felmer et al. [96] and G_0 BEF14 cells (data not shown). However, M-phase somatic cells developed poorly in comparison to their G_0 counterpart and Felmer et al.'s G_0 somatic cells. If this difference is reflective of the donor cell cycle stage used, then G_0 ePSCs should have an even greater development rate. In contrast, Tani et al. achieved blastocyst development of 17% from M-phase somatic cells using DMAP activation, higher than achieved in this thesis [46]. Somatic donor cell type and preparation differed between these two studies: Tani et al. used cumulus cells arrested for 20-24 hr with 10 µM nocodazole and this thesis used embryonic fibroblasts arrested for 1-2 hr with 0.5-1.67 µM nocodazole. It would be hypothesised that the concentration and

incubation length used by Tani et al. would be more detrimental to development based on nocodazole studies [98]. A greater number of reconstructs were cultured for this thesis (173 vs 23), making this data set more robust. However, somatic cell trials were not contemporaneous with ePSC NT, introducing variation in comparing these results.

The increase in transferrable grade blastocysts observed in PG trials on day 8 was compared with NT reconstructs. Overall, there was a trend of decreased blastocyst development and grade from day 7 to day 8. This was emphasised by tracking individual blastocyst grades. On day 8, nearly all grade 2 embryos had degraded to grade 3. The lack of statistical significance likely occurred due to the power of analysis being reduced by the small number of blastocysts that survived to day 8 (total blastocysts from ePSC and somatic donor cells on day 8 N=23). This drop in transferrable grade blastocysts is significant as it confirmed that NT blastocysts do not survive well to day 8 *in vitro*, and culturing them for this length reduced the number of embryos that could be used for embryo transfer. This means that anisomycin may be an unsuitable activation agent as day 7 development of PGs was poor, or that overall development with anisomycin is delayed and development of NT reconstructs on day 8 would be similar to day 7 CHX. This is in contrast to Felmer et al.'s results with total blastocyst development with somatic G_0 donor cells [96]. However, the proportion of blastocysts that were of transferrable quality was not stated in that study.

5.3.5.3 NT into preactivated cytoplasts

The development of blastocysts from ePSC donor cells was attempted to be increased by using preactivated cytoplasts. Tani et al. showed that activating cytoplasts with ionomycin and incubating in CHX for up to five hours prior to cell fusion with M-phase cells had a trend of increased development [46]. As DMAP had resulted in the highest development using standard MII-phase cytoplasts, it was used in place of CHX. A one hour preactivation was used to limit the difference in nocodazole arrest timing of donor cells and prevent compromising

donor cell quality after single cell isolation. A trend of lower total development was observed using one hour preactivated cytoplasts, and a significant decrease in transferrable grade. Contrary to Tani et al., NT into ionomycin/DMAP preactivated cytoplasts decreased blastocyst development.

5.3.5.4 Summary of NT with DMAP activation

Overall, development with DMAP was higher than CHX and reconstructs from ePSC donor cells developed better than somatic donor cells. Preactivation of cytoplasts did not improve development rate with DMAP activation. Therefore, the most efficient NT cloning protocol was using ePSC donor cells with MII-phase cytoplasts, activated with ionomycin and DMAP. The remaining question with this activation protocol was whether embryos still contained double the amount of DNA (2N/4C). Without the expulsion of a pseudo-polar body, embryos should have twice the normal amount of DNA, appearing as 'tetraploid' when karyotyped. Karyotype analysis was necessary to assess whether embryos were still 4C or if they were able to regulate their ploidy through cell cycling.

5.4 Objective 4: Validating different activation and NT cloning protocols by karyotype analysis

The normal diploid bovine karyotype contains 29 pairs of acrocentric autosomes and 2 metacentric sex chromosomes [109]. Blastocyst chromosome spreads were generated from a range of activation protocols. PGs activated with standard DMAP or CHX protocol had a bimodal distribution, with chromosome spreads containing around 60 or 120 chromosomes. The expected number of chromosomes of a standard PG blastocyst would be 30 chromosomes; 1N/2C oocyte would replicate its DNA following activation, maintaining the double chromosome content which appears as 60 chromosomes when karyotyped. The presence of spreads with 120 was unexpected. These were unlikely to be caused by multiple cells being mistaken for a single spread as only those spreads that were clearly identifiable as coming from a single cell were analysed. The bimodal

distribution was hypothesised to be caused by different chromosome numbers in TE vs ICM cells. Polyploidy is common in TE cells, whereas ICM cells maintain diploid chromosome content to generate the embryo proper [110; 111]. The distribution of polyploid cells was addressed by bisecting PG embryos to separate TE cells, and immunosurgically isolating ICM cells, to karyotype each population of cells separately. The chromosome spreads analysed from each cell population supported this hypothesis; ICM cells had spreads around 60 chromosomes, and TE cells produced a spread of over 100 chromosomes. Unfortunately, only a small number of embryos were able to be processed in this manner. To confirm this hypothesis more embryos should be analysed from each isolated cell population.

