Improving bovine donor cell culture conditions for embryo-derived cell transfer cloning

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Masters of Science (Research)

in Molecular and Cellular Biology

at

The University of Waikato

by

Shaan Harker Wilson

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2021

Abstract

Cloning by cell transfer offers an efficient method for increasing genetic gain in livestock populations. High-quality genes are routinely induced into embryos with artificial selection-based *in vitro* fertilisation, or genetic engineering using microinjection. The capacity for large-scale amplification of these superior genes with cell culture is unique to cloning due to its ability to use a single cultured cell as a donor for producing new embryos. Thus, cell transfer cloning could become the primary tool for genetic advancement in the livestock industry if its application is improved. Currently, cell transfer cloning is inefficient in producing viable offspring that survive to term compared to methods such as *in vitro* production. This is largely caused by epigenetic modifications that arise from the donor cell and the epigenetic abnormalities carried over to the blastocyst generated. Cloning with pluripotent embryonic cells rather than the more widely researched somatic cells may remediate cases of genetic abnormalities. Pluripotent embryo-derived cells are less differentiated than their somatic cell counterparts and can generate cell types from all three of the germ layers, requiring much less reprogramming by the cytoplast. However, the elucidation of optimal conditions to culture pluripotent embryo-derived cells has proved challenging. The primary aim of this research project was to advance methodologies involved in the culture of pluripotent embryo-derived cells in order to increase the efficiency of cell transfer cloning.

A series of comparative culture experiments were executed on blastocysts produced through *in vitro* generation. Experiments focused on the effect of substrate and selection of media components on the generation of an embryo-derived outgrowth. Substrate implementation was found to be essential to initial outgrowth generation as well as further growth. Minimal media with cytokines and small molecules removed was unable to produce outgrowths, indicating the importance of the small molecules and cytokines in attachment, cell proliferation, and survival. Immunofluorescent assays were completed on outgrowths to demonstrate proliferation and cell type markers. Pluripotency marker OCT4 was found in 48% of cell nuclei stained 4 days after plating and in 5% of those stained on day 6. Hypoblast marker Gata6 was not detected in any of the outgrowth cells. The DNA synthesis marker EdU was

incorporated into the DNA of 49% of cells after 30 min, which is highly relevant to cloning as donors in S-phase are incompatible with the MII arrested cytoplasts used.

A robust protocol was formed for generating outgrowths from bovine blastocysts. Outgrowths demonstrated proliferation and presence of pluripotency markers was illustrated. Cell transfer cloning was completed successfully using embryo-derived donor cells. However, exact donor cell conditions for improved cell transfer efficiency were not elucidated. Further cell transfer experiments should be conducted with a range of donor cell conditions. Karyotyping of the embryos produced would be required before any *in vivo* implantation of blastocysts into recipients is attempted.

Acknowledgements

I would like to start by thanking the University of Waikato for the opportunity to gain my undergraduate degree and the financial aid that allowed me to work toward a master's degree. Also, to AgResearch for providing this project and the facilities to complete my research.

A huge thank you to my supervisor at AgResearch, Dr Björn Oback, for your unwavering passion and enthusiasm for science, your level of involvement and guidance in my project, and your ability to find humour in any situation. Thank you to my university supervisor Dr Pawel Olszewski for taking me onboard and for your insights into my thesis. A special mention to Ryan Martinus for being a wonderful lecturer and connecting me with AgResearch for this project. I would also like to thank Cheryl Ward for all the time she puts into helping postgraduate students through the thesis writing process.

To the other members of the AgResearch animal biotech team: Dave, Brigid, Goetz, Dan, and Fan Li for being so welcoming and making me feel at home. A big thank you to Blaise and Sally for your patience in teaching me skills throughout my time at AgResearch and answering my endless questions. Jingwei, you have my utmost gratitude for your many hours enucleating that made this project possible and for always pushing me to work hard.

My masters buddy Shantaya, you kept me sane throughout this process and I have learned stress is more manageable when shared. I am grateful for your friendship and wish you all the best in your upcoming writing.

I would not have made it to this point without the endless support from my parents Carol and Tony and my brother Ayden. Thank you for always telling me I can do anything I set my mind to and being there to support me every step of the way, I am eternally grateful to be a part of such a wonderful family. I hope I make you proud.

Finally, a special mention to my partner Thorne, for believing in me and my ability, and for the snacks you provided to get me through the many hours of writing. I appreciate you.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

0C/1C/2C/4C	DNA quantity of cell
0N/1N/2N	Chromosomal quantity of cell
2i	Double inhibitor combination (PD0325901 and CHIR99021)
AC	Alternating current
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AOS	Abnormal offspring syndrome
bEPSC	Bovine embryonic pluripotent stem cell
BSA	Bovine serum albumin
Cas9	CRISPR associated protein 9
CDK	Cyclin-dependent kinases
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CT	Cell transfer
DC	Direct current
DMAP	6-dimethylaminopurine
ECT	Embryo-derived cell transfer
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2’-deoxyuridine
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
G_0	Non-proliferative phase
G_1	Primary growth phase
G_2	Secondary growth phase
Gata6	GATA-binding factor 6

HSD	Honestly significant difference
ICM	Inner cell mass
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
IVP	*In vitro* production
KSR	Knockout serum replacement
LOS	Large offspring syndrome
M	Mitosis
MI	Metaphase I
MII	Metaphase II
mOsm	milli-osmoles per kilogram
MPF	Maturation promoting factor
mRNA	messenger RNA
n	Number of technical replicates
N	Number of biological replicates
NANOG	Homeobox protein NANOG
Nocodazole	Methyl N-[6-(thiophene-2-carbonyl)-1H-benzimidazol-2-yl] carbamate
OCT4	Octamer-binding transcription factor 3/4
P	Statistical P-value
PBS	Phosphate buffered saline
PC2	Physical containment level 2
pEPSC	Porcine embryonic pluripotent stem cell
PFA	Paraformaldehyde
PG	Parthenote
PMEL	Premelanosome protein
PRL	Prolactin

PVA	Polyvinyl alcohol
ROCK	Rho-associated kinase
S	DNA synthesis
SCT	Somatic cell transfer
SD	Standard deviation
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SOX2	Sex determining Region Y-Box 2
Src	Proto-oncogene tyrosine-protein kinase
TALENS	Transcription activator-like effector nucleases
TE	Trophoblast
Wnt	Wingless-related integration site family

Chapter 1
Introduction

Climate change is an ongoing issue that requires human response, as a substantial proportion of the changes seen are anthropogenically driven. Climate changes involving increasing global temperatures have been accelerating, and studies estimate this accelerated trend will continue during the next decades [1]. The response to global climate change by many species is geographical relocation and altering the timing of biological events such as reproduction [2]. These responses are not sustainable as climate change continues, and many natural and domestic populations are unable to acclimate in this way [2; 3].

Rising temperatures have negative influences on health, causing adverse effects such as heat stress and heat stroke, and have been shown to affect human populations as well as the livestock we rely on for food and economic value [4; 5]. New Zealand's bovine exports for 2020 were $3.8 billion in beef and $17.6 billion for dairy products, making them New Zealand's largest exports [6]. This means the condition and production of bovine livestock in New Zealand is essential for a stable economy. Heat stress has been demonstrated to decrease milk production by 25 to 40% and reduce oocyte and embryo quality, which presents short and long-term concerns for the bovine livestock industry [5; 7].

One method to limit the negative impact of climate change on bovine livestock is through cloning with gene-edited, embryo-derived cells. Selective breeding of high-quality animals remains a common practice to increase livestock production and welfare [8]. This practice is highly limited due to the chance events of independent assortment in meiosis, which removes any assurance of the high-quality alleles being inherited by the offspring. If insemination is done *in vivo*, the process is also bottlenecked by the high-quality cows, which have a gestation period of 9 months. Techniques such as Embryonic cell transfer (ECT) and *in vitro* production (IVP) can overcome this, as the blastocysts are generated *in vitro* with high-quality genetic material and transferred to recipient cows. Recipient cows are not required to possess high-quality genetic makeup, as there is no genetic contribution to the developing fetus [9].

ECT could be a valuable replacement for selective breeding, as the utility of embryo-derived donor cells as the sole provider of genetic information ensures inheritance of the genes by the offspring [10]. Pairing ECT with gene editing of the donor cells establishes the introduction of target alleles which are inherited by the new, genetically elite animal. Genes related to heat tolerance in cattle can be edited, and cloning used to amplify the genetic information to generate many clones [11]. There is evidence that using embryo-derived cells that display pluripotency increases the rate of cloned embryo implantation and survival in mammals [12]. However, cloning techniques still have low efficiency, and animals that do survive to term are sometimes found to possess epigenetic abnormalities [13]. Cloning with pluripotent embryo-derived donor cells requires culturing of bovine blastocysts into outgrowths. Successful methodologies for this are only recently being realised and more work in the field is required to advance the technique and the understanding behind it. All cells cultured from blastocysts in this research project will be referred to as embryo-derived cells, as their pluripotency was not functionally defined, and molecular characterisation was limited.

The objective of this research project was to improve upon the bovine blastocyst culturing process and gain a deeper understanding of the state of the cells during culture. These developments may lead to improvements in blastocyst generation efficiency and proportion of successful blastocyst transfers into recipient cattle. Progress in the cloning field could elevate its use in the commercial setting and specifically drive the improvement of bovine welfare in terms of climate change.

Chapter 2

Literature Review

2.1 Blastocyst development

The field of embryology gained its biggest advancements since the early 17th century when microscopy allowed for the first observation of an embryo [14]. At the start of the 19th century, each cell division from oocyte to blastocyst was recorded, giving a visual representation of blastocyst development [14]. This knowledge was harnessed and used to develop the first live offspring as a result of *in vitro* fertilisation (IVF) in 1959 [15]. Since then, the technology has been improved, and embryo transfer derived from IVF has occurred in other mammalian species including humans [16]. *In vitro* production (IVP) involves the production of blastocysts from oocytes entirely *in vitro,* which was completed in bovine livestock in 1987 [17]. This technology is now commonly used to improve livestock genetics through selective breeding and to aid people with fertility issues to induce viable pregnancies (Figure 2.1) [18; 19].

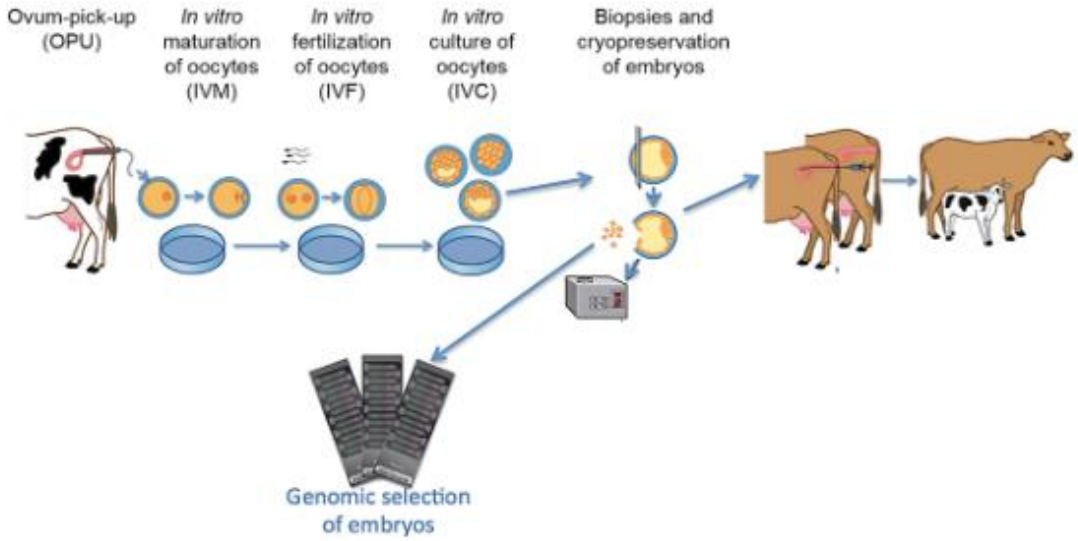


Figure 2.1 *In vitro* production of a bovine embryo resulting in embryo transfer. Oocytes are cultured *in vitro* and fertilised with bovine semen. Embryos are left to develop into an expanded or hatched blastocyst. Biopsy allows for genomic selection of blastocysts with high quality alleles by examining single nucleotide polymorphisms (SNPs). Cryopreservation is employed to optimise the timing of embryo transfer. Image from [18].

To prepare for fertilisation, an oocyte must mature to metaphase II (MII). While premature, the oocyte enters metaphase I (MI) and replicates its DNA, leaving it with 2 homologous chromosomes (2N), one from each parent, and double the normal DNA quantity (4C). The oocyte then matures, undergoing anaphase I which divides the chromosomes (Figure 2.2). During the division, one chromosome set is expelled and creates a primary polar body, and the oocyte enters MII. That leaves a haploid oocyte (1N) with a sister chromatid still attached by cohesin (2C), and a primary polar body between the oocyte and the zona pellucida. During this process, cytoplasmic maturation occurs, which includes arrangement of organelles and degradation of maternal mRNA to prepare for fertilisation. Once in MII, the oocyte becomes arrested until fertilisation [20].

Fertilisation begins with the binding of the sperm (1N/1C) to the oocyte's membrane. This elevates Ca^{2+} levels in the oocyte which leads to a hardening of the zona pellucida through cortical granule exocytosis [21]. This prevents further sperm from entering the zona pellucida to ensure a diploid zygote. Fertilisation of the oocyte by sperm causes meiosis of the oocyte to proceed, signalled by the rise in cytosolic Ca^{2+}. The oocyte enters anaphase II where spindles pull apart the sister chromatids and one set of chromatids is expelled, creating a second polar body and leaving an oocyte with one set of DNA (1N/1C; Figure 2.2). The fertilised oocyte is now considered a zygote with 2 sets of DNA (2N/2C). The two DNA sets migrate toward each other while beginning DNA replication and align along one plane. The first mitotic division occurs, commencing blastocyst development [21; 22].

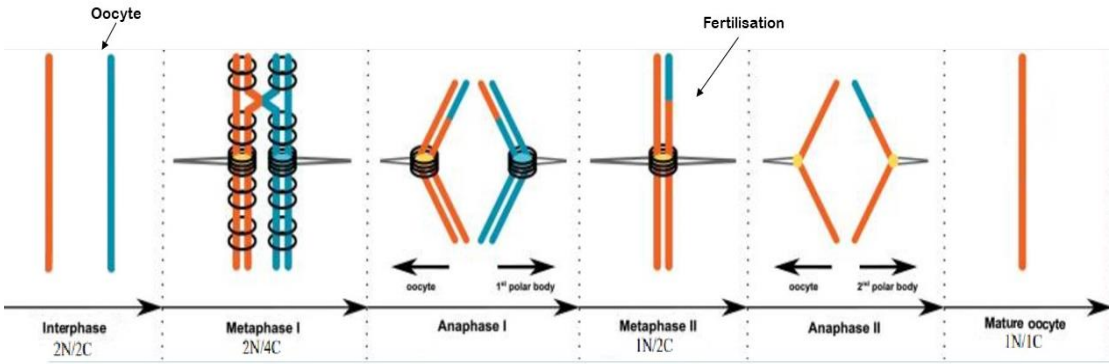


Figure 2.2. Meiotic progression of oocyte. During Interphase, the oocyte contains two sets of homologous chromosomes prior to replication (2N/2C; blue, orange). The MI-arrested oocyte contains two sets of replicated chromosomes (2N/4C). Each chromosome is now a pair of sister chromatids held together by cohesion (black ring). A maturing oocyte undergoes anaphase I, where chromosomes sets are separated by spindles (grey lines) forming an oocyte and a polar body. The oocyte is now in metaphase II a contains 1 set of replicated chromosomes (1N/2C), as does the polar body. When fertilisation of the oocyte occurs, meiosis is resumed and the oocyte experiences anaphase II, where the sister chromatids segregate. One set of sister chromatids is dispelled and forms a second polar body, leaving the mature oocyte with 1 set of chromatids (1N/1C). Image modified from [23].

Embryos go through multiple stages during development to a blastocyst (Figure 2.3). The first cleavage event brings the embryo from a one-cell zygote to a two-cell embryo. At this stage, the cells are totipotent and can give rise to a full organism from a single cell [24]. The cells (blastomeres) continue to cleave to a four and eight-cell stage and then form a compact morula. The tight morula then undergoes blastulation, forming an early blastocyst with a small cavity called a blastocoele. At this stage, the blastocyst has two cell types, the trophoblasts (TB) which reside around the periphery of the blastocyst, and the inner cell mass (ICM), found on the inside of the blastocyst. During the next stage of development to a late blastocyst, the size of the blastocoele increases and the ICM differentiates into epiblast and hypoblast (primitive endoderm), which form a layer between the epiblast and the blastocoele. A late-stage blastocyst will enlarge to a point of breaking through the zona pellucida, assisted by the secretion of a protease enzyme that breaks down the zona pellucida. This process is known as hatching, and allows further growth of the blastocyst in addition to attachment to the uterine wall for implantation. If development is conducted *in vitro*, morulae and hatched blastocysts are commonly used for ECT, further cell amplification, or preservation. Hatched blastocysts can also be implanted in recipient donors at this

stage. The TB cells go on to form extraembryonic tissue while the epiblast forms the embryo proper, and an embryonic yolk sac is derived from the hypoblast [25-27].

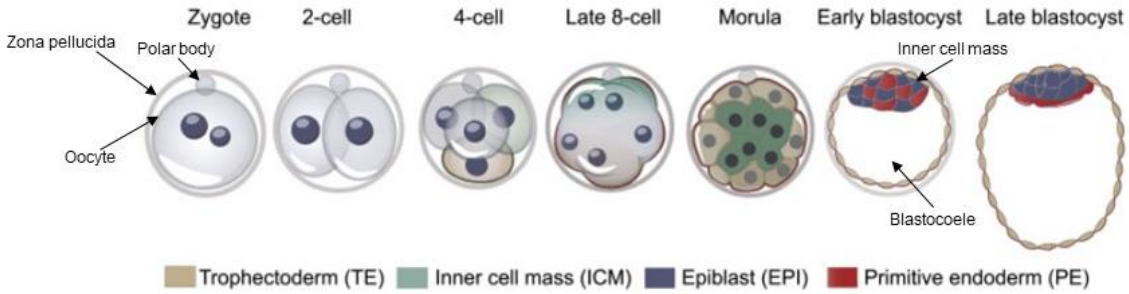


Figure 2.3. Development of blastocyst from zygote. Fertilisation induces cleavage to begin in the zygote. The blastomeres begin to compact between the 16 and 32-cell stage in bovine embryos and continue on to form a tight morula. Totipotency of the blastomeres is lost around the eight-cell stage. Cell differentiation in the morula leads to the different cell fates of the early and late blastocyst; trophoblasts (trophectoderm), epiblasts, and hypoblasts (primitive endoderm). In bovine development, zygote to late-stage blastocyst takes ≈ 8 days. Image modified from [28].

2.1.1 IVP

Blastocysts developed using IVP require certain techniques to best emulate *in vivo* conditions to achieve optimal development rates. Generation of blastocysts from oocytes *in vitro* still show lower rates of cleavage and blastulation than *in vivo* counterparts, but techniques for IVP are continuously being improved to better mimic *in vivo* conditions and increase these rates [29]. It has been demonstrated that blastocyst generation is decreased when oocyte maturation occurs *in vitro* as opposed to *in vivo* and this reduction is also maintained in fertilisation events *in vitro*, however, cleavage was similar between *in vitro* and *in vivo* treatments in both experiments. Both experiments saw 15-20% fewer blastocysts generated in the *in vitro* treatment compared to *in vivo* [30]. The most significant loss in the IVP procedure is the decrease in successful implantation of *in vitro* produced blastocysts compared to *in vivo*. One study reported a 37% pregnancy rate on day 53 for *in vitro* blastocysts transferred and

a 79% rate for *in vivo* [31]. More recently, a 34% pregnancy rate on day 60 for *in vitro* blastocysts and 42% for *in vivo* blastocysts was reported [32].

The improvements to *in vitro* methods begin with *in vitro* maturation (IVM), as early as aspiration where the size of quality follicles has been narrowed down to 2 to 8mm in diameter, and then a grading system is used to select high-quality oocytes [29]. It is also crucial to regulate oxygen to low levels in culture stages as conditions *in vivo* are hypoxic [33]. Fetal bovine serum (FBS) has been commonly used in embryo development media but is controversial due to possible genetic deviations it may cause. Common deviations are large offspring syndrome (LOS) and abnormal offspring syndrome (AOS). Features often observed in LOS/AOS include enlarged organs, skeletal defects, excessive birth weight, and difficulty with birthing, and are caused by epigenetic modification [34]. LOS and AOS occur in offspring produced from both IVP and ECT, leading to higher mortality rates of the pre-natal fetus and organ developmental failure can cause death shortly after birth [35]. Generation of large offspring has been regularly observed in blastocysts cultured *in vitro* and this has been linked to the use of serum in the culture medium [36; 37]. It has now been shown that FBS can be replaced with a serum-free formula such as bovine serum albumin (BSA) and still show similar or higher development rates [18].

Embryos that develop into blastocysts are further distinguished by their developmental grade, morphologically. Morphological grading is typically based on, size, cell compaction, relative size of the blastocoele, and degeneration of cells. International grading standards have formed a grading system from 1-3 with grade 1 as the highest quality. Both grade 1 and 2 embryos are considered high-quality and are routinely used for transfer into recipient animals. Pregnancy rates are found to be elevated in high-grade blastocysts compared to low-grade [38; 39].

2.1.2 Genetic editing

Genetic modification can be introduced artificially through several methods including Clustered regularly interspaced short palindromic repeats (CRISPR), CRISPR-associated 9s (Cas9), and Transcription activator-like effector nucleases (TALENS). These techniques act by creating a double-strand break in the DNA which is then repaired either with a homologous sequence to compliment, called homology-directed repair, or without, which is termed non-homologous end joining. TALENS are

engineered to resemble plant pathogenic bacteria and pinpoint target sequences on DNA before creating a break. CRISPR/Cas9 are engineered from sequences found in bacteriophages which allows for the detection of target sequences by guide RNAs and further cleavage of the region by Cas9 endonucleases [40]. Gene editors can be introduced to the cell through microinjection or transfection of vectors via electroporation [41; 42]. Both techniques can be used in somatic or embryo cells, however, gene editing in embryo cells allows for use of the edited cells as donors in ECT.

Transfection via electroporation is completed in a buffered solution containing DNA vectors with editors and the cells to be edited. A pulse of high-voltage electricity is sent through the solution and shocks the cells, permeabilising the membrane via the formation of pores, allowing the DNA to enter the cell. This technique is often used to introduce entire genes to the cells [41; 43].

Microinjection of early-stage embryos can be used to edit target genes in zygotes or blastomeres. Microinjection can be completed on recently fertilised embryos at the single-cell stage or after cleavage, up to the 8-cell stage. A mixture of reagents to modify the genome including Cas9 with guide RNAs or TALENS are injected into the cell using a fine needle to reduce damage [42]. If CRISPR/Cas9 reagents are successful in modifying the target site on the genome, subsequent cell divisions will include the modified allele [44]. Microinjection can lead to blastocysts/animals with 100% of cells modified or it can create genetic mosaicism. Genetic mosaicism refers to the presence of cells with differing allele combinations within an organism, and indicates not all cells underwent successful genetic modification [45].

Genetic modification techniques can be used to obtain genetic gain in target regions. With increased temperatures due to climate change, genes involved in thermotolerance in cattle are important for maintaining welfare in livestock populations [46]. Some genes involved in thermotolerance include premelanosome protein gene (*PMEL)* which is crucial for the formation of fibrils in melanosomes in bovine skin and allows for organised accumulation of pigment in the cell and Prolactin *(PRL)* which codes for factors involved in hair length, hair follicle density, and sweat excretion rates [47; 48]. *PMEL* has a wide variety of alleles that lead to a range of coat colours. A three-base-pair deletion within the gene generates a leucine deletion which creates a mutant

PMEL allele. A single copy of the allele causes a diluted colour in the offspring and the dilution is enhanced when two copies of the mutant allele are present [49; 50]. The *PRL* allele termed *slick* is caused by a range of mutations, including a well-defined single base deletion, leading to a premature stop codon that removes amino acids from the C-terminus of the PRL receptor. Mutation of the PRL receptor causes a truncated protein, influencing signal transduction. The *slick* allele is dominant and causes lower hair follicle density and shorter hair, in addition to increased rates of sweating [51].

Embryos microinjected to engineer the desired *PMEL* or *slick* alleles can be transferred to recipients to generate live offspring with the desired phenotype or cultured to form outgrowths and amplify the high-quality genetic information through cell division [44].

2.2 Blastocyst culture

A late-stage blastocyst can be generated approximately eight days from fertilisation. After the development of blastocysts through IVP is completed, they can be cryopreserved or used to generate embryo-derived outgrowths with cell culture techniques. Blastocyst culture is useful to amplify cells with valuable genetic makeup. Cultured cells can then be employed as donors for embryonic-derived cell transfer (ECT). Culture occurs in dishes or wells filled with optimised media and substrate (Figure 2.4). This helps the blastocyst attach to the substrate and form an outgrowth where cells can continue to grow and divide.

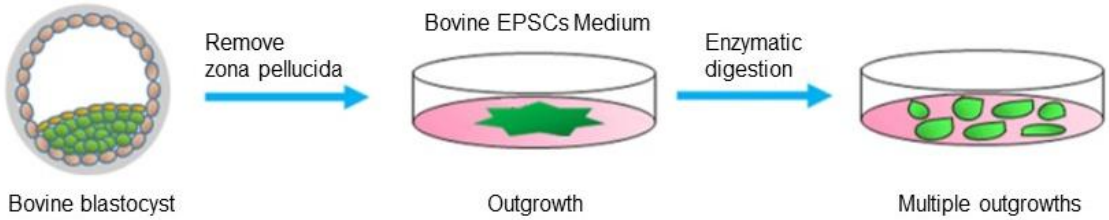


Figure 2.4. Bovine blastocyst culture. Before the blastocyst is plated the zona is removed with pronase if it has not happened naturally through hatching, and the blastocyst is given time to re-expand. The plated blastocyst is left to form an outgrowth. When the outgrowth grows to 70-80% confluency it is passaged using enzymatic digestion to dissociate the cells which are then replated, forming new outgrowths. Image modified from [52].

2.2.1 Pluripotent cell culture

Pluripotency refers to a cell's ability to differentiate into any of the three primary germ layers in an organism leading to all cells in an adult, though, pluripotent cells can not generate extraembryonic tissues such as placenta and umbilical cord [53]. Embryonic cell transfer has been shown to have higher rates of success for the blastocysts implanting in the recipient animal and developing to term if the donor cells used are pluripotent stem cells [12]. Although embryonic stem cells have been cultured from mouse epiblasts successfully since 1981, stable pluripotent embryo-derived stem cell lines have not been possible in bovine biology until the last few years [54; 55].

The *in vivo* conditions between mice (murine) and bovine embryo development vary and signalling pathways start to differ between them as early as MI to MII phase in oocytes. Examples of this include protein synthesis being crucial to begin maturation and germinal vesical breakdown (which allows chromosomes to condense and the oocyte to advance toward MII) in bovine oocytes but not in murine. Additionally, adenosine monophosphate-activated protein kinase (AMPK) which is involved in metabolic responses in addition to energy and appetite balancing, influenced oocyte maturation. In murine oocytes, the addition of AMPK activators induced germinal vesicle breakdown and the resumption of meiosis, whereas, AMPK activators delayed germinal vesicle breakdown in bovine oocytes and inhibited meiotic progression [56]. This hindered the ability to employ the same techniques for non-murine species. Further in-depth study on porcine blastocysts demonstrated that signalling networks responsible for embryonic pluripotency in porcine cells followed different patterns compared to mouse embryos. An example of this was the discovery that the receptor for Leukemia Inhibitory Factor (LIF) was not present in ICM or epiblasts of porcine blastocysts which could suggest the addition of LIF would not be beneficial in culturing porcine pluripotent embryonic stem cells (pEPSCs), while it is critical in murine embryo-derived culture to maintain pluripotency [57; 58]. Consequently, the methodologies were not completely transferrable, hindering the progression of pluripotent embryonic cell culture in bovine and other livestock animals.

Approximately a decade ago, there was some breakthrough in establishing stem cell-like blastocyst cultures in porcine embryology. Porcine ICM cells were cultured and showed self-renewal in addition to indicating the presence of pluripotency protein

markers Homeobox protein NANOG (NANOG), octamer-binding transcription factor 3/4 (OCT4), also known as POU domain, class 5, transcription factor 1 (POU5F1), but these were not completely pluripotent and failed some of the pluripotency tests such as chimera formation [59]. Bovine pluripotent embryonic stem cells (bEPSCs) were finally cultured in 2018, helped by earlier advances in porcine culture literature. This was the first bovine study to pass the *in vivo* teratoma assay as well as establishing the maintenance of pluripotency markers over many passaging events. However, the stem cells in this study were shown to be in a primed state where the pluripotent cells are primed toward a specific cell line, in contrast to the naïve state where cells are unbiased toward all lineages. The primed state was demonstrated with histone methylation signatures specific to primed cells and the lack of naïve pluripotency markers [54]. This means germline chimeras could not be established as naïve pluripotent stem cells are required and so complete pluripotency was not demonstrated. In 2019, pEPSCs were cultured and maintained through 50 passages over a year, and showed stability of pluripotent markers and self-renewal. This study demonstrated the presence of naïve pluripotency markers such as stage-specific embryonic antigen-1^{+}. Successful formation of embryoid bodies and teratomas that differentiated into cells from all three germline layers was also shown [60].

Media components and substrate type are both critical in developing outgrowths and maintaining pluripotency. It has been demonstrated that gelatin, fibronectin, and laminin can all successfully be used as a substrate to generate outgrowths [60; 61]. There is no one substrate yet proven to be the gold standard. Understanding of molecular pathways that play a role in differentiation is crucial in developing a growth media that encourages pluripotency. Wingless-related integration site family (Wnt) proteins are crucial in cell differentiation and proliferation and have a big role in an organism's development [62]. Binding of the Wnt ligand to its receptor causes stabilisation of β-catenin. β-catenin can then enter the nucleus and regulates gene expression, although the result of this regulation can lead to opposite outcomes and it is proposed that this is based on cell type [63]. Thus, the importance of inhibiting the Wnt signalling pathways to maintain pluripotency was recognised and Wnt inhibitors IWR1 or XAV-939 (XAV) were added to culture mediums. XAV inhibits Wnt signalling by inducing the β-catenin destruction complex [61]. Interestingly, many recent, successful studies also include CHIR99021 (CHIR) in growth media which is a Glycogen synthase kinase-3 (GSK3) inhibitor [52]. The inhibition of GSK3 leads to

promotion of the Wnt/β-catenin pathway and stabilises β-catenin. It has not been explained why XAV and CHIR work together in bovine embryonic stem cells (ESCs) but may be due to the difference in cell-to-cell responses where Wnt/β-catenin promotes pluripotency in cells with high levels of β-catenin in the cytoplasm and promotes differentiation in cells where β-catenin has relocated to the nucleus [63]. WH-4–023 is another inhibitor and targets proto-oncogene tyrosine-protein kinase (Src) pathways [64]. Src is a family of tyrosine kinases that influence signal transduction across the cell membrane and are responsible for cellular processes such as differentiation, proliferation, and survival [64]. WH-4-023 binds important domains on the kinase such as the Lck binding domain, holding it in an inactive state, inhibiting activity. The WH-4–023 inhibitor is widely used in small concentrations for sustaining pluripotency in embryo-derived outgrowths [52]. LIF and Interleukin-6 (IL-6) are cytokines from the same IL-6 family and are involved in signalling pathways for self-renewal. The cytokines encourage conversion of the cell to a naïve pluripotent state rather than the partially differentiated primed pluripotent state in mice [65]. When LIF and IL-6 act on their respective receptors multiple signalling pathways are activated which lead to the homodimerization of signal transducer and activator of transcription 3 (STAT3). STAT3 is then able to activate target genes involved in proliferation and pluripotency. LIF has been actively recognised and utilised since early pluripotent stem cell studies. Rho-associated kinase (ROCK) inhibitor decreases cell apoptosis by increasing cell-cell adhesion via gap junctions and was first demonstrated in human ESCs [66]. Use of this became valuable to bEPSC culture in growth media, and is especially notable in media during dissociation events such as passaging or ECT [61].

Knowledge of small molecules and cytokines has been the key component to advancing the competency of ESC culture and further work is required to elucidate key conditions to best maintain outgrowth survival and pluripotency. In the last three years, understanding of these components lead to several studies successfully culturing embryo-derived bovine cells that demonstrated pluripotency at various levels [52; 54; 61]. The differences in cytokines and small molecules between the literature suggests room for improvement to highlight the optimal medium. Improving the quality of blastocyst culture will, in turn, increase the success of ECT in generating viable offspring.