PG blastocysts that produced more than one clear chromosome spread were analysed for mixoploidy: most cells being diploid with a few cells having differing chromosome number (e.g. haploid or tetraploid) [112]. Mixoploid embryos support the hypothesis that high chromosome counts are being caused by a subpopulation of cells within the embryo, whereas embryos with multiple tetraploid spreads may suggest errors in artificial activation causing irregularities in chromosome number. Of the blastocysts analysed, only a single blastocyst had multiple tetraploid spreads, while 28% were mixoploid and 67% were solely diploid. This further supports the idea that tetraploid spreads (~120 chromosomes) were from TE cells and diploid spreads (60 chromosomes) were from ICM cells.

PG blastocysts produced by ionomycin activation with a delay into DMAP to allow for a pseudo-polar body to be expelled were also assessed. The expected karyotype was 30 chromosomes, as the expulsion of a pseudo-polar body would halve the number of chromosomes (1N/1C) which appears as 30 chromosomes when karyotyped. A small number of spreads analysed were haploid, however, the majority of spreads contained chromosome numbers ranging from between 30-60 chromosomes to 120 chromosomes. As this activation protocol was proven to be detrimental to NT blastocyst development, experiments generating pseudo-polar body expelled PG blastocysts were halted before analysis of separate TE and ICM populations could be completed. Therefore, it is not known whether the

unexpected chromosome numbers were located solely in TE cells, or if they were caused by unequal segregation of chromosomes in the pseudo-polar body, affecting the ploidy of the whole embryo.

NT generated blastocysts did not display the tight bimodal distribution of their PG counterparts. Of the blastocysts karyotyped, only one group (CHX pseudo-polar body expelled blastocyst from ePSC donor cell) was expected to have 60 chromosomes (2N/2C), whereas the other three groups (DMAP from somatic donor cell, DMAP from ePSC donor cell, and CHX from ePSC donor cell without a pseudo-polar body expelled) did not have a pseudo-polar body expelled, so should have appeared to have 120 chromosomes (2N/4C). In general, most chromosome spreads analysed contained over 100 chromosomes. The spread from CHX activated, pseudo-polar body expelled blastocyst was 60 chromosomes, and the blastocyst that did not expel a pseudo-polar body had 120 chromosomes. These observations matched the expected outcome of pseudo-polar body expulsion. This indicated that blastocyst development with CHX would likely have been higher if chromosome segregation in pseudo-polar body was correct. However, low development rates meant only a small number of blastocysts were available for karyotyping. This resulted in only a single clear spread being analysed for each blastocyst type. This reduces the significance of this finding, as there may be more variation in chromosome numbers observed with a greater sample size.

DMAP-activated NT reconstructs had a small number of spreads with around 60 chromosomes. This was unexpected as reconstructs had not been allowed to expel a pseudo-polar body, so all cells should have appeared to have 120 chromosomes (4C). A postulated mechanism for this occurring was that the cells within the embryo were able to regulate their DNA content, perhaps by detecting the amount of DNA present and preventing DNA replication before cell division, thus returning DNA to the normal amount. Studies by Parmenter showed that the occurrence of diploidy in pseudo-polar body extruded PGs was caused by delaying the cell cycle to enable an extra round of DNA replication, correcting

DNA content [113]. If the opposite is possible, then a reduction in the cell cycle due to bypassing DNA synthesis would correct DNA content from M-phase donor cells. To prove this, a live chromatin image analysis would be required, which was not available.

The main issue with karyotype analysis was achieving spreads that had exactly 30, 60, or 120 chromosomes. Selection of chromosome spreads was focused on quality: having few overlapping chromosomes and being separate enough from other spreads to be able to identify chromosomes from a single cell. The chromosome spreads that were the best for analysis often had a few chromosomes scattered further away from the main cluster, introducing some subjectivity as to which spread the chromosomes originated from. To account for these fluctuations in chromosome number, the ploidy of the cell was calculated based on ± 10%.

Chapter 6: Conclusion and future recommendations

To summarise, ePSCs were successfully cultured and isolated into single cells through serial treatment with dispase, pronase, and trituration in Ca-Mg-free dissociation media containing CB. Overnight incubation of 500 nM nocodazole was the minimal concentration identified to arrest the maximal number of ePSCs into mitosis. This incubation arrested ~40% of the colony, as shown through condensed chromatin and immunofluorescent staining of phosphorylated H3, and reduced the number of cells in S-phase, shown though staining of EdU and Ki-67. Immunofluorescent staining of NANOG and SOX2 revealed that 43% of the cultured cells were expressing the pluripotency genes expected of ePSCs, and these cells were enriched in the central dome-shaped colony. Of the cells arrested in mitosis that would be morphologically selected as donor cells for NT cloning, 64% were SOX2 positive. NT cloning protocols were optimised for use with these M-phase cells. Use of MII-phase cytoplasts, pronase treatment of donor cells, fusion in isoosmolar fusion buffer, and activation with ionomycin followed by DMAP incubation produced the highest blastocyst development. Comparison of donor cell type under these conditions revealed three-fold higher development with ePSCs than the more differentiated somatic donor cells. Validation of correct ploidy of blastocysts was achieved using karyotype analysis. This showed a bimodal distribution of spreads around 60 or 120 chromosomes, with higher chromosome numbers apparently restricted to TE cells.