2.2.1.1 Demonstrating pluripotency

Pluripotency must be demonstrated both molecularly and functionally for a cell to be defined as pluripotent. Molecularly, this is illustrated through three main pluripotency markers, NANOG, OCT4, and sex determining Region Y-Box 2 (SOX2; Figure 2.5). *NANOG*, *OCT4*, and *SOX2* are genes for transcription factors that are critical in inducing self-renewal gene expression and suppressing genes involved in cell differentiation [67]. *NANOG* is activated by SOX2 and OCT4 proteins which synergise as transcription factors. These Oct-Sox enhancers are also essential in self-promotion of the *OCT4* and *SOX2* genes [68]. The NANOG protein is expressed solely in undifferentiated cells, so its presence is strongly indicative of a pluripotent epiblast [67]. OCT4 protein is produced in totipotent blastomere and pluripotent epiblasts and downregulates as cells differentiate to trophoblast or primitive endoderm (hypoblast) cells [69]. The SOX2 protein is expressed in pluripotent cells such as the ICM and extraembryonic ectodermal cells. Expression of all three genes is required to form and maintain cell pluripotency [69]. Testing for the presence of NANOG, OCT4, and SOX2 is one of the first steps in identifying cell pluripotency and is typically completed with immunofluorescence assays. Immunofluorescence assays consist of the addition of a primary antibody against a marker such as OCT4, which binds to the marker. A secondary antibody with a fluorescent tag is later added and attaches to the primary antibody and allows for visualisation of regions where the primary antibody bound to its target. Extensive washes after each step are essential to remove any unbound antibodies to reduce off-target fluorescence [70]. Immunofluorescence has successfully illustrated the presence of all three markers in bEPSCs [61]. In addition to immunofluorescence, expression of pluripotency genes can be investigated by mRNA levels through microarray analysis [71]. High levels of expression of genes such as *OCT4*, *SOX2*, and *NANOG* are a good indication that cells are pluripotent and can verify immunofluorescent results. Expression of all three genes has been demonstrated in bEPSCs [52]. Molecular assays are typically completed before functional investigations including embryoid bodies, germline chimeras, teratoma formation, and tetraploid complementation.

Other cell types present in the blastocyst can also be revealed by testing for transcription factors such as Gata6. *Gata6* codes for the transcription factor Gata6, involved in cell outcomes with fibroblast growth factor FGF signalling [72]. Gata6 is

found in the primitive endoderm (hypoblast). This molecular marker can help to elucidate differentiation occurring in the embryo-derived outgrowths.

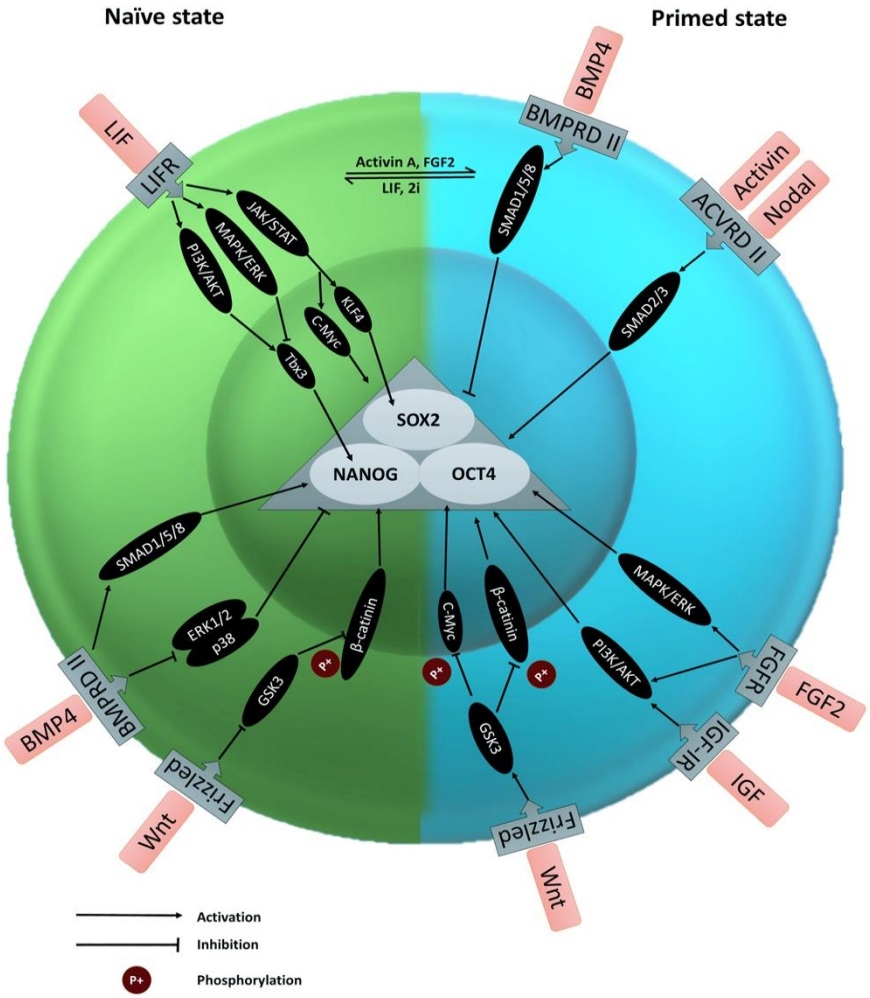


Figure 2.5. Cellular pathways influential in pluripotency. Pluripotency genes *NANOG, OCT4, and SOX2* are induced and inhibited by a number of molecular pathways. The pathways differ in the naïve state where cells can unbiasedly create all cell lineages or primed state where cell is primed toward germ line lineages. Image from [73].

Functional pluripotency measures are demonstrated with four main benchmarks, of which all are required to ensure complete pluripotency. The least stringent of these benchmarks is an *in vitro* embryoid body assay. Embryoid bodies are formed by placing the cultured cells in a liquid suspension at a high concentration where they aggregate and are left for 27-35 days. Specialised equipment can also be utilised to aid this technique. Embryoid bodies are then assayed for genes specific to each of the three germ layers (endoderm, ectoderm, mesoderm), unless a more stringent histological examination is completed, involving sectioning of the embryoid body and investigation of cell morphology, localisation of protein expression, and tissue

organization using staining. Histological methods highlight morphological markers of each of the germ layers [74].

The formation of teratomas is another key factor in illustrating pluripotency and is commonly used, especially for human embryonic stem cells as chimera assays cannot be done due to ethical reasons. A teratoma is a tumour that is comprised of cells from the three germ layers and is not malignant [53]. When a possible embryonic stem cell is transplanted into a growth-permissive site on a host animal, if the cell is completely pluripotent, it will produce a benign tumour that includes all three germ layers [75]. This assay has recently been completed with successful results in bovine ESCs [54; 61; 76].

Another of these measures is the ability to form germline chimeras which was first demonstrated in mice [53; 68]. This experiment involves a chimeric embryo in which the proportion of the cells being evaluated for pluripotency express a target gene. If the resulting animal is illustrated to be expressing this gene in the somatic cells and germline cells, it indicates the cells were pluripotent as they were still able to differentiate into germline cells or somatic cells [52]. Pluripotent cells that are in the primed state are not able to generate germline chimeras [73]. Germline chimeras have not been generated in bEPSCs, however, bovine chimeras have been produced where the bEPSCs contributed to both embryonic and extraembryonic cell lineages which indicates expanded pluripotent stem cells, as extraembryonic lineages such as placenta cells cannot be derived from regular primed pluripotent cells [52].

The final benchmark practiced is tetraploid complementation, which is the most stringent experiment [77]. Tetraploid complementation requires the injection of the diploid ESCs in question into tetraploid embryos which were created using cell fusion. The tetraploid cells are only capable of forming extraembryonic tissue as they lack an ICM. ESC-derived embryos can be transferred to recipient animals and if a functioning, live offspring can be generated from the embryo it demonstrates that the diploid ESCs are pluripotent as they generated all cell types to form an entire animal [78]. This has not yet been shown in bEPSCs.

2.3 Cloning

2.3.1 History of cell transfer cloning techniques

Cell transfer (CT) was first successful in amphibians in around 1950, referred to as nuclear transfer as only nuclear portions of the cell were transferred into the recipient cytoplast. The North American leopard frog (*Rana pipiens)* was the pioneer species for this technique. The large size of the frog's oocytes allowed for easy micromanipulation including enucleation and nucleus introduction. Development into an adult frog was achieved in *Xenopus laevis*, using nuclear information extracted from an early stage embryo [79]. It proved more difficult to successfully transfer the nucleus of a somatic cell and this was not accomplished until 1975 [80]. Tadpoles were cloned using adult somatic cells and development to tadpoles was observed, although sexual maturity was never reached in these experiments. The study illustrated the potential of somatic cells to act in a pluripotent manner as the donor [81].

The first mammalian clones from cell transfer required the blastomere donor nucleus technique to be revisited and were completed in the 1980s [81]. A somatic cell was finally used for the cloning of mammals in 1996 when 'Dolly' the sheep was created with a completely differentiated cell taken from a mammary gland [82]. This discovery encouraged the use of the technique in an array of mammals and success was found in a range of animals, importantly; mice, cattle, and pigs [83].

2.3.2 Embryonic cell transfer techniques (cloning)

Development of a blastocyst *in vitro* using IVF techniques has limitations for amplifications of elite genetics. This is based on the need for both high-quality maternal and paternal gametes in the process and the limited number that can be given by the selected parent animals. Techniques to circumvent these limitations such as cloning were developed, driven by economic and medical possibilities [83]. The simplest cloning technique is achieved by embryo splitting, which involves the separation of blastomeres early in embryo development, typically after the first or second cleavage event. Each of the blastomeres is then developed into a full organism [84]. A more widely used and sought-after technique is cell transfer and iterations of this such as ECT [85].

ECT involves three active steps. Mature oocytes are selected for ECT, and enucleation is used for complete nucleus removal from the cell (0N/0C; Figure 2.6). The next step is to transfer the high-value genetic information of the donor cell to the enucleated oocyte. The oocyte's cytoplasm reprograms the donor nucleus back toward totipotency, allowing it to create embryonic cells through cleavage. Lastly, activation of the reconstruct is used to kickstart the mitotic process. Successfully activated reconstructs will undergo cleavage events, following the same embryonic stages as IVF embryos (Figure 2.3).

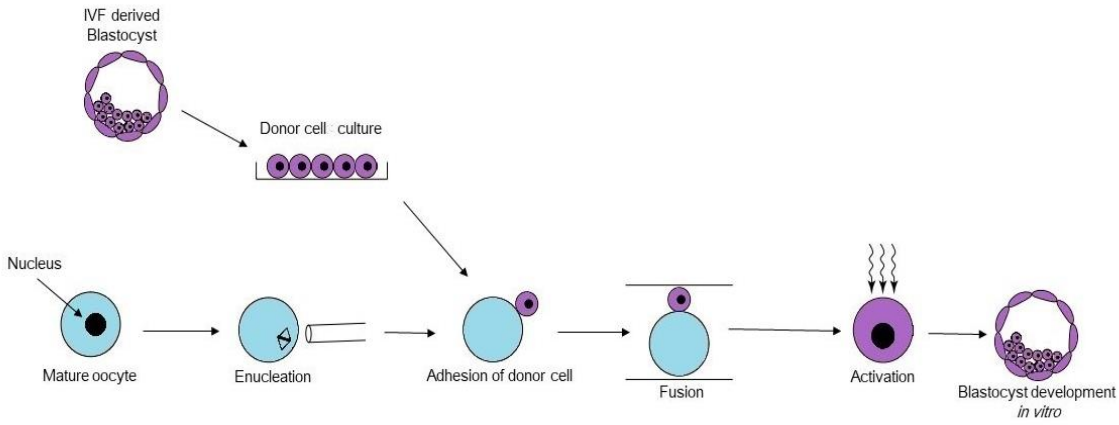


Figure 2.6. Embryo-derived cell transfer. Blastocysts are cultured to generate outgrowths from which donor cells can be utilised. Oocytes matured *in vitro* are enucleated to form cytoplasts. Donor cells are dissociated and adhered to the cytoplast making a couplet which is electrically fused. The nucleus of the donor cell is reprogrammed inside of the cytoplast and artificial activation of the reconstruct causes mitosis to proceed. Cell cleavage events lead to development of a late-stage blastocyst. Modified from [84].

2.3.2.1 Enucleation of oocytes

Enucleation can occur in both zona-intact and zona-free oocytes, but the zona-free method will be focused on. The oocyte's DNA is visualised by adding the oocytes to a medium containing Hoechst dye directly before enucleation. Enucleation is then completed on a microscope with a UV light that can be switched on for a period to visualise the DNA but not long enough to damage the oocyte [85]. During enucleation, a finely pulled pipette is inserted into the oocyte and the DNA is aspirated along with a small amount of cytoplasm [86]. Zona-intact enucleation does not require removal of the zona pellucida, compared to zona-free. However, zona-free enucleation is much

quicker and easier to replicate reliably. Zona-free reconstructs are at risk of sticking to each other, so culturing is done with each reconstruct in a single medium drop, or groups of reconstructs are separated by dimples within a drop [87]. Group culture, which is defined by multiple reconstructs placed in a single drop of culture medium, has been shown to have a small positive effect on embryo development due to the growth factors excreted by neighbouring reconstructs [88].

2.3.2.2 Donor cell transfer

When enucleation has concluded, the new cytoplasts can be fused with the donor cells to receive their genetic information. The donor cells are first prepared by dissociation to single cells which can be done enzymatically with pronase or trypsin as well as mechanically [89]. Pronase is preferred due to the increase in fusion rates it adds. Improvement may be a result of removal of protein surface charges, therefore gaining adhesion of the donor to the cytoplast [90]. Media with added lectin allows attachment between the donor cell and the cytoplast to form a couplet [84]. Lectin encourages cell-cell attachment through the recognition and binding of membrane carbohydrates and assists in fusion by aiding adhesion between the cytoplast and donor cell throughout the fusion process. It is recognised that lectin can be toxic and has been shown to decrease cleavage rates and blastocyst development after a 1 h incubation of cells. To counter this, time in lectin is managed by transferring the couplet from lectin to a wash plate with H199 and BSA after attachment [91]. The other key factor for fusion is fusion buffer, specifically osmolarity. Osmolarity is a measure of the concentration of particles or solutes in the liquid being dissolved into. The osmolarity of a typical mammalian cell resides around 280 milli-osmoles per kilogram (mOsm). Hypoosmolar buffer has been illustrated to cause swelling in the donor cell-cytoplast pair, which is thought to increase membrane permeability and flexibility [92]. Increased membrane permeability is utilised to increase fusion rates. The couplet is added to fusion buffer and aligned parallel to the current's direction. Alignment can be done mechanically with a pipette tip or with an alternating current (AC). The couplet is then stimulated by two direct current (DC) pulses which permeabilise membranes of both cells, causing them to fuse. Fusion of the cells causes transfer of the donor cell DNA to the cytoplast and becomes a reconstruct [93].

The cytoplasm of the original cytoplast begins to reprogram the DNA to a totipotent state to generate all cell types necessary for an adult organism [10]. Reprogramming is still poorly understood but occurs as long as the cell cycle stages of the cytoplast and donor are in compatible phases. Reprogramming of donor epigenetic modification is thought to be driven by the cytoplast and begins with an increase in chromatin accessibility in the donor nucleus [94]. Reprograming can reverse epigenetic modifications that have occurred throughout the donor organism's life. The modifications target access to the DNA sequence, allowing transcription factors to bind DNA. Epigenetic reprogramming includes removal and addition of DNA methylation and modification of histones, to encourage expression of genes linked to pluripotency [95]. Reprogramming of these epigenetic marks allows formation of a complete animal with all cell types rather than multiplication of the cell type of the donor cell prior to transfer [94].

2.3.2.3 Reconstruct activation to complete meiosis

Reconstructs that fused successfully need to undergo artificial activation which causes a rise in cytoplasmic Ca^{2+}. The heightened levels occur during one long release during artificial activation as opposed to the series of pulses that are observed *in vivo*, but the desired effect is maintained [79]. Increased cytoplasmic Ca^{2+} pulls the reconstruct out of meiotic arrest and meiosis resumes. Artificial activation can be done chemically or with electrical pulses [94; 96]. Activation with electric field pulses generates pores in the reconstructs membrane which subsequently increases the cytoplasmic Ca^{2+} levels through external entry. Chemical activators such as ionomycin stimulates Ca^{2+} release from the smooth endoplasmic reticulum in addition to forming pores for extracellular Ca^{2+} influx. The latter method provides higher pronuclear formation in cattle and is more commonly used for the bovine species as the cells are less sensitive to electrical stimulation than some other species [13]. Pronuclear formation is essential to embryo development and is dependent on the inhibition of protein kinases. Artificial activation decreases activity of the maturation promoting factors (MPFs) including the B1-CDK1 complexes, allowing release of the reconstruct from MII and meiotic progression. To ensure the MPFs are not reactivated, reconstructs are treated with a cytokinetic activity inhibitor such as 6-dimethylaminopurine (DMAP) [97]. The downside of this method is that DMAP has been illustrated to cause some chromosomal abnormalities and incorrect ploidy in reconstructs with non-diploid donors [98; 99].

2.3.2.4 Parthenotes

During cell transfer experiments it is useful to have a positive control to ensure activation is functioning correctly. A parthenote (PG) is an organism formed from an unfertilised oocyte. Parthenogenesis is denoted by the production of an organism with no male genetic contribution. In nature, it is commonly used by insects such as flies and aphids and is also possible in vertebrates including fish and lizards [100]. The process was first done artificially *in vitro* in 1899 in frogs and sea urchins by either needle pricking the oocyte or influencing the surrounding salt concentration [101].

Parthenogenesis can be used during cell transfer as a positive control, through activation of an oocyte with all nuclear components still intact. In most mammals, a parthenote contains one set of replicated DNA (1N/2C). After activation, the cell does not complete meiosis and a second polar body is never extruded, resulting in a diploid pronucleus with two sister chromatid sets [100]. Parthenogenesis does not result in formation of a full organism in mammals as the lack of paternal genomic information inhibits the extraembryonic tissue from developing, which is influenced by the paternal DNA alongside many other embryonic genes [102]. However, cleavage still occurs, and embryos can be generated, which means any unexpected defect of activation will be demonstrated on the PG and is separated from any responses due to the addition of a donor cell or processes surrounding this such as fusion and enucleation. This is a useful tool for identifying where unexpected developments during cell transfer originated [103].

2.4 Cloning efficiency

Cloning efficiency can be defined as the proportion of embryos from cell transfer that are transferred to recipients and develop into a healthy animal. Efficiency is affected by multiple *in vitro* factors as cloning is a multistep process [104]. Even the widely established somatic cell transfer still has a low cloning efficiency due to epigenetic abnormalities caused by incomplete epigenetic reprogramming and cell cycle incompatibilities [105].

One such genetic concern is incorrect chromosomal ploidy. Incorrect ploidy can be found in up to 20% of blastocysts generated in CT [106]. Aneuploidy (state of missing chromosomes or having extra) and polyploidy (possessing abnormally high quantity

of complete chromosome sets) are commonly both found in the same individual in a mosaic fashion. This indicates the issues in ploidy result from cleavage events of the developing embryo rather than from the donor cell. Mitotic spindles are responsible for separating and distributing chromosome pairs and sister chromatids so breakdown in spindles is thought to be the main determinant [107]. Embryos with incorrect ploidy can still develop into late-stage blastocysts, thus, impacts to cloning efficiency generally happen at the implantation or viable offspring phase. This is a welfare concern and so chromosomal abnormalities should be screened for before implantation.

Donor cell cycle phase is important in cell transfer as certain phases are incompatible with the cytoplast. The cell cycle has four main stages (Figure 2.7). When mitosis is complete, the cell enters the first growth phase. Cells in this phase can become quiescent and undergo no further growth or DNA preparation (G_0) until this phase is departed. Cells that do not become quiescent, enter the G_1 stage where there is an increase in size, and components required for DNA replication are synthesised. If a donor is in G_1 or G_0 when it is fused with the cytoplast the correct ploidy is achieved as the DNA has not been replicated. The reconstruct replicates its DNA and undergoes the first cleavage successfully [108]. After G_1, cells enter S-phase where DNA is replicated. If a donor cell is introduced into the cytoplast during S-phase, the ploidy can be incorrect, or partially replicated DNA can cause genetic fragmentation, depending on the stage of replication. This makes S-phase incompatible for cell transfer. G_2 follows S-phase and prepares the cell for mitosis with rapid growth and protein synthesis. When a donor in G_2 is fused with a cytoplast, there is twice the DNA content (4C). This can be remediated with the expulsion of a second polar body containing half of the genetic information. However, if DMAP is being used this polar body will not occur, leaving incorrect ploidy. During mitosis (M-Phase) the chromosomes are segregated by spindles and two nuclei are formed before the cell cleaves completely, generating two daughter cells. Donors in the start of M-phase are compatible in the same way G_2 donors are [108; 109]

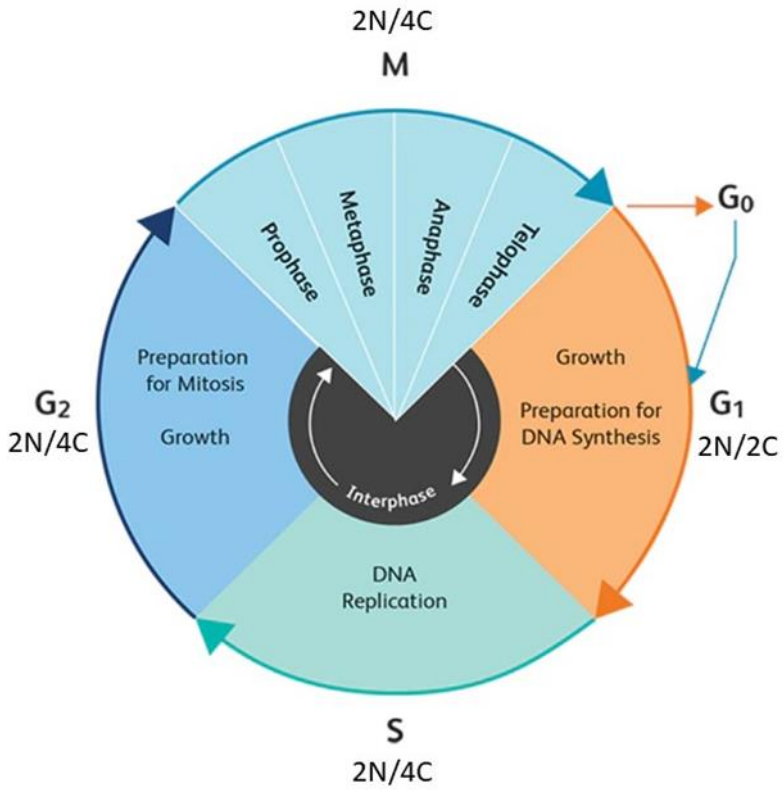


Figure 2.7. Phases of the cell cycle. If cells are arrested after mitosis, they enter the G_0 phase. Cells that go on to grow and produce components for DNA synthesis are in G_1. S-phase entails the replication of DNA. In G_2 phase, cells undergo a second growth and prepare for cell division. Interphase is now complete, and cells divide in mitosis. Image from [110].

The epigenetic state of the donor cell influences cloning efficiency. When the donor cell is introduced into an enucleated cytoplast, donor DNA is reprogrammed by factors in the cytoplast. Reprogramming must entirely reverse the epigenetic state of the genetic contents to revert to an early embryonic state, allowing the correct genes to be expressed for embryonic development [111]. Reprogramming must therefore be completed before the first mitotic event in the reconstruct, or epigenetic abnormalities may occur. Somatic cells have undergone additional epigenetic modifications during differentiation from embryonic cells. Additional modifications increase the difficulty and time required for reprogramming by the cytoplast. Thus, somatic cell transfer often encounters incomplete epigenetic reprogramming which leads to low viability in offspring and phenotypic abnormalities, reducing cloning efficiency [95]. Pluripotent embryo-derived cells have undergone less epigenetic modification and have an epigenetic state more like that of the cytoplast, hence, less epigenetic reprogramming is required, causing fewer epigenetic abnormalities.

2.4.1 Cloning with pluripotent ESCs

Donor cell type selected for cloning has a substantial impact on the outcomes of cloning. Somatic cell cloning (SCT) in mammals is a widely established technique, pioneered in sheep and commonly used in mice as well as animals in the livestock industry such as cattle [105]. SCT has a good rate of blastocyst generation, however, issues with the blastocysts and offspring are often generated, including abnormal gene expression due to epigenetic modifications, large offspring, and early death [105; 112].

Pluripotent ESCs were first used to successfully clone mice, in 1999 [11]. Studies show that initial methods to use ESC donors in cloning did not improve overall cloning rates (number of cloning reconstructs that generated healthy, live offspring) compared to somatic cell cloning [112]. The initial blastocyst development was found to be lower compared to SCT but the proportion of embryos transferred to recipients that developed to term was higher [113]. Improvement in *in vivo* development to term in ECT may be due to the epigenetic abnormalities observed in SCT. Improvement in the initial embryo development of ECT would lead to increased cloning efficiency compared to SCT.

G_1 is shorter in pluripotent embryonic cells than in somatic cells which means fewer ESCs are in this compatible phase at the time of cell transfer. Bovine ESCs have displayed a higher proportion of cells in S-phase within the outgrowth than somatic cell outgrowths, a phase discordant with cell transfer and which typically does not lead to blastocyst generation [54]. In somatic cells, donors in incompatible cell cycle stages such as S-phase can be remediated using serum starvation to hold the cells in G_0/G_1. However, embryo-derived cells are much more sensitive to this technique, and it may result in extensive cell death. Synchronisation to M-phase can be achieved in ESCs to overcome the risk of S-phase cells during cloning with a drug called nocodazole [114]. However, this method cannot be used if activation during cloning is done with ionomycin and 6-dimethylaminopurine (DMAP). The use of DMAP prevents the second polar body from being extruded which leaves the reconstruct with double the regular amount of DNA (2N/4C) [115]. These tetraploid reconstructs will produce blastocysts that cannot form healthy offspring.

2.5 Research aims and objectives

The aim of this research project was to advance the current field of cell transfer cloning with bovine embryo-derived cells, by improving protocols surrounding embryo-derived outgrowth formation, and the use of these cells as donors in cloning. Progressing current methodologies could increase the viability of cloning with embryo-derived cells at a commercial level. Increased viability would accelerate the introduction of genes for thermotolerance in cattle populations, improving welfare in the world of climate change.

The research project aims were broken down into three main objectives.

- Increasing the efficiency of generating pluripotent outgrowths from blastocysts by improving the current methodologies.
- Characterising the cells in outgrowth culture with immunofluorescence to gain a better understanding of cell type and rates of proliferation.
- Optimising embryo-derived cell transfer cloning techniques.

Chapter 3
Materials and Methods

3.1 Ethics statement and relevant approvals

All research was conducted under guidelines from New Zealand's Biosecurity Act 1993. Experiments with genetic modification were approved under the New Zealand Hazardous Substances and New Organisms Act (HSNO) 1996. Animal ethics approval was not required as study conducted was limited to development of late-stage blastocysts.

3.2 Materials

All consumables used for this thesis were contained in sterile packaging and kept in sterile conditions until use. Heat resistant components such as glassware were washed and autoclaved at 121°C for 20 min between uses. Recipes and constituents unique to AgResearch Ruakura are defined in Appendix I. Equipment suppliers are listed in text and reagent suppliers are listed in Appendix I. Milli-Q water (15-18 MΩ·cm) was used in the making of all solutions. Solutions using non-sterile ingredients were passed through a 0.2µm Supor® Hydrophilic polyethersulfone membrane filter (Supor®, Pall laboratories, USA) for sterilisation. All surfaces were sterilised using 70% ethanol prior to lab work.

3.3 Generation of bovine blastocysts

3.3.1 *In vitro* production of blastocysts (IVP)

All IVP information was registered on an IVP record sheet (Appendix II). Blastocyst production was done on-site at AgResearch Ltd, Ruakura, Hamilton, New Zealand under physical containment level 2 (PC2) of the embryology lab. All live-cell work was completed on 36-38°C warm stages. Embryo plates were made in a sterile laminar flow hood (Model CF, Gelman Sciences, Australia) and placed in the chosen gassing conditions two h prior to any cell work. Wash plates were made up of 35 mm Petri dishes with 2 ml of solution.

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

IVM plates were assembled 2 h prior to aspiration. Cysteamine was first added to the maturation media at a 0.1 mM concentration. IVM plates were then made by pipetting 12 x 40 µl drops of this into a 60 mm Petri dish (Appendix III, Figure III.1) and covered by 8 ml of mineral oil in the laminar flow hood with a pipette aid and 10 ml serological pipette. Plates were left to gas in a 5% CO_2 incubator (Contherm biocell 1000 incubator, Contherm, New Zealand) at 38°C to equilibrate. H199, B199, and aspiration media are also warmed for ≈ 2 h and B199 was gassed by loosening the lid to obtain a suitable pH for oocytes.

The bovine ovaries used for aspiration were provided by abattoirs from both Auckland and Morrinsville and placed into a thermos containing 0.9% saline at 29-30°C. When the ovaries were on site, three washes with 30.5°C 0.9% saline were done to clear external blood and ample saline was applied to cover the ovaries in a holding container. An 18-gauge needle on a 15 ml conical tube containing aspiration media was used with a 48-mmHg vacuum (IVF Ultra Quiet VMAR-5100, Cook veterinary products, Switzerland) to aspirate follicles between 2-8 mm in diameter. The follicle was pierced, and fluid and oocytes were collected.

The aspiration tube contents were then added to a 90 mm Petri dish with aspiration media just covering the base (15-20 ml) and this was placed on a grid under the microscope. The entire dish was searched for high-quality oocytes which present with a light cumulus layer that surrounds the oocyte entirely (Appendix IV). Oocytes of good quality were moved into the first of two wash plates containing H199 + 10% FBS and screened once more before transfer to the next plate. Oocytes were then washed through a final plate containing B199 + 10% FBS. A 10 µl pipette was implemented to transfer 10-12 oocytes in 10 µl of B199 + 10% FBS to each drop in the IVM plate. The IVM plate was returned to the incubator for 20-22 h for maturation.

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

IVF plates were made a minimum of 2 h before IVF. To complete the IVF media, 10 mg/ml heparin, 0.2 mM penicillamine, 0.1 mM hypotaurine, and 0.1 mM pyruvate were added to 10 ml of IVFSOF medium. The IVF plates consisted of a 60 mm Petri dish with 12 x 30 µl drops of IVF media covered with 8 ml of mineral oil. The extra IVF media, 10 ml HSOF and IVF plates were transferred to a 5% CO_2 incubator at 38°C to warm, and the IVF media and plate to equilibrate (IVF media lid loosened to allow this).

Sperm prep was executed using frozen bull semen (SRB Monowai Debonair). The sperm were separated using a gradient. Gradient was formed by adding 1 ml 80% BoviPure™ solution to a conical centrifuge tube and then slowly pipetting 1 ml of 40% BoviPure™ solution on top of this, aiming the pipette at the side of the tube so the solutions do not mix, and an interphase is created between them. The straw of semen was removed from its vial in liquid nitrogen and placed in a 35°C water bath for 30 seconds. The straw was removed and dried manually with tissue paper and wiped down with 70% ethanol for sterilisation. The straw was held over the BoviPure™ gradient and the top end cut, causing the sperm to release from the straw into the BoviPure™ gradient. The gradient containing sperm was then centrifuged at 300 x g for 20 min at room temperature, during which time 1 ml of HSOF was poured into a 15 ml centrifuge tube and left at room temperature.

During this time oocytes were transferred to and subsequently washed through two wash plates containing HSOF. During the second wash, the oocytes are gently pipetted up and down to loosen the cumulus cells and then placed in a final wash dish containing IVF media. Oocytes were transferred to the IVF plate in groups of five with 10 µl of IVF media. The IVF plate was finally placed in 5% CO2 incubator at 38°C until sperm prep was finished.

When centrifugation was complete, the pellet was immediately removed using a pipette and inserted into a new 15 ml tube to prevent sperm from swimming up. HSOF was slowly added to this tube while gently flicking the tube to prevent dilution shock. The sperm were once again centrifuged at 300 x g for 5 min. Supernatant was removed using a sterile pasture pipette and the pellet was resuspended by slowly dripping in 200 µl of IVF media with a pipette while gently flicking the tube.

To count the sperm, 10 µl of sperm solution was added to 190 µl of pre-prepared water in a glass tube and mixed. Remaining sperm solution volume was measured with a Pasteur pipette at the end of a 1 ml syringe and placed in a dark area at 38°C. A haemocytometer (Bürker Counting Chamber, Neubauer, Weber, UK) was used for sperm counting. The haemocytometer was wiped down with ethanol prior to use and a coverslip was applied with gentle, constant pressure. A pipette was filled with 10 µl of the sperm dilution and placed against the edge of the glass coverslip at an angle, pointing toward the junction between the coverslip and grid surface, allowing the solution to flow under the coverslip, filling the area. The same was done on the other side. The grid contained 25 central squares and counting was done under 400x magnification (Figure 3.1, Figure 3.2). Counting was done on sperm heads to negate issues with multiple tailed sperm and heads touching the top and right edge lines of the grid were counted while heads touching the bottom and left edge lines were not. Sperm counts were done on each side of the chamber and the average calculated. Average sperm count was used in an equation to find the required end volume of media.

$$\frac{\text{Volume of sperm measured (A)x Average of sperm count (B)}}{37.5}$$

$$= \text{Total IVF media volume (C)}$$

$$\text{Total volume IVF media} - \text{volume sperm measured}$$

$$= \text{Volume IVF media to be added}$$

Sperm dilution was calculated to give a final concentration of 1.5 million sperm per ml and 10 µl of the sperm dilution was pipetted into each of the drop in the IVF plate containing oocytes. Sperm motility within the drops was assessed before the IVF plates were placed back in the 5% CO_2 incubator at 38°C for 18-24 h for fertilisation.

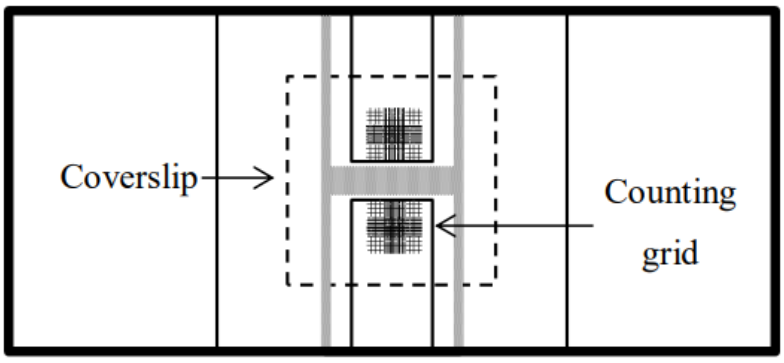


Figure 3.1. Aerial view of Haemocytometer. Diagram demonstrates coverslip placement and position of the counting grid on the haemocytometer. Modified from [116].

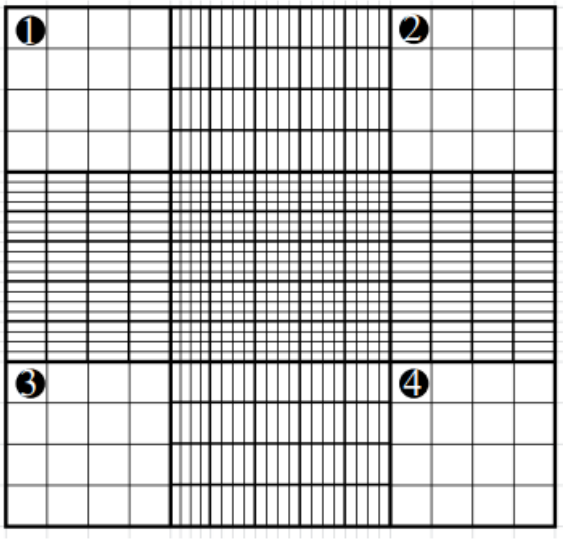


Figure 3.2. Counting grid. Grid with triple lines in the central square was used for sperm counts. Sperm heads touching the top and right edge lines were counted and those touching bottom and left edge lines were ignored. Modified from [116].

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

IVC plates were assembled a minimum of 2 h before IVC commenced. IVC plates were made using a 35 mm Petri dish with 6 x 20 µl drops surrounding 2 x 40 µl of ESOF, overlaid with 3mls of Squibbs mineral oil (Appendix III, Figure III.1). Each drop would hold 10-15 embryos. Equilibration of plates was done in a modular incubator chamber (QNA International Pty Ltd., Australia) with Milli-Q water at the base as a humidifier, inside a 38°C Contherm incubator. These conditions best mirror conditions *in vivo.* A B199 indicator dish was contained within the incubator chamber to monitor pH of adjacent IVC dishes. Conditions inside the chamber are generated by feeding in a mixture of 5% CO2, 7% O2, 88% N2 gasses for 5 min before airtight capping of the chamber. The modular incubator chamber was then placed in a 38°C incubator. HSOF (tightly capped) for washing embryos was stored in a 38°C incubator to warm for use (15ml).

Approximately 24-26 h after IVF the oocytes were removed from the IVF plates with a pipette tip and transferred to an HSOF wash dish. The oocytes were moved (with minimal media carry-over) to a sterile micro test tube (Eppendorf) containing 500 µl of 1mg/ml hyaluronidase in HSOF + BSA. The tube was vortexed at 2000 rpm for 2 min on minishaker (SM1 minishaker IKA®, Germany), and spun for 5 seconds in a mini centrifuge (Spectrafuge mini C1301, Labnet International INC., USA). A pulled Pasteur pipette attached to a mouth pipette was used to transfer oocytes back to the HSOF followed by a second HSOF wash before being relocated to the central wash drop of the IVC plate. Oocytes were transferred to the culture drops in groups of 8-12 and the plate returned to the 38°C incubator in the incubation chamber after gassing with 5% CO2, 7% O2, 88% N2 for 5 min and sealing. The indicator dish was checked ≈ 2 h later for an orange colour indicating pH is optimal.

3.3.1.4 Day 5 – Culture medium change (Change-over)

On day 5, LSOF plates were prepared at least 2 h before change-over. Plates were 35mm Petri dishes, made with 2x 40 µl central wash drops surrounded by 6x 20 µl drops of LSOF, covered with 3 ml of mineral oil. Plates were equilibrated in a modular incubator chamber, gassed with a mix of 5% CO2, 7% O2, and 88% for 5 min, then sealed airtight for storage in a 38°C incubator.

Embryos were transferred using a pulled Pasteur pipette, from IVC plates to the central wash drop of new LSOF plates. The embryos were washed through the second wash drop before transfer into culture drops in groups of 10, according to development, leaving uncleaved oocytes in the central wash drop. Cleavage was recorded, with each embryo assigned to a developmental stage of 1-cell, less than 8 cell, greater than 8 cell, or tight morula. Embryo degradation was also recorded. The plates were then returned to the incubation chamber and equilibrated by gassing with 5% CO2, 7% O2, and 88% for 5 min and sealed before placing in the 38°C.

Change-over was done in order to provide similar conditions to the changes that occur *in vivo* during the developmental stages. Movement into LSOF medium is necessary for further development of the embryos.

3.3.1.5 Day 8 – Grading

On day 8 of IVP, the embryos were assessed for grade and development. Developmental stage, grade, and total number of blastocysts was established. These factors were assessed using a standard guide (Appendix V). Blastocysts were then processed for outgrowth generation and cell culture, with high grade blastocysts taking priority.

3.4 Blastocyst culture

3.4.1 Generation of embryo-derived outgrowths

3.4.1.1 Culture plate preparation

The day before blastocyst plating, complete growth media was prepared to aid in cell growth and pluripotency. Culture of embryo-derived outgrowths was executed on a 96-well plate (Nunc 96-well plate, Thermo Scientific, NZ), with one blastocyst per well. If gelatin coating was utilised, gelatin was first passed through a 0.2µm Supor® (Hydrophilic polyethersulfone) membrane filter for sterilisation followed by adding 100 µl of gelatin into each well to aid attachment of the blastocyst. This was completed 2 h prior to blastocyst plating. After ≈ 30 min, excess gelatin was removed from the well with a pipette. PBS was also added to all edge wells to stop evaporation of growth media occurring. An h prior to blastocyst plating, the growth media was placed in a 5% CO2 incubator at 38°C with an unscrewed lid to warm and equilibrate to the optimal pH for bovine cells. Once equilibrated the media was added to the culture plate at 100 µl per well, and the plate is then placed into a 5% CO2 incubator at 38°C until use for plating.

3.4.1.2 Blastocyst plating

To create outgrowths and increase cell numbers, bovine blastocysts were plated on 96-well plates. The blastocysts utilised were all plated on day 7 or 8 of IVP. Blastocysts cultured were produced with IVF protocols and were either wild type or edited to include *PMEL* or *slick* alleles. Prior to plating, a curved Pasture pipette was formed by heating the glass over a Bunsen burner flame and pulling the glass at an angle, creating a bend. This allows for pipette work directly under a microscope lens.

Blastocysts were removed from their zona pellucidas, which either occurred naturally through hatching, or artificially with short exposure to pronase for 1-5 min until the zona loosens and begins to detach from the blastocyst. The blastocysts were transferred back into LSOF wash drops to remove excess pronase before being placed back into LSOF culture droplets covered with oil while they re-expanded. In a laminar flow hood (Hera guard, Heraeus, Germany), blastocysts were transferred from LSOF to the growth-media-filled wells using the pulled Pasteur pipette at the end of a mouth pipette. The plate was then left in a 38°C incubator with 5% CO2.

3.4.2 Embryo-derived cell culture

Cell culture was completed in the tissue culture laboratory at AgResearch Ltd, Ruakura, Hamilton, New Zealand, under PC2 conditions. Culture work was performed in a laminar flow hood using aseptic technique. All surfaces and non-sterile equipment were wiped with 70% ethanol prior to use. Cell cultures were left in a 38°C incubator with 5% CO2 (Series II water-jacketed CO2 incubator, Therma Forma, Thermo Scientific, USA).

Media changes were done at regular intervals of 3-5 days. During the first 5 days after plating, half-media changes were completed. Half-media changes consisted of gently removing 50 µl of old media from each well by holding the pipette tip barely under the surface. 50 µl of fresh media was then added to each well. After 5 days from plating, full media changes were undergone which consisted of removing all the old media from the well (≈ 100 µl) and adding 100 µl of fresh media to each well.

3.4.2.1 Cell passaging

When outgrowths reached 78-80% confluency in the well, passaging was completed to transfer the cells into a well with more surface area. All media and enzymatic solutions were pre-warmed 30 min prior to passaging. A 48-well plate (Nunc 48-well plate, Thermo Scientific, NZ) was pre-treated by adding 200 µl gelatin 1 h before passaging and removing excess gelatin 30 min later. Passaging was complete under sterile conditions in a laminar flow hood. Growth media was removed from the wells using a 200 µl sterile pipette tip followed by washing the outgrowth once with PBS (twice if growth media contained FBS). 50 µl of TrypLE™ Select was added to each well and the plate was put back in 5% CO2 38°C incubator to keep cells at optimal pH and temperature and wait until outgrowth has dissociated from the gelatin substrate (around 2-5 min). Dissociation was confirmed using a phase-contrast microscope (Nikon TMS, Nikon, Japan), and gentle tapping of the plate was used to dislodge any remaining cells. 100 µl of growth media was added to the well, followed by pipetting the solution up and down 10 times with a 200 µl pipette to mechanically dissociate the outgrowth into smaller fragments and single cells. The contents were then transferred to a well in the 48-well plate, followed by adding another 250 µl growth media to the new well to bring the final concentration to 400 µl. If passaging was performed on an

outgrowth in a 48-well plate, the cells were passaged and divided between two wells on a 48-well plate and all volumes were adjusted appropriately.

3.5 Cell characterisation

3.5.1 DNA staining

Hoechst dye was applied to assess the presence of nuclear DNA. The state of DNA condensation was also evaluated to gauge cells in mitosis or meiosis. Cells were incubated in 5 µg/ml Hoechst 33342 in PBS or added straight to current media for 10-15 min. EVOS microscope (Advanced microscopy group, Millennium Science, Australia) was employed to visualise the Hoechst stain with UV at 361 nm.

3.5.2 EdU staining

The proportion of cells undergoing proliferation in an outgrowth can be elucidated using a 5-ethynyl-2'-deoxyuridine (EdU) stain. Cells in S-phase are replicating their DNA will incorporate EdU. Fluorescent probes can be bound to this and used to detect nuclei that have incorporated EdU with the Click-iT® EdU Imaging Kit (Invitrogen, Life Technologies, USA). All solutions were made in a laminar flow hood and incubations were done at room temperature in dark conditions to avoid photo-bleaching of fluorophores.

Embryo-derived outgrowths were grown for four to seven days before staining. Bovine fibroblasts were employed as a positive control and grown for two days prior to staining on a 35 mm tissue culture dish with 2 mL of fibroblast growth media. On the day of staining, 20 µM EdU solution was made and placed in the 5% CO2 38°C incubator to equilibrate. Once warmed, half the growth media (50 µl) was removed from the wells with a 100 µl pipette and 50 µl of Edu solution added (including positive controls). The cells were incubated at 38°C in 5% CO2 for the allotted time (30 min or 24 h).

After incubation, all media was removed, and outgrowths were washed twice with PBS or donkey serum. 100 µl of depolymerised 3.7% paraformaldehyde (PFA) with sucrose in PBS was added and the plate was left at room temperature to fix the cells for 15 min, which prevents cellular component degradation and cell death. Solution was removed and followed with 2 washes (as previously stated) before adding 100 µl of

0.5% Triton X-100 to the well and left for 20 min which permeabilises the cell membrane to allow the fluorescent dye to enter the cell. During this incubation period, the Click-iT® reaction cocktail was prepared (Table 3.1). Components were added following the table order to enhance reaction proficiency and the solution protected from the light as were all subsequent steps. Permeabilization buffer was removed, and cells washed twice before adding 100 µl of the reaction cocktail to each well, including the negative control which contained no EdU. This is left for 30 min in darkness before the solution is removed, and 5 µl/ml Hoechst in PBS added for 10-15 min. Hoechst was removed, and one wash performed before viewing on Leica microscope (Leica DMI6000 B, Leica Microsystems, Danaher, Germany).

Table 3.1: Click-iT® reaction components

Reaction Component	Volume
1X Click-iT® EdU reaction buffer	1.72 mL
100 mM CuSO4	80 µl
Alexa Fluor® azide (diluted in DMSO)	5 µl
10X Click-iT® reaction buffer additive	200 µl
Total	**2 mL**

3.5.3 Antibody staining

Cell staining with primary and secondary antibodies can elucidate proliferation and cell type markers. The primary antibody is included to bind the marker protein, and the secondary antibody is fluorescently labelled and binds the primary antibody (Table 3.2). Volumes used in antibody steps were calculated to completely cover the outgrowth and consider evaporation, approximately 100 µl per well in the plate.

Table 3.2: Primary antibodies applied in antibody staining

Protein antibody	Host	Clonality	Dilution required	Manufacturer
Ki-67 (Mitosis marker)	Rabbit	Polyclonal	1/200	Abcam
Gata-6 (Hypoblast marker)	Rabbit	Polyclonal	1/100	Santa Cruz
Oct 3/4 (Pluripotency marker)	Goat & Rabbit	Polyclonal	1/200	Santa Cruz

Table 3.3: Secondary antibodies applied in antibody staining

Secondary Antibody	Host	Reactivity	Fluorescent emission (nm)	Dilution required	Life Technologies number
DαG 568	Donkey	Goat	568	1/2000	A-11057
DαG 488	Donkey	Goat	488	1/2000	A-11055
DαRab 488	Donkey	Rabbit	488	1/2000	A-21206
GαRab 568	Goat	Rabbit	568	1/2000	A-11036

Before antibody binding; washing, fixing, and permeabilisation steps were followed as stated in the EdU protocol (3.5.2). After permeabilisation, outgrowths were blocked in 5% donkey or goat serum for one h. The serum was chosen to reflect the host of the secondary antibody which prevents false positives in the stain. During blocking a dilution of the primary antibody was made in blocking solution and kept on ice (Table 3.2). If there were two proteins of interest, a double stain was chosen and the primary antibody for each was raised in a different host. Blocking serum was removed after 1 h, the outgrowth washed once with PBS and replaced with 100 µl of antibody/antibodies in blocking serum to be left overnight in a 4°C fridge. A negative control outgrowth was left only in blocking serum.

The following day, the secondary antibody solutions were made at the necessary dilution in blocking solution with Hoechst added at 5 µl/ml and kept on ice in dark

conditions to protect the fluorophores from light damage (Table 3.3). The secondary antibody was selected with reactivity to the host of the primary. The primary antibody dilution was removed, and the outgrowths washed three times in PBS. 100 µl of the secondary antibody/antibodies were added to wells including negative control and incubated at 38°C for 30 min, covered to block any light. The negative control was used to indicate any off-target binding that may have occurred. The secondary antibody solution was removed after incubation and outgrowths washed three times with blocking solution followed by a final wash with Milli-Q water and this was replaced with donkey serum for imaging/viewing. Plates were subsequently stored in a 4°C fridge for further visualisation with Leica microscope.

3.5.4 Cell and outgrowth sizing

Cells or outgrowths to be sized were imaged on EVOS microscope at either 4x, 10x, 20x, or 40x zoom. Images were uploaded to ImageJ (Public domain, Github) and sized using the measure feature with the microscope's inbuilt reticule as reference. Diameter measurements were taken, and area calculated where relevant.

3.6 Embryo-derived cell transfer cloning

Plates for cell transfer were made in a sterile laminar flow hood two h prior to use and left in a 38°C incubator with 5% CO2 to warm and equilibrate. All live-cell work was completed on 36-38°C warm stages. Wash plates were made up of 35mm Petri dishes with 2-3ml of solution. Drop plates were covered with oil to improve sterile conditions and prevent evaporation. Finely pulled Pasteur pipettes were used for cell work during cloning unless otherwise stated. Record sheets (Appendix VI) were used to record embryo-derived cell transfer (ECT) details including embryo culture. Information on fusion was recorded on fusion sheets (Appendix VII). Some ECT plates were made the afternoon prior to ECT (Table 3.4), and other plates made the day of ECT (Table 3.5). Overnight plates were stored at 4°C in a fridge and taken out the following morning and placed on a 38°C warm stage to heat up. Fusion buffer was removed from the fridge at the same time and left on a bench to warm to room temperature. Same day plates were produced the morning of ECT and stored in the 38°C incubator with 5% CO2 alongside ECT solutions. 'ESOF' plates were transferred to a modular incubator chamber and gassed before being stored for a minimum of 2 h before use.

Table 3.4: Plates prepared the day prior to cell transfer

Plate Name	Solution	Drop Configuration	Number of Plates
Oocyte	H199 +3mg/ml BSA	12 x 30 µl	1
Cytoplast	H199 +3mg/ml BSA	12 x 30 µl	1
Couplet	H199 +3mg/ml BSA	12 x 30 µl	1 per treatment
Post Fusion	H199 +3mg/ml BSA	12 x 30 µl	1 per treatment
ESOF – Ca	ESOF - Ca + 10% FBS	12 x 30 µl	1 per treatment
ESOF	ESOF	3 x 40 µl, 30 x 5 µl	1 per treatment
Post Disaggregation	H199 +3mg/ml BSA + Y-27632	6 x 40 µl	1

Table 3.5: Plates prepared the morning of cell transfer

Plate Name	Solution	Drop Configuration	Number of Plates
Lectin	1980 µl H199 + 3mg/ml BSA + 20 µl Lectin	12 x 30 µl	1 per treatment
Stain	995 µl H199 + 3mg/ml BSA + 5 µl Hoechst	12 x 30 µl	1
Pronase	H199 + 0.5% pronase	2 x 50 µl	1
Dissociation	Dissociation media	6 x 40 µl	1
DMAP	1992 µl ESOF + 8 µl DMAP	3 x 40 µl, 32 x 5 µl	1 per treatment

Oocytes were aspirated and added to IVM plates using the same methodologies outlined in 3.3.1.1. Oocytes were matured for 20-22 h before use for cloning to give them ample time to reach MII arrest.

3.6.1 Oocyte preparation

Oocytes were removed from the IVM plate after maturation was completed (20-22 h post IVM) using a 200 µl pipette and transferred into H199 + 10% FBS in a 35 mm

wash dish. The oocytes were then vigorously pipetted up and down in a 200 µl pipette tip to loosen the cumulus cells. The oocytes were then moved to a 1.5 ml Eppendorf tube with 500 µl 1 mg/mL hyaluronidase (in H199) in a small volume of media. Eppendorf tube was vortexed for 2 min at approximately 2000 rpm on minishaker to dislodge the cumulus cells from the oocyte. The tube was spun on mini centrifuge for approximately 5 sec, creating a loose pellet of oocytes at the base of the tube. The contents of the pellet were returned to H199 + 10% FBS wash plate in little media volume. The remaining contents of the tube were then transferred to a 35 mm plate lid by creating 3 large droplets with a 200 µl pipette. The sides and lid of Eppendorf tube were rinsed with H199 + 10% FBS which was transferred to the droplets. Droplets were searched for any remaining oocytes which were subsequently transferred to the wash plate. The oocytes were then washed through one more H199 + 10% FBS plate. Washes dilute remaining hyaluronidase solution which prevents further hyaluronidase activity. Finally, the oocytes were collected and moved to a 35 mm plate containing H199 + 3 mg/ml BSA for polar body searching.

Oocytes were inspected for a polar body. The presence of a polar body is evidence that the oocyte has matured through to MII. The polar body is found in the region between the oocyte and the zona pellucida. Searching involves using the tip of a finely pulled Pasteur pipette to slowly rotate the oocyte to view it from all angles and check for the presence of a polar body. The proportion of oocytes with a polar body was recorded and oocytes with a polar body were pronased. Oocytes were left in pronase until zona pellucida begins to fall off, before being returned to H199 + 3mg/ml BSA to dilute any remaining pronase. When the wash was completed, the zona-free oocytes were moved to the ‘Oocyte’ plate containing H199 + 3mg/ml BSA, with 30 zona-free oocytes per drop. Oocytes stayed in the ‘Oocyte’ plate until their shape prior to pronasing had been restored. Several of the remaining oocytes with zona still intact were placed in a labelled drop to be later used as a positive control for activation events.

3.6.2 Oocyte enucleation

3.6.2.1 Development of enucleation tools

Enucleation requires two glass tools which includes a blunt enucleation pipette and a close-ended separation needle. Tools were made prior to the day of ECT and can be cleaned in trypsin and reused for multiple cell transfer runs. The tools were formed by

pulling borosilicate capillaries (GC100T-15, Harvard Apparatus Ltd, UK) with a horizontal puller (P-87, Sutter Instruments, USA). Blunt enucleation pipettes ware produced by breaking the pipette tip where the outer diameter is ≈ 25-30 µm using heat from a microforge (MP-9, Narishige, Japan) and bent at a 25-35° angle at the point where the capillaries diameter was 80-90 µm thick. The tips of separation needles were broken with a wide outer diameter of 100-150 µm. The tip of the separation needle was melted closed, and a 30° angle was introduced where the diameter was 200 µm thick. Breaks were made by direct contact with a heated bead and bends were made by holding the bead ≈ 10 µm under the pipette.

3.6.2.2 Enucleation

Groups of 30 oocytes we moved from the 'Oocyte' plate to a drop in the 'Stain' plate which contained Hoechst dye. The oocytes were incubated for 10 min to allow the stain to bind to DNA, enabling UV light to fluoresce the DNA so enucleation could be done accurately. Stained oocytes were transferred to a 90 mm Petri dish lid containing multiple drops of H199 + 10% FBS just covered with mineral oil. Enucleation was completed in the lid drops, on the warm stage of the micromanipulation microscope (MO-188, Nikon Narishige, Japan). The enucleation pipette and separation needle were attached to and guided by three-axis oil hydraulic hanging joystick micromanipulators with a 0.2 mL Gilmont® micrometer syringe (Cole-Parmer Instruments, USA). A UV light was used at the lowest level to illuminate the nuclear contents of the oocyte for long enough to line up the DNA to the enucleation needle then the UV light closed off to prevent any UV damage. The DNA was gently pulled into the enucleation pipette and the newly made cytoplast pinched off using the separation needle. Cytoplasts were stored in 'Cytoplast' plate until further use.

3.6.2.3 Aged cytoplasts

To create aged cytoplasts, after pronase and enucleation steps were completed, a select number of cytoplasts were removed from the 'Cytoplast' plate and placed in fresh drops within the 'Oocyte' plate and labelled accordingly. At 35 h post IVM oocytes were transferred into a 40 µl drop of embryo hold, covered by 8 mL of mineral oil in a 60 mm Petri dish. Dish was then left in a 15°C water bath overnight to age the cytoplasts. At 44 h post IVM, the cytoplasts were removed from embryo hold and

washed through a 30 mm dish filled with H199 + 3mg/ml BSA before being placed into a lectin plate where the attachment and fusion processes were resumed.

3.6.3 Donor cell preparation

Donor cell preparation was commenced in the tissue culture laboratory at AgResearch Ltd, Ruakura, Hamilton, New Zealand under PC2 conditions. Embryo-derived cells were cultured for varying amounts of time leading up to ECT (4–40 days). Outgrowths were chosen before ECT was initiated and selection was based on the apparent number of cells in the outgrowth and their quality unless a specific experiment was being run such as comparing effect of donor cell age. On the day of cell transfer, the outgrowths were lifted and dissociated into single cells. This was completed by removing the growth media from the well and adding 50 µl of dissociation media. The plate was moved back to the 5% CO2 38°C incubator for 2-5 min to keep cell conditions optimal. The plate was removed, and a phase-contrast microscope used to verify lifting of the outgrowth. If lifting was not completely achieved, the plate was gently tapped to dislodge the cells. A 200 µl pipette was used to transfer the cells and media to a 35 mm Petri dish and carried to the embryology lab. The cells were transferred to the drops in the dissociation plate and a pipette with a small diameter (≈ 100 µm) was used to break the outgrowth into smaller fragments. The fragments were transferred to the 'post disaggregation' plate in a small volume of media and subsequently smaller diameter pipettes (≈ 70, 50, and 30 µm) were used to dissociate the fragments to a single cell suspension.

3.6.4 Cytoplast and donor cell adhesion

Cytoplasts and donor cells were attached in 'Lectin' plates. Lectin encourages cell-cell adhesion through carbohydrate binding. Donor cells from the single-cell suspension were transferred to drops in the lectin plate. Cytoplasts were then transferred in small groups of 5 and placed strategically to avoid unwanted contact with multiple donor cells or other cytoplasts that may cause adherence. A single cytoplast is pushed with the tip of a pulled pipette and rolled onto a single donor cell. The cytoplast is gently pushed onto the donor to increase surface area of the attachment site. The new reconstruct is left in the lectin for 2 mins before the adherence is assessed by pipetting the couplet up and down 3-5 times vigorously and inspecting whether the two cells remain attached. The couplets were ready for fusion and transferred to H199 + 3 mg/ml

BSA after 5 min to prevent excess lectin activity. Couplets were placed into the ‘Couplet’ plate in small groups separated from each other until fusion.

3.6.5 Fusion

Couplets were fused using electrical stimulation to fuse the donor cell to the cytoplast. The equipment used included a parallel-plate fusion chamber that was custom made and joined to an electro cell manipulator (BTX ECM 200, Biotechnologies and Experimental Research Inc, USA). This setup allowed group fusion of multiple couplets.

Groups of 20 couplets were moved across to a 35 mm Petri dish filled with fusion buffer to equilibrate to the osmolarity. The fusion chamber was filled with fusion buffer. Any cell couplets that detached in the fusion buffer were returned to lectin and re-adhered. Couplets that were still attached and had not lysed were placed in the fusion chamber in groups of 5, in the 3 mm gap between electrodes. The couplets were lined up along the gap between electrodes and an alternating current (AC) was applied at an amplitude of 21.2 A to align the couplets so that the donor cell is pointing perpendicular to the electrode. Couplets that did not align with this method were manually aligned using a pulled pipette.

When couplets were aligned, two direct currents (DC) pulses were applied for 10 µsec at an amplitude of 600 A was applied to fuse the couplet. Couplets were returned to the fusion buffer wash plate and the process repeated until all 20 couplets had been fused, and then all couplets were moved to the ‘Post Fusion’ plate. This method was repeated until all couplets had completed fusion.

Around 20-40 min after fusion, fusion was scored in the couplet groups. Couplets with cell lysis, an unincorporated donor cell, or an uncoupled donor were scored as unfused and did not advance further into the cell transfer process. Couplets that fused successfully (reconstructs) were put through an HSOF – Ca + 10% FBS wash plate and divided between the top row of drops in the ‘ESOF – Ca’ plate in groups of 20. Reconstruct groups were transferred through the top and middle row as wash steps and settled in the bottom row, spaced to avoid unwanted adhesion. ‘ESOF – Ca’ plates were placed in 5% CO2 incubator at 38°C for 3 h from the halfway point of fusion, which was calculated using the fusion sheet.

3.6.6 Activation of reconstructs

Artificial activation of reconstructs was used post-fusion, to trigger mitosis to resume in the cytoplast which leads to embryo development. Three different media were required for activation and were added to 35 mm plates prior to activation. HSOF + 1 mg/mL BSA and HSOF + 30 mg/mL BSA were prepared the afternoon before CT. Immediately before activation, ionomycin was thawed and added to half of the HSOF + 1mg/mL BSA at 1 µl/mL. Ionomycin solution was vortexed at 2000 rpm for 20 sec on minishaker to mix completely. 1 h after the midway point of fusion, the reconstructs were removed from 'ESOF – Ca' plate and placed in the HSOF + 1 mg/ml BSA wash plate with a pulled Pasteur pipette at the end of a mouth pipette, for at least 1 min but no more than 30. Reconstructs were then transferred to the Ionomycin dish for 4 min and 30 sec before being moved to the HSOF + 30 mg/ml BSA dish for at least 3 min. Reconstructs were transferred to the first wash drop in the 'DMAP' plate and washed through the second and third wash drops. Reconstructs were then divided between the 5 µl drops to end with one reconstruct per drop. This process was repeated for each treatment group with fresh dishes of media. Zona-intact parthenotes (PGs) were activated following the same protocol. 'DMAP' plates were returned to the 5% CO2 incubator at 38°C for 4 hrs.

3.6.7 Embryo culture

After 4 h of post-activation culture in 'DMAP' plates, the reconstructs and PGs were cultured for embryo development following the same technique as in 3.3.1.3 and 3.3.1.4. The main difference was single culture was applied instead of group culture, using a different drop layout in the plate (Appendix III). Both reconstructs and PGs were transferred to a 35 mm HSOF wash dish and left for 2 min before being moved to the second HSOF wash dish. Reconstructs and PGs were transferred to the first wash drop of pre-gassed 'ESOF' plates and washed through subsequent wash drops. Reconstructs and PGs were relocated into individual 5 µl culture drops. Once this process was completed for all treatments and the PGs, the 'ESOF' plates were placed in the modular incubation chamber and gassed with 5% CO2, 7% O2, and 88% N2 for 5 min before the chamber was sealed and stored in an incubator at 38°C. Five days after the groups were placed in ESOF, LSOF was prepared by pre-gassing in 5% CO2, 7% O2, and 88% N2 for 10 min to prevent precipitation build-up in the drops. LSOF

was then used to prepare ‘LSOF’ plates which were gassed in modular incubation chamber with 5% CO2, 7% O2, and 88% N2 for 5 min before the chamber was sealed and stored in an incubator at 38°C for 2 h before change-over. During change-over the embryo development was recorded as either compact morula, greater than 8-cells, less than 8 cells, or 1-cell, and degradation was recorded simultaneously (Appendix VI). Embryos were transferred to a wash drop in the ’LSOF’ plate with a pulled Pasteur pipette and washed through the two remaining wash drops before being placed in single culture drops. The plates were then returned to the incubation chamber and gassed with 5% CO2, 7% O2, and 88% N2 for 5 min before sealing the chamber and placing it in a 38°C incubator. On both day seven and day eight, the plates were removed from the chamber and the grade and developmental stage of the blastocysts were assessed using visual criteria (Appendix V) and recorded. After day eight grading the blastocyst were prepared for plating or vitrification.

3.6.8 Statistical analysis

All data analysis was completed in Microsoft Excel®, version 2202. Embryo development was calculated as a proportion of embryos in IVC. Error bars were calculated with standard error of the mean (SEM) unless stated otherwise. Data sets under objective 1 do not contain error bars as this was a preliminary study and the minimum number of replicates required was not met, based on the time constraints of a master’s thesis. Significance was calculated with Fisher’s exact test of independence unless stated otherwise. Fisher’s exact test of independence is used to compare whether proportions of one nominable variable differ significantly among values of a second nominal variable and is appropriate for experiments with a small number of observations. The null hypothesis states that the variables are independent with no association and the alternative hypothesis is that the variables are dependant. Fisher’s exact test of independence was chosen for embryo development as the proportion of blastocyst development was low. It was also selected for cell characterisation development as number of colonies was small without a high number of replicates. ANOVA and Tukey’s HSD post hoc test was chosen for cell sizing data as ANOVA calculates statistical significance when comparing more than two treatments against each other. If significance is found, Tukey’s HSD is appropriate to calculate which variable pairs are significantly different from each other. Tukey’s HSD can be used when sample sizes between variables are different. An unpaired t-test was applied to

experiments where the aim was to compare the means between treatments to elucidate any significant difference. Significance was always given as an exact P-value and considered significant if $P = < 0.05$.

Chapter 4

Results

4.1 Objective 1: Optimising embryo-derived outgrowth conditions

4.1.1 Blastocyst generation through IVP

Conducting IVP runs allowed for the generation of blastocysts for plating, in which higher grade blastocysts were prioritised (replicates *n*=7, total oocytes into IVC *N*=944). After *in vitro* fertilisation of the oocytes, cleavage (>1 cell) was tracked and shown to be 88 ± 1% (Figure 4.1). Blastocyst development and grade was determined on day 8 as a proportion of oocytes that went into IVC. Development of all blastocysts (grade 1-3) was 36 ± 5%. Grade 1-2 blastocysts are considered high grade and are prioritised for plating and vitrification. High-grade blastocyst development was 14 ± 3%.

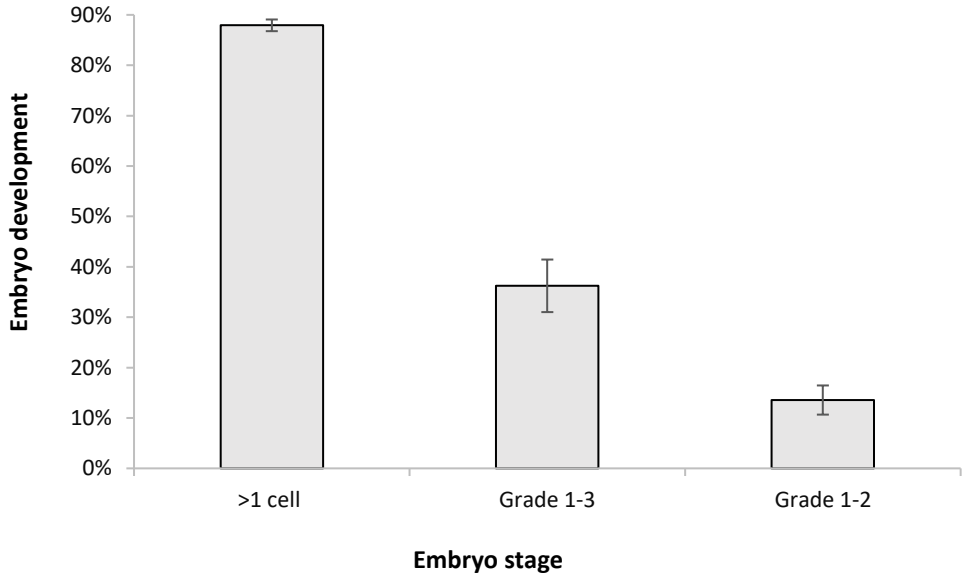


Figure 4.1: IVP development. IVP was used to develop blastocysts through *in vitro* fertilisation. The proportion of fertilised cells that underwent cleavage by day 5 is referred to as >1 cell. Embryo grade was determined on day 8. Grade 1-3 illustrates all development to the blastocyst stage and grade 1-2 includes high grade blastocysts. Replicates *n*=7, oocytes in IVC *N*=944, error bars = SEM.