Two main areas of investigation would be beneficial to further validate these findings. Firstly, further IF characterisation of isolated single ePSCs actually used for NT, rather than analysing whole colonies, should be assessed. This would ensure the population of cells used for NT were correctly identified as mitotic, and they were from the subset of the colony that was expressing pluripotency markers. Alternatively, a comparison could be made between outgrowth and inner colony cells for cloning efficiency. Secondly, further analysis of karyotypes from NT

blastocysts, specifically, investigating the ploidy in TE and ICM cells through bisection and immunosurgical isolation of the two subpopulations. This will be important to address before *in vivo* trials due to issues with continued development from 4C embryos; if 4C spreads are found in isolated ICM cells, then the optimised *in vitro* protocol may result in poor development once embryos are transferred to a surrogate.

For the practical applications of using ePSCs in cloning, assessment of the number of blastocysts that can be achieved from a single colony should be performed. Work for this thesis focused on optimising procedures, so multiple ePSC cultures were pooled together to generate single donor cells. However, the practical aim of cloning with ePSCs is to multiply the genetics of a single embryo. Therefore, it will be important to know how many blastocysts, and eventually live offspring, can be generated from a single original blastocyst. Also, it will be beneficial to know if this efficiency is affected by taking TE biopsies and freezing embryos prior to generation of ePSC cultures. For practical applications, blastocysts would be biopsied for genotyping and frozen until results were returned. High quality blastocysts would then be thawed and used to create ePSCs.

If results from this work show that cloning efficiency is too low from a single blastocyst ePSC colony, other techniques can be tested with the optimisations already achieved. For example, fusing two MII cytoplasts with a single donor cell (double cytoplast cloning) has been shown to improve blastocysts development [114]. Also, decreasing interval between fusion and activation has been shown to improve NT efficiency. Alternatively, the lower concentration of 3.8 µM anisomycin, shown to result in higher development [96], could be trialled against 10 µM. If the development achieved to day 7 can be replicated with PGs, then this activation procedure could be compared with DMAP activation of NT with ePSCs.

Finally, once procedures have been optimised for efficiency and proven to be consistent with frozen-thawed embryos, embryo transfers should be completed. This is the final step and the main aim of this research: to know how many live calves can be generated from a single elite blastocyst. Embryos should be screened for karyotype abnormalities non-invasively and efficiency compared with somatic donor cell derived embryos to assess if there is an overall improvement in cloning with ePSCs.

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Appendices

Appendix I: Materials

Acetic Acid
AnalaR, VWR International Ltd. (England). Stored at room temperature.

Alexa Fluor® azide
Alexa Fluor® azide 488 diluted in DMSO as per kit instructions. Click-iT® EdU Imaging Kit, Invitrogen, Life Technologies (USA). Stored at -4°C.

Anisomycin
26.53 mg/ml (100mM) in ethanol. Anisomycin from *Streptomyces griseolus*, Sigma Aldrich (USA). Stored at -20°C.

Aspiration media
H199 medium with 925 IU/ml Heparin (CP Pharmaceuticals, UK) and 2% (w/v) FCS (Life Technologies, USA). Stored at 4°C.

B199
Bicarbonate-buffered medium M199 with 25 mM sodium bicarbonate, 0.2 mM pyruvate, and 0.086 mM kanamycin monosulfate. Stored at 4°C.

B199 + 10% FCS
B199 medium with 10% FCS. Stored at 4°C.

Bovine Serum Albumin (BSA)
Fatty-acid free bovine albumin. MP Biologicals, (NZ). Stored at 4°C.

Click 10X Click-iT® reaction buffer additive
Click-iT® EdU Imaging Kit, Invitrogen, Life Technologies (USA). Stored at -4°C.

Copper sulphate ($CuSO_4$)
100mM Cupric Sulphate (D3247) dissolved in Milli-Q water. Ajax chemicals (Australia). Stored at -4°C.

Cycloheximide
10mg/ml Cycloheximide (C1988-1G) in DMSO. Sigma Aldrich (USA). Stored at -20°C.

Cytochalasin B (CB)
5mg/ml Cytochalasin B from *Drechslera dematioidea* (C6762) in DMSO. Sigma Aldrich (USA). Stored at -20°C.

Diamond antifade mounting media
ProLong Diamond Antifade, Life Technologies (USA). Stored at 4°C.

Dimethyl sulfoxide (DMSO)
Dimethyl sulfoxide (D2650), Sigma Aldrich (USA). Stored at room temperature.

Dimethylpolysiloxane
Poly(dimethylsiloxane) (9016-00-6), Sigma Aldrich (USA). Stored at room temperature.

Dispase
0.5 mg/ml dispase powder (17105-041) in PBS. Life Technologies (USA). Stored at 4°C.

Dissociation media
HSOF – Ca + 10% FCS with 0.1mg/ml PBS/PVA and 0.02% EDTA. Stored at 4°C for 1 – 2 weeks.

DMAP
74.62 mg/mL 6-dimethylaminopurine in DMSO. Sigma Aldrich (USA). Stored at -20°C.

Donkey Serum
5% donkey serum in PBS. Sigma Aldrich (USA). Stored at 4°C.

EdU
10 mM EdU, Click-iT® EdU Imaging Kit, Invitrogen, Life Technologies (USA). Stored at 4°C.

Embryo Hold – BSA
107.7 mM sodium chloride, 3.99 mM potassium chloride, 1.20 mM monopotassium phosphate, 25 mM sodium bicarbonate, 0.33 mM sodium pyruvate, 1.71 mM calcium chloride dihydrate, 0.49 mM magnesium chloride hexahydrate, 3.32 mM sodium lactate, 0.04 mM kanamycin sulfate, 1.5 mM glucose, 1 mM DNP (2-, 4-dinitrophenol), 0.081 g/L Non Essential Amino Acid, 1 mM Gluta-Max, 0.22 g/L BM Essential Amino Acid, 17.37 g/L MOPS free acid, and 38.61 g/L MOPS sodium salt. Stored at 4°C.

ESOF
107.7 mM sodium chloride, 7.15 mM potassium chloride, 0.30 mM monopotassium phosphate, 25 mM sodium bicarbonate, 0.33mM sodium pyruvate, 1.71 mM calcium chloride dihydrate, 0.15 mM glucose, 3.32 mM sodium lactate, 0.04 mM kanamycin sulfate, 0.081 g/L Non Essential Amino Acid, 1 mM Gluta-Max, and 8 mg/ml BSA. Stored at 4°C.

ESOF – Ca
107.7 mM sodium chloride, 7.15 mM potassium chloride, 0.30 mM monopotassium phosphate, 25 mM sodium bicarbonate, 0.33mM sodium pyruvate, 0.15 mM glucose, 3.32 mM sodium lactate, 0.04 mM kanamycin sulfate, 0.081 g/L Non Essential Amino Acid, 1 mM Gluta-Max, and 8 mg/ml BSA. Stored at 4°C.

Ethanol
70% ethanol in Milli-Q water. Fisher Chemicals (USA). Stored at room temperature.

Fetal calf serum (FCS)
Fetal calf serum, GIBCO, Life Technologies, (USA). Stored at 4°C.

Gelatin
0.1% gelatin powder in Milli-Q water. Sigma Aldrich (USA). Stored at room temperature.

Giemsa
KaryoMAX® Giemsa Stain Solution (10092-013), Life Technologies, (USA). Stored at room temperature.

Goat serum
5% goat serum in PBS. Sigma Aldrich (USA). Stored at 4°C.

Guinea Pig Complement (GP)
Minimum 80 CH50 units per mL Guinea Pig Complement Serum (S1639) in deionized water. Sigma Aldrich (USA). Stored at -80°C.

Gurr buffer
1 Gurr buffer tablet (331932D) in 100 ml Milli-Q water, pH 6.8. VWR International Ltd. (England). Stored at room temperature.

H199
Hepes-buffered M119 with 15mM Hepes, 5 mM sodium bicarbonate, and 0.086 mM kanamycin monosulfate. Stored at 4°C.

H199 + 0.5% FCS + nocodazole
H199 medium with 0.5% FCS and 100nM nocodazole. Stored at 4°C.

H199 + 10% FCS
H199 medium with 10% FCS. Stored at 4°C.

H199 + 3 mg
H199 medium with 3 mg/ml BSA. Stored at 4°C.

Hoechst 33342
1 mg/ml Bis-Benzimide (B2261) in Milli-Q water. Sigma Aldrich (USA). Stored at 4°C.

HSOF
107.7 mM sodium chloride, 7.15 mM potassium chloride, 0.3 mM monopotassium phosphate, 5 mM sodium bicarbonate, 3.32 mM sodium lactate, 0.069 mM kanamycin monosulfate, 20 mM Hepes, 0.33 mM pyruvate, 1.71 mM calcium chloride dihydrate, and 3 mg/mL BSA. Stored at 4°C.