4.1.2 **Plate centrifugation post blastocyst plating**

Blastocyst plating consisted of transferring a blastocyst from culture medium to a well in a 96-well plate containing growth medium. Centrifugation of the plate at 200 x g for 3 min directly after blastocyst plating, was assessed for effects on outgrowth efficiency against a no-centrifuge control. Outgrowth efficiency was determined by the proportion of blastocysts that generated an outgrowth at the time of visualisation. This was performed on *N*=34 blastocysts equally divided between the two treatments. On day 2 the outgrowth efficiency was similar for the centrifuge group compared to the control group (18% and 24%, respectively; Figure 4.2.1). There was no difference in outgrowth efficiency between the centrifuge group and the control group on day 6 (100% and 100%, respectively; *P*=1.0). It was also noted that blastocysts from the centrifuge group attached at the edge of the well, whereas the control group attached randomly, commonly in the centre of the well (Figure 4.3).

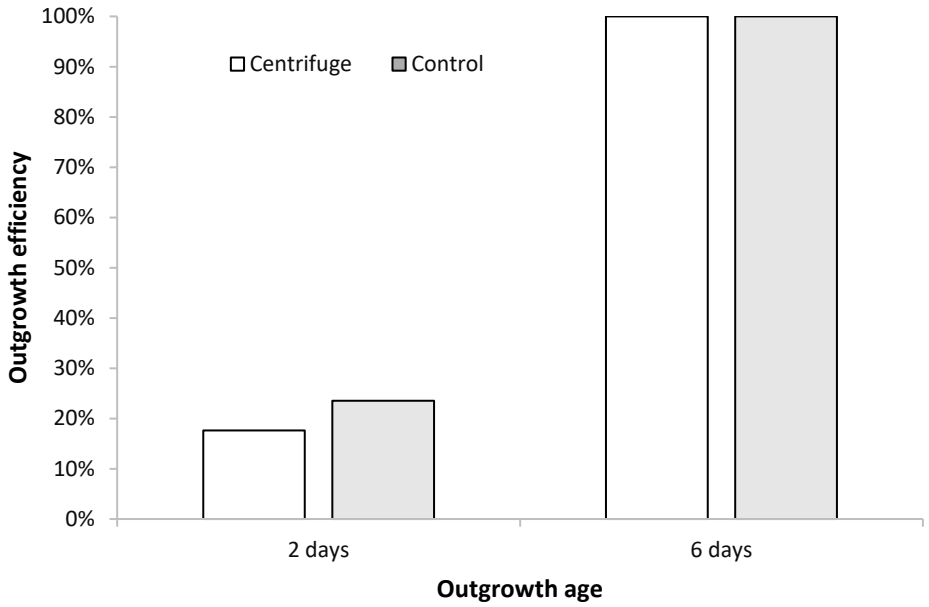


Figure 4.2: Effect of centrifugation after blastocyst plating on outgrowth efficiency. Centrifuge plates were spun at 200 g for 3 min after the blastocysts were plating. Outgrowth age refers to number of days since blastocyst plating. Outgrowth efficiency is calculated by the proportion of blastocysts plated into wells that form an outgrowth. Blastocysts were assessed for outgrowths on day 2 and day 6. Total blastocysts *N*=34, replicates *n*=1.

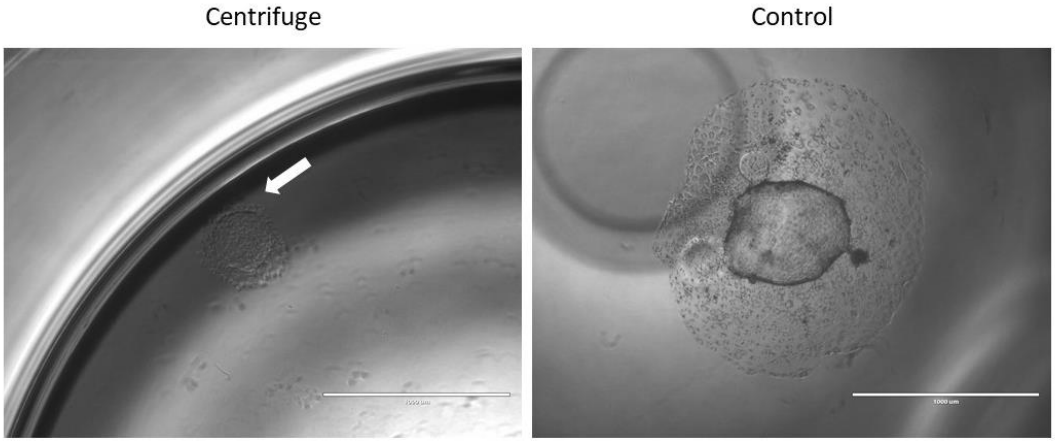


Figure 4.3: Outgrowth positioning from centrifuge and control group. Arrow demonstrates attached embryo-derived outgrowth, growing next to edge of the well. Centrifuge image taken one day post plating. Control image taken three days post plating. Scale bar = 1000 μm. Images taken under phase contrast on EVOS microscope.

4.1.3 **Gelatin coating 96-well plate**

The difference in outgrowth efficiency between blastocysts plated on wells containing a gelatin coating or no gelatin coating was evaluated. Before blastocyst plating, the bottom of the gelatin group wells were coated in a layer of gelatin. On day 3, the gelatin coated group (replicates n=1, total blastocysts N=16) were compared to the no-gelatin control (replicates n=1, total blastocysts N=16) and were shown to have a higher proportion of blastocysts that formed outgrowths (56% and 0%, respectively; P= 0.0008; Figure 4.4). On day 8, the proportion of blastocysts derived outgrowths was greater in the gelatin group (replicates n=2, total blastocysts N=26) compared to the control (replicates n=2, total blastocysts N=26), (50% and 15%, respectively; P=0.017). It was also noted that the outgrowths from the control group had a significantly smaller area on average 1.5 mm^2 as opposed to 9.4 mm^2 (P=0.002) in the gelatin group (Figure 4.5).

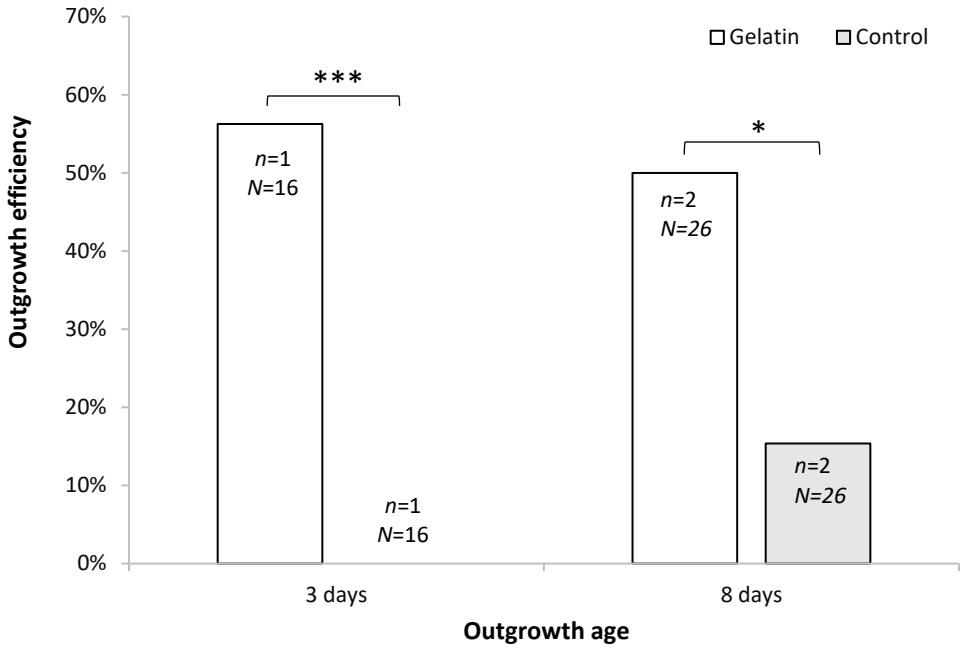


Figure 4.4: Influence of gelatin coating on blastocyst outgrowth efficiency. Gelatin coating was performed at least an h before blastocyst plating. Significance * = $P < 0.05$, *** = $P < 0.001$. *N* value on graph refers to blastocysts observed, *n* on graph refers to replicates.

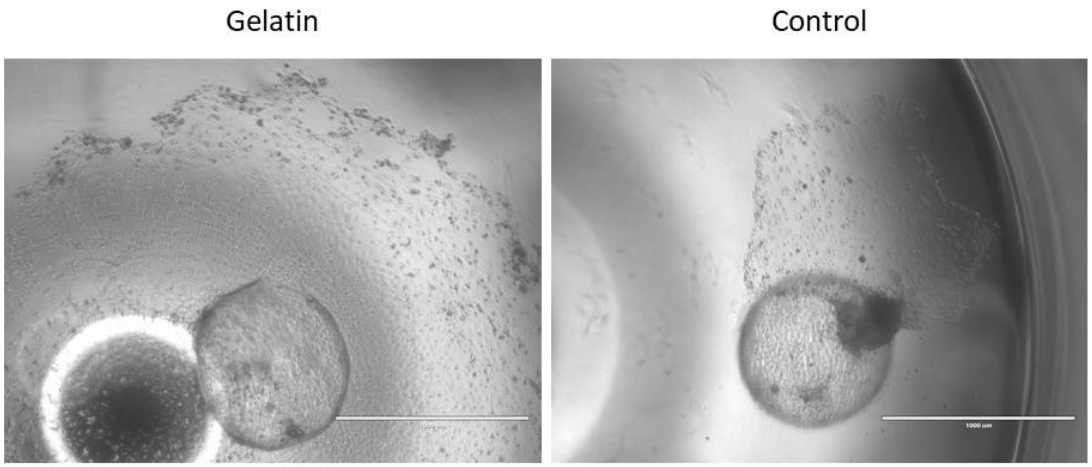


Figure 4.5: Outgrowth size comparison between gelatin treatment and control. Outgrowths measured *N*=8. Scale bar = 1000 μm. Images taken six days after plating, on EVOS microscope with phase contrast. *P*-value calculated using an unpaired t-test.

4.1.4 **Media volume**

Media was added to wells before blastocyst plating at 50 µl or 100 µl to determine any impact media volume may have on blastocysts' ability to form outgrowths. There was no notable difference to outgrowth efficiency between 50 µl (replicates *n*=2, total blastocysts *N*=20) and 100 µl (replicates *n*=2, total blastocysts *N*=20) on day 2 (65% and 60%, respectively; *P*=1; Figure 4.6). Day 3 results similarly showed no significant difference between 50 µl and 100 µl (80% and 75%, respectively; *P*=1). Despite the initial similarity in outgrowth efficiency shown here, it was found that the increased rate of evaporation in the 50 µl wells led to greater cell necrosis in the outgrowths, and eventually death of the entire outgrowth.

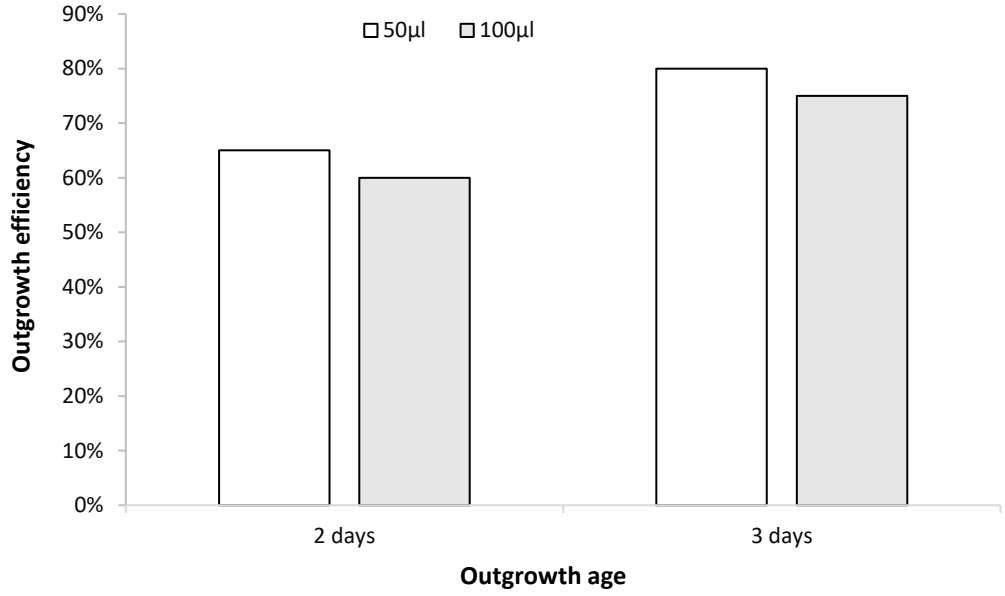


Figure 4.6: Impact of media volume on initial outgrowth efficiency. Blastocyst plating was trialled in 50 and 100 µl and outgrowths were checked for on day 2 and 3. Total blastocysts *N*=40, replicates *n*=2.

4.1.5 **Optimisation of media components**

To identify optimal media conditions for blastocysts, five media were trialled. The media differed by their bases (DMEM/F-12 or pEPSC) and/or by their serum type and concentration, FBS or knockout serum replacement (KSR). The blastocysts were left to grow in the various media for 3-4 days and then assessed for outgrowth presence. DMEM/F-12 bases consisted solely of DMEM/F-12 nutrient mixture while pEPSC base included DMEM/F-12 with N2 and B27 supplement media, Penicillin-Streptomycin antibiotics, Non-Essential Amino Acids, and beta-mercaptoethanol antioxidant. Compared with pEPSC 10%FBS (replicates n=3, total blastocysts N=40), pEPSC 0.3%FBS (replicates n=1, total blastocysts N=10) had a lower rate of producing outgrowths (85% and 20%, respectively; P=0.0004; Figure 4.7). The pEPSC 0.3%FBS also had a lower rate of producing outgrowths than the pEPSC 3%FBS (83%, P=0.001; replicates n=2, total blastocysts N=30). Examining serum types revealed that pEPSC 10%FBS did not give significantly different results than pEPSC 10%KSR (89%, P=0.98; replicates n=2, total blastocysts N=19). The effect of the bases (DMEM/F-12 and pEPSC) could be distinguished by directly comparing the DMEM 10%FBS and pEPSC 10%FBS treatments. There was no significant difference in initial outgrowth efficiency between the bases (100% and 85%, respectively; P= 0.48).

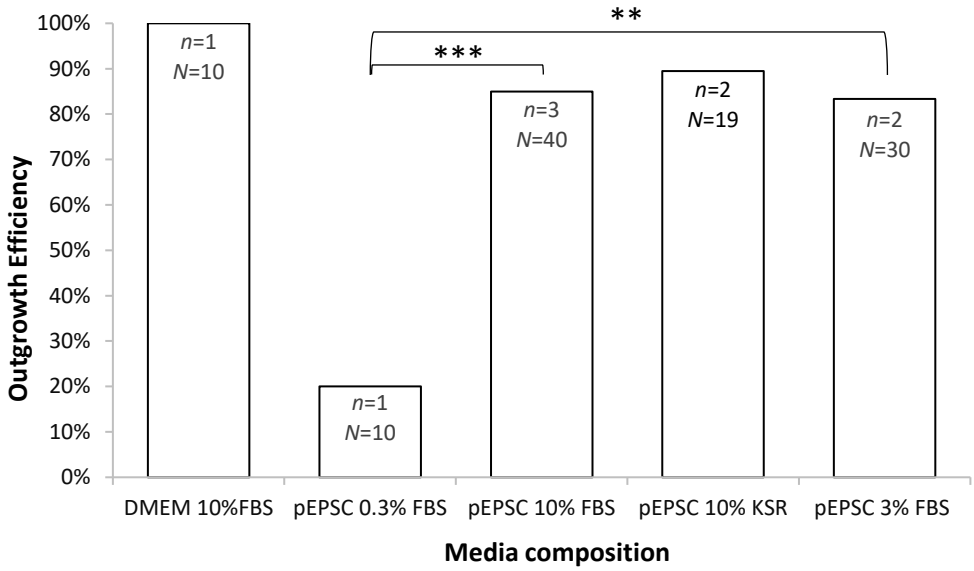


Figure 4.7: Effect of media base, serum type and concentration on blastocyst outgrowth efficiency. Blastocysts were plated into wells containing one of five media variations to assess their impact on plated blastocysts which was analysed on days 3-4. Significance ** = $P < 0.01$, *** = $P < 0.001$. Total blastocysts N=109. N value on graph refers to blastocysts observed, n value on graph refers to replicates.

4.1.6 Investigating importance of cytokines and small molecules

To further establish that the small molecules and cytokines chosen for media are imperative to the growth of embryo-derived cells, the complete media was assessed against a minimal media with small molecules and cytokines removed. Minimal media consisted of pEPSC base media with CHIR, PD0325901 (PD), and IL-6 while complete media also had pEPSC base media with CHIR and IL-6, in addition to WH-4-023, XAV, Ascorbic acid, Activin A, ROCK inhibitor and KSR. Safety and efficacy of the cytokine activin A in the media was also investigated through a comparison of two brands (Sigma and Prospec). On day 3, the outgrowth efficiency of the Sigma activin group (60%; replicates *n*=2, total blastocysts *N*=12) was not significantly higher than the Prospec activin group (50%, *P*=1.0; replicates *n*=1, total blastocysts *N*=10; Figure 4.8). Blastocysts in the minimal media were unable to produce any outgrowths (0%, replicates *n*=2, total blastocysts *N*=11) and this was significantly less than both the Complete Sigma and Complete Prospec media (*P*=0.011 and *P*=0.033, respectively). These results were reflected in the day 5 measurements.

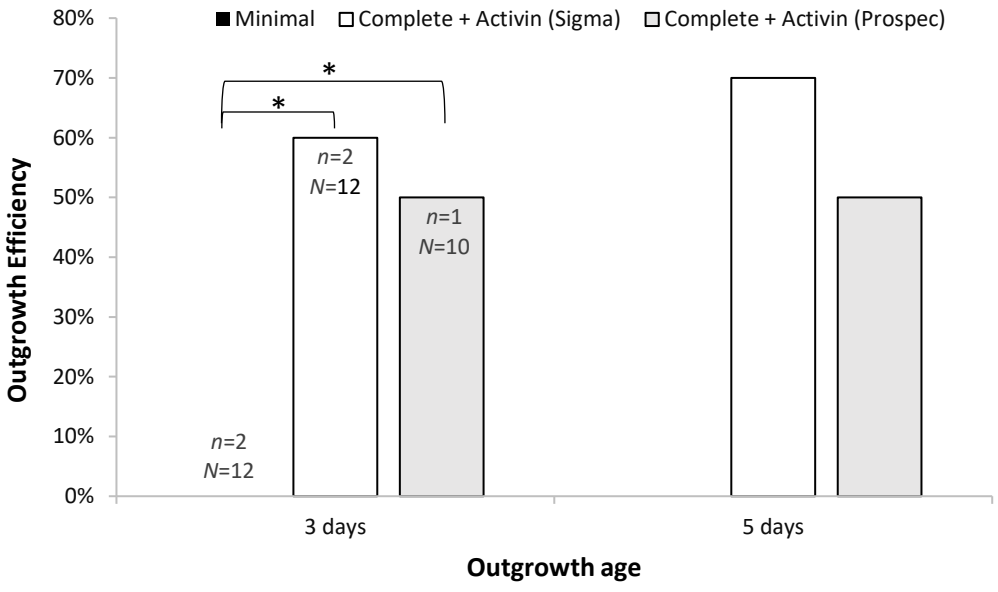


Figure 4.8: Proportion of blastocysts that formed outgrowths in media conditions with cytokine and small molecule variability. Blastocysts were plated into wells containing one of three media conditions and assessed for outgrowths on day 3 followed by day 5. Significance * = $P < 0.05$. Total blastocysts *N*=34. *N* value on graph refers to blastocysts observed, *n* value on graph refers to replicates, all *N/n* values are constant to day 5.

4.2 Objective 2: Characterisation of embryo-derived outgrowths

4.2.1 Elucidation of proliferation markers by immunofluorescence

4.2.1.1 EdU

An EdU assay was conducted on embryo-derived outgrowths to reveal the proportion of cells in S phase over a 30-min and 24-h period. After the allotted time in EdU, the Click-iT™ reagent was added to stain the cells, which were then visualised (Figure 4.9). Negative controls were used in all immunofluorescent staining, to display background fluorescence. Negative controls contained no primary fluorescent component (EdU or primary antibody) and received the secondary fluorescent component (Click-iT® reaction cocktail or secondary antibody) to illustrate the level of fluorescence with no incorporation of the primary. Total nuclei counted N=2536. The 30-min EdU group (replicates n=3, total blastocysts N=7) averaged 49 ± 5% of cells positive for the EdU stain (Figure 4.10). The proportion of cells positive for the Edu stain in the 24-h group (replicates n=3, total blastocysts N=4) was higher than this, averaging 88 ± 6%.

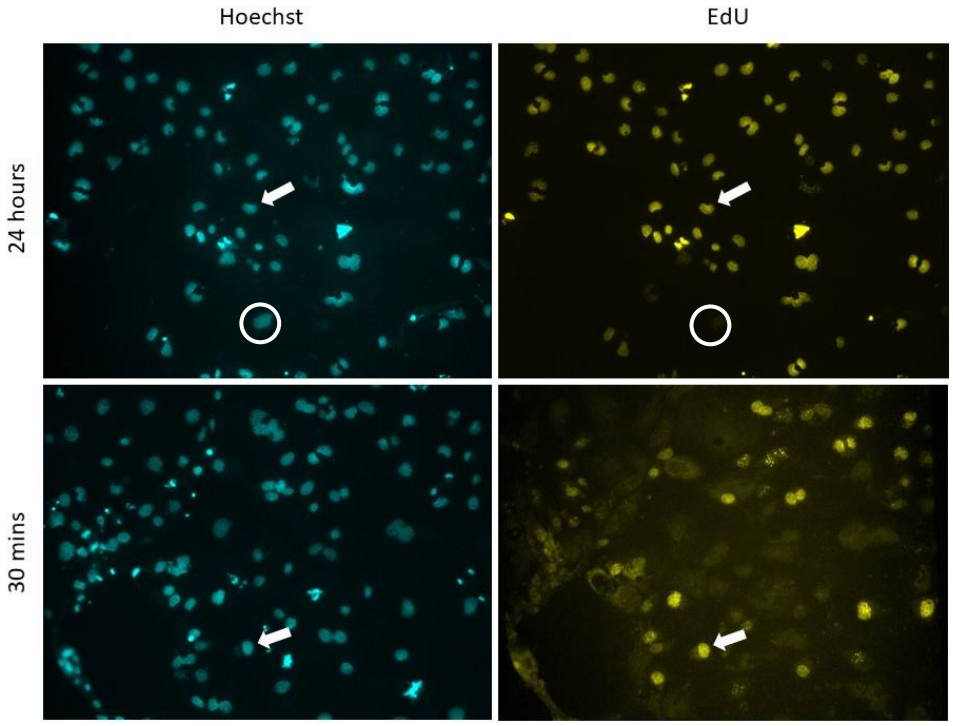


Figure 4.9: Embryo-derived outgrowth with EdU staining. Outgrowths were left in media containing Edu for 30 min or 24 h. Nuclei positive for EdU incorporation are demonstrated with bright yellow fluorescence, (illustrated by arrow in figure). Nuclei negative for EdU illustrated with a surrounding ring in figure. Images taken on Leica microscope at 20x magnification, Hoechst stain visualised with UV light at 361 nm and EdU at 488 nm.

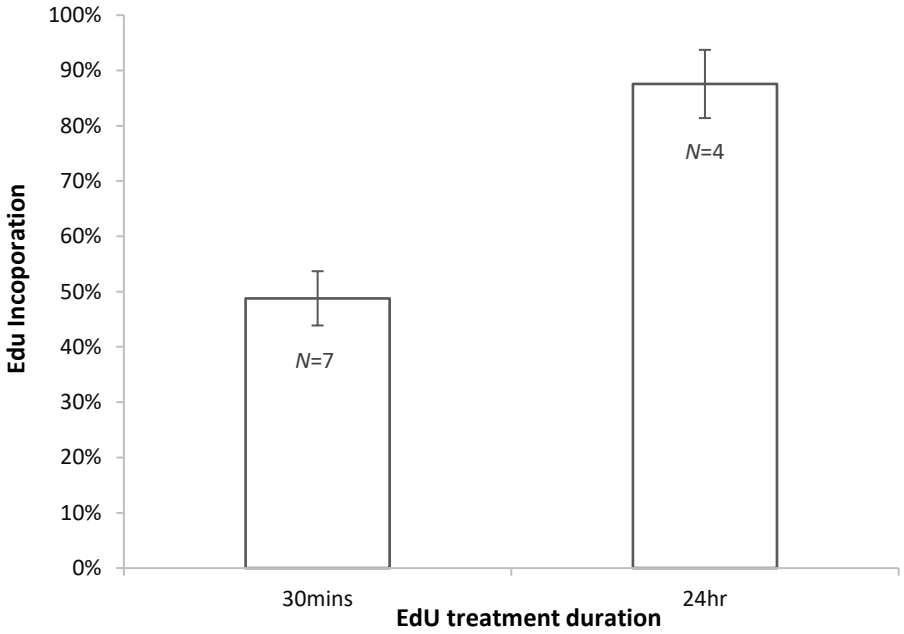


Figure 4.10: Proportion of cells positive for EdU after varying stain lengths. EdU was added to embryo-derived outgrowths for varying lengths of time, followed directly by staining. *N* value on graph refers to outgrowths counted. Replicates *n*=3, error bars = SEM, total nuclei counted *N*=2536.

4.2.1.2 Ki-67

Immunofluorescent staining was employed to detect Ki-67 in the embryo-derived outgrowths. This was chosen to predict the proportion of cells proliferating, as Ki-67 is a proliferation marker and is found during active cell cycle phases. Staining is done after the outgrowth has been fixed and permeabilised (Figure 4.11). Negative controls contained only secondary antibodies without primaries. Total nuclei counted *N*=1526. Total blastocysts counted *N*=9, replicates *n*=3. The counts showed that the proportion of nuclei positive for Ki-67 was 77 ± 3%.

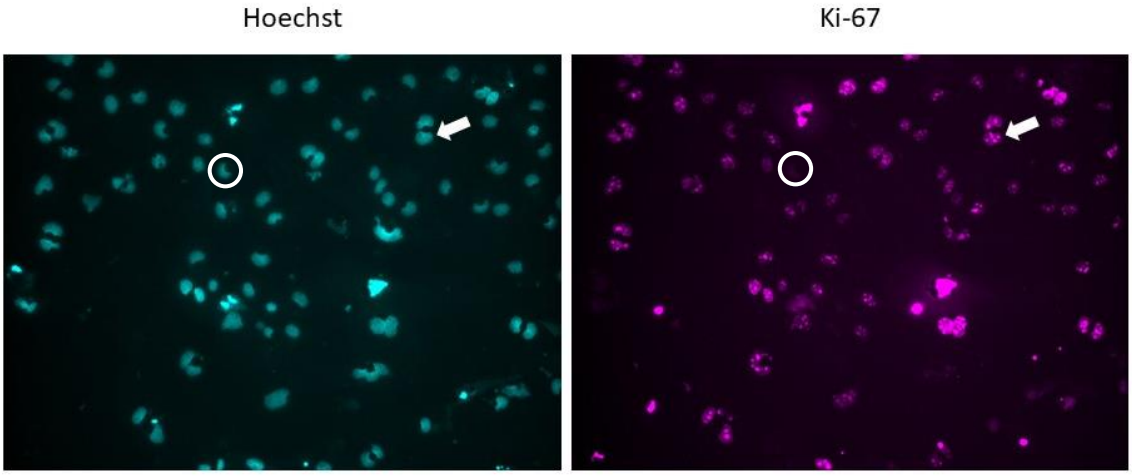


Figure 4.11: Ki-67 staining on embryo-derived outgrowth. Outgrowths were first fixed and permeabilised, followed by a primary antibody against Ki-67. Total nuclei counted *N*=1526. Nuclei positive for Ki-67 are shown with bright magenta fluorescence, (illustrated by arrow in figure). Nuclei negative for Ki-67 are illustrated with a surrounding ring. Images taken on Leica microscope at 20x magnification. Hoechst stain visualised with UV light at 361 nm and Ki-67 at 568 nm.

4.2.1.3 Mitotic cells

Mitotic cell count was accomplished on fixed outgrowths, imaged after staining with Hoechst dye to fluoresce DNA. Hoechst staining was done in combination with other fluorescent dyes. Mitotic cells were distinguished by bright compacted lines of fluorescence that occur due to condensed chromosomes that line up along the metaphase plate during mitosis. Total nuclei counted *N*=1076. Total blastocysts counted *N*=5, replicates *n*=3. Mitotic counts revealed that 5.7% of cells were in mitosis at the time of fixation.

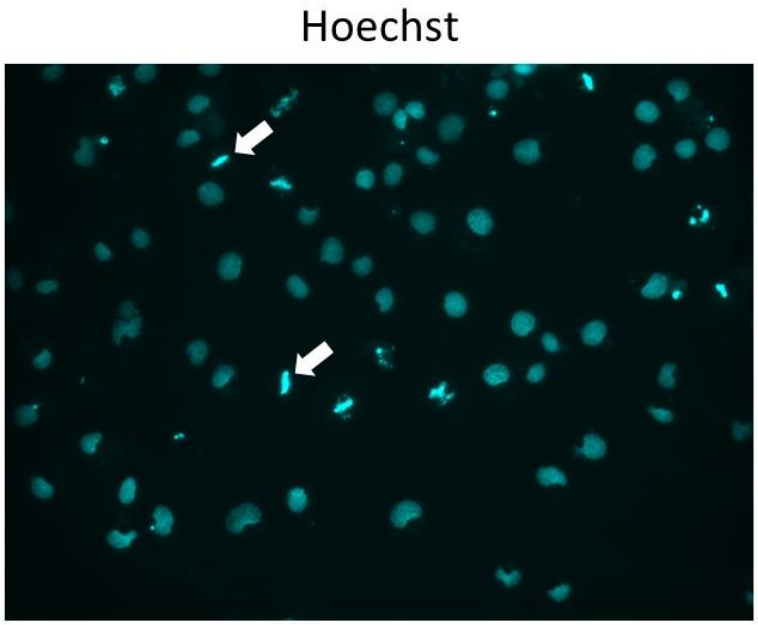


Figure 4.12: Hoechst staining to elucidate cells in mitosis. Outgrowths were first fixed and permeabilised, followed by staining with Hoechst dye. Total nuclei counted *N*=1076. Nuclei in mitosis have bright cyan fluorescence and are condensed in a line (examples illustrated by arrows in figure). Images taken on Leica microscope at 20x magnification. Hoechst stain visualised with UV light at 361 nm.

4.2.2 Characterisation of cell type markers by immunofluorescence

4.2.2.1 OCT4

Immunofluorescent staining was used to elucidate cells positive for the OCT4 transcription factor. OCT4 is a marker of epiblast cells and is mostly absent in trophoblast and hypoblast cells. Fixed outgrowths were left in OCT4 primary antibody for ≈ 24 h before secondary antibody staining (Figure 4.12). Fixing and staining of the 4-day and 6-day old outgrowths were done at the same time to remove procedural variability. Total nuclei counted N=1107. The 4-day-old group (replicates n=3, outgrowths N=4) were counted at an average of 195 nuclei per outgrowth (Figure 4.13). The 6-day-old group (replicates n=3, outgrowths N=3) were counted at an average of 108 nuclei per outgrowth. The 4-day old group had a significantly higher proportion of cells positive for OCT4 than the 6-day-old group (48 ± 7%, 5 ± 3%, respectively; $P<0.0 \times 10^{6}$).

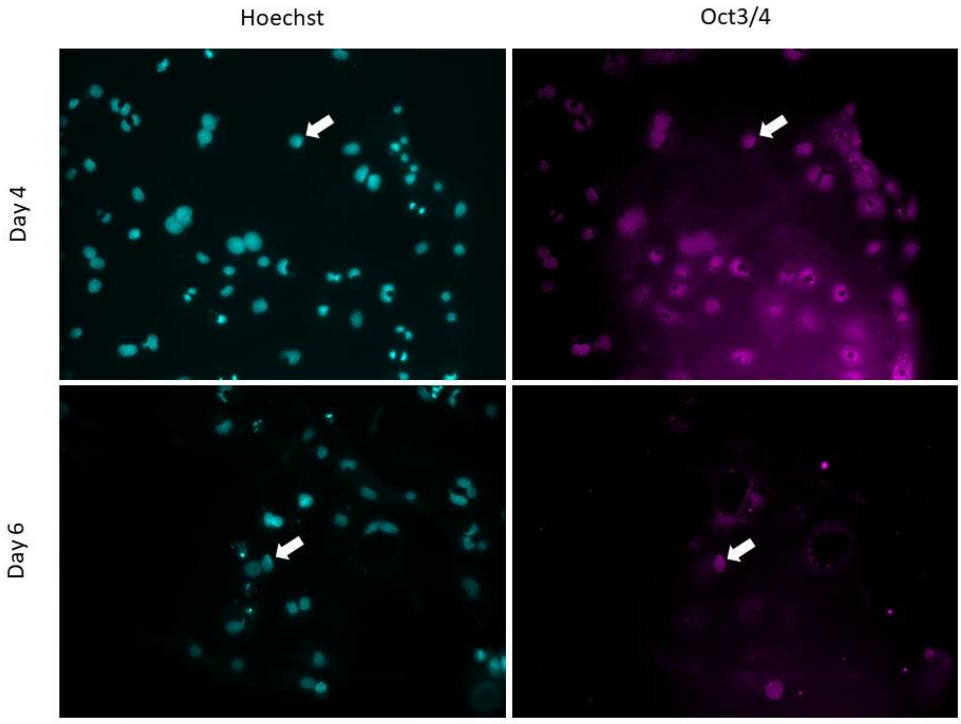


Figure 4.13: Embryo-derived outgrowths with OCT4 staining. Outgrowths were fixed and permeabilised followed by media containing OCT4 primary antibody for 24 h. Nuclei positive for OCT4 are demonstrated by magenta fluorescence, (emphasised by arrows in figure). Images taken on Leica microscope at 20x magnification, Hoechst stain visualised with UV light at 361 nm and OCT4 at 568 nm.

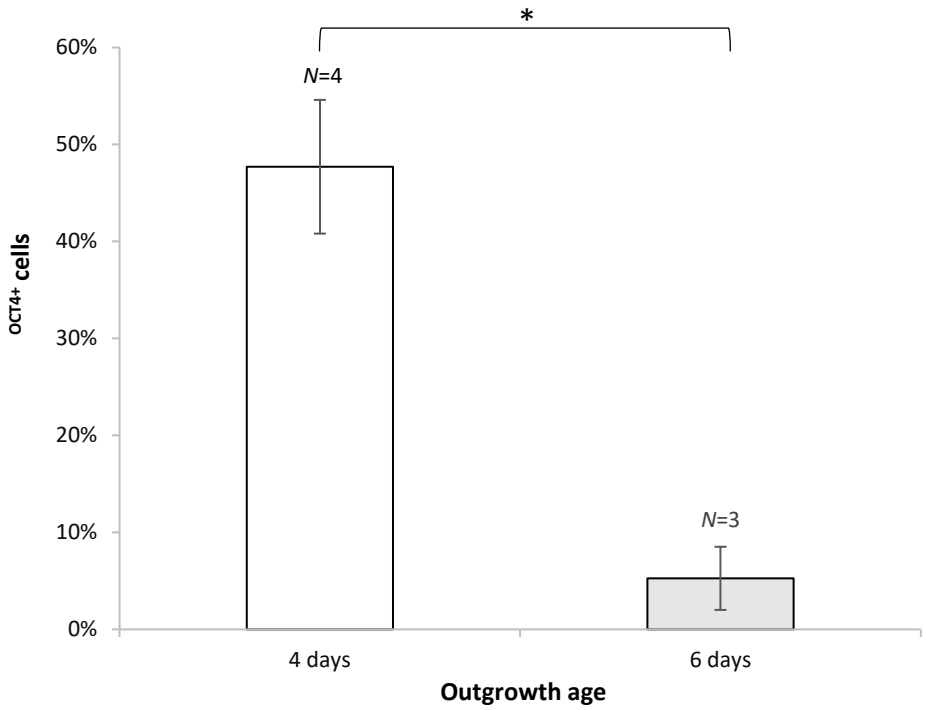


Figure 4.14: Proportion of cells positive for OCT4 at different outgrowth ages. OCT4 primary antibody was added to fixed, permeabilised outgrowths and then stained. Significance * = $P < 0.05$. *N* value on graph refers to outgrowths counted. Replicates *n*=3, error bars = SEM, total nuclei counted *N*=1107.

4.2.2.2 Gata6

Immunofluorescent staining was completed on embryo-derived outgrowths against the Gata6 transcription factor. Gata6 is a marker for hypoblast cells. Gata6 stain was used on 8 separate outgrowths N=8, and each count was replicated 3 times n=3. These stainings returned completely negative results with no nuclei stained positively for Gata6 (Figure 4.14). A positive control with blastocysts was used to ensure the negative result was not due to procedural error or the products used and yielded positive nuclei.

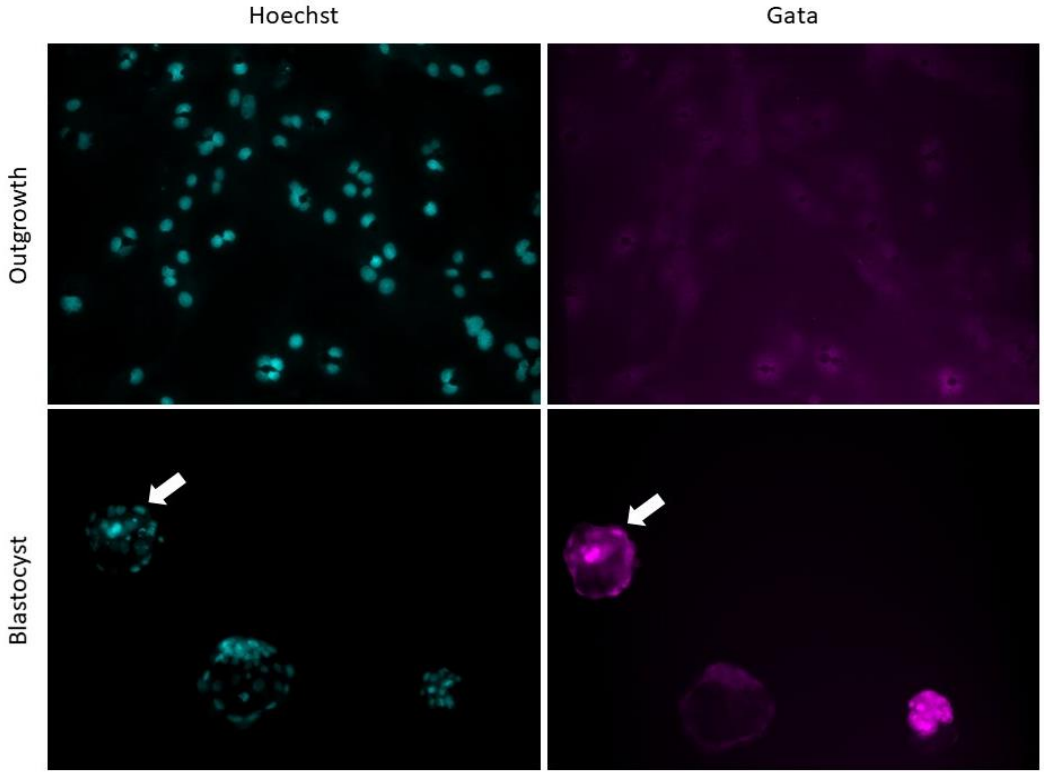


Figure 4.15: Gata6 staining on embryo-derived outgrowth and blastocysts. Outgrowths and blastocysts were first fixed and permeabilised, followed by a primary antibody against Gata6 for 24 h. Nuclei positive for Gata6 are shown with bright magenta fluorescence (example shown with arrow). No positive nuclei were visualised for the outgrowth stain. Images taken on Leica microscope at 20x magnification, Hoechst stain visualised with UV light at 361 nm and Gata6 at 568 nm.

4.2.3 **Outgrowth phenotype characterisation**

4.2.3.1 Outgrowth sizing over time

Outgrowth areas were calculated to better understand the growth patterns of the blastocysts plated. Measurements were taken on all blastocysts that had successfully formed outgrowths each day and average size calculated. For group 2 (total blastocysts *N*=13) the most rapid growth occurred between day 3 and day 5 where there was a 3.1 mm^2 increase in outgrowth area (Figure 4.15). This period was also the most rapid growth for group 4 (total blastocysts *N*=14) which showed a 6.9 mm^2 increase in outgrowth area. Both group 2 and group 1 (total blastocysts *N*=6) had decreases in average size from day 5 to 6 due to cell death in the outgrowths (2.5 mm^2 and 0.9 mm^2, respectively).

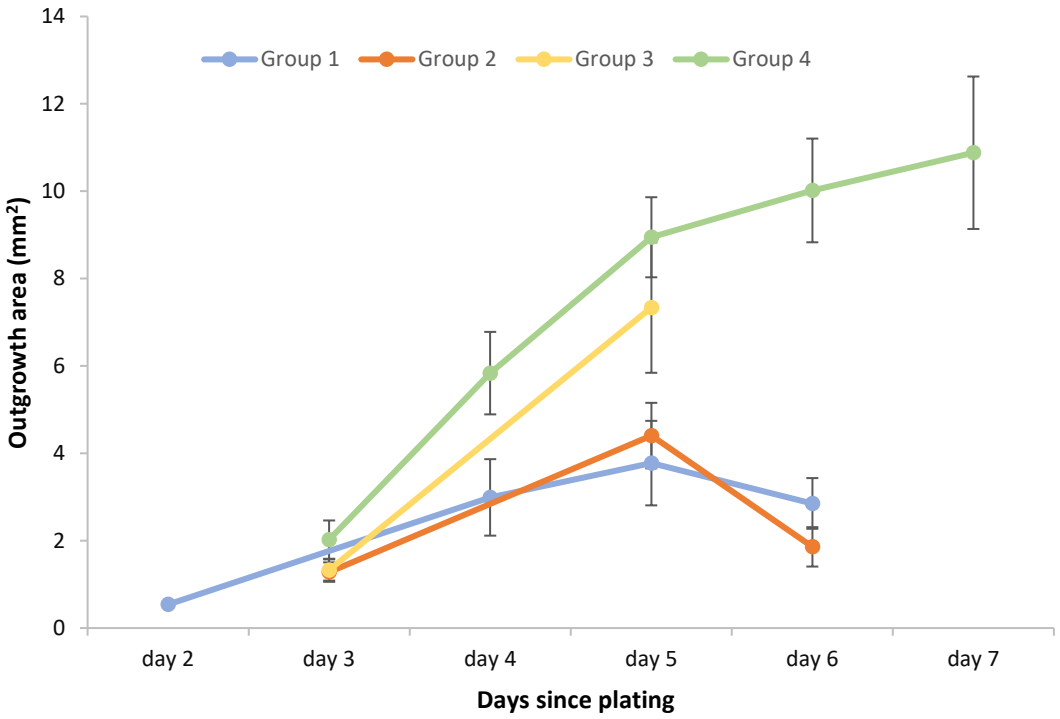


Figure 4.16: Changes to outgrowth area over time. Blastocysts were plated under standard conditions and outgrowths were imaged with EVOS microscope and measured. Total outgrowths measured *N*=42. Error bars = SEM.

4.2.3.2 Hoechst staining of large donors

Hoechst staining on large donor cells was completed to further evaluate the reconstruct death after fusion. Staining was done after fusion, on large, dissociated cells not used as donors. Hoechst staining revealed that some of these large cells contained multiple nuclei (Figure 4.18).

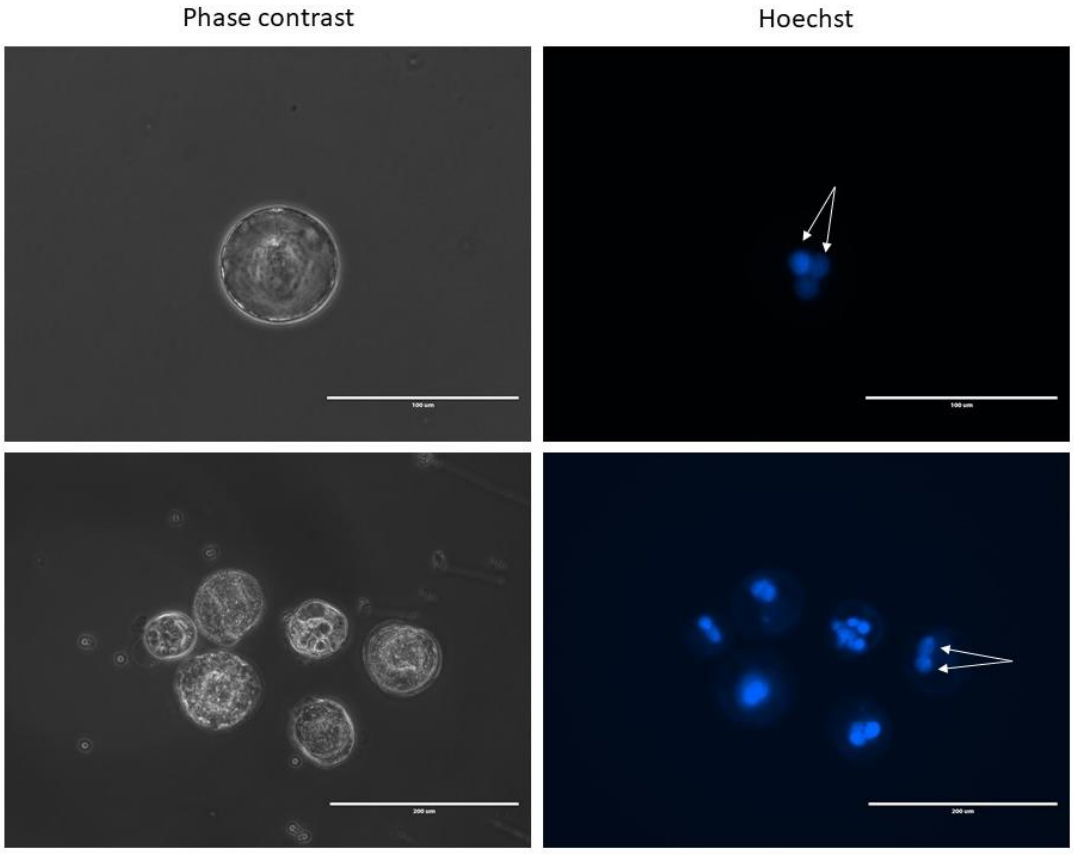


Figure 4.17: Hoechst staining of large donor cells. Large cells, not selected for fusion were stained with Hoechst dye and visualised using phase-contrast on EVOS microscope and UV at 361 nm to visualise Hoechst. Multiple nuclei within a single cell indicated with arrows. Scale bar = 100 μm for top images and 200 μm for bottom images.

4.2.3.3 Cell sizing

Outgrowth cell size was investigated against age of outgrowth to elucidate whether the outgrowth age could impact the average cell size and range. Cells from four outgrowths were sampled with an average of 16 cells measured per outgrowth. The average cell diameter of the 6-day old treatment (13 ± 2; cells measured *N*=10) was found to be significantly smaller than the average cell size of the 26-day old group (35 ± 19, *P*=0.0006; cells measured *N*=27) and the 39-day old treatments average cell size (28 ± 11, *P*=0.04; cells measured *N*=10; Figure 4.19). The average sizes for all other treatments were not significantly different from each other. The older treatments (26 and 39 days) also showed much more variability in their sizes than the two younger treatments (6 and 7 days).

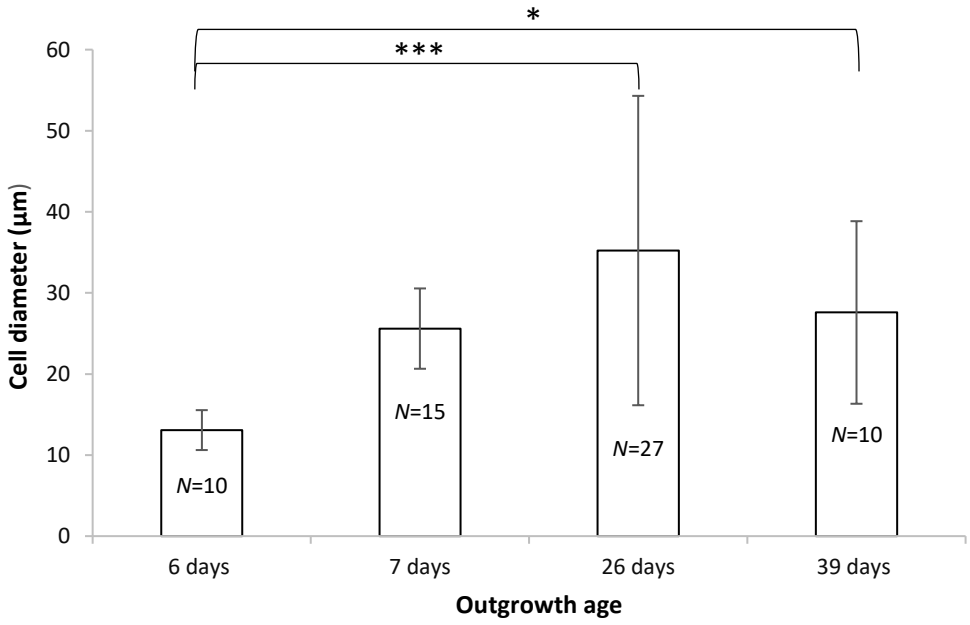


Figure 4.18: Effect of outgrowth age on cell diameter. Outgrowths were dissociated to single cells at varying ages. Cells were subsequently imaged on EVOS microscope and measured. Significance * = $P < 0.05$, *** = $P < 0.001$. Total cells measured *N*=62. *P*-values calculated using one-way ANOVA and Tukey's HSD post hoc test. Error bars = SD.

4.3 Objective 3: Optimisation of ECT conditions

4.3.1 Cytoplast age

Aged and young cytoplasts were compared to examine whether using aged cytoplasts during embryonic derived cell transfer (ECT) has a positive impact on blastocyst development. Cytoplasts from the aged group were left in embryo hold in a 15°C water bath overnight (9.5 h) to age them while limiting further maturation. PGs were also used as a positive control for activation and ESOF/LSOF conditions. The young group (replicates *n*=7, total reconstructs *N*=243) was fused with donor cells ≈ 22 h post IVM, while the aged group (replicates *n*=4, total reconstructs *N*=100) underwent fusion ≈ 44 h post IVM (Figure 4.16). The proportion of reconstructs that cleaved into two or more cells was greater in the young group than the aged group (63 ± 5% and 53 ± 5%, respectively; *P*=0.097), but not significantly. Development was significantly higher with young cytoplasts than aged for grade 1-3 embryos (11 ± 3% and 3 ± 3%, respectively; *P*=0.02) but the difference was not significant for grade 1-2 embryos (2 ± 1% and 0 ± 0%, respectively, *P*=0.5), between young and aged.

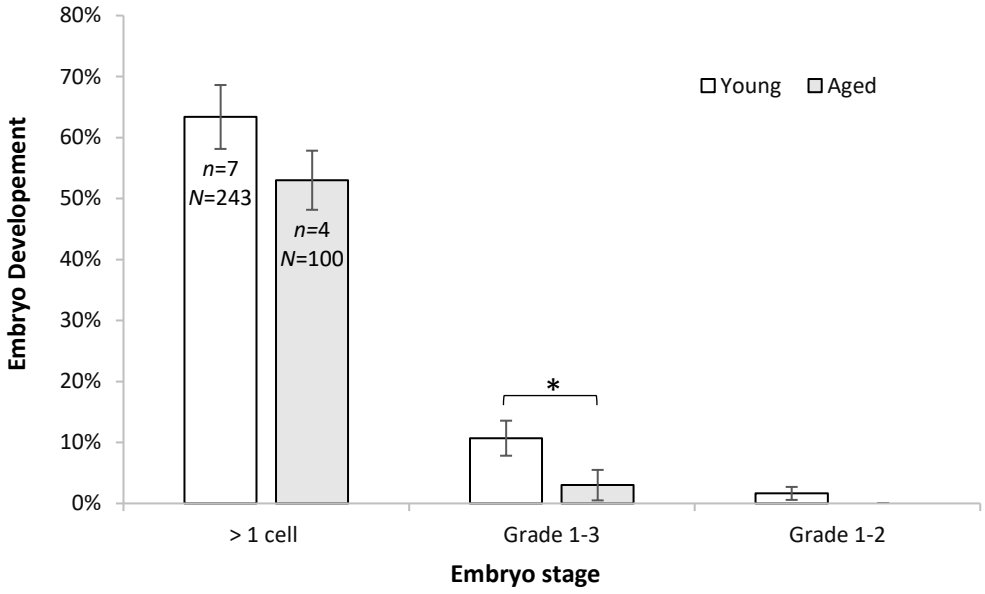


Figure 4.19: ECT development with aged or young cytoplasts. Cleavage was determined on day 5 and embryo grade on day 8. Significance * = $P < 0.05$. *N* on graph refers to reconstructs into IVC per group, *n* on graph refers to replicates, total reconstructs in IVC *N*=343, error bars = SEM.

4.3.2 **Donor cell size**

Donor cells used for ECT display phenotypic variance, including size. To gain a better understanding on whether this impacts the development of embryos, large donor cells were evaluated against small donor cells during ECT. The large group (replicates *n*=3, total reconstructs *N*=20) and small group (replicates *n*=3, total reconstructs *N*=65), were distinguished by relative size during each ECT run as opposed to absolute size, as the time taken to measure could compromise the donors, reducing the success of the ECT experiment. Cleavage was lower in the large group than the small group (55 ± 19% and 74 ± 9%, respectively), but this was not found to be significant. There was no distinction in grade 1-3 embryos between large and small (5 ± 3% and 6 ± 2%, respectively), or in grade 1-2 embryos (0 ± 0% and 2 ± 1%, respectively; Figure 4.17). It was noted, but not quantified, that there was substantial lysis in the large group between fusion and activation, hence the low number of reconstructs into IVC.

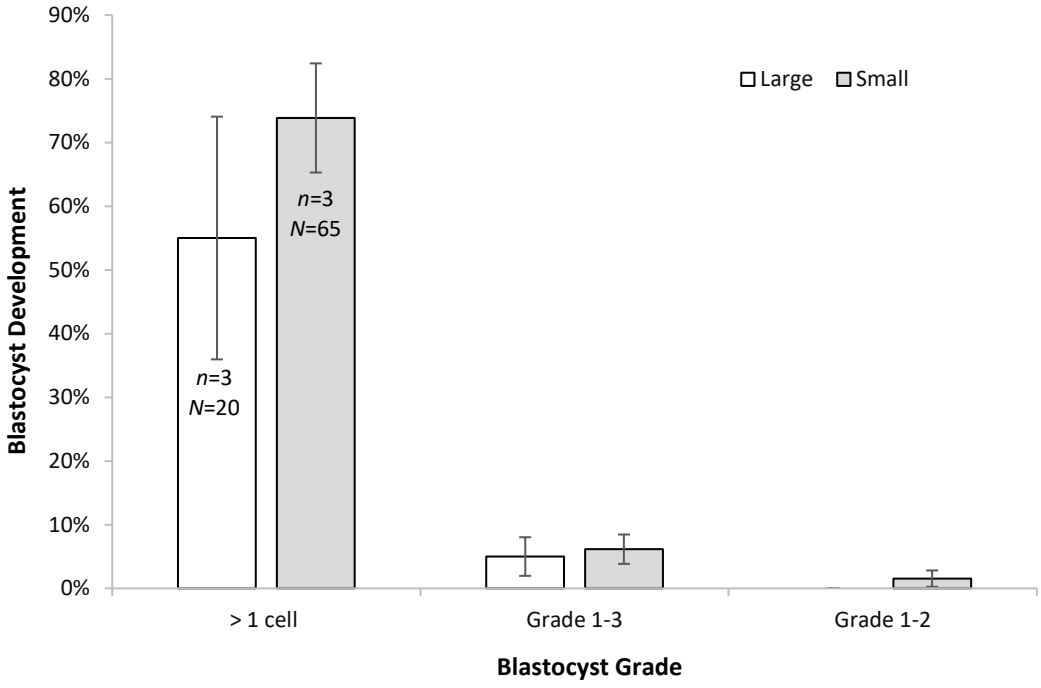


Figure 4.20: Effect of donor cell size on ECT development. Cleavage was determined on day 5 and embryo grade on day 8. *N* on graph refers to reconstructs into IVC per group, *n* on graph refers to replicates, total reconstructs in IVC *N*=85, error bars = SEM.

Chapter 5
Discussion

The primary aim of this study was to improve the efficiency of embryo-derived cell transfer cloning by focusing on protocols surrounding generation, maintenance, and selection of embryo-derived donor cells. Increased cloning efficiency would broaden the application of this technique for rapid genetic gain in livestock populations. Cell transfer cloning enables a controlled introduction of relevant alleles related to thermotolerance into a population, promoting livestock welfare in response to rising temperatures. The overall aim of the project was broken down into three main research objectives. The first objective was to form a reliable methodology for generating pluripotent embryo-derived outgrowths. This was accomplished with comparative tests and assessment against newly published literature surrounding the topic. The second objective involved characterisation of the cultured outgrowths by implementing immunofluorescence and sizing to gain a better understanding of the cell types present and their activity. The third objective was to assess and optimise cell transfer cloning, using embryo-derived cells as donors, which was executed with comparative cell transfer experiments.

Plating trials with various treatments and controls allowed for the establishment of components essential to the generation of outgrowths by day-8-late-stage blastocysts. Embryo-derived outgrowths were maintained in culture for up to 39 days and through 4 passaging events. Despite this, outgrowths were prone to dying between the 7-10-day mark while following the final protocol. When reliable methodologies were ascertained, the cells were stained for a variety of markers. Staining with Ki-67 illustrated that on average 77% of the outgrowth cells were proliferating which has implications for cell transfer cloning. Further investigation with EdU showed 49% of cells were in S-phase at the time of ECT, a phase incompatible with the enucleated cytoplasts. Immunofluorescence for the OCT4 protein showed a substantial portion of the cells were positive for the pluripotency marker OCT4. This is the first step in demonstrating cells are in a pluripotent state, but further tests are required before claiming pluripotency. Implementing the embryo-derived cells as donors during cell transfer cloning was successful, and late-stage blastocysts were generated. Comparison of donor cell sizes during cell transfer showed no significant effect on

developmental rates of reconstructs in IVC. However, the large group had notable reconstruct lysis between fusion and IVC which was not recorded in the data set. Further investigation illuminated many of the larger donor cells from the experiment contained multiple nuclei.

5.1 Objective 1: Optimising embryo-derived outgrowth conditions

Testing outgrowth conditions required the production of late-stage blastocysts. Blastocysts were developed using IVF. Cleavage rate of the fertilised oocytes was 88% and blastocyst development was 36% which is within the normal range for IVF based on previous literature [117]. Bovine outgrowths with stability and long-term pluripotency were first reported only three years prior to this research project, in 2018 [54]. Thus, the technique is a work in progress, and conditions between research groups still vary [52]. Establishing competent methodologies for the generation of embryo-derived outgrowths is essential for the amplification of high-quality genotypes through cloning This includes blastocysts gene edited for alleles such as *slick* and mutated *PMEL*. Protocols should also encourage pluripotency in the outgrowth cells, as reducing epigenetic reprogramming required is the main driving factor for using embryo-derived cells rather than somatic cells in cloning [95].

In this study, outgrowths were generated using the whole blastocyst, compared to just the ICM, both of which are utilised in numerous studies [54; 118]. Isolating the ICM is commonly completed with immunosurgery which removes trophoblast cells with anti-bovine antibodies, leaving the ICM intact. Plating and culturing of the isolated ICM allows for amplification of only the desired pluripotent epiblasts adjacent to hypoblast and competition with trophoblast does not occur. However, immunosurgery is a time-consuming process which would be amplified by the number of embryo-derived outgrowth experiments completed in this study. Immunosurgery also provides a small risk of damaging the ICM through the immunological reaction to break down trophoblasts and is increased by any defect or tear in the trophectoderm [119]. Research on bEPSCs demonstrated ESC lines were generated with similar efficiency between immunosurgery and whole blastocyst treatments which indicated immunosurgery is not required for optimal outcomes of outgrowth derivation [54]. Another study found cells in ICM outgrowth cultures exhibited trophoblast

characteristics including a cystic structure and lipid vesicles in the cytoplasm [120]. Together, these findings suggest successfully generating pluripotent outgrowths relies more on the conditions of culture than isolation of the ICM.

Composing and improving the process of generating pluripotent embryo-derived outgrowths concluded with a methodology that created outgrowths from 82% of blastocysts plated on average (data not shown). This efficiency is higher on average than what was reported in a 2013 study based on finite ICM-derived outgrowths that saw 59-85% outgrowth efficiency [121]. Understanding of small molecules and cytokines that are essential for culture medium has since been improved and many were not utilised in this study, thus, the efficiencies cannot be directly compared. Literature in the last three years followed protocols similar to this thesis, however, the focus was on deriving stable cell lines and so initial outgrowth efficiency was not reported, and therefore, comparisons cannot be made. One such paper reported 52-100% efficiency in deriving stable cell lines which indicates this is the minimum possible outgrowth efficiency for the study [54]. Interestingly, the lowest cell line efficiency of 52% was seen when the embryo source was from IVM-produced embryos which are also used in this research project. This finding could indicate that the embryo source employed here is a limiting factor in increasing outgrowth efficiency. Establishing cell lines was not required for this research project, as the aim was rapid genetic multiplication and utilising the cells as donors for ECT. If embryos with valuable genetic editing were utilised, establishing a cell line where pluripotency and survival remain stable over many passaging events would be imperative.

5.1.1 Plate centrifugation post blastocyst plating

It was considered that centrifugation of the 96-well plate after plating the blastocysts might increase adherence of the blastocyst to the well, aiding the outgrowth formation process. The control group received no centrifugation and achieved 100% outgrowth efficiency, suggesting centrifugation is unnecessary for outgrowth formation. Bovine embryo-derived outgrowths have been established in studies without centrifugation and so centrifuge use was removed from the protocol without replication of the experiment [61]. Spinning from the centrifuge also pushed the blastocysts to the edge of the well, where they attached and formed outgrowths. This placement was not ideal as growth was restricted in the direction of the well edge.

5.1.2 Gelatin coating 96-well plate

Gelatin is formed from the hydrolysis of native collagen, and crosslinking of the molecules can lead to 3D structures that aid in adhesion of cells to the substrate [122]. When the effect of gelatin was investigated, blastocysts plated in wells with no gelatin had 0% outgrowth efficiency on day 3 which was significantly lower than the 56% observed in gelatin-coated wells. The gelatin-free wells also displayed a smaller increase in outgrowth size, which is detrimental if number of cells is reduced. Substrate is crucial for initial cell adhesion in addition to survival ability and preserving cell phenotype, which meant this result was expected [123]. Gelatin was added in all subsequent plating. Other substrates such as Matrigel™, fibronectin, and laminin have been used with varying levels of success [124]. Matrigel™ is a membrane extract and contains factors that have a positive effect on growth and maintaining pluripotency, however, Matrigel™ is chemically undefined, causing increased variation between experiments [125]. The need for consistency in this study meant that despite the stated upsides, gelatin was utilised over other options. Many studies also utilise feeder cells in addition to substrate [52]. Feeder cells form a layer on the substrate and provide growth factors and extracellular matrix secretions to the outgrowth, but do not proliferate. Feeder cells can aid in attachment and support long-term proliferation, however, the use of feeder cells reduces reproducibility as the nature of feeders are undefined [126]. Feeders pose the risk of introducing xenogeneic contamination such as viruses which can interfere with cells in the embryo-derived blastocyst and ultimately the ECT process [127]. During donor cell preparation for ECT, feeder cells may also be lifted, requiring extra measures to ensure feeder cells are not selected as donors. For these reasons, the use of feeder cells was not fitting for this research project.

5.1.3 Media volume

The bottom of a well in 96-well plates can sufficiently be covered with 50 µl of medium. Blastocysts were initially plated into wells containing 50 µl of growth media and to ensure this was sufficient, an evaluation was conducted comparing 50 µl with 100 µl of growth media. On day 2, 65% of the blastocysts from the 50 µl treatment and 60% from the 100 µl treatment had successfully generated outgrowths. By day 3 the proportion had increased to 80% and 75% respectively. The lack of notable difference

between the treatments indicates both 50 and 100 µl of medium are sufficient to support the formation of an outgrowth. However, wells with only 50 µl experienced accelerated evaporation due to an increased surface area to volume ratio which lead to outgrowths dying 3-5 days after plating. Cell metabolic activity dictates that as the cells grow and divide, metabolic by-products acidify the media pH, harming the cells if the change is extreme [128]. Acidification would occur more rapidly in lower medium volumes due to concentration of metabolic byproducts, requiring more frequent media changes. Rapid acidification and increased relative evaporation were, therefore, the main drivers behind applying 100 µl to all ensuing experiments. Further action to limit evaporation included adding 100 µl of PBS to all edge wells in the plate and using central wells for blastocyst plating.