HSOF – Ca
107.7 mM sodium chloride, 7.15 mM potassium chloride, 0.3 mM monopotassium phosphate, 5 mM sodium bicarbonate, 3.32 mM sodium lactate, 0.069 mM kanamycin monosulfate, 20 mM Hepes, 0.33 mM pyruvate, and 3 mg/mL BSA. Stored at 4°C.

HSOF – Ca + 10% FCS
HSOF - Ca medium with 10% FCS. Stored at 4°C.

HSOF + 1 mg
HSOF medium with 1 mg/ml BSA. Stored at 4°C.

HSOF + 30 mg
HSOF medium with 30 mg/ml BSA. Stored at 4°C.

Hyaluronidase (in H199)
0.1% Bovine Testicular Hyaluronidase in H199-BSA. Sigma Aldrich (USA). Stored at -20°C.

Hyaluronidase (in HSOF)
0.1% Bovine Testicular Hyaluronidase in HSOF. Sigma Aldrich (USA). Stored at -20°C.

Hypoosmolar Fusion buffer (164)
165 mM mannitol, 50 µM calcium chloride, 100 µM magnesium chloride, 500 µM Hepes, and 0.05% bovine albumin (ABIVP, ICP), pH 7.3. Stored at 4°C.

Ionomycin
1 mg Ionomycin salt in 268 µL DMSO. Sigma Aldrich (USA). Stored at -20°C.

Isoosmolar Fusion buffer (270)
270 mM mannitol, 50 µM calcium chloride, 100 µM magnesium chloride, 500 µM Hepes, and 0.05% bovine albumin (ABIVP, ICP), pH 7.3. Stored at 4°C.

IVF media
107.7 mM sodium chloride, 7.15 mM potassium chloride, 0.3 mM monopotassium phosphate, 25 mM sodium bicarbonate, 0.33 mM sodium pyruvate, 3.32 mM sodium lactate, 1.71 mM calcium chloride dihydrate, 8 mg/ml fatty-acid free bovine serum albumin (BSA; MP Biologicals, Auckland, NZ), with 0.04 mM kanamycin sulfate, and supplemented with 0.1 mM pyruvate, 10 mg/ml heparin, 0.2 mM penicillamine and 0.1 mM hypotaurine. Stored at 4°C.

Laminin
1 mg/ml natural laminin mouse protein. Invitrogen, Life Technologies (USA). Stored at -20°C.

Lectin
2 mg/mL Phytohaemagglutinin PHA-P in H199 + 3 mg/mL BSA. Sigma Aldrich (USA). Stored at -20°C.

LSOF
107.7 mM sodium chloride, 3.99 mM potassium chloride, 1.20 mM monopotassium phosphate, 25 mM sodium bicarbonate, 0.33 mM sodium pyruvate, 1.71 mM calcium chloride dihydrate, 0.49 mM magnesium chloride hexahydrate, 3.32 mM sodium lactate, 0.04 mM kanamycin sulfate, 1.5 mM glucose, 1 mM DNP (2-, 4-dinitrophenol), 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. Stored at 4°C.

M199
Medium 199 with L- glutamine, Earle's salts, Life Technologies (USA). Stored at 4°C.

Maturation media
B199 with 10 µg/mL ovine follicle stimulating hormone, Ovagen; Immuno-Chemical Products (New Zealand), 1 µg/mL ovine luteinizing hormone, and 1 µg/mL 17-β-estradiol. Supplemented with 0.1 mM cysteamine (2-Mercaptoethylamine). Stored at 4°C.

Methanol
Mallinckrodt chemicals (USA). Stored at 4°C.

Milli-Q water
Millipore H2O-production unit Milli-Q plus, Bio Lab, (USA).

Mineral Oil
M5310, Sigma Aldrich (USA). Stored at 38°C or room temperature.

N-2/B-27 media
1:1 ratio of N-2 and B-27 medias:
N-2: DMEM/F12 Hepes (12400-24) with 10 µl/ml N-2 100X stock (117502-048), Life Technologies (USA).
B-27: Neurobasal (21103-049) with 20 µl/ml B-27 (17504-044) and 10 µl/ml 200mM L-Glutamine, Life Technologies (USA).
Stored at 4°C.

NH_4Cl
50 mM NH_4Cl in PBS. Stored at room temperature.

Nocodazole
1 mg/ml nocodazole (M1404) in DMSO. Sigma Aldrich (USA). Stored at -20°C.

Paraformaldehyde (PFA)
4% depolymerised (w/v) PFA, 4% (w/v) sucrose, and 1 M sodium hydroxide in PBS with phenol red indicator. Sigma Aldrich (USA). Stored at 4°C.

Percoll 45%
2 ml 90% Percoll Plus (GE Healthcare) diluted with 2 ml 1x SPTL. Stored at room temperature.