5.1.4 Optimisation of media components

There are several crucial major components to a growth medium including base media, small molecules, cytokines, and serum. Literature on successful bEPSC culture is limited as bovine embryo-derived pluripotent outgrowths have only been maintained in the last few years, and studies are still divided on many of the components, but this is a useful benchmark to investigate culture conditions further [54; 129]. Blastocysts were plated in two alternate base mediums with a range of serum types and concentrations. Both bases allowed for ample outgrowth efficiency with 100% of blastocyst forming outgrowths in DMEM/F-12 10%FBS and 85% in pEPSC 10%FBS. It was rationalised that the additional components in the pEPSC base may be advantageous for further replication in the outgrowths, hence, this base was used going forward. Examples include Penicillin/Streptomycin to avoid contamination in the wells and beta-mercaptoethanol to reduce the number of toxic oxygen radicals from building up in the media [130]. Another base used in successful bEPSC derivation is mTeSR1^{TM} which is designed for human ESCs and contains basal medium and growth factors such as recombinant human basic fibroblast growth factor intended to replicate *in vivo* conditions [52]. In comparison pEPSC which includes DMEM/F-12 with N2/B27 supplement used in this research is also a basal medium that utilised growth factors such as progesterone often found in tumours in addition to BSA for cell nutrition. Both bases are serum-free and recommend addition of FBS or KSR. DMEM was also employed successfully as a base medium to culture bEPSCs [61]. DMEM typically has high glucose but is lacking several amino acids provided in DMEM/F-12

such as L-Alanine and L-Aspartic acid. DMEM/F-12 is a mixture of DMEM and Ham F-12 so also contains Linoleic Acid, Sodium Pyruvate, and Thymidine not found in DMEM. Literature has not conclusively shown an advantage of any of these bases over the other and so DMEM/F-12 in the pEPSC base was appropriate for this research.

FBS is extracted from bovine fetuses and is the liquid separated from coagulating blood. It contains prominent levels of growth factors, amino acids, and sugars which promote rapid cell growth and proliferation. In contrast, KSR is a serum-free formula based on FBS and is thought to cause less cell differentiation than FBS, making it appropriate for use in bEPSC culture [131]. By examining the serum type and concentration, it was revealed that 0.3%FBS was insufficient in assisting blastocysts to survive and generate outgrowths. Blastocysts in the 0.3%FBS treatment had a 20% rate of producing outgrowths which was significantly lower than both 3%FBS and 10%FBS treatments. The serum FBS was also compared against KSR. With each at 10% concentration, FBS resulted in an 85% outgrowth efficiency compared to 89% for KSR, which was not significantly different. Ultimately KSR was selected as the desired serum choice due to its chemically defined nature, removing experiment to experiment variation which can occur in FBS due to its biological origins which also lead to moral ambiguity [132]. Research has also found that KSR can improve the generation and maintenance of pluripotent cells in long-term outgrowth culture, reinforcing this decision [131].

5.1.5 Investigating importance of cytokines and small molecules

Small molecules and cytokines are added to growth media to regulate cellular signalling and pathways, which can discourage differentiation, holding a pluripotent state in the cells. Minimal medium was compared against a complete growth medium Minimal media was based on 2i media and has had success in generating stable outgrowths and maintaining pluripotency in some species [133]. 2i includes two inhibitors CHIR and PD. CHIR was also utilised in the complete media as it is shown to be essential to maintaining pluripotency due to its role in promoting the Wnt/β-catenin pathway through the inhibition of GSK3 [63]. PD is important for mouse pluripotency through its inhibitory effect on Mitogen-activated protein kinase which blocks a signalling pathway involved in cell cycling, inhibiting activity of the

fibroblast growth factor [134]. However, in bovine embryo-derived outgrowths, some studies saw an increased differentiation into non-pluripotent trophoblast lineages in presence of PD and this contrast may be caused by differences in cellular determination into trophoblasts in bovine and murine species [61; 135; 136]. PD is not used by any of the groups successful in generating bEPSCs which corroborates the importance of the absence of this small molecule for bovine outgrowth culture [52; 54; 61].

The minimal media does not include many small molecules or cytokines, and recent studies on pluripotent bovine cultures contain a wide range of them involved in cell differentiation pathways including WH-4-023, XAV939, and Activin A [52]. To ensure these additions were not superfluous, the complete growth media was trialled against minimal media. The complete media did not include PD found in the minimal media but contained XAV, Activin A, ROCK inhibitor, and WH-4-023 not found in the minimal media as well as the antioxidant Ascorbic acid to reduce free radicles [137]. The addition of XAV to outgrowth medium has been a breakthrough in maintaining pluripotency in bovine ESCs. XAV was not typically included in bovine outgrowth media due to reports on murine and human naïve state pluripotency of ESCs requiring Wnt proteins which would be blocked by XAV [138]. However, a naïve state has not been demonstrated in bEPSCs bringing its relevancy into question, and a combination of XAV and CHIR has since been shown to maintain pluripotency and is implemented in recent studies [52]. Activin A targets activin receptors, leading to phosphorylation of SMAD2 which can then translocate to the nucleus and regulate expression of genes related to cell differentiation. Necessity for Activin A in maintaining pluripotency has been demonstrated in human ESCs and the cytokine is used to produce bEPSCs [61; 139].

Blastocysts in minimal media produced no outgrowths by day 3 or day 5 which is significantly less than both the complete media trialled against it. This result is a strong indication that the additional small molecules and cytokines used in the complete growth media are essential in maintaining healthy embryo-derived cells and supporting the generation of outgrowths in culture, however, which of the additional molecules is essential could not be elucidated from such a comparison.

In addition to this, tests were run on two brands of Activin A to establish their safety and efficacy. No significant difference was demonstrated between Sigma and Prospec Activin A, with 60% and 50% outgrowth efficiency on day 3, respectively. A difference was not expected; however, commercial Activin A is produced *in vivo* which can lead to differences in bioactivity which may be exacerbated by products from separate suppliers.

Many additional adjustments were made to the methodologies surrounding the complete growth media that were not displayed in the results. One such change was increasing media changes from once every 4-5 days to every 2-3 days to ensure factors required for growth were always present in the medium as well as removing metabolic bioproducts. Recent bEPSC literature reported media changes of 2-3 days or every day, indicating the original 4–5-day media changes were not sufficient [52; 54]. Increased media changes increase the risk of cells/outgrowths lifting from the substrate. During the first 5 days after plating, half media changes, where only the top 50 µl of media were pipetted off carefully and 50 µl of fresh media added. This avoided detachment of blastocysts or new outgrowths when adherence was not strong. Activin A and Beta-mercaptoethanol were replenished in the media with increased frequency following the understanding of their instability in solution. LIF was utilised in the original media recipe and was removed for redundancy adjacent to IL-6 as they are from the same cytokine family and act on the same signalling pathways [65].

Recent papers have reported the establishment of cell lines proliferating through 70 passages while outgrowths in this research paper did not observe proliferation past four passages and often died around day 7 [54]. Because the recent literature successful in generating bEPSCs are not methodological papers, many technical methods such as removal of zona pellucida, plating techniques, and passaging techniques are not completely described [52; 54; 61]. Disparity in the technical methods between this research project and recent literature may account for some of the limits of success in long-term culture in this research project.

5.2 Objective 2: Characterisation of embryo-derived outgrowths

When a satisfactory methodology for producing embryo-derived outgrowths was obtained, further information about the cells in culture was gathered. This was crucial to elucidate the effect of the implemented culture conditions on the cells, in addition to checking their compatibility with the cell transfer cloning process. Immunofluorescence was employed to demonstrate cell proliferation and pluripotency through specific cell markers. Rate of size increase in outgrowths was also calculated to gain an indication of when amplification rates were highest and when passaging or cell transfer would be most appropriate.

5.2.1 Elucidation of proliferation markers by immunofluorescence

When EdU is added to growth media, it is incorporated into DNA as a thymidine analog during DNA synthesis which allows for the identification of cells in S-phase [140]. Reactions with fluorescent azides in the Click-iT® reagent cause nuclei containing EdU to fluoresce. A 30-min EdU labelling indicates the proportion of cells in S-phase during this time interval, which is highly relevant for cell transfer experiments. Labelling for 24 h gives an estimation of the total proportion of cells in the outgrowth proliferating. The 24-h label revealed 88% of cells were positive for EdU, and therefore, are proliferating. This observation indicates that the other 12% of cells are in the inactive G_0 stage of the cell cycle for minimum of a 24-h period or entered G_1 from G_0 but did not reach S-phase. Cells in $G1/G_0$ are compatible with the MII-arrested cytoplasts used in cell transfer cloning and generate embryos with the correct ploidy, making them ideal candidates for donor cells. Completion of a 30-min EdU label registered 49% of cells positive for EdU, illustrating they are in S-phase of the cell cycle. S-phase donors are incompatible with cytoplasts in MII, as the partially replicated DNA typically leads to DNA fragmentation within the reconstruct or inability to replicate DNA completely as chromosomes condense incorrectly [141]. Such a high proportion of donors in S-phase will have a substantial negative impact on *in vitro* cloning efficiency. A commonly used solution is chemically arresting the cells in mitosis (M-phase) using a drug such as nocodazole [142]. However, the application of DMAP in this methodology meant M-phase arrest was not appropriate

in these experiments as the second polar body is not extruded, inducing incorrect ploidy. Reconstructs with double the DNA content that do not extrude a secondary polar body can form blastocysts with tetraploidy but these will not typically generate live animals [143]. S-phase cells are also larger than those in the ideal G_0/G_1 and so selecting smaller donors may improve chances of selecting a donor in G_0/G_1 rather than S-phase [144].

The presence of the protein Ki-67 is an indication of cell proliferation. Ki-67 is found in all active stages of the cell cycle (G_1, S, G_2, and M) and absent in the non-proliferative, quiescent G_0 stage. The expression of Ki-67 increases as the cell progresses through the active cell cycle stages with mRNA levels peaking during G_2, and protein levels maximised in M-phase [145]. Ki-67 staining is completed on fixed cells and therefore demonstrates the proportion of cells proliferating at the time of fixation. Approximately 77% of cells in the outgrowths were positive for Ki-67. This was higher than the 49% positive in the 30-min EdU, which is expected as EdU only captures the S-phase of the cell cycle. The frequency of positive cells was higher in 24-h EdU than Ki-67 (88% and 77%, respectively) which can be explained by the proportion of cells that exited G_0 during the 24-h period and reached S-phase as G_1 lasts for approximately 9-10 hours in somatic cells and is reduced in pluripotent cells [146; 147].The difference between the 30-min EdU and Ki-67 experiments indicates that around 28% of total cells are in G_1, G_2, and M-phase and 49% in S-phase. G_2 is extremely short and G_1 is reduced in pluripotent embryonic cells which could indicate a high proportion of mitotic cells in the outgrowth culture [54]. Mitotic counts showed 5.7% of cells were in mitosis at once, which is within the range for bovine embryonic cells seen in past studies [148; 149]. The remaining 23% of cells are estimated to be in G_0 at the time of fixation which is ideal for cell transfer cloning along with G_1.

5.2.2 Characterisation of cell type markers by immunofluorescence

OCT4 protein is one of the three main cell pluripotency markers, along with SOX2 and NANOG proteins. *OCT4* is expressed in totipotent blastomeres and remains in the inner cell mass in epiblasts while being downregulated in cells differentiated to trophoblast or primitive endoderm (hypoblast) cells. *SOX2* is regulated alongside *OCT4* by many of the same regulators including Oct-Sox enhancers which self-

promote the genes [69]. The characterisation of these genes is the standard protocol for demonstrating pluripotency molecularly. Immunofluorescence can also be implemented to stain for cell type markers such as Gata6 protein which is involved in cell fate determination and primarily found in hypoblast cells [72].

Antibody staining against the OCT4 protein was completed in multiple outgrowths of varying ages. Outgrowths that were 4 days old demonstrated an average of 48% of cells positive for OCT4 and in 6-day old outgrowths a 5% proportion of positive cells was observed. The difference illustrated could be a sign that pluripotency is being drastically reduced during this period. Outgrowths of both ages were stained in the same plate at the same time, thus, variance in proportion of positive cells between outgrowth ages was not a result of procedural errors or variability. Increased cloning efficiency in pluripotent embryonic cells dictates that using younger outgrowths could be beneficial as rates of pluripotency are higher, however, the trade-off being smaller cell numbers in the outgrowth at this stage. If culture conditions were improved to maintain pluripotency for extended periods, this trade-off would not be required. SOX2 stains were also completed, and no positive cells were visualised (data not shown). This data was not analysed in the results, as a positive control with blastocysts is essential to ensure results are not due to technical failures, such as defective antibody stocks, and completion of this control was not possible. Recent studies on the generation of bEPSCs have shown OCT4, SOX2, and NANOG in outgrowths with immunofluorescence [52; 54].

Outgrowths were investigated for the presence of hypoblast cells by antibody staining for Gata6 proteins. A total of 8 outgrowths were stained from a variety of plating experiments and in all outgrowths, 0% of cells were positive for Gata6. This suggests culture conditions are not encouraging differentiation of pluripotent cells to hypoblasts or supporting the growth and proliferation of the original hypoblast cells from the plated blastocysts. Such a result could indicate pluripotency is being maintained in the epiblast cells plated. Alternatively, it could indicate hypoblasts have already undergone further differentiation into endoderm cells.

5.2.3 Outgrowth phenotype characterisation

It was important to analyse the rate of proliferation and expansion of the outgrowth to understand patterns of growth. Knowing when outgrowths undergo the most expansion

and when this is reduced can help to pinpoint ideal times for outgrowth passaging. Visualising growth rates also helps optimise amplification of high-quality target genes before use in cell transfer cloning.

Expansion was characterised by measuring the diameter of the outgrowths and calculating the area. The greatest increase in growth across the groups was visualised between day 3 and 5 and all groups experienced growth at this time. Between day 5 and 6, groups 1 and 2 experienced decreases in average outgrowth size due to cell death concentrated at the periphery of the outgrowth. Group 4 had reduced growth at this stage, from an increase of 3.1 mm^2 from day 4-5 to a 1.1 mm^2 increase from day 5-6. This means generally, the highest rates of amplification and growth occur before day 5. Therefore, the first round of passaging occurring between day 5 and 7 would be optimal in case reduced growth is a result of lack of room or inadequate growth factors for the number of cells. Application of outgrowths as donor cells could also be done as early as day 5 as a substantial portion of amplification has already occurred.

5.2.4 Concerns with characterisation of embryo-derived outgrowths

The pluripotency genes *OCT4* and *SOX2* are commonly co-expressed in cells as they share many of the same regulatory factors. The positive OCT4 stains adjacent to negative SOX2 stains is alarming as it would be expected that these proteins are typically present together. Double staining for OCT4 and SOX2 was not completed, but stainings were performed on similar outgrowths grown in identical culture conditions. The capability of SOX2 stain was not verified which could be the cause of this discrepancy. Current pluripotency characterisation was not sufficient to claim pluripotency of the outgrowths. Issues involved in pluripotent characterisation could be solved with further staining in addition to gene expression analysis to clarify the immunofluorescence results.

Another issue was the lighting of the images from Leica microscope which caused increased exposure at the bottom centre of the image. Irregularities may have affected quantification of immunofluorescent experiments, but this effect should have been minor as contrast between nuclei and background could still be observed. The correct adjustments to Leica microscope could eliminate this inconsistency in the future.

Finally, groups of outgrowths used in outgrowth sizing experiment were generated over a period of 2 months. Although the main culture protocol had already been decided on, micro-adjustments were made during this time such as adding activin A and beta-mercaptoethanol more frequently. The data is still valid as this was not a comparative study, but protocol changes may explain some of the differences between groups such as group 4 maintaining growth after day 5, as this was the most recent group generated.

5.2.5 Summary of characterisation of embryo-derived outgrowths

EdU staining demonstrated that around 49% of cells were in S-phase at once, a phase incompatible with MII-arrested cytoplasts, in cell transfer cloning approaches that implement DMAP. Cells in all active stages of the cell cycle were elucidated by staining for Ki-67 and 77% of cells were positive. The proportions indicate ≈ 28% of cells are in G_1 or G_2/M-phase and 23% of cells are in the inactive G_0 stage, which is compatible with cell transfer cloning along with G_1. Antibody staining against OCT4 revealed 48% of cells were positive in 4-day-old outgrowths and 5% positive in day-6 outgrowths. OCT4 positivity is an indicator of pluripotency, however, the negative SOX2 results put this into question. The OCT4 staining was validated with both positive and negative controls while the SOX2 stain did not receive a positive control, giving more weight to the OCT4 results. Further pluripotency testing is required to confirm either result. The decrease in proportion of cells positive for OCT4 between day 4 and 6 suggests pluripotency is declining rapidly during this period. Observing the rates of outgrowth size increase showed the most rapid growth occurs by day 5, thus, day 5 may be optimal for use as donor cells in cloning, as amplification of high-quality genetics has been achieved, and pluripotency may be higher than in older outgrowths.

Maintaining pluripotency is important, as the use of pluripotent ESCs as donors in cloning has been shown to improve development post-implantation and survival of the animal to adulthood compared to somatic cell donors in mice [11; 150]. This illustrates the importance of pinpointing protocols that reliably maintain pluripotency in outgrowth culture. These studies also reported a decrease in embryo development when ESCs were implemented as donors, but this may be caused by cell cycle

incompatibility and the shortened length of the compatible G_1 phase in embryonic cells [54; 150]

5.3 Objective 3: Optimisation of ECT conditions

The final objective was to illustrate the ability to successfully utilise the embryo-derived cultured cells in cell transfer cloning and optimise *in vitro* conditions to increase the cloning efficiency. Improving rates of blastocyst and live animal generation from cloning is the ultimate goal and the reason investigation with pluripotent embryo-derived cells is crucial [12]. Understanding phenotypic markers of compatible donor cells such as size could also be employed through donor selection to optimise the results of cell transfer.

5.3.1 Cytoplast age

The importance of donor cell cycle stage has been discussed and the molecular state of the cytoplast is also crucial. During the cell cycle, M-Cdk levels increase as M-cyclin forms a complex with Cdk1, resulting in M-Cdk. Abundant M-Cdk propels the cell into mitosis by provoking chromosome condensation, mitotic spindle formation, and nuclear envelope breakdown [151]. MII-arrested cytoplasts are naturally high in M-Cdk. When donor cells in S-phase are fused with the cytoplast, the M-Cdk encourages condensation of the partially replicated DNA which leads to condensed chromatin with multiple breaks along it [141]. Gaps in condensation results in the inability for replication to resume correctly and chromosome fragmentation can occur, leading to reconstructs that are not capable of generating blastocysts.

It has been shown that aging the cytoplast can decrease cellular M-Cdk levels. Decreased levels could suppress premature chromosome condensation and allow the DNA in S-phase donors to successfully complete replication before the first cleavage event [152]. Enabling the successful use of S-phase donor cells would significantly increase overall embryo development rates, illustrated by the high numbers of donors visualised in S-phase with EdU. To assess this, one group of recipient cytoplasts were aged overnight in embryo hold before fusion. Both cleavage and blastocyst generation rates were slightly higher in the young group than the aged group but not significantly so. This result could imply the aging process did not significantly reduce levels of M-Cdk. The past cytoplast aging study was completed with blastomeres rather than

embryo-derived outgrowths used in this research project [152]. The blastomeres may require less epigenetic reprogramming before completing DNA synthesis of S-phase, which may explain why cytoplast aging was sufficient in proving ample time for synthesis completion in blastomeres but not embryo-derived outgrowth donors. Pre-activation of the cytoplast may be more beneficial than aging. Pre-activation is executed by introducing the cytoplast to maturation medium supplemented with a calcium ionophore A2318 and followed by medium with cycloheximide for 5 h. Pre-activation has been shown to reduce M-Cdk and therefore increase compatibility with S-phase donors. It was considered that chemical pre-activation may prove more competent in substantially reducing M-Cdk levels than aging. One study demonstrated pre-activated cytoplasts improved the blastocyst generation rate in embryonic cell cloning [153]. Once again, the donor cells were from blastocysts rather than embryo-derived outgrowths, thus, improvement from implementing this technique is not assured.

5.3.2 Donor cell size

Donor cell size can be an indication of cell cycle stage as the cell increases in size as it moves through cell cycle from G_1 to mitosis [144]. It was thought that larger donors within an outgrowth are more likely to be in S-phase or G_2/mitosis, which are both incompatible with MII-arrested cytoplasts when DMAP is utilised due to donor ploidy and will not create live animals. However, donors in G_2/mitosis can often still generate tetraploid blastocysts [143]. One study that used bEPSCs as donors in embryo-derived cell transfer cloning, suggested that the low proportion of reconstructs that generated blastocysts may be due to the reduced number of G_1 cells in embryo-derived outgrowths [54]. Thus, it was considered that selecting for G_1 cells using a phenotypic marker such as size would increase blastocyst generation rates. An alternative method to assessing donor cell size would be the use of cycloheximide instead of DMAP during activation protocols, which would allow the extrusion of a secondary polar body to correct ploidy reconstructs with G_2/M-phase donors. Use of cycloheximide has successfully produced live bovine offspring with M-phase donors [154].

Donor cell size was distinguished between small and large as relative size between the donors used in the cloning experiment, as trying to measure cells before adhering them to a cytoplast could compromise the cells due to the length of time taken. The

proportion of reconstructs cleaved, blastocysts generated, and high-grade blastocysts generated were recorded. Cleavage in the small treatment was 74% compared to 55% in the large treatment, however, this was not found to be significant, influenced by the low number of reconstructs into IVC in the large treatment. Proportion of reconstructs in IVC that generated blastocysts was similar between the small and large groups, 6 and 5% respectively. No clear difference was observed between the two treatments as a proportion from IVC, though, if larger cells were in G_2/M-phase, these blastocysts would not be appropriate for transfer to recipient animals based on incorrect ploidy [143]. This would mean small donors would still be more appropriate even if blastocyst generation was equal between the treatments.

Another study that used mouse ESCs as donors also compared small and large donors in cell transfer. The difference between the study and this research project was the study only used a cytokinetic inhibitor such as DMAP on reconstructs from small donors in the hopes that both groups would end with correct ploidy. Using this method, the study achieved higher blastocyst generation rates in the large donor cell group as they were able to extrude a secondary polar body which was visualised [11]. Interestingly, using this technique allowed for a proportion of blastocysts developed up to 31%, which is high for embryo-derived donors. This may signal that donor cell size is a good indication of cycle stage and that conditions for different size treatments can be designed accordingly, boosting the overall rate of blastocyst generation. Further investigation in this area may prove helpful in increasing blastocyst generation from embryo-derived donors. Karyotyping blastocysts generated from a donor cell size experiment could elucidate whether there is a difference in ploidy between the large and small donor treatments, indicating cell cycle stages of the two groups.

Importantly, it was noted that many reconstructs from the large treatment lysed after fusion and were not transferred into IVC, hence the relatively low number of cloned reconstructs. This reconstruct lysis was alarming and called for further analysis. Cell lysis is the breakdown of the cell membrane, and it is possible that the introduction of such a large donor by fusion disrupted the cytoplasts membrane enough to compromise it, causing cell lysis.

Large cells from embryo-derived outgrowths that were not attached to a cytoplast were stained with Hoechst dye to visualise the DNA content and assess for any

abnormalities. Many multinucleated cells were observed within this group. Multinucleation can occur in embryo-derived cells when trophoblasts undergo amitosis where nuclear multiplication occurs without cell division, leaving the cell binucleated or even trinucleated for long periods before the cell divides [155]. Another cause of multinucleation is the fusion of multiple trophoblast cells forming a syncytiotrophoblast with multiple nuclei. Syncytiotrophoblasts are critical in implantation events of pregnancy and form a boundary for maternal exchange to the developing fetus [156-158]. Many large donor cells were observed with around 5 nuclei suggesting the formation of syncytiotrophoblasts is the reason for multinucleation rather than amitosis which tends to only increase the nuclei number by 1 or 2 [155]. One study found culturing trophoblast outgrowths in the presence of Activin A and absence of FGF increased the expression of the syncytiotrophoblast gene *Gcm1* [159]. This literature is highly relevant as the complete media used in this research project included Activin A without FGF. The study also noted the increased *Gcm1* expression began on day 2 of culture, which would impact the compatibility of these cells as donors early in culture. These findings indicate culture conditions could also be used to downregulate the expression of syncytiotrophoblast genes which may be important in the culture of whole blastocysts for use as donor cells. Large multinucleated, potential donor cells often had a blastocyst-like appearance which could be used as a key phenotypic characteristic to avoid the use of multinucleated cells, in addition to selecting smaller cells.

It was considered that multinucleation would be enhanced in older outgrowths which aligned with observations of abnormally large cells after dissociation of older outgrowths. To evaluate this, outgrowths of various ages were dissociated, and cell sizes measured. Cells from both older outgrowths (26 and 39 days old) were significantly larger on average than the youngest 6-day-old outgrowth. It was also demonstrated that the 2 older outgrowths had a greater range of cell sizes and a larger maximum size. These results align with the idea that rates of multinucleation increase over time in outgrowths, as this could account for the upper range in cell sizes which was not observed in the younger outgrowths. This rationale was further investigated by Hoechst staining the cells in the 26-day-old group which illustrated many of the largest cells were multinucleated and the majority of smaller cells stained were uninucleate (images not shown). This indicates the upper size range of cells in aged outgrowths includes many multinucleated cells which would be unsuitable as donor

cells in cloning. Multinucleation and the risk of S-phase or G_2/M-phase are important justifications for avoiding selection of the larger donor cells during cell transfer cloning. Increased multinucleation with outgrowth age could be a key argument against the use of older outgrowths for donor cells and culture conditions to reduce multinucleation should be investigated.

5.3.3 Concerns with optimisation of ECT conditions

Selection of large and small donors were distinguished by relative size within the single-cell suspension. Selection was conducted by more than one person which introduces human bias and decreases congruency. These conditions were necessary for feasibility of the cloning experiment but may affect the reliability of the comparison.

The water bath in which the cytoplasts were aged was set to 15°C, however, thermometers registered different temperatures after the water bath was used to age the cytoplasts. This could indicate the water bath was faulty and may influence the results if specific temperature is essential in the aging process.

5.3.4 Summary of optimisation of ECT conditions

Aging cytoplasts before fusion with a donor was attempted to induce compatibility with donors in S-phase, of which EdU stains illustrated a high proportion. No significant difference between aged and young cytoplasts was found, which may suggest the cytoplasmic concentrations targeted were not lowered a significant amount during aging. Large donor cells were considered to potentially be in S or G_2/M-phase which would have a lower rate of development than donors in G_0/G_1. Testing demonstrated no significant difference in blastocyst development between the two groups for reconstructs that survived to IVC, however, notable reconstruct death after fusion occurred, spurring further analysis. It was illustrated that many of the larger cells were multinucleated which may explain the reconstruct death. Having a wide range of cell sizes with a larger upper limit was linked to increased age of outgrowths, and staining demonstrated that many of the large cells in one of the older treatments were multinucleated. These results suggest avoiding larger donor cells may increase cloning efficiency, and that older outgrowths may be less suitable for cell transfer than younger outgrowths, due to increased multinucleation.

On average, cloning experiments in this research project were successful in producing blastocysts from ≈ 10% of reconstructs that went into IVC (data not shown). This is lower than early ESC mouse studies which achieved development rates of 15%, however, compact morulae were also included in the count, inflating the proportion of development [150]. In 1994, cloning with bovine cultured ICM donors generated blastocysts at a rate of 15% from reconstructs produced [118]. This was followed by a study in 2003, that utilised bovine ESCs as donor cells and saw a 4% development rate from fused reconstructs [160]. Recently bEPSCs have been implemented as donors and blastocyst development from reconstructs into culture between 10-20% was demonstrated [52; 54]. Results in this research project are within the range of this literature but on the lower end. This could be partially explained by control/treatment experiments where one condition selected was considered to be suboptimal such as donor cell size.

Chapter 6
Future Recommendations

To expand on the findings of this study, four key inquiries should be made. The first is improving the consistency of achieving around 90% outgrowth efficiency. Gelatin was employed as the substrate throughout this study to maintain consistency, but other substrates may benefit cell attachment, survival, and pluripotency to a higher degree and should be explored. Matrigel is made with a laminin and collagen base and has been reported to increase cell adherence in addition to maintaining pluripotency and aiding in cell survival compared to gelatin, which may also reduce the cell death observed [161]. Matrigel is undefined due to the animal-derived products used to compose it, so if defined media is important, laminin or fibronectin may be preferable. Laminin forms a mesh-like matrix structure as a substrate, which can aid cell attachment. Laminin has been demonstrated to assist in maintaining long-term culture in addition to encouraging pluripotency [124; 161]. Fibronectin is a glycoprotein that assists in cytoskeletal organisation and has adhesive properties. Studies have illustrated the ability of fibronectin to elevate cell adherence while encouraging proliferation and self-renewal of the cells to maintain pluripotency, which may make the addition of fibronectin to gelatin substrate helpful in maintaining healthy outgrowth cultures [162]. Comparisons between the four treatments may elucidate a resolution to the inconsistency in outgrowth formation and early cell death.

Secondly, further testing is required before pluripotency of the embryo-derived cells can be claimed. It would be important to repeat SOX2 staining with a verified positive control to properly illustrate the presence or absence of this marker. NANOG staining would be required, as NANOG is the third molecular pluripotency marker and is found solely in pluripotent epiblast cells [67]. Another widely used staining protocol not completed in this research project is alkaline phosphatase and is suggested for further research. Alkaline phosphatase levels are significantly higher in pluripotent cells and are downregulated during lineage differentiation [163]. Although alkaline phosphatase is also found in differentiated cells, the contrast in levels is sufficient for its use as a pluripotency marker. Assessment of expression of key pluripotency genes such as *OCT4*, *SOX2*, and *NANOG* can also be explored via mRNA levels [71]. This technique has been used to illustrate pluripotency of bovine ESCs and is also important to

corroborate data from immunofluorescent staining [52]. If the other pluripotency markers are demonstrated, a final step would be illustrating pluripotency functionally through *in vivo* chimera generation. This experiment involves a chimeric embryo partially composed of cells from the outgrowth in question, edited with a target gene. If an animal is generated that expresses the gene in embryonic and extraembryonic cells it indicates pluripotency, as the outgrowth cells were able to differentiate into both cell types. *In vivo* chimeras have been produced using bovine embryonic stem cells, making it a viable technique for future study [52].

It is also suggested that karyotype analysis is conducted on blastocysts produced by cell transfer cloning to highlight their ploidy. Classic karyotyping requires the whole blastocysts, as only cells in mitosis have condensed replicated chromosomes that can be successfully karyotyped, and a small proportion are in mitosis at once [149; 164]. Karyotyping could be implemented to determine polyploidy of blastocysts in a comparison between large and small donors. If the assumption that larger donors are more likely to be in S/G2/M-phase is true, karyotyping would reveal higher rates of tetraploidy in the large treatment. This finding would encourage the avoidance of selecting large donors in cloning when DMAP is being employed. A less invasive method called karyomapping requires only a biopsy of the blastocyst, as SNP analysis is implemented to calculate ploidy and can be completed in cells at any cycle stage [165]. This means the blastocyst can still be utilised if euploidy is showcased. Karyomapping of blastocysts produced from cloning can detect aneuploidy even if only a single chromosome is missing or extra. This would be important in analysis of blastocysts generated from small donors presumed to be diploid to determine if this assumption is correct, indicating that the use of these donors would increase the proportion of blastocysts generated that would be appropriate for transfer to recipient animals.

Finally, when all previous suggestions have been completed, high-quality, euploid blastocysts should be transferred into recipient animals. The rate of blastocysts that produce healthy live offspring can be recorded and overall cloning efficiency calculated. Cloning efficiency with pluripotent embryo-derived donor cells can then be compared to efficiency with somatic cell donors, revealing any improvements in the embryo-derived treatment. This is the final step in demonstrating the positive impact of implementing pluripotent embryo-derived donor cells in cloning.

Chapter 7
Conclusions

To conclude, a suitable methodology for generating outgrowths from blastocysts was established. This allowed for outgrowths to survive up to 39 days and through four passaging events, although, moderate rates of cell death around day 7 were still observed. Initial passaging expanded the number of potential donor cells for ECT; however, proliferation was reduced after the second passaging event. Immunofluorescent staining was implemented on the generated blastocysts and EdU showed ≈ 49% of cells were in S-phase over a 30-min period which is incompatible with MII-arrested cytoplasts in standard cloning protocols. Staining for OCT4 illustrated 48% of cells were positive for OCT4 in outgrowths 4 days old and this proportion decreased drastically by day 6. Cell transfer cloning using cells from the generated embryo-derived outgrowths was completed successfully and blastocyst generation observed. Compatibility of S-phase donors and MII-arrested cytoplasts was attempted with aging cytoplasts, but no positive effect was observed. Larger donor cells were found to cause lysis in reconstructs, although, surviving reconstructs produced a similar proportion of blastocysts as small donor cells. Hoechst staining of large donor cells revealed multinucleation was common. The largest cells were found in older outgrowths which is likely due to multinucleation and illustrates younger outgrowths may be more suitable for use as donor cells which is endorsed by the increased OCT4 positive cells found in younger outgrowths.

A recent review corroborated IVP data to illustrate that a blastocyst generated from IVP had ≈ 30% chance of generating a live calf [166]. In comparison, following the technique of this research paper, the IVP blastocyst could be used to generate an outgrowth with 82% efficiency. These outgrowths consisted of up to 1000 cells that could be used as donors (data not shown). When using the embryo-derived outgrowths as donor cells, 10% of reconstructs formed blastocysts. Past literature has shown blastocysts generated from cloning with embryonic cells can be transferred to recipient animals and generate live calves at 10% efficiency [152]. This indicates that following the procedure in this thesis, if all donors were used to create reconstructs, on average, 8.2 calves could be generated from the starting material of one IVP blastocyst, compared to 0.3 calves on average from direct implantation of the IVP blastocyst.

However, ECT is a laborious technique, and it is more common that 100 donors are utilised in one experiment which would generate an average of one live calf. This demonstrates the importance of improving the efficiency of blastocyst generation from ECT as this would greatly increase the overall cloning efficiency. In and of itself, this amplification of genetic material may not be enough to warrant the time involved in the cloning process, but an increase in blastocyst generation from ECT would change this. The combination of gene editing techniques with ECT can ensure the elite genetics are inherited and is a desirable reason to continue advancing cloning practices and outcomes.

References

[1] Smith, S. J., Edmonds, J., Hartin, C. A., Mundra, A., & Calvin, K. (2015). Near-term acceleration in the rate of temperature change. *Nature Climate Change, 5*(4), 333-336.

[2] Hoffmann, A. A., & Sgrò, C. M. (2011). Climate change and evolutionary adaptation. *Nature, 470*(7335), 479-485.

[3] Jezkova, T., & Wiens, J. J. (2016). Rates of change in climatic niches in plant and animal populations are much slower than projected climate change. *Proceedings of the Royal Society B: Biological Sciences, 283*(1843), 20162104.

[4] McMichael, A. J., & Lindgren, E. (2011). Climate change: present and future risks to health, and necessary responses. *Journal of internal medicine, 270*(5), 401-413.

[5] Tao, S., Orellana, R., Weng, X., Marins, T., Dahl, G., & Bernard, J. (2018). Symposium review: The influences of heat stress on bovine mammary gland function. *Journal of dairy science, 101*(6), 5642-5654.

[6] Beef and Lamb New Zealand. (2021). Compendium of New Zealand farm facts 2021. In (45 ed.). New Zealand: Beef and Lamb.

[7] Abdelatty, A. M., Iwaniuk, M. E., Potts, S. B., & Gad, A. (2018). Influence of maternal nutrition and heat stress on bovine oocyte and embryo development. *International journal of veterinary science and medicine, 6*, S1-S5.

[8] Oltenacu, P. A., & Broom, D. M. (2010). The impact of genetic selection for increased milk yield on the welfare of dairy cows. *Animal welfare, 19*(1), 39-49.

[9] Kaniyamattam, K., Block, J., Hansen, P. J., & De Vries, A. (2017). Comparison between an exclusive in vitro–produced embryo transfer system and artificial insemination for genetic, technical, and financial herd performance. *Journal of Dairy Science, 100*(7), 5729-5745.

[10] Tachibana, M., Amato, P., Sparman, M., Gutierrez, N. M., Tippner-Hedges, R., Ma, H., Kang, E., Fulati, A., Lee, H.-S., & Sritanaudomchai, H. (2013). Human embryonic stem cells derived by somatic cell nuclear transfer. *Cell, 153*(6), 1228-1238.

[11] Wakayama, T., Rodriguez, I., Perry, A. C. F., Yanagimachi, R., & Mombaerts, P. (1999). Mice cloned from embryonic stem cells. *Proceedings of the National Academy of Sciences, 96*(26), 14984-14989.

[12] Rideout III, W. M., Eggan, K., & Jaenisch, R. (2001). Nuclear cloning and epigenetic reprogramming of the genome. *Science, 293*(5532), 1093-1098.

[13] Polejaeva, I. A. (2021). 25th Anniversary of cloning by somatic cell nuclear transfer: Generation of genetically engineered livestock using somatic cell nuclear transfer. *Reproduction, 162*(1), F11-F22.

[14] Iyer, S., Mukherjee, S., & Kumar, M. (2021). Watching the embryo: Evolution of the microscope for the study of embryogenesis. *BioEssays, 43*(6), 2000238.

[15] Chang, M. C. (1959). Fertilization of rabbit ova in vitro. *Nature, 184*(7), 466-7.

[16] Eppig, J. J., O'Brien, M., & Wigglesworth, K. (1996). Mammalian oocyte growth and development in vitro. *Molecular Reproduction and Development, 44*(2), 260-273.

[17] Lu, K., Gordon, I., Chen, H., & McGovern, H. (1987). In vitro culture of early bovine embryos derived from in vitro fertilization of follicular ovocytes matured in vitro. In *Colloque Scientifique de l'Association Europeenne de Transfert Embryonnaire, Lyon (France), 4-5 Sep 1987*: AETE.

[18] Ferré, L., Kjelland, M., Strøbech, L., Hyttel, P., Mermillod, P., & Ross, P. (2020). Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. *Animal, 14*(5), 991-1004.

[19] Biggers, J. D. (2012). IVF and embryo transfer: historical origin and development. *Reproductive BioMedicine Online, 25*(2), 118-127.

[20] He, M., Zhang, T., Yang, Y., & Wang, C. (2021). Mechanisms of Oocyte Maturation and Related Epigenetic Regulation. *Frontiers in Cell and Developmental Biology, 9*.

[21] Clift, D., & Schuh, M. (2013). Restarting life: fertilization and the transition from meiosis to mitosis. *Nat Rev Mol Cell Biol, 14*(9), 549-62.

[22] Cooper, G. M. (2000). *The cell: A molecular approach*. (2nd ed.). Sinauer Associates.

[23] MacLennan, M., Crichton, J. H., Playfoot, C. J., & Adams, I. R. (2015). Oocyte development, meiosis and aneuploidy. *Seminars in cell & developmental biology, 45*, 68-76.

[24] Boiani, M., Casser, E., Fuellen, G., & Christians, E. S. (2019). Totipotency continuity from zygote to early blastomeres: a model under revision. *Reproduction, 158*(2), R49-R65.

[25] Wei, Q., Zhong, L., Zhang, S., Mu, H., Xiang, J., Yue, L., Dai, Y., & Han, J. (2017). Bovine lineage specification revealed by single-cell gene expression analysis from zygote to blastocyst†. *Biology of Reproduction, 97*(1), 5-17.

[26] Sawada, H., Yamazaki, K., & Hoshi, M. (1990). Trypsin-like hatching protease from mouse embryos: Evidence for the presence in culture medium and its enzymatic properties. *Journal of Experimental Zoology, 254*(1), 83-87.

[27] Leonavicius, K., Royer, C., Preece, C., Davies, B., Biggins, J. S., & Srinivas, S. (2018). Mechanics of mouse blastocyst hatching revealed by a hydrogel-based microdeformation assay. *Proceedings of the National Academy of Sciences, 115*(41), 10375-10380.

[28] Zhu, M., & Zernicka-Goetz, M. (2020). Principles of self-organization of the mammalian embryo. *Cell, 183*(6), 1467-1478.

[29] Wrenzycki, C., & Stinshoff, H. (2013). Maturation Environment and Impact on Subsequent Developmental Competence of Bovine Oocytes. *Reproduction in Domestic Animals, 48*(s1), 38-43.

[30] Rizos, D., Ward, F., Duffy, P., Boland, M. P., & Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development, 61*(2), 234-248.

[31] Farin, P. W., & Farin, C. E. (1995). Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development. *Biology of reproduction*, *52*(3), 676-682.

[32] Pontes, J., Nonato-Junior, I., Sanches, B., Ereno-Junior, J., Uvo, S., Barreiros, T., Oliveira, J., Hasler, J., & Seneda, M. (2009). Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (Bos indicus) donor cows. *Theriogenology*, *71*(4), 690-697.

[33] Hirao, Y. (2011). Conditions affecting growth and developmental competence of mammalian oocytes in vitro. *Animal science journal*, *82*(2), 187-197.

[34] Li, Y., Sena Lopes, J., Fuster, P. C., & Rivera, R. M. (2022). Spontaneous and ART-induced large offspring syndrome: similarities and differences in DNA methylome. *Epigenetics*, 1-20.

[35] Mangiavacchi, P. M., Caldas-Bussiere, M. C., Mendonça, M. d. S., Dias, A. J. B., & Rios, Á. F. L. (2021). Multi-locus imprinting disturbances of Beckwith-Wiedemann and Large offspring syndrome/Abnormal offspring syndrome: A brief review. *Theriogenology*, *173*, 193-201.

[36] Behboodi, E., Anderson, G. B., BonDurant, R. H., Cargill, S. L., Kreuscher, B. R., Medrano, J. F., & Murray, J. D. (1995). Birth of large calves that developed from in vitro-derived bovine embryos. *Theriogenology*, *44*(2), 227-232.

[37] Thompson, J. G., Gardner, D. K., Anne Pugh, P., McMillan, W. H., & Robin Tervit, H. (1995). Lamb Birth Weight is Affected by Culture System Utilized during in Vitro Pre-Elongation Development of Ovine Embryos. *Biology of Reproduction*, *53*(6), 1385-1391.

[38] Lindner, G. M., & Wright, R. W. (1983). Bovine embryo morphology and evaluation. *Theriogenology*, *20*(4), 407-416.

[39] Stringfellow, D. A., Seidel, S. M., & International Embryo Transfer Society. (2007). *Manual of the International Embryo Transfer Society a procedural guide and general information for the use of embryo transfer technology, emphasizing sanitary procedures*. Savory, Ill: International Embryo Transfer Society.

[40] Lee, K., Uh, K., & Farrell, K. (2020). Current progress of genome editing in livestock. *Theriogenology*, *150*, 229-235.

[41] Potter, H. (2003). Transfection by electroporation. *Current protocols in molecular biology, Chapter 9*, Unit-9.3.

[42] Li, M., Bui, M., & Akbari, O. S. (2017). Embryo Microinjection and Transplantation Technique for Nasonia vitripennis Genome Manipulation. *Journal of visualized experiments : JoVE*(130), 56990.

[43] Chang, D. C., & Reese, T. S. (1990). Changes in membrane structure induced by electroporation as revealed by rapid-freezing electron microscopy. *Biophysical journal*, *58*(1), 1-12.

[44] Gu, B., Posfai, E., & Rossant, J. (2018). Efficient generation of targeted large insertions by microinjection into two-cell-stage mouse embryos. *Nature biotechnology*, *36*(7), 632-637.

[45] Lamas-Toranzo, I., Galiano-Cogolludo, B., Cornudella-Ardiaca, F., Cobos-Figueroa, J., Ousinde, O., & Bermejo-Álvarez, P. (2019). Strategies to reduce genetic mosaicism

following CRISPR-mediated genome edition in bovine embryos. *Scientific reports*, *9*(1), 1-8.

[46] Osei-Amponsah, R., Chauhan, S. S., Leury, B. J., Cheng, L., Cullen, B., Clarke, I. J., & Dunshea, F. R. (2019). Genetic selection for thermotolerance in ruminants. *Animals*, *9*(11), 948.

[47] National Library of Medicine. (2021). *PMEL premelanosome protein [Bos taurus (cattle)]*. Retrieved April 26, 2022, from https://www.ncbi.nlm.nih.gov/gene/281487#:~:text=Indication%20of%20Premelanosome%20Protein%20(PMEL,red%2C%20yellow%2C%20and%20white.

[48] National Library of Medicine. (2022). *PRL prolactin [Bos taurus (cattle)]*. Retrieved April 26, 2022, from https://www.ncbi.nlm.nih.gov/gene/280901.

[49] Schmutz, S. M., & Dreger, D. L. (2013). Interaction of MC1R and PMEL alleles on solid coat colors in Highland cattle. *Animal Genetics*, *44*(1), 9-13.

[50] Laible, G., Cole, S. A., Brophy, B., Wei, J., Leath, S., Jivanji, S., Littlejohn, M. D., & Wells, D. N. (2021). Holstein Friesian dairy cattle edited for diluted coat color as a potential adaptation to climate change. *BMC Genomics*, *22*(1), 856.

[51] Littlejohn, M. D., Henty, K. M., Tiplady, K., Johnson, T., Harland, C., Lopdell, T., Sherlock, R. G., Li, W., Lukefahr, S. D., Shanks, B. C., Garrick, D. J., Snell, R. G., Spelman, R. J., & Davis, S. R. (2014). Functionally reciprocal mutations of the prolactin signalling pathway define hairy and slick cattle. *Nat Commun*, *5*, 5861.

[52] Zhao, L., Gao, X., Zheng, Y., Wang, Z., Zhao, G., Ren, J., Zhang, J., Wu, J., Wu, B., Chen, Y., Sun, W., Li, Y., Su, J., Ding, Y., Gao, Y., Liu, M., Bai, X., Sun, L., Cao, G., Tang, F., *et al.* (2021). Establishment of bovine expanded potential stem cells. *Proceedings of the National Academy of Sciences*, *118*(15), e2018505118.

[53] Romito, A., & Cobellis, G. (2016). Pluripotent stem cells: current understanding and future directions. *Stem cells international*, *2016*.

[54] Bogliotti, Y. S., Wu, J., Vilarino, M., Okamura, D., Soto, D. A., Zhong, C., Sakurai, M., Sampaio, R. V., Suzuki, K., Belmonte, J. C. I., & Ross, P. J. (2018). Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. *Proceedings of the National Academy of Sciences*, *115*(9), 2090-2095.

[55] Evans, M. J., & Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *nature*, *292*(5819), 154-156.

[56] Bilodeau-Goeseels, S. (2011). Cows are not mice: The role of cyclic AMP, phosphodiesterases, and adenosine monophosphate-activated protein kinase in the maintenance of meiotic arrest in bovine oocytes. *Molecular Reproduction and Development*, *78*(10-11), 734-743.

[57] Hall, V. J., & Hyttel, P. (2014). Breaking down pluripotency in the porcine embryo reveals both a premature and reticent stem cell state in the inner cell mass and unique expression profiles of the naive and primed stem cell states. *Stem cells and development*, *23*(17), 2030-2045.

[58] Hall, V. J., Christensen, J., Gao, Y., Schmidt, M. H., & Hyttel, P. (2009). Porcine pluripotency cell signaling develops from the inner cell mass to the epiblast during early development. *Developmental Dynamics*, *238*(8), 2014-2024.

[59] Haraguchi, S., Kikuchi, K., Nakai, M., & Tokunaga, T. (2012). Establishment of self-renewing porcine embryonic stem cell-like cells by signal inhibition. *Journal of Reproduction and Development*.

[60] Choi, K.-H., Lee, D.-K., Kim, S. W., Woo, S.-H., Kim, D.-Y., & Lee, C.-K. (2019). Chemically defined media can maintain pig pluripotency network in vitro. *Stem cell reports, 13*(1), 221-234.

[61] Kinoshita, M., Kobayashi, T., Planells, B., Klisch, D., Spindlow, D., Masaki, H., Bornelöv, S., Stirparo, G. G., Matsunari, H., & Uchikura, A. (2021). Pluripotent stem cells related to embryonic disc exhibit common self-renewal requirements in diverse livestock species. *Development, 148*(23), dev199901.

[62] Kestler, H. A., & Kühl, M. (2008). From individual Wnt pathways towards a Wnt signalling network. *Philosophical Transactions of the Royal Society B: Biological Sciences, 363*(1495), 1333-1347.

[63] Kim, H., Wu, J., Ye, S., Tai, C.-I., Zhou, X., Yan, H., Li, P., Pera, M., & Ying, Q.-L. (2013). Modulation of β-catenin function maintains mouse epiblast stem cell and human embryonic stem cell self-renewal. *Nature Communications, 4*(1), 2403.

[64] Meyn, M. A., 3rd, & Smithgall, T. E. (2008). Small molecule inhibitors of Lck: the search for specificity within a kinase family. *Mini Rev Med Chem, 8*(6), 628-37.

[65] Hirai, H., Karian, P., & Kikyo, N. (2011). Regulation of embryonic stem cell self-renewal and pluripotency by leukaemia inhibitory factor. *The Biochemical journal, 438*(1), 11-23.

[66] Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J. B., Nishikawa, S., Nishikawa, S.-i., & Muguruma, K. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature biotechnology, 25*(6), 681-686.

[67] Swain, N., Thakur, M., Pathak, J., & Swain, B. (2020). SOX2, OCT4 and NANOG: The core embryonic stem cell pluripotency regulators in oral carcinogenesis. *Journal of oral and maxillofacial pathology : JOMFP, 24*(2), 368-373.

[68] Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A. A., Ko, M. S. H., & Niwa, H. (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nature Cell Biology, 9*(6), 625-635.

[69] Yeo, J.-C., & Ng, H.-H. (2013). The transcriptional regulation of pluripotency. *Cell Research, 23*(1), 20-32.

[70] Li, W., Zhou, H., Abujarour, R., Zhu, S., Young Joo, J., Lin, T., Hao, E., Schöler, H. R., Hayek, A., & Ding, S. (2009). Generation of human-induced pluripotent stem cells in the absence of exogenous Sox2. *Stem cells (Dayton, Ohio), 27*(12), 2992-3000.

[71] Sperger, J. M., Chen, X., Draper, J. S., Antosiewicz, J. E., Chon, C. H., Jones, S. B., Brooks, J. D., Andrews, P. W., Brown, P. O., & Thomson, J. A. (2003). Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. *Proceedings of the National Academy of Sciences, 100*(23), 13350-13355.

[72] Schrode, N., Saiz, N., Di Talia, S., & Hadjantonakis, A.-K. (2014). GATA6 levels modulate primitive endoderm cell fate choice and timing in the mouse blastocyst. *Developmental cell, 29*(4), 454-467.

[73] Devika, A. S., Wruck, W., Adjaye, J., & Sudheer, S. (2019). The quest for pluripotency: a comparative analysis across mammalian species. *Reproduction, 158*(3), R97-R111.

[74] Sheridan, S. D., Surampudi, V., & Rao, R. R. (2012). Analysis of Embryoid Bodies Derived from Human Induced Pluripotent Stem Cells as a Means to Assess Pluripotency. *Stem Cells International, 2012*, 738910.

[75] Gropp, M., Shilo, V., Vainer, G., Gov, M., Gil, Y., Khaner, H., Matzrafi, L., Idelson, M., Kopolovic, J., Zak, N. B., & Reubinoff, B. E. (2012). Standardization of the teratoma assay for analysis of pluripotency of human ES cells and biosafety of their differentiated progeny. *PloS one, 7*(9), e45532.

[76] Pillai, V. V., Koganti, P. P., Kei, T. G., Gurung, S., Butler, W. R., & Selvaraj, V. (2021). Efficient induction and sustenance of pluripotent stem cells from bovine somatic cells. *Biology open, 10*(10), bio058756.

[77] Zhao, X.-y., Li, W., Lv, Z., Liu, L., Tong, M., Hai, T., Hao, J., Guo, C.-l., Ma, Q.-w., & Wang, L. (2009). iPS cells produce viable mice through tetraploid complementation. *Nature, 461*(7260), 86-90.

[78] Li, T.-D., Feng, G.-H., Li, Y.-F., Wang, M., Mao, J.-J., Wang, J.-Q., Li, X., Wang, X.-P., Qu, B., & Wang, L.-Y. (2017). Rat embryonic stem cells produce fertile offspring through tetraploid complementation. *Proceedings of the National Academy of Sciences, 114*(45), 11974-11979.

[79] Wilmut, I., Bai, Y., & Taylor, J. (2015). Somatic cell nuclear transfer: origins, the present position and future opportunities. *Philosophical Transactions of the Royal Society B: Biological Sciences, 370*(1680), 20140366.

[80] Wabl, M. R., Brun, R. B., & Du Pasquier, L. (1975). Lymphocytes of the Toad Xenopus laevis Have the Gene Set for Promoting Tadpole Development. *Science (American Association for the Advancement of Science), 190*(4221), 1310-1312.

[81] Yang, X. (2006). History and Current Status of Nuclear Transfer Cloning. *Biotechnology and Genetic Engineering Reviews, 23*(1), 63-70.

[82] Campbell, K. H. S., McWhir, J., Ritchie, W. A., & Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. *Nature, 380*(6569), 64-66.

[83] Niemann, H., & Lucas-Hahn, A. (2012). Somatic Cell Nuclear Transfer Cloning: Practical Applications and Current Legislation. *Reproduction in Domestic Animals, 47*(s5), 2-10.

[84] Wells, D. N., Oback, B., & Laible, G. (2003). Cloning livestock: a return to embryonic cells. *Trends in biotechnology (Regular ed.), 21*(10), 428-432.

[85] Gil, M. A., Maside, C., Cuello, C., Parrilla, I., Vazquez, J. M., Roca, J., & Martinez, E. A. (2012). Effects of Hoechst 33342 staining and ultraviolet irradiation on mitochondrial distribution and DNA copy number in porcine oocytes and preimplantation embryos. *Molecular reproduction and development, 79*(9), 651-663.

[86] Russell, D. F., Ibáñez, E., Albertini, D. F., & Overström, E. W. (2005). Activated bovine cytoplasts prepared by demecolcine-induced enucleation support development of nuclear transfer embryos in vitro. *Molecular reproduction and development, 72*(2), 161-170.

[87] Ji, W., & Bavister, B. (2000). Development of zona-free hamster ova to blastocysts in vitro. *Theriogenology, 54*(6), 827-834.

[88] O'doherty, E., Wade, M., Hill, J., & Boland, M. (1997). Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology, 48*(1), 161-169.

[89] Nasr-Esfahani, M. H., Hosseini, S. M., Hajian, M., Forouzanfar, M., Ostadhosseini, S., Abedi, P., Khazaie, Y., Dormiani, K., Ghaedi, K., Forozanfar, M., Gourabi, H., Shahverdi, A. H., Vosough, A. D., & Vojgani, H. (2011). Development of an Optimized Zona-Free Method of Somatic Cell Nuclear Transfer in the Goat. *Cellular reprogramming, 13*(2), 157-170.

[90] Oback, B., & Wells, D. N. (2003). Cloning Cattle. *Cloning and stem cells, 5*(4), 243-256.

[91] Du, F., Shen, P.-c., Xu, J., Sung, L.-Y., Jeong, B. S., Lucky Nedambale, T., Riesen, J., Cindy Tian, X., Cheng, W. T. K., Lee, S.-N., & Yang, X. (2006). The cell agglutination agent, phytohemagglutinin-L, improves the efficiency of somatic nuclear transfer cloning in cattle (Bos taurus). *Theriogenology, 65*(3), 642-657.

[92] Oback, B., & Wells, D. N. (2003). Cloning cattle. *Cloning Stem Cells, 5*(4), 243-56.

[93] Kim, E. Y., Park, M. J., Park, H. Y., Noh, E. J., Noh, E. H., Park, K. S., Lee, J. B., Jeong, C. J., Riu, K. Z., & Park, S. P. (2012). Improved cloning efficiency and developmental potential in bovine somatic cell nuclear transfer with the oosight imaging system. *Cellular Reprogramming (Formerly" Cloning and Stem Cells"), 14*(4), 305-311.

[94] Matoba, S., & Zhang, Y. (2018). Somatic Cell Nuclear Transfer Reprogramming: Mechanisms and Applications. *Cell stem cell, 23*(4), 471-485.

[95] Wang, X., Qu, J., Li, J., He, H., Liu, Z., & Huan, Y. (2020). Epigenetic Reprogramming During Somatic Cell Nuclear Transfer: Recent Progress and Future Directions. *Frontiers in Genetics, 11*.

[96] Nasr-Esfahani, M. H., Hosseini, S. M., Hajian, M., Forouzanfar, M., Ostadhosseini, S., Abedi, P., Khazaie, Y., Dormiani, K., Ghaedi, K., Forozanfar, M., Gourabi, H., Shahverdi, A. H., Vosough, A. D., & Vojgani, H. (2011). Development of an optimized zona-free method of somatic cell nuclear transfer in the goat. *Cellular Reprogramming, 13*, 157+.

[97] de Macedo, M. P., Glanzner, W. G., Rissi, V. B., Gutierrez, K., Currin, L., Baldassarre, H., & Bordignon, V. (2019). A fast and reliable protocol for activation of porcine oocytes. *Theriogenology, 123*, 22-29.

[98] Oliveira, R., Mantovani, M., Da Silva, A., Pesarini, J., Mauro, M., & Ribeiro, L. (2014). Compounds used to produce cloned animals are genotoxic and mutagenic in mammalian assays in vitro and in vivo. *Brazilian Journal of Medical and Biological Research, 47*, 287-298.

[99] Felmer, R., & Arias, M. (2015). Activation treatment of recipient oocytes affects the subsequent development and ploidy of bovine parthenogenetic and somatic cell nuclear transfer (SCNT) embryos. *Molecular reproduction and development, 82*(6), 441-449.

[100] Hipp, J., & Atala, A. (2004). Tissue engineering, stem cells, cloning, and parthenogenesis: new paradigms for therapy. *Journal of experimental & clinical assisted reproduction*, *1*(1), 3-3.

[101] Loeb, J. (1899). On the nature of the process of fertilization and the artificial production of normal larvae (plutei) from the unfertilized eggs of the sea urchin. *American Journal of Physiology-Legacy Content*, *3*(3), 135-138.

[102] Surani, M. A. H., & Barton, S. C. (1983). Development of gynogenetic eggs in the mouse: implications for parthenogenetic embryos. *Science*, *222*(4627), 1034-1036.

[103] Fernandes, C. B., Devito, L. G., Martins, L. R., Blanco, I. D. P., de Lima Neto, J. F., Tsuribe, P. M., Gonçalves, C. G. P., & da Cruz Landim-Alvarenga, F. (2014). Artificial activation of bovine and equine oocytes with cycloheximide, roscovitine, strontium, or 6-dimethylaminopurine in low or high calcium concentrations. *Zygote*, *22*(3), 387-394.

[104] Oback, B. (2008). Climbing Mount Efficiency – Small Steps, Not Giant Leaps Towards Higher Cloning Success in Farm Animals. *Reproduction in Domestic Animals*, *43*(s2), 407-416.

[105] Akagi, S., Matsukawa, K., & Takahashi, S. (2014). Factors affecting the development of somatic cell nuclear transfer embryos in cattle. *Journal of Reproduction and Development*, *60*(5), 329-335.

[106] Bureau, W. S., Bordignon, V., Léveillée, C., Smith, L. C., & King, W. A. (2003). Assessment of Chromosomal Abnormalities in Bovine Nuclear Transfer Embryos and in Their Donor Cells. *Cloning and stem cells*, *5*(2), 123-132.

[107] Hanada, H., Takeda, K., Tagami, T., Nirasawa, K., Akagi, S., Adachi, N., Takahashi, S., Izaike, Y., Iwamoto, M., & Fuchimoto, D. I. (2005). Chromosomal instability in the cattle clones derived by somatic cell nuclear-transfer. *Molecular Reproduction and Development: Incorporating Gamete Research*, *71*(1), 36-44.

[108] Wells, D., Laible, G., Tucker, F., Miller, A., Oliver, J., Xiang, T., Forsyth, J., Berg, M., Cockrem, K., & L'Huillier, P. (2003). Coordination between donor cell type and cell cycle stage improves nuclear cloning efficiency in cattle. *Theriogenology*, *59*(1), 45-59.

[109] Urakawa, M., Ideta, A., Sawada, T., & Aoyagi, Y. (2004). Examination of a modified cell cycle synchronization method and bovine nuclear transfer using synchronized early G1 phase fibroblast cells. *Theriogenology*, *62*(3-4), 714-728.

[110] Biosciences. (2022). *Overview of cell cycle and cell proliferation*. Retrieved April 28, 2022, from https://www.bdbiosciences.com/en-us/learn/research/cell-biology/cell-cycle.

[111] Giraldo, A. M., Hylan, D. A., Ballard, C. B., Purpera, M. N., Vaught, T. D., Lynn, J. W., Godke, R. A., & Bondioli, K. R. (2008). Effect of Epigenetic Modifications of Donor Somatic Cells on the Subsequent Chromatin Remodeling of Cloned Bovine Embryos1. *Biology of Reproduction*, *78*(5), 832-840.

[112] Wakayama, S., & Wakayama, T. (2010). Improvement of mouse cloning using nuclear transfer-derived embryonic stem cells and/or histone deacetylase inhibitor. *Int J Dev Biol*, *54*(11-12), 1641-8.

[113] Oback, B., & Wells, D. (2002). Donor Cells for Nuclear Cloning: Many Are Called, but Few Are Chosen. *Cloning and stem cells, 4*(2), 147-168.

[114] Amano, T., Tani, T., Kato, Y., & Tsunoda, Y. (2001). Mouse cloned from embryonic stem (ES) cells synchronized in metaphase with nocodazole. *Journal of Experimental Zoology, 289*(2), 139-145.

[115] Lan, G.-C., Han, D., Wu, Y.-G., Han, Z.-B., Ma, S.-F., Liu, X.-Y., Chang, C.-L., & Tan, J.-H. (2005). Effects of duration, concentration, and timing of ionomycin and 6-dimethylaminopurine (6-DMAP) treatment on activation of goat oocytes. *Molecular Reproduction and Development, 71*(3), 380-388.

[116] Sigma-Aldrich. (2010). *Cell Quantification*. Cook Book Sept 2010 (Vol. Volume 12). Sigma-Aldrich.

[117] Ferré, L. B., Kjelland, M. E., Strøbech, L. B., Hyttel, P., Mermillod, P., & Ross, P. J. (2020). Review: Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. *Animal, 14*(5), 991-1004.

[118] Sims, M., & First, N. L. (1994). Production of calves by transfer of nuclei from cultured inner cell mass cells. *Proceedings of the National Academy of Sciences of the United States of America, 91*(13), 6143-6147.

[119] Chen, A. E., & Melton, D. A. (2007). Derivation of human embryonic stem cells by immunosurgery. *Journal of visualized experiments : JoVE*(10), 574-574.

[120] Wu, X., Song, M., Yang, X., Liu, X., Liu, K., Jiao, C., Wang, J., Bai, C., Su, G., & Liu, X. (2016). Establishment of bovine embryonic stem cells after knockdown of CDX2. *Scientific reports, 6*(1), 1-12.

[121] Verma, V., Huang, B., Kallingappa, P. K., & Oback, B. (2013). Dual Kinase Inhibition Promotes Pluripotency in Finite Bovine Embryonic Cell Lines. *Stem cells and development, 22*(11), 1728-1742.

[122] Davidenko, N., Schuster, C. F., Bax, D. V., Farndale, R. W., Hamaia, S., Best, S. M., & Cameron, R. E. (2016). Evaluation of cell binding to collagen and gelatin: a study of the effect of 2D and 3D architecture and surface chemistry. *Journal of materials science. Materials in medicine, 27*(10), 148-148.

[123] Hagbard, L., Cameron, K., August, P., Penton, C., Parmar, M., Hay, D. C., & Kallur, T. (2018). Developing defined substrates for stem cell culture and differentiation. *Philosophical Transactions of the Royal Society B: Biological Sciences, 373*(1750), 20170230.

[124] Hayashi, Y., & Furue, M. K. (2016). Biological Effects of Culture Substrates on Human Pluripotent Stem Cells. *Stem Cells International, 2016*, 5380560.

[125] Trujillo, S., Gonzalez-Garcia, C., Rico, P., Reid, A., Windmill, J., Dalby, M. J., & Salmeron-Sanchez, M. (2020). Engineered 3D hydrogels with full-length fibronectin that sequester and present growth factors. *Biomaterials, 252*, 120104.

[126] Soto, D. A., Navarro, M., Zheng, C., Halstead, M. M., Zhou, C., Guiltinan, C., Wu, J., & Ross, P. J. (2021). Simplification of culture conditions and feeder-free expansion of bovine embryonic stem cells. *Scientific Reports, 11*(1), 11045.

[127] Li, Z., Leung, M., Hopper, R., Ellenbogen, R., & Zhang, M. (2010). Feeder-free self-renewal of human embryonic stem cells in 3D porous natural polymer scaffolds. *biomaterials, 31*(3), 404-412.

[128] Michl, J., Park, K. C., & Swietach, P. (2019). Evidence-based guidelines for controlling pH in mammalian live-cell culture systems. *Communications Biology, 2*(1), 144.

[129] Pillai, V. V., Koganti, P. P., Kei, T. G., Gurung, S., Butler, W. R., & Selvaraj, V. (2021). Efficient induction and sustenance of pluripotent stem cells from bovine somatic cells. *Biol Open, 10*(10).

[130] Gonzalez, R., Loring, J. F., esselschmidt, R. L., & Schartz, P. H. (2007). CHAPTER 1 - Human Embryonic Stem Cell Culture. In J. F. Loring, R. L. Wesselschmidt & P. H. Schwartz (Eds.), *Human Stem Cell Manual* (pp. 3-17). Oxford: Academic Press.

[131] Cheng, J., Dutra, A., Takesono, A., Garrett-Beal, L., & Schwartzberg, P. L. (2004). Improved generation of C57BL/6J mouse embryonic stem cells in a defined serum-free media. *genesis, 39*(2), 100-104.

[132] Subbiahanadar Chelladurai, K., Selvan Christyraj, J. D., Rajagopalan, K., Yesudhason, B. V., Venkatachalam, S., Mohan, M., Chellathurai Vasantha, N., & Selvan Christyraj, J. R. S. (2021). Alternative to FBS in animal cell culture - An overview and future perspective. *Heliyon, 7*(8), e07686-e07686.

[133] Tamm, C., Pijuan Galitó, S., & Annerén, C. (2013). A comparative study of protocols for mouse embryonic stem cell culturing. *PloS one, 8*(12), e81156.

[134] Van der Jeught, M., O'Leary, T., Ghimire, S., Lierman, S., Duggal, G., Versieren, K., Deforce, D., Chuva de Sousa Lopes, S., Heindryckx, B., & De Sutter, P. (2013). The combination of inhibitors of FGF/MEK/Erk and GSK3β signaling increases the number of OCT3/4- and NANOG-positive cells in the human inner cell mass, but does not improve stem cell derivation. *Stem cells and development, 22*(2), 296-306.

[135] Harris, D., Huang, B., & Oback, B. (2013). Inhibition of MAP2K and GSK3 Signaling Promotes Bovine Blastocyst Development and Epiblast-Associated Expression of Pluripotency Factors1. *Biology of Reproduction, 88*(3).