Percoll 90%
4.5 ml Percoll Plus (GE Healthcare), 0.525 ml 10x SPTL, 0.05 ml 100x SPAD, and 0.05 ml 250 mM sodium bicarbonate. Stored at room temperature.

Phosphate buffered saline (PBS)
8.4 mM disodium hydrogen orthophosphate 2-hydrate, 1.9 mM sodium dihydrogen orthophosphate 1-hydrate, and 150 mM sodium chloride in Milli-Q water, pH 7.4. Prepared from powder and autoclaved prior to use. Stored at room temperature.

Polyvinyl alcohol in PBS (PBS/PVA)
1 mg/ml Polyvinyl alcohol in PBS. Stored at 4°C.

Pronase
0.5% Protease in HSOF + 1 mg/ml PVA. Sigma Aldrich (USA). Stored at -20°C.

Rabbit Anti-Bovine Serum (RαB)
0.01M Anti-Bovine Serum developed in Rabbit (B3759) in deionised water. Sigma Aldrich (USA). Stored at -80°C.

Saline
0.9% sodium chloride in Milli-Q water. AnalaR, VWR International Ltd. (England). Stored at 30°C.

Sodium citrate
0.9% sodium citrate (MC6922) in Milli-Q water. May and Baker (England). Stored at room temperature.

Standard culture media (DMEM F12 10% FCS)
DMEM/F12+GlutaMAX I 1x, GIBCO, Life Technologies (USA), with 10% FCS. Stored at 4°C.

Tris buffered saline
100mM Tris-Cl, 1.5 M sodium chloride, pH 7.4, in distilled water. Stored at room temperature.

Triton X-100
0.1% or 0.5% Triton X-100 in PBS. Sigma Aldrich (USA). Stored at 4°C.

TrypLE
TrypLE Express, GIBCO, Life Technologies, (Denmark). Stored at 4°C.

$2i^+$ media
N-2/B-27 media with 3 µM CHIR99021 (04-0004-02, Stemgent), 10 µM PD0325901 (04-0006, Stemgent), 10 µM Forskolin (11018, Cayman Chemical), and 10 ng/ml recombinant bovine Interleukin-6 (RP0014B-025, Kingfisher Biotech). Stored at 4°C.

3:2:1 fixative solution
3 ml 100% methanol, 2 ml glacial acetic acid, and 1 ml Milli-Q water. Stored at -20°C.

Appendix II: IVP record sheet

Experiment : **Run Number :**

IVM	IVF	IVC	C/O
Date/time : Monday, 18 May 2015	Tuesday, 19 May 2015	Wednesday, 20 May 2015	Sunday, 24 May 2015
Source/collector:	No. oocytes :	No. oocytes :	No. oocytes :
No. ovaries : No. oocytes : Quality :	Bull :	Treatment : E SOF	Treatment : L SOF
Treatment :	IVF treatm :		deg 1 cell <8 >8
notes :	notes :	notes :	notes :

End of Culture Date : Wednesday, 27 May 2015

Total	1-cells	Total	1-cells	Total	1-cells

Appendix III: Plate layout diagrams

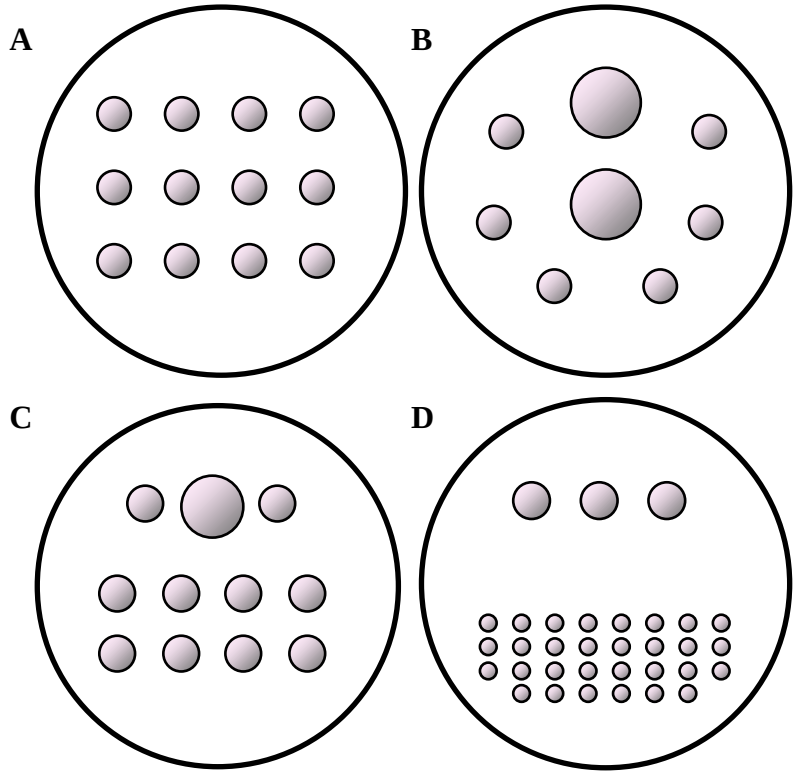


Figure III.1: Layout of plates. A) 12x 40 µl or 12x 30 µl drops. B) ESOF and LSOF layout with two central 40 µl drops and six 20 µl outer culture drops. C) RαB and GP layout. Top row wash drops, centre 40 µl with one 20 µl either side. Second and third rows 20 µl culture drops. D) Single embryo culture plate layout. Top row three 40 µl wash drops, followed by three rows of 8x 5 µl culture drops and a final row of 6.

Appendix IV: Development and grading of bovine embryo

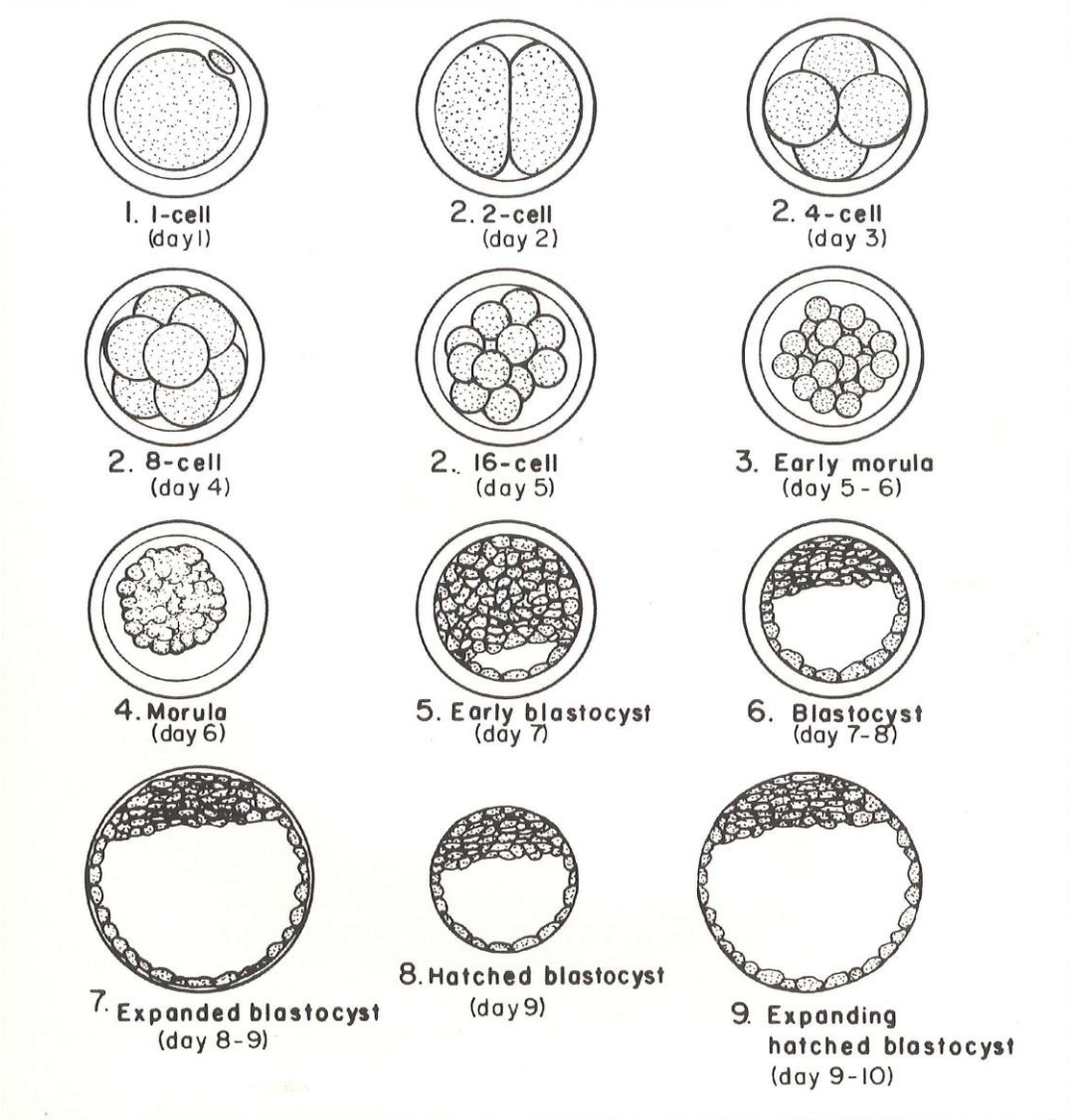
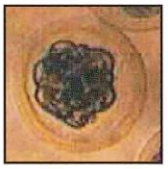


Figure IV.2: Developmental stages of bovine embryo. Development starts from IVC (Day 1) through to hatched blastocyst. Image from international guide to embryo grading [115].

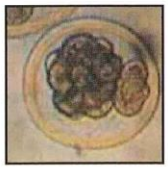
BOVINE EMBRYOS: EXAMPLES OF DEVELOPMENTAL STAGE AND QUALITY

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

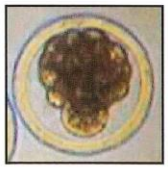


Cycle day: 6.5
Stage Code: 3
Quality Code:1
Comments: a

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

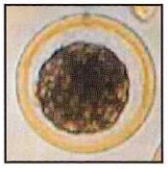


Cycle day: 6.5
Stage Code: 3
Quality Code: 2
Comments: a, b

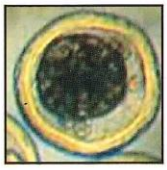


Cycle day: 6.5
Stage Code: 4
Quality Code: 1
Comments: b,c,d

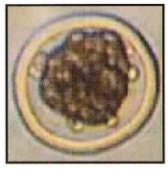
Cycle day: 7
Stage Code: 4
Quality Code: 1
Comments:



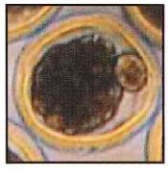
Cycle day: 7
Stage Code: 4
Quality Code: 1
Comments:



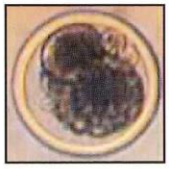
Cycle day: 7
Stage Code: 4
Quality Code:l
Comments: d



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



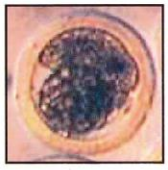
Cycleday: 7
Stage Code: 4
Quality Code: 1
Comments: d



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

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

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



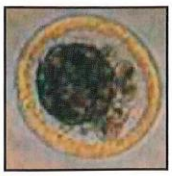
Cycle day: 7
Stage Code: 4
Quality Code:2
Comments: b, e

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

Comments:

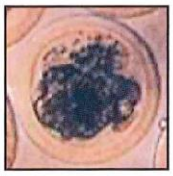
a. If this embryo is collected on day 7 or later, the stage is not consistent with the expected stage of development and, therefore, should be lowered one quality code.
b. Large cells that were extruded from the embryo mass prior to the 16-cell stage easily make up more than 15% of the total cellular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

BOVINE EMBRYOS: EXAMPLES OF DEVELOPMENTAL STAGE AND QUALITY

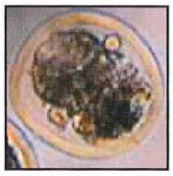


Cycle day: 7
Stage Code: 4
Quality Code:2
Comments:

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



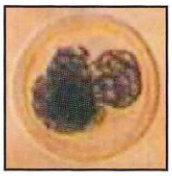
Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g



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

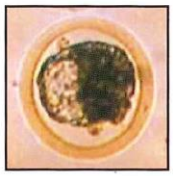
Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g

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

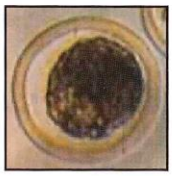


Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g, h

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



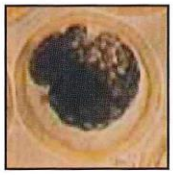
Cycle day: 7
Stage Code: 5
Quality Code: 1
Comments: d



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

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

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



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

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

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

Comments:

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

Figure IV.3: Grading of bovine embryos. Examples of different grade embryos as determined by international embryology guide [115].

Appendix V: Cloning record sheet

FBA Zona-Free

Experiment:_____

Date:_____

Cell Data:_____

Drop No.	No. Cytos	Time Fused	No. Fused	No. not fused	Comments
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					

Fusion Parameters

	Parameter 1	Parameter 2	Parameter 3
Amplitude			
µsec			

Fusion Rate

	No. fused	Total	Percent
1st Fusion			
Total			

	No. into ESOF –Ca	Time into ESOF –Ca	No. for Activation	Time HSOF + 1mg/ml	Time Ionomycin	Time into DMAP	No. into DMAP	Time into IVC	No. into IVC
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									

Appendix VI: NT record sheet

Experiment : **Run Number :**

IVM	NT	IVC	C/O
Date/time : Monday, 18 May 2015	Tuesday, 19 May 2015	Tuesday, 19 May 2015	Sunday, 24 May 2015
Source/collector:	No. oocytes :	No. oocytes :	No. oocytes :
No. ovaries : No. oocytes : Quality :		Treatment : E SOF	Treatment : L SOF
Treatment :			deg / 1 cell / <8 / >8 / TM

End of Culture Date : Day 7: Tuesday, 26 May 2015 Day 8: Wednesday, 27 May 2015