[136] Berg, D. K., Smith, C. S., Pearton, D. J., Wells, D. N., Broadhurst, R., Donnison, M., & Pfeffer, P. L. (2011). Trophectoderm Lineage Determination in Cattle. *Developmental Cell, 20*(2), 244-255.

[137] Olson, S. E., & Seidel, J. G. E. (2000). Culture of In Vitro-Produced Bovine Embryos with Vitamin E Improves Development In Vitro and After Transfer to Recipients1. *Biology of reproduction, 62*(2), 248-252.

[138] ten Berge, D., Kurek, D., Blauwkamp, T., Koole, W., Maas, A., Eroglu, E., Siu, R. K., & Nusse, R. (2011). Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. *Nature cell biology, 13*(9), 1070-1075.

[139] Xiao, L., Yuan, X., & Sharkis, S. J. (2006). Activin A Maintains Self-Renewal and Regulates Fibroblast Growth Factor, Wnt, and Bone Morphogenic Protein Pathways in Human Embryonic Stem Cells. *Stem Cells, 24*(6), 1476-1486.

[140] Flomerfelt, F. A., & Gress, R. E. (2016). Analysis of Cell Proliferation and Homeostasis Using EdU Labeling. *Methods in molecular biology (Clifton, N.J.), 1323*, 211-220.

[141] Stevens, J. B., Abdallah, B. Y., Regan, S. M., Liu, G., Bremer, S. W., Ye, C. J., & Heng, H. H. (2010). Comparison of mitotic cell death by chromosome fragmentation to premature chromosome condensation. *Molecular Cytogenetics, 3*(1), 20.

[142] Liu, T. M., Wu, C. J., Yu, X. M., & Li, X. Y. (2001). Effects of cell-cycle arrest agents on cleavage and development of loach (Misgurnus anguillicaudatus) embryos. *Fish physiology and biochemistry, 25*(1), 3-9.

[143] Eakin, G. S., & Behringer, R. R. (2003). Tetraploid development in the mouse. *Developmental Dynamics, 228*(4), 751-766.

[144] Singhania, R., Sramkoski, R. M., Jacobberger, J. W., & Tyson, J. J. (2011). A hybrid model of mammalian cell cycle regulation. *PLoS computational biology, 7*(2), e1001077.

[145] Sobecki, M., Mrouj, K., Colinge, J., Gerbe, F., Jay, P., Krasinska, L., Dulic, V., & Fisher, D. (2017). Cell-cycle regulation accounts for variability in Ki-67 expression levels. *Cancer research, 77*(10), 2722-2734.

[146] Boward, B., Wu, T., & Dalton, S. (2016). Concise review: control of cell fate through cell cycle and pluripotency networks. *Stem cells, 34*(6), 1427-1436.

[147] Singh, A. M., & Dalton, S. (2009). The cell cycle and Myc intersect with mechanisms that regulate pluripotency and reprogramming. *Cell stem cell, 5*(2), 141-149.

[148] Xu, K. P., Yadav, B. R., King, W. A., & Betteridge, K. J. (1992). Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. *Molecular Reproduction and Development, 31*(4), 249-252.

[149] Avery, B., Madison, V., & Greve, T. (1991). Sex and development in bovine in-vitro fertilized embryos. *Theriogenology, 35*(5), 953-963.

[150] Rideout, W. M., Wakayama, T., Wutz, A., Eggan, K., Jackson-Grusby, L., Dausman, J., Yanagimachi, R., & Jaenisch, R. (2000). Generation of mice from wild-type and targeted ES cells by nuclear cloning. *Nature Genetics, 24*(2), 109-110.

[151] Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Intracellular control of cell-cycle events. In *Molecular Biology of the Cell. 4th edition*. Garland Science.

[152] Misica-Turner, P. M., Oback, F. C., Eichenlaub, M., Wells, D. N., & Oback, B. (2007). Aggregating Embryonic but Not Somatic Nuclear Transfer Embryos Increases Cloning Efficiency in Cattle1. *Biology of Reproduction, 76*(2), 268-278.

[153] Du, F., Sung, L.-Y., Tian, X. C., & Yang, X. (2002). Differential cytoplast requirement for embryonic and somatic cell nuclear transfer in cattle. *Molecular Reproduction and Development, 63*(2), 183-191.

[154] Tani, T., Kato, Y., & Tsunoda, Y. (2001). Direct Exposure of Chromosomes to Nonactivated Ovum Cytoplasm Is Effective for Bovine Somatic Cell Nucleus Reprogramming1. *Biology of Reproduction, 64*(1), 324-330.

[155] Kuhn, E. M., Therman, E., & Susman, B. (1991). Amitosis and endocycles in early cultured mouse trophoblast. *Placenta, 12*(3), 251-261.

[156] Mi, S., Lee, X., Li, X.-p., Veldman, G. M., Finnerty, H., Racie, L., LaVallie, E., Tang, X.-Y., Edouard, P., Howes, S., Keith, J. C., & McCoy, J. M. (2000). Syncytin is a

captive retroviral envelope protein involved in human placental morphogenesis. *Nature, 403*(6771), 785-789.

[157] Huppertz, B., Bartz, C., & Kokozidou, M. (2006). Trophoblast fusion: Fusogenic proteins, syncytins and ADAMs, and other prerequisites for syncytial fusion. *Micron, 37*(6), 509-517.

[158] Ma, Z., Sagrillo-Fagundes, L., Tran, R., Parameshwar, P. K., Kalashnikov, N., Vaillancourt, C., & Moraes, C. (2019). Biomimetic micropatterned adhesive surfaces to mechanobiologically regulate placental trophoblast fusion. *ACS applied materials & interfaces, 11*(51), 47810-47821.

[159] Natale, D. R. C., Hemberger, M., Hughes, M., & Cross, J. C. (2009). Activin promotes differentiation of cultured mouse trophoblast stem cells towards a labyrinth cell fate. *Developmental Biology, 335*(1), 120-131.

[160] Saito, S., Sawai, K., Ugai, H., Moriyasu, S., Minamihashi, A., Yamamoto, Y., Hirayama, H., Kageyama, S., Pan, J., Murata, T., Kobayashi, Y., Obata, Y., & Yokoyama, K. K. (2003). Generation of cloned calves and transgenic chimeric embryos from bovine embryonic stem-like cells. *Biochemical and Biophysical Research Communications, 309*(1), 104-113.

[161] Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D., & Carpenter, M. K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nature Biotechnology, 19*(10), 971-974.

[162] Hunt, G. C., Singh, P., & Schwarzbauer, J. E. (2012). Endogenous production of fibronectin is required for self-renewal of cultured mouse embryonic stem cells. *Experimental cell research, 318*(15), 1820-1831.

[163] Štefková, K., Procházková, J., & Pacherník, J. (2015). Alkaline phosphatase in stem cells. *Stem cells international, 2015*, 628368-628368.

[164] Johnson, D. S., Gemelos, G., Baner, J., Ryan, A., Cinnioglu, C., Banjevic, M., Ross, R., Alper, M., Barrett, B., Frederick, J., Potter, D., Behr, B., & Rabinowitz, M. (2010). Preclinical validation of a microarray method for full molecular karyotyping of blastomeres in a 24-h protocol. *Human Reproduction, 25*(4), 1066-1075.

[165] Turner, K. J., Silvestri, G., Black, D. H., Dobson, G., Smith, C., Handyside, A. H., Sinclair, K. D., & Griffin, D. K. (2019). Karyomapping for simultaneous genomic evaluation and aneuploidy screening of preimplantation bovine embryos: The first live-born calves. *Theriogenology, 125*, 249-258.

[166] Ealy, A. D., Wooldridge, L. K., & McCoski, S. R. (2019). BOARD INVITED REVIEW: Post-transfer consequences of in vitro-produced embryos in cattle. *Journal of animal science, 97*(6), 2555-2568.

[167] Hawk, H. W., & Wall, R. J. (1994). Improved yields of bovine blastocysts from in vitro-produced oocytes. I. Selection of oocytes and zygotes. *Theriogenology, 41*(8), 1571-1583.

[168] Gordon, I. (2003). *Laboratory Production of Cattle Embryos*. CAB International.

Appendices

Appendix I: Materials

Alexa Fluor™ azide
Alexa Fluor™ azide 488 diluted in 70 µl DMSO. From Click-iT™ EdU Cell Proliferation for Imaging Kit, Invitrogen, Thermo Fisher (NZ). Stored at -20°C.

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

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

Bovine Serum Albumin (BSA)
Fatty-acid-free bovine albumin. Sigma Aldrich (USA). Stored at 4°C.

BoviPure™ 40%
200 µl BoviPure™ with 300 µl BoviDilute. Nidacon (Sweden). Stored at room temperature.

BoviPure™ 80%
400 µl BoviPure™ with 100 µl BoviDilute. Nicadon (Sweden). Stored at room temperature.

Click 10X Click-iT® reaction buffer additive
Click 10X Click-iT® reaction buffer additive diluted in 2 mL milli-Q water. Click-iT® EdU Imaging Kit, Invitrogen, Thermo Fisher (NZ). Stored at -20°C.

Complete growth media
pEPSC base media with 3 mM CHIR99021 (41368, Stemgent), 0.3 mM WH-4-023 (R&D systems, USA), 2.5 mM XAV939 (R&D systems, USA), 65 µg/mL ascorbic acid (Sigma Aldrich, USA), 20 ng/mL activin A (Prospec Bio, Israel), 10 ng/mL IL-6, 10 ng/mL Y27632 (Reagents Direct, USA), 10% KSR. Stored at 4°C.

Copper sulphate (CuSO4)
100mM Copper Sulphate dissolved in Milli-Q water. Stored at 4°C.

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

Dissociation media
HSOF – Ca – BSA with 0.1 mg/ml PVA and 0.02% EDTA, 0.1% Cyotochalasin B, 0.1% ROCK inhibitor and 5.0 mg/ml pronase. Stored at 4°C for up to 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 -20°C or 4°C for 2 weeks.

EdU
10 mM EdU diluted in 2 mL DMSO, Click-iT® EdU Imaging Kit, Invitrogen, Life Technologies (USA). Stored at 4°C.

Embryo Hold
107.8 mM sodium chloride, 3.99 mM potassium chloride, 1.20 mM monopotassium phosphate, 0.33 mM sodium pyruvate, 0.49 mM magnesium chloride hexahydrate, 38.61 g/L MOPS sodium salt, 25 mM sodium bicarbonate, 1.71 mM calcium chloride dihydrate, 3.32 mM sodium lactate, 0.04 mM kanamycin sulfate, 1.5 mM glucose, 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. Stored at 4°C.

ESOF
107.8 mM sodium chloride, 0.33mM sodium pyruvate, 7.15 mM potassium chloride, 1.71 mM calcium chloride dihydrate, 0.30 mM monopotassium phosphate, 25 mM sodium bicarbonate, 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
107.8 mM sodium chloride, 0.33mM sodium pyruvate, 7.15 mM potassium chloride, 0.30 mM monopotassium phosphate, 25 mM sodium bicarbonate, 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 bovine serum (FBS)
Fetal bovine serum, Gibco, Thermo Fisher, (USA). Stored at 4°C.

Fibroblast growth media
DMEM/F-12 with 10% FBS. Gibco, Thermo fisher, USA

Fusion buffer (205)
36.4 g/L Mannitol, 7.7 mg/L calcium chloride, 24.9 mg/L magnesium chloride, 2ml/L Hepes, and 0.5 g/L BSA. Stored at 4°C.

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

Goat serum
5% goat serum in PBS. Gibco, Thermo Fisher (NZ). Stored at -20°C or 4°C for up to 2 weeks.

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

Hoechst
bisBenzimide H 33342 trihydrochloride in Milli-Q water. Sigma Aldrich (USA). Stored at 4°C.

HSOF
107.8 mM sodium chloride, 5 mM sodium bicarbonate, 7.15 mM potassium chloride, 0.3 mM monopotassium phosphate, 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.

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

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

IVF media
107.8 mM sodium chloride, 0.33 mM sodium pyruvate, 7.10 mM potassium chloride, 0.3 mM monopotassium phosphate, 25 mM sodium bicarbonate, 3.32 mM sodium lactate, 1.71 mM calcium chloride dihydrate, 8 mg/ml BSA, with 0.04 mM kanamycin sulfate, and supplemented on the day of use with 10 µg/ml heparin, 1 mM pyruvate, 20 µM penicillamine and 10 µM hypotaurine. Stored at 4°C.

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

LSOF
107.8 mM sodium chloride, 0.33 mM sodium pyruvate, 1.20 mM monopotassium phosphate, 25 mM sodium bicarbonate, 1.71 mM calcium chloride dihydrate, 0.50 mM magnesium chloride hexahydrate, 3.32 mM sodium lactate, 0.04 mM kanamycin sulfate, 1.5 mM glucose, 1 mM DNP (2-, 4-dinitrophenol), 1 mM Gluta-Max, 0.22 g/L BM Essential Amino Acid, 0.081 g/L Non-Essential Amino Acid, and 8 mg/ml BSA. Stored at 4°C.

Maturation media
10 µg/mL ovine follicle-stimulating hormone in B199, OVAGEN; ICP Bio (NZ), 1 µg/mL ovine luteinizing hormone, and 1 µg/mL 17-β-estradiol. Supplemented on day of use with 0.1 mM cysteamine (2- Mercaptoethylamine). Stored at 4°C.

Milli-Q water
Produced in Millipore Milli-Q plus unit, Bio Lab, (USA). Stored at room temperature.

Mineral Oil
M5310, Sigma Aldrich (USA). Stored at room temperature or 38°C in 5% CO_2.

Minimal media
pEPSC base media with 3 µM CHIR99021 (41368, Stemgent), 10 µM PD0325901 (pzo162-5mg, Sigma Aldrich), and 10 ng/ml IL-6. Stored at 4°C.

Paraformaldehyde (PFA)
PBS with 3.7% depolymerised (w/v) paraformaldehyde, 3.7% (w/v) sucrose, 1 M sodium hydroxide and phenol red indicator. Sigma Aldrich (USA). Stored at 4°C.

pEPSC base media
DMEM/F-12 (Gibco, Thermo Fisher, NZ) with 5 µl/mL 100X N2 (Gibco, Thermo Fisher, NZ), 10 µl/mL B27 (Gibco, Thermo Fisher, NZ), 10 µl/mL Penicillin-Streptomycin (Gibco, Thermo Fisher, NZ), 10 µl/mL Non-Essential Amino Acid, and 0.1 mM beta-mercaptoethanol (Sigma Aldrich, USA). Stored at 4°C.

Phosphate buffered saline (PBS)
8.4 mM disodium hydrogen orthophosphate 2-hydrate, 150 mM sodium chloride, and 1.8 mM sodium dihydrogen orthophosphate 1-hydrate dissolved in Milli-Q water, pH 7.4. Stored at room temperature

Pronase
0.5% Protease in H199. Sigma Aldrich (USA). Stored at -20°C.

Saline
0.9% sodium chloride in Milli-Q water. Sigma Aldrich (USA). Stored at 32°C.

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

TrypLE™ Select
TrypLE™ Select, GIBCO, Thermo Fisher (USA). Stored at 4°C.

Appendix II: IVP record sheet

Experiment : | **Run Number :**

IVM	IVF	IVC	C/O
Date/time : Wednesday, 18 May 2022	Thursday, 19 May 2022	Friday, 20 May 2022	Tuesday, 24 May 2022
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 : Friday, 27 May 2022

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

Appendix III: Plate layout examples

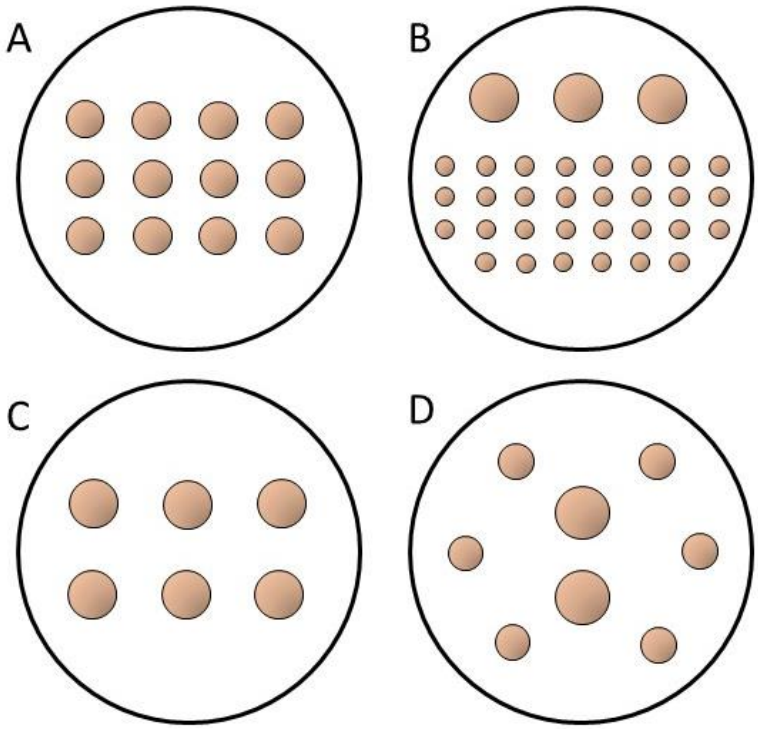


Figure III.1: Plate layouts. A) 12 x 30 or 12 x 40 µl drops. B) Culture plate for single embryos. Top row 3 x 40 µl wash drops, lower rows make up 30 x 5 µl drops. C) Disaggregation-related plates. 6 x 40 µl drops. D) ESOF and LSOF plates. 2 x 40 µl central was drops surrounded by 6 x 20 µl culture drops.

Appendix IV: Bovine oocyte grading

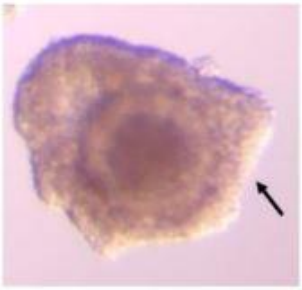
I. Cumulus cell morphology

Cat. 1) oocyte is surrounded by **> 5 layers of compact granulosa cells**

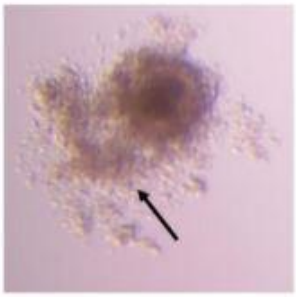
Cat. 2) oocyte is surrounded by **> 5 layers of less compact granulosa cells**

Cat. 3) oocyte is surrounded by **> 5 layers of expanded granulosa cells**

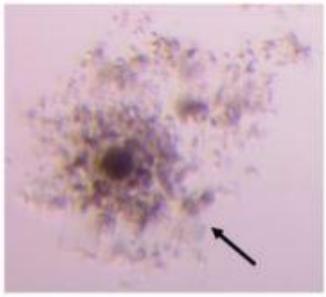
Cat. 4) oocyte is surrounded by **≤ 5 layers of granulosa cells and/or not completely surrounding the oocyte**



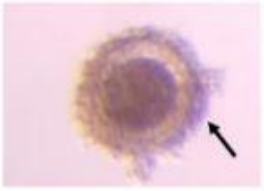
Cat. 1) Cumulus consists of more than 5 layers. **Granulosa cells are compact and dense.**



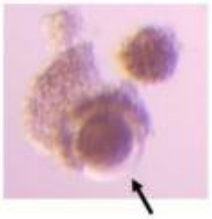
Cat. 2) Cumulus consists of more than 5 layers. **Granulosa cells are less compact and start to expand.**



Cat. 3) Cumulus consists of more than 5 layers. **Granulosa cells are expanded**



Cat. 4) Cumulus consists of **less than 5 layers.**



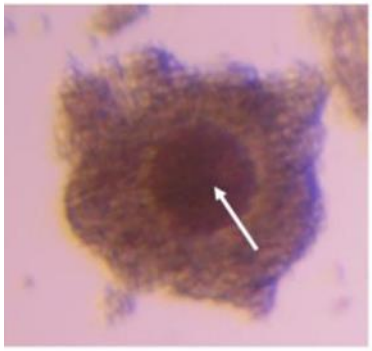
Cat. 4) Granulosa cells are **not completely surrounding the oocyte.**

II. Ooplasm morphology

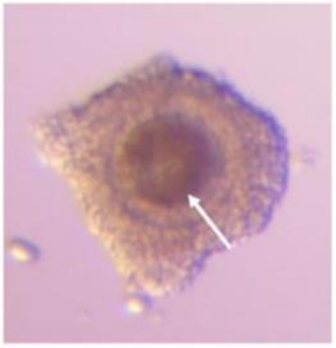
Cat. 1) Ooplasm is **homogeneously dark**

Cat. 2) Ooplasm is **dark and slightly granular** (acceptable granularity)

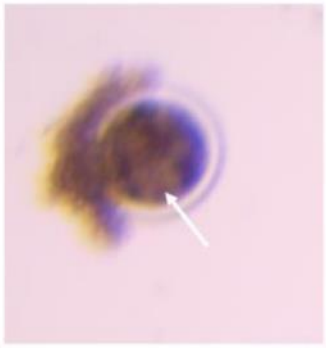
Cat. 3) Ooplasm is **heterogeneous** (mix of dark and pale areas, the granularity is non-acceptable).



Cat. 1) Ooplasm is **homogeneously dark**



Cat. 2) Ooplasm is **dark and slightly granular**



Cat. 3) Ooplasm is a **heterogeneous** mix of dark and pale areas

Grade	Description
1	Many layers of tight cumulus surrounding the oocyte. Cytoplasm is homogeneous with fine granulation in a light even greyish colour.
2	Cumulus layers are less compact and fewer. The ooplasm is slightly coarser, still even in coloration.
3	Corona only, zona pellucida may be exposed. The edge of the cytoplasm can be darker, uneven in colour.
4	Naked Oocyte with little to no cumulus cells. Cytoplasm is still ok, but usually uneven, can be lightly spotted with moderate size granules or with a darker zone around the periphery
5	Expanded cumulus with degenerated cells as satellites, the cytoplasm looks very pale with a darker edge, very pigmented dark black, brown, or dark clusters (clumping of organelles), and indicates a progression towards degeneration.
6	Degenerate. Oocyte cytoplasm is degenerate/absent/lysed. This grade also includes atretic, expanded oocytes, and any empty zonas

Figure IV.1: Grading of bovine oocytes. Categories of cumulus and ooplasm morphologies with overall grading guide. In house grading constructed from [167; 168]

Appendix V: Bovine embryo development and grading

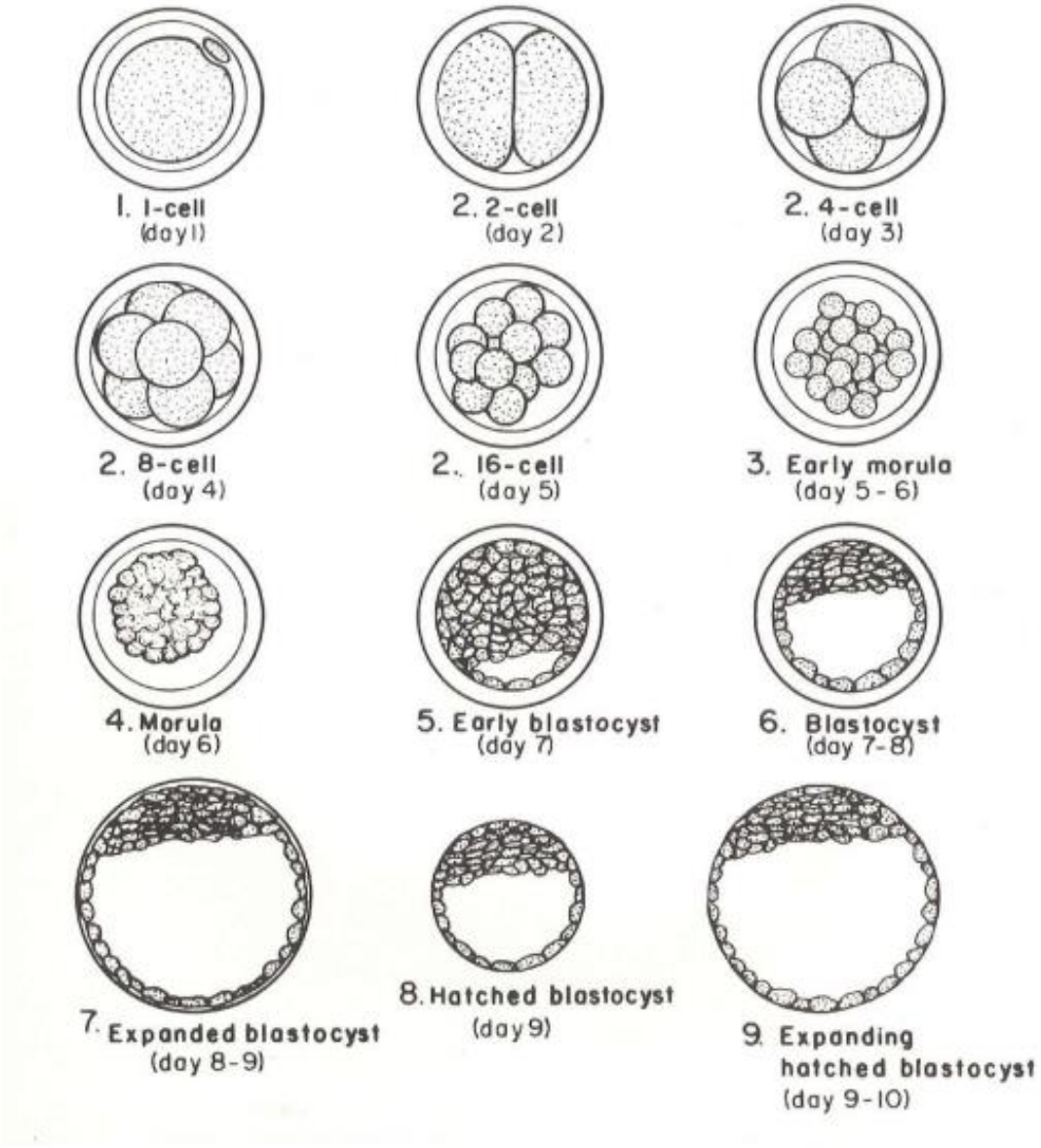
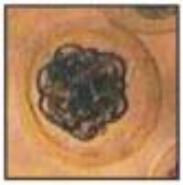


Figure V.1: Developmental stage of bovine embryo. Development starting from IVC (day 1) to an expanded hatched blastocyst. Image from international embryo grading guide [39].

BOVINE EMBRYOS: EXAMPLES OF DEVELOPMENTAL STAGE AND QUALITY

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

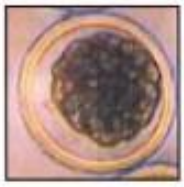


Cycle day: 6.5
Stage Code: 3
Quality Code:1
Comments: a

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

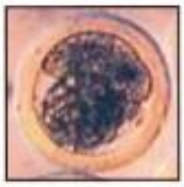
Cycle day: 7
Stage Code: 4
Quality Code: 1
Comments: d

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

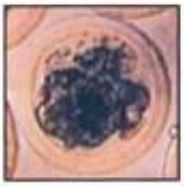
Comments:

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.

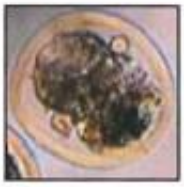
BOVINE EMBRYOS: EXAMPLES OF DEVELOPMENTAL STAGE AND QUALITY

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

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



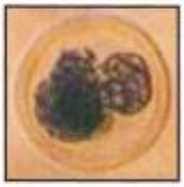
Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g



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



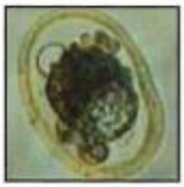
Cycle day: 7
Stage Code: 4
Quality Code: 3
Comments: f, g, h

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:

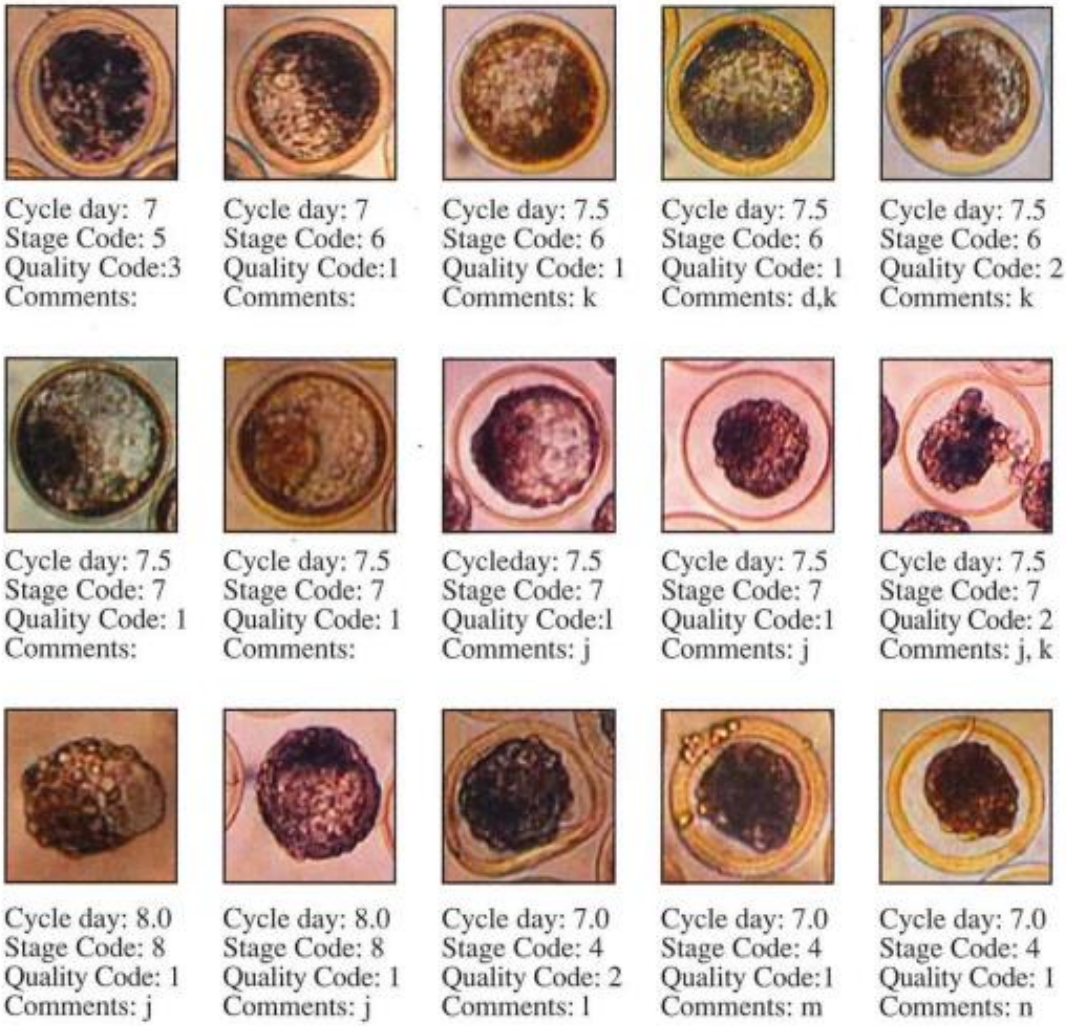
Cycle day: 7
Stage Code: 5
Quality Code:2
Comments:

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.

BOVINE EMBRYOS: EXAMPLES OF DEVELOPMENTAL STAGE AND QUALITY



Comments:

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

Figure V.2: Examples of bovine embryos at various stages and qualities. Images with an affiliated grade as a guide to standardise grading. Grading determined by international embryo grading guide [39].

Appendix VI: Cell transfer record sheet

Experiment : **Run Number :**

IVM 19/05/2022	**Cloning solutions**					**NT** 20/05/2022
Time :	Solution	Fresh ?	Date Thawed	Date made	Batch person	Time out IVM:
						Oocyte breakdown
Person(s) :	H199 - FCS					Homo +PB:
No. ovaries :	HSOF					Homo -PB:
	HSOF-BSA					Hetero:
No. oocytes :	HSOF-Ca-BSA					Dead:
	ESOF-Ca-BSA					Enucleator(s):
Mother age :	SOF					
	FCS					
Abattoir:	BSA					Culture system:
	Scriptaid					
Approx. collection time :	Lectin					
	6-DMAP					Experiment comments:
No. with CL:	Ionomycin					
	Hyaluronidase					
Comments:	Pronase					
	FB					

24hr C/O 21/05/2022							**D5 C/O** 25/05/2022						
Group	Lysed	1-c	2-c	3-c	>3c	Frag	Group	Lysed	1-c	>1-c	Frag	TM	B

End of Culture Date : 26/05/2022 Grader:

	Total		1-cell	Total		1-cell	Total		1-cell

Appendix VII: Fusion record sheet

FBA Zona-Free

Experiment:_____

Date:_____

Cell information:_____

Cell cycle synchronisation: _____

Drop	Plate	SC/ DC	No. Cytos	Time Fused	Cytoplast lysed PF	Donor lysed PF	No. Fused	No. not fused	Comments

Fusion Parameters

	Parameter 1	Parameter 2	Parameter 3
Amplitude			
µsec			
PF AC time			

Fusion rate

Group	No. fused	Total	Percent
Total			

Group	No. into ESOF –Ca	Time into ESOF – Ca	No. for Act.	Time HSOF + 1mg/ml	Time Iono.	No. into DMAP	Time into DMAP	No. into IVC	Time into IVC
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		:
		:		:	:		:		: