The Derivation and Characterisation of Bovine Embryonic Stem Cells

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By Nicole Waldron

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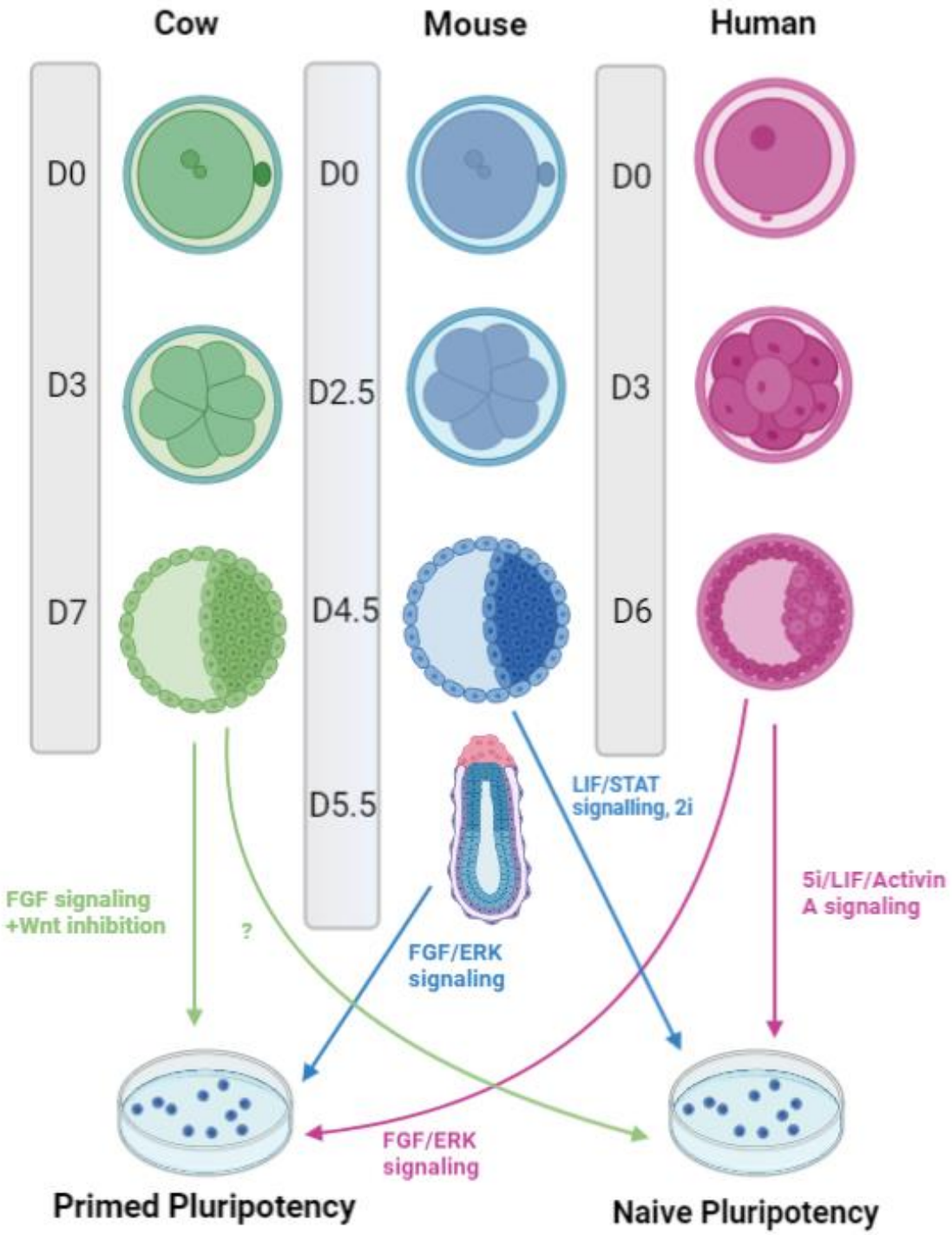
Abstract

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of preimplantation embryos. ES cells exhibit two common characteristics: the ability to differentiate into all three germ layers, and the ability to self-renew. Scientists first discovered how to derive embryonic stem cells from mouse embryos 40 years ago, in 1981. Almost 20 years later, in 1998, embryonic stem cells were first isolated from humans. Mouse and human embryonic stem cells have been used in a vast array of important research, including regenerative medicine and cancer research. However, the successful isolation and subsequent culture of bovine embryonic cells has proven more difficult. For a long time, embryonic stem-like cells cultured from bovine embryos showed poor derivation efficiencies, limited proliferation, and progressive loss of pluripotency markers. Following decades of attempts, embryonic stem cells were derived from cattle and maintained in a pluripotent state for the first time in 2018.

This thesis presents an exploration into the methods used to derive bovine embryonic stem cells, and the culture conditions needed to sustain their self-renewal in a pluripotent state. Outgrowths of the ICM were cultured in conditions which promoted proliferation, and were able to be sustained for upwards of 25 days. Characterisation of these cells using immunohistochemistry and RT-qPCR analysis showed expression of pluripotency related genes, however this expression was limited and appeared to reduce with passaging. The results of this research suggest that bovine ES cells are able to be maintained in similar culture conditions to porcine expanded potential stem cells, however pluripotency is only able to be maintained for a limited number of passages. Consequently, further research is needed in order to fully understand the regulatory mechanisms surrounding pluripotency states in bovine species.


Cow
Mouse
Human
D0
D3
D7
D0
D2.5
D4.5
D5.5
D0
D3
D6
FGF signaling
+Wnt inhibition
?
FGF/ERK
signaling
LIF/STAT
signalling, 2i
5i/LIF/Activin
A signaling
FGF/ERK
signaling
Primed Pluripotency
Naive Pluripotency

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List of Abbreviations

Abbreviation	*Term*
ESC	Embryonic stem cell
iPSC	Induced pluripotent stem cell
bESC	Bovine embryonic stem cell
hESC	Human embryonic stem cell
mESC	Murine embryonic stem cell
EPSC	Expanded potential stem cell
EpiSC	Epiblast-primed stem cell
pEPSC	Porcine expanded potential stem cell
bEPSC	Bovine expanded potential stem cell
mESC	Murine expanded potential stem cell
IVM	In vitro maturation
IVF	In vitro fertilisation
IVC	In vitro culture
COC	Cumulus-oocyte complex
TE	Trophectoderm
ICM	Inner cell mass
BSA	Bovine serum albumin
SCNT	Somatic cell nuclear transfer
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Complimentary deoxyribonucleic acid
FITC	Fluorescein isothiocyanate
DAPI	4', 6-Diamidino-2-phenylinodole
PBS	Phosphate-buffered saline
qPCR	Quantitative polymerase chain reaction
DMSO	Dimethyl sulfoxide

Chapter One: Introduction

1.1 Overview of Stem Cells

Stem cells are founder cells capable of developing into many different types of specialised (differentiated) cells in the body (Gepstein 2002, Evans and Kaufman 1981, Martin 1981, Thomson 1998). They have the ability to divide and replicate themselves for an extended, or indefinite, period of time. All stem cells are able to self-renew and differentiate, however, beyond these two capabilities the various types of stem cells have distinct potencies. There are three main types of stem cells: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells (Table 1). ESCs and iPSCs are both pluripotent, meaning they are capable of differentiating into cells of all three germ layers in the body (Zakrzewski et al. 2019, Beddington and Robertson 1989). In contrast, adult stem cells are multipotent, exhibiting limited differentiation abilities, as well as limited self-renewal capabilities (Verfaillie 2002). Understanding the mechanisms underlying the maintenance of stem cells, and the reprogramming of somatic cells into a pluripotent state is important for the safe and effective utilization of stem cells in clinical applications. However, our knowledge regarding these mechanisms is incomplete, and the nature of this remarkable pluripotent cellular state has become one of the central questions of developmental biology (Liu et al. 2020).

Adult stem cells are tissue specific stem cells which can be found throughout the body. These multipotent cells are capable of forming only a limited amount of cell types, which develop into a specific tissue or organ (Cable et al. 2020). Adult stem cells have been used therapeutically since the 1960's, with the most common procedure performed being bone marrow transplantation (Verfaillie 2002). In this procedure stem cells are extracted from the bone marrow and transplanted into another area of the patient's body; this procedure has been successfully utilised to treat some types of cancer including leukaemia and lymphoma, as well as other hematopoietic conditions (Prentice 2019, Dessie et al. 2020). Adult stem cells have also been used to treat neurological conditions and are showing promising results in clinical trials of patients with type I diabetes, spinal cord injuries and multiple sclerosis (Aly 2020). The remarkable success seen by the therapeutic use of adult stem cells hints at how much more powerful pluripotent stem cell technology could be therapeutically, given that these cells are able to give rise to a greater variety of cells. Consequently, pluripotent stem cell research is currently one of the most up-and-coming areas of biomedical research.

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are both pluripotent stem cells which exhibit the same characteristics, however, the two types of cells are derived from different sources (Figure 1). Embryonic stem cells are derived from the inner cell mass of a blastocyst

stage embryo, whereas iPSCs are derived from somatic cells which have been reprogrammed into a pluripotent state (Liang and Zhang 2013). Somatic cells can be induced to acquire a pluripotent identity through somatic cell nuclear transfer (SCNT), cell fusion, or the ectopic expression of pluripotency transcription factors (Takahashi and Yamanaka 2006). Both types of pluripotent cells have the potential to differentiate into derivatives of all three germ layers: endoderm, ectoderm, and mesoderm (Mahla 2016). This ability has been harnessed to direct pluripotent cells to differentiate into specialised cell types such as mature neurons (Reubinoff et al. 2001) and cardiomyocytes (Kehat et al. 2001).

From a clinical perspective, iPS cells pose some potential advantages over ES cells. First, the ability to reprogram somatic cells may allow for the production of pluripotent cells for a specific individual and their subsequent use in personalised medicine. Embryonic stem cells pose the risk of autologous rejection if used therapeutically, as they will have been derived from an embryo in the lab, rather than from the patient being treated (Deinsberger, Reisinger and Weber 2020). In one clinical trial which used hESCs to treat ischemic left ventricular dysfunction, 50% of the patients experienced alloimmunity (Menasché et al. 2018). In this case the alloimmunity was clinically silent and caused no complications, however it would be preferable to use iPSCs, derived from the patients themselves, to avoid this risk. The further advancement of iPSC technology may see the generation of organ transplants for individual patients, removing the risk of immune rejection and eliminating the need for organ donors (Platt and Cascalho 2013). Secondly, the use of iPSCs poses fewer ethical concerns than the use of embryonic stem cells as they can be extracted from adult cells, rather than embryos (Zacharias et al. 2011). Currently clinical trials using pluripotent cells are being conducted, many of them focused on treating ophthalmologic diseases such as macular degeneration (Mandai et al. 2017, Mehat et al. 2018). As of 2021, very few clinical trials have published results as this is a very new field of regenerative medicine, however this number will increase as more trials come to completion (Deinsberger et al. 2020).

Type	Characteristic	Example
Totipotent	Able to differentiate into all cell types including the extra-embryonic tissue, therefore able to form a fully functional complete organism	Blastomeres composing the zygote
Pluripotent	Able to differentiate into all three germ layers (endoderm, ectoderm, mesoderm), therefore able to form any of the foetal or adult cell types	ES cells, iPS cells
Multipotent	Able to differentiate into only a limited number of cell types	Adult stem cells
Oligopotent	Able to terminally differentiate into cells of a specific tissue	Lymphoid stem cell
Unipotent	Able to differentiate into a single type of cells	Muscle stem cells

Table 1. Characterisation of Stem Cells Based on Their Potency and Differentiation Potential.

1.2 The Origin of Embryonic Stem Cells: Early Mammalian Embryonic Development

To understand the nature of embryonic stem cells it is necessary to understand the early development of an embryo. Embryonic development begins when an oocyte is fertilised by a sperm in the oviduct. The first cleavage divisions occur rapidly as the embryo travels down the oviduct and into the uterus (Valadão, Moreira Da Silva and Moreira Da Silva 2019). Each cell of the cleavage-stage embryo, termed a blastomere, is totipotent, meaning it has the potential to form all cell types of not only of the embryo (i.e. the three germ layers), but also all cell types constituting the extra-embryonic tissues. This has been demonstrated by showing that individual blastomeres from a 4-cell or 8-cell embryo are able to develop normally and form an adult (Willadsen 1981, Johnson et al. 1995). At the 8-cell stage in mice, or the 32-cell stage in cattle, the blastomeres maximise contact with one another and compact to form a morula (Soom et al. 1997, Turlier and Maître 2015). After compaction a cavity, termed the blastocoel, develops and expands resulting in the inner cells being pushed to one side of the embryo. At this stage in development the embryo is known as a blastocyst. The inner cells, termed the inner cell mass (ICM), are apolar and are morphologically different from the outer cells (Pfeffer 2018). The outer cells, termed the trophectoderm (TE), are polarised and form an epithelium. The decision to form either an ICM or TE cell is known as the first lineage decision, and represents the first restriction in developmental potential (Mihajlović and Bruce 2017). The cells which compose the ICM are pluripotent and possess the ability to form all three germinal layers (Smith 2017). The trophectoderm cells are fated to give rise to the extra-embryonic placenta, and are unable to give rise to cells which will form embryonic tissue (Valadão et al. 2019). Upon the second lineage decision the ICM lineage bifurcates to form either the hypoblast or the epiblast, which form the yolk sac and the embryo proper, respectively (Rossant 2018). The blastocyst continues to grow and will hatch out of the zona pellucida, and prepare for gastrulation.

Embryonic stem cells are derived from the inner cell mass or epiblast of the embryo (Figure 1.) When these cells are cultured under appropriate conditions, they can continue to proliferate indefinitely, whilst maintaining capacity for pluripotency. There are distinct phases of stem cells which correlate with different development stages of the embryo, and with a reduction of developmental potential as cellular fate becomes more restricted.

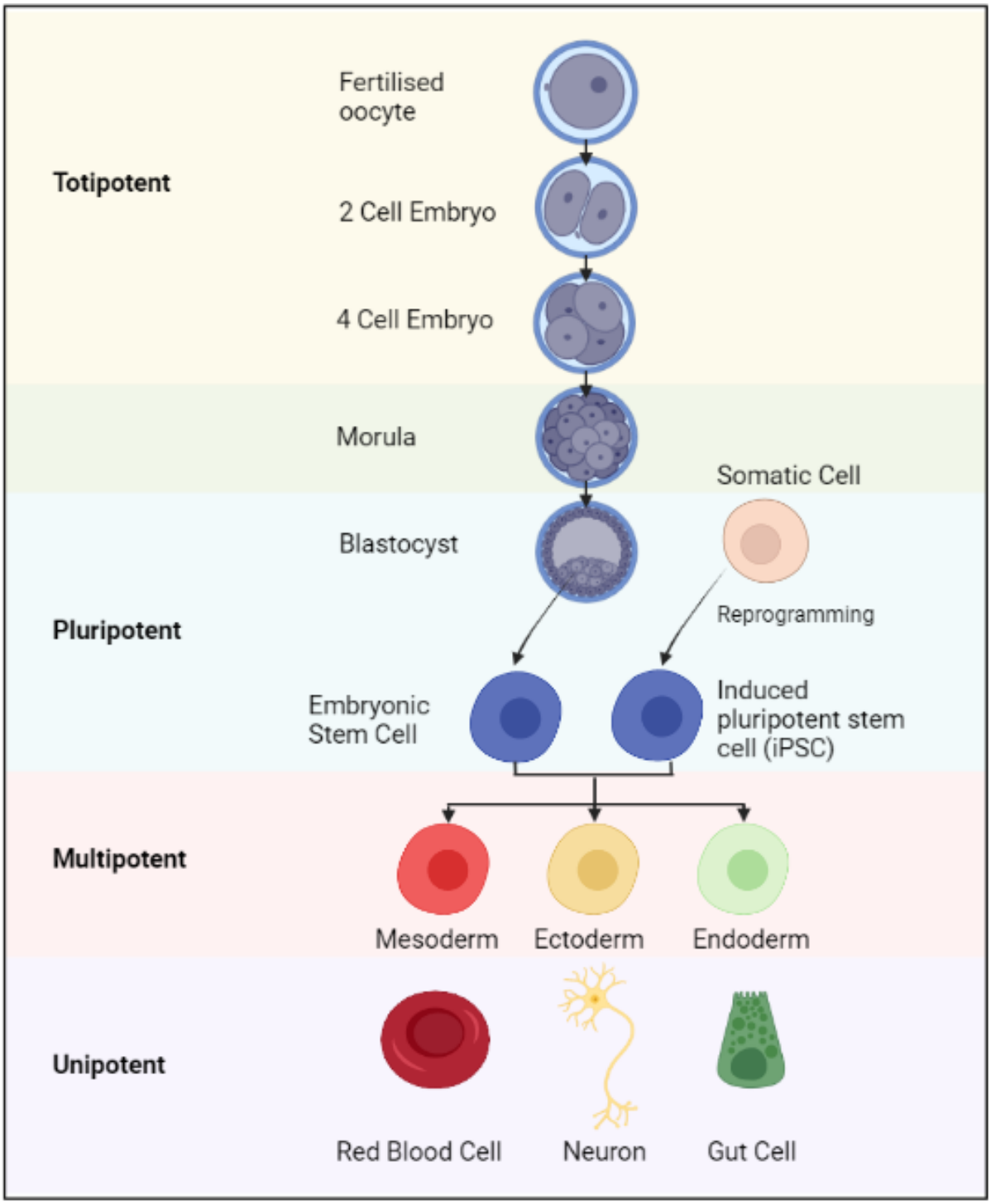

Figure 1. Diagram depicting early embryonic development up until the blastocyst stage and the subsequent derivation of embryonic stem cells (ESCs).

1.3 Naïve Vs Primed Stem Cells

There are two distinct states of pluripotent cells: naïve and primed. Both naïve and primed stem cells are able to self-renew and are capable of differentiating into the three germ layers in vitro (Brons et al. 2007, Theunissen et al. 2014). However, primed stem cells are more limited in their developmental potential, as unlike naïve stem cells, they are unable to give rise to germline chimeras, and are slightly fate-biased (Kumari 2016, Smith 2017). Characteristic of the late epiblast, primed stem cells are "primed" for gastrulation, and thus for generation of endoderm and mesoderm (Beddington and Robertson 1989).

There are several morphological and physiological differences between naïve and primed pluripotent stem cells (Table 2), and these can be used as hallmarks to help categorise stem cells (Boroviak and Nichols 2017). Morphologically they can be easily distinguished, as naïve cells tend to grow as small, compact, domed colonies, whereas primed stem cells tend to grow in large monolayer colonies (Brons et al. 2007). The change that is seen in DNA methylation patterns throughout development is reflected by naïve and primed state cells. Representative of the early epiblast, naïve state stem cells exhibit global DNA hypomethylation. The progression of embryonic development, and the subsequent exit from naïve pluripotency, is accompanied by progressive restriction of chromatin accessibility, and a greater prevalence of repressive histone marks (Kumari 2016). Consequently, primed state stem cells exhibit patterns of DNA hypermethylation. Over prolonged growth and expansion, naïve stem cells maintain global transcriptome proximity to the early epiblast, whereas the transcriptome of primed stem cells is more similar to the late epiblast (Smith 2017, Guo et al. 2021). Another epigenetic distinction that can be made between female pluripotent stem cells is the status of the X chromosome. An early developmental event that occurs in female embryos is the inactivation of one X chromosome (Geens and Chuva De Sousa Lopes 2017). Consequently, primed cells have one inactivated X chromosome whereas, both X chromosomes of naïve state cells remain transcriptionally active. Additionally, naïve and primed stem cells utilise different methods of generating energy; naïve stem cells use oxidative phosphorylation and glycolysis, whereas primed stem cells generate energy though glycolysis only (Kumari 2016).

The different states of stem cells are best characterised in mice; naïve stem cells represent the state of the pre-implantation epiblast, whereas primed stem cells represent the state of the post-implantation epiblast (Boroviak and Nichols 2017). Murine naïve stem cells are able to form cell types of all three germ layers; however they do not have the ability to follow the trophectoderm lineage, and have a strongly reduced ability to differentiate into hypoblast (Posfai et al. 2021,

Beddington and Robertson 1989). Therefore, such embryonic stem cells are thus developmentally equivalent to the late ICM/early epiblast (Smith 2017). In contrast, murine primed stem cells are more characteristic of the late epiblast, where the embryo is about to begin gastrulation.

	Naïve	**Primed**
Developmental equivalence	Early epiblast	Late epiblast
Chimera-forming ability	Yes	No
Cellular respiration profile	Oxidative phosphorylation, glycolysis	Glycolysis
X-chromosome status	XaXa (both active)	XaXi (one active, one inactive)
Global DNA methylation	Hypomethylation	Hypermethylation

Table 2. Characteristics of naïve and primed pluripotent cell states

1.4 Progression of Embryonic Stem Cell Research

Embryonic stem cells were first derived from mice in 1981 (see Figure 2 for a timeline). These cells were demonstrated to have the ability to form cell types of all three germ layers; however they did not possess the ability to follow the trophectoderm lineage, and had a reduced ability to differentiate into the hypoblast (Evans and Kaufman 1981, Martin 1981). Although the terminology had not yet been established, these cells would later be characterised as naïve pluripotent cells. In 1998 ES cells were derived from humans (Thomson 1998). From the start it was clear that there were some differences between these cells and mESCs; they had a markedly different morphology, were less amenable to passaging, and had different growth requirements (Eguizabal et al. 2019). Initially, it was believed that these differences were species specific, and consequently hESCs were thought to be equivalent to mESCs (Takashima et al. 2014). However, it didn't take long to retract this assumption, as it became evident that hESCs rely on different signaling pathways than mESCs to maintain pluripotency (Nichols and Smith 2009, Vallier, Alexander and Pedersen 2005).

In 2007, an alternative state of murine pluripotent stem cells was derived, these cells were observed to share many characteristics with hESCs, and reflected the late-epiblast stage of the mouse embryo (Brons et al. 2007, Tesar et al. 2007). It was at this time that the terminology for naïve and primed cells was developed; this newly derived state of cells was classified as epiblast primed (EpiSCs), as transcriptome analysis showed them to reflect the late-epiblast stage of embryonic development (Nichols and Smith 2009). During the same year, induced pluripotent stem cells (iPSCs) were derived from human fibroblasts, and these cells showed remarkably similar characteristics to hESCs (Takahashi et al. 2007). Similar to murine EpiSCs, hESCs and human iPSCs are unable to efficiently contribute to chimeras following blastocyst injection, and have reduced expression of genes associated with ground state pluripotency (Kojima et al. 2014). Therefore, the current consensus is that human ES cells and human iPSCs are primed stem cells as they more closely resemble murine EpiSCs than naïve mESCs.

2010 saw the first report of hESCs being reverted to a naïve state (Hanna et al. 2010). The cells had been epigenetically modified to have a similar gene expression prolife and active signaling pathways to naïve mESCs, as well as two active X chromosomes in female cells; however they required continual transgene expression to remain in this state (Hanna et al. 2010). Several lab groups have since generated culture conditions that support the retention of the human pluripotent stem cells in the naïve state (Gafni et al. 2013, Theunissen et al. 2014, Takashima et al. 2014, Ware et al. 2014). These cells represent the early epiblast, and meet the mouse criteria for naivety (Dodsworth et al. 2020, Ware et al. 2014).

In 2017, the first report of expanded potential stem cells being derived was published. Expanded potential stem cells have the developmental potential of a totipotent blastomere, and can consequently differentiate to form both the embryo proper and the extraembryonic exoderm (Yang et al. 2017a). These cells were first established in mice and humans, and two years later there were further reports of porcine expanded potential cells being derived (Gao et al. 2019, Yang et al. 2017b, Yang et al. 2017a). However in 2021, further studies analysing the transcriptome of these cells showed that these 'expanded potential' cells were in fact more similar to epiblast primed stem cells (Posfai et al. 2021, Guo et al. 2021).

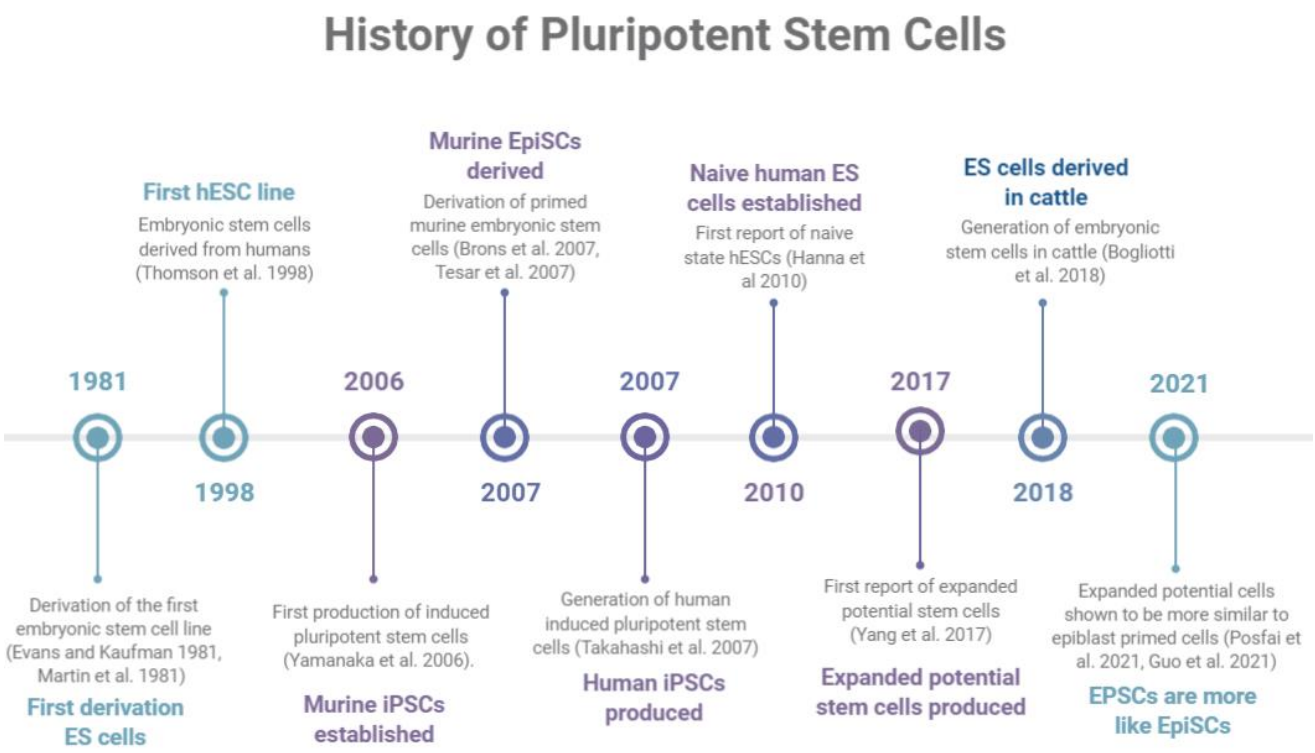

Figure 2. Timeline showing many of the major milestones associated with the culture of pluripotent stem cells.

1.5 Culture Conditions for Pluripotent Cells

The combination of factors required for undifferentiated proliferation is distinct for different states of pluripotent stem cells, each has dependence on distinct growth factors. Naïve mESCs rely on leukaemia inhibitory factor (LIF) and inhibitors for MEK and GSK3 for maintenance of naïve pluripotency, a condition known as LIF/2i. LIF supports the self-renewal of naïve mESCs by activating STAT3, which in turn activates the transcription of several genes involved in inhibiting differentiation (Graf, Casanova and Cinelli 2011). The essential function of STAT3 is evident as mESCs are able to self-renew without the addition of LIF when STAT3 is overexpressed, indicating that it is the activation of STAT3 via LIF acting to promote self-renewal (Bourillot et al. 2009).

After the establishment of mESCs, the same methods and culture conditions were used to attempt the derivation of embryonic stem cells from humans. However, the combination of factors used to culture mESCs was found to be ineffective for human ES cells and did not result in cell proliferation (Moore 2006). After many years it was found that the LIF/STAT3 pathway is inactive in hESCs, and that instead activin/nodal and FGF2 signaling is required to maintain pluripotency. Later, similar culture conditions were found to support the growth of murine pluripotent cells in a primed state (Tesar et al. 2007, Brons et al. 2007). FGF is thought to support the self-renewal of primed pluripotent cells via its downstream upregulation of MEK. In contrast to primed cell types, FGF2-induced activation of MEK signaling causes differentiation of naïve mESCs (Kumari 2016). Consequently, naïve and primed pluripotent cells can be distinguished by their ability or failure to maintain self-renewal upon inhibition of MEK signaling. When MEK signaling is inhibited, naïve pluripotent cells will maintain self-renewal, whereas primed pluripotent cells will not maintain self-renewal (Weinberger et al. 2016).

It took many attempts to establish the culture conditions required to sustain the growth of human pluripotent stem cells in a state which resembles naïve murine embryonic stem cells (Table 3.) Gafni, Chan, and Ware were the first labs to formulate culture conditions which were able to maintain human pluripotent stem cells in their naïve state. Such conditions used media supplemented with FGF2 and various inhibitors for MEK, GSK3, BMP, JNK and p38 (Gafni et al. 2013, Chan et al. 2013, Ware et al. 2014). However, all of these culture conditions included FGF2, a growth factor associated with primed pluripotency. The first demonstration of naïve pluripotency in conditions without FGF2 was reported by Theunissen in 2014. This culture condition is referred to as 5i/LIF, and consists of a N2B27 medium supplemented with inhibitors for MEK, GSK3, ROCK, SRC and BRAF kinases, as well as supplementation with LIF and activin (Theunissen et al. 2014).

The concept of expanded potential stem cells was introduced in 2017 when the Deng and Lui labs published reports of the derivation of mouse cells having bi-potential for embryonic and extra-embryonic lineages in vivo (Yang et al. 2017b, Yang et al. 2017a). This was achieved using a chemical cocktail which sought to inhibit blastomere differentiation by inhibiting MAPK, Src and Wnt/Hippo/TNKS1/2; pathways thought to be involved in the first lineage decision. In 2019, the Liu lab published another report of expanded potential cells being derived in humans and porcine species (Gao et al. 2019). However, in 2021 further analysis of the 'expanded potential' cells derived in the Lui and Deng labs revealed that they were in fact more similar to epiblast-primed cells (Posfai et al. 2021). Posfai et al. performed bulk RNA sequencing (RNA-seq) and single-cell RNA sequencing to compare the transcriptome of the 'expanded potential stem cells' to ESCs and embryos. The analysis showed that the transcriptional signature of the murine cells derived by the Lui lab matched embryonic day 3.5-4.5 of development, similar to that of naïve state embryonic stem cells. The majority of cells derived by the Deng laboratory showed a transcriptional signature similar to embryonic day 5.5 of development, correlating with the transcriptome of late epiblast and primed stem cells. However, a small number of cells were positioned within the extraembryonic and hypoblast cluster. It is possible that the transcriptome of this small number of cells caused the researchers to believe that the cells had a bipotential for both the embryonic and extra embryonic lineages, despite stating in their own paper that the inclusion of LIF in the culture media can promote the rare expression of totipotent cells (Yang et al. 2017a). The analysis carried out by Posfai et al. suggests that these cells align with a pluripotent cell state, rather than a totipotent state (Posfai et al. 2021).

Regarding cattle stem cells, in 2018 Bogliotti and colleagues published the first report of embryonic stem cells being derived in bovine species, using a medium containing FGF2 and a canonical WNT inhibitor, IWR1. These conditions differ from naïve human and murine pluripotent cells, which activate WNT signaling through inhibition of GSK3, an inhibitor of WNT. The cells derived by Bogliotti are more characteristic of epiblast primed cells, as evidenced by their cell morphology and transcriptome analysis (Bogliotti et al. 2018).

Species	Term/Author	Definition
Mouse	2i/LIF conditions	Defined naive pluripotency growth conditions for naïve mESCs. Medium supplemented with LIF, containing inhibitors for MEK and GSK3.
Mouse	FGF2/Activin A conditions	Defined primed pluripotency growth conditions for mouse epiblast stem cells, composed of recombinant fibroblast growth factor 2 (FGF2) and Activin A cytokines.
Mouse	FGF2/IWR1 conditions	Defined primed pluripotency growth conditions for mouse epiblast stem cells, containing recombinant fibroblast growth factor 2 (FGF2) and the small-molecule tankyrase inhibitor, IWR1.
Human	Gafni	Naïve pluripotency growth conditions for human pluripotent stem cells developed by Gafni et al. 2013. Medium supplemented with FGF2, TGFB, LIF and containing inhibitors for MEK, GSK3, JNK, p38, PKC and ROCK.
Human	Ware	Naïve pluripotency growth conditions for human pluripotent stem cells developed by Ware et al. 2014. Medium supplemented with FGF2 and LIF, and containing inhibitors for MEK and GSK3.
Human	3i/LIF conditions	Naïve pluripotency growth conditions for human pluripotent stem cells developed by Chan et al. 2013. Medium supplemented with LIF and FGF2 and containing three inhibitors for MEK, GSK3 and BMP4.
Human	5i/LIF/FA conditions	Naïve human pluripotency growth conditions developed by Theunissen et al 2014. The medium contains 5 inhibitors for MEK, GSK3, ROCK, SRC and BRAF, is supplemented with LIF, FGF2, and activin A.
Mouse	Yang	Expanded potential growth conditions developed by Yang et al. 2017. Base medium supplemented with LIF and inhibitors for GSK3, ERK, SRC, JNK, p38 and tankyrases.
Mouse + Human	LCDM conditions	Expanded potential growth medium developed by Yang et al. 2017. Base medium supplemented with LIF, a GSK3 inhibitor, and small molecules dimethindene maleate and minocycline hydrochloride.
Bovine	Bogliotti	Bovine embryonic stem cell growth conditions. DMEM/F12 medium supplemented with FGF2 and IWR1.
Porcine	Gao	N2B27 base medium supplemented with LIF, activin A, and inhibitors for SRC, GSK3, and tankyrases.

Table 3. Growth mediums developed for pluripotent/totipotent cell types.

1.6 Markers of Pluripotency

With any new embryonic stem cell line, it is important to characterise the line and confirm pluripotency. Common methods used for pluripotency confirmation and characterisation are chimera and teratoma assays, immunofluorescence, and quantitative PCR, which looks for the upregulation of genes associated with pluripotency (Nelakanti, Kooreman and Wu 2015).

1.6.1 Teratoma Formation

A teratoma is a non-malignant tumour composed of cells from all three of the germ layers (Wesselschmidt 2011). A classical method used in mice for evaluating pluripotency is to test the in vivo capabilities of the cell line to form all three germ layers by performing a teratoma assay (Nelakanti et al. 2015). The putative pluripotent cells are injected into an immunocompromised mouse where they may proliferate to form a teratoma. After several weeks, the growth is extracted and analysed by immunofluorescent staining to verify that cell types of all three germ layers have formed (Nelakanti et al. 2015).

1.6.2 Chimera Assay

A chimera is an organism composed of two or more distinct populations of cells, derived from different zygotes (Polejaeva and Mitalipov 2013). In a chimera assay small clumps of cells are injected into embryos, either in vivo or in vitro. The level of genetic contribution to a chimera depends on the developmental potency of the cells. Totipotent cells are able to contribute to both extra-embryonic and embryonic lineages, whereas pluripotent stem cells are only able to give rise to the embryonic lineages (Beddington and Robertson 1989). The more restricted developmental potential of epiblast primed stem cells is reflected in their more limited capacity to be able to contribute to chimeras, therefore this is one way to differentiate between naïve and primed stem cells (Polejaeva and Mitalipov 2013).

Figure 3. Example of a chimeric mouse

1.6.3 Marker Expression

Transcription factors impact the transcriptional and epigenomic landscape, and control the establishment and maintenance of gene regulatory networks active in individual cell types. Oct4, Sox2, and Nanog are key transcription factors associated with pluripotency; the expression of these markers seems to be conserved in all mammalian pluripotent cells. Together, these three genes control ground state pluripotency, therefore their expression must be established before characterizing a cell as pluripotent. The knockdown of any of these genes causes the differentiation of ES cells; Oct4 or Sox2 knockdown directs cells down the trophectoderm lineage, whereas knockdown of Nanog causes differentiation to the endoderm (Khan et al. 2012). Upregulation of these transcription factors can also hinder pluripotency, as demonstrated by the overexpression of Oct4 in mESCs inducing the cells to adopt a primitive endoderm activity (Niwa, Miyazaki and Smith 2000).

In mice, Oct4 is ubiquitously expressed in all cells of the embryo until the blastocyst stage, where it becomes downregulated in the trophectoderm, and eventually expression is restricted to the inner cell mass (Khan et al. 2012). If Oct4 expression is lost in early development this causes the cells of the embryo to acquire a trophectodermal fate (Kim et al. 2009). Oct4 and Cdx2, a trophectoderm marker, autoregulate themselves by upregulating the expression of themselves, and turning off the expression of the opposing gene. However, bovine embryos are distinct from mouse embryos in the way that Oct4 expression is not restricted to the inner cell mass until later in development (Berg et al. 2011). A likely explanation is that implantation into the endometrium takes place at a later stage in cattle, consequently there is no need to specify the outer cells early on.

Sox and Oct4 dimerize, and induce the expression of Nanog, the expression of which is restricted to the inner cell mass in bovine embryos (Khan et al. 2012). Nanog, along with Sox2, are two genes which are among the first to be uniquely expressed in the inner cells of 16-24 cell “morula-stage” embryos. Cells comprising the inner cell mass initially co-express both the epiblast marker Nanog and the hypoblast marker Gata6 (Saiz et al. 2016). At the late blastocyst stage the expression of these markers becomes restricted so that each ICM cell now either exclusively expresses Nanog or Gata6. The expression of these markers does not seem to be localised and can be described as a ‘salt and pepper’ type pattern (Pfeffer 2018). Culturing cells positive for only one marker under alternative signaling environmental conditions does not change the expression of Nanog or Gata6, suggesting that commitment to either the epiblast or hypoblast is final once an ICM cell expresses one marker in a mutually exclusive fashion (Saiz et al. 2016, Xenopoulos et al. 2015).

1.6.4 Alkaline Phosphatase Activity

Alkaline phosphatase (AP) is a membrane bound enzyme found in almost all living organisms. The enzyme is expressed at a high level in the inner cell mass of an embryo, from which embryonic stem cells are derived. Consequently, a high level of AP is expressed in embryonic stem cells and this makes alkaline phosphatase activity a suitable marker of pluripotency (Štefková, Procházková and Pacherník 2015). A high level of AP correlates with an undifferentiated pluripotent phenotype. As developmental potential becomes restricted, the level of alkaline phosphatase activity decreases, and eventually becomes absent. Therefore reduced or depleted levels of AP denotes the restriction of pluripotency (Tesar et al. 2007). Alkaline phosphatase activity has been shown to be present in the late epiblast, however upon culture of epiblast cells AP activity becomes undetectable. Therefore a lack or reduced expression of AP activity may be representative of epiblast-primed stem cells, and does not necessarily denote a lack of pluripotency (Brons et al. 2007).

1.7 Rationale and Objectives

Producing embryonic stem cells from large livestock species such as cattle is important for genome engineering and use in developmental models. Bovine ES cells have the potential to dramatically improve breeding of cattle in a short amount of time if research advances enough that bovine sperm and egg cells can be generated from stem cell lines. Cattle with genetically superior traits such as greater production of milk, emission of less methane, or greater resistance to disease will be able to be generated in a much shorter length of time than using classical breeding methods.

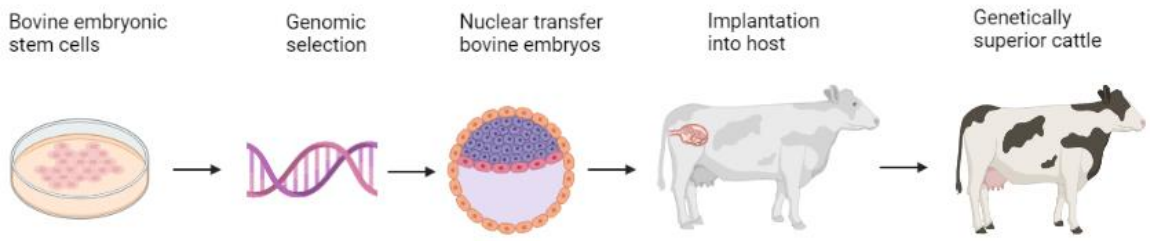

Figure 4. Diagram showing the strategy of using bESCs for genomic selection to produce animals of superior genetic value through bESC derivation and nuclear transfer.

Research using human embryos is limited due to ethical restrictions. In New Zealand, only non-viable embryos produced through IVF are able to be used for research (Auckland University, 2018). Consequently, animal models are frequently used as a model of human development. Much of developmental research has been focused on the easily kept laboratory models: mouse, rat, and rabbit. The mouse model especially has been the model organism of choice due to the mice's easy accessibility and small size. However, advancement of molecular technologies is revealing that early mouse development is atypical of most mammals, and indicates that alternative animal models may be helpful to learn more about human development (Pfeffer 2018). Similarities between human and cattle embryonic development, such as the almost identical gestation period of around 280 days, as opposed to the 22-day mouse gestation period suggest that cattle can be used as a good model of human embryonic development. Similarly to humans, livestock species are outbred and have organs of a similar size and function, which means that the pathogenesis of many human diseases is often better reflected by a livestock model than a rodent (Roth and Tuggle 2015). Because ES cells can proliferate indefinitely and differentiate into any cell type, bovine ES cells offer an unprecedented access to tissues from the bovine body, making them an attractive source to address developmental questions.

The primary objective of this project was to derive embryonic stem cells from the inner cell mass of bovine blastocysts. As described above, establishing pluripotent stem cells in bovine species is important, however it is equally important to characterise these cells. Establishing the conditions under which naïve and primed bovine pluripotent cells are able to be maintained is essential for our further understanding of the regulatory mechanisms regarding totipotent and pluripotent cell states. Having a well-established and characterised bovine embryonic cell line, and an understanding of the signaling mechanisms, which control the state of these cells, is essential for the advancement of embryonic stem cell research in cattle. We therefore aim to:

1. Isolate stem cells from bovine blastocysts
2. Establish conditions which support the proliferation of these cells in their undifferentiated state
3. Test the efficacy of various methods of passaging such cells
4. Characterise the cells in terms of gene expression (qPCR) and protein translation (immunohistochemistry)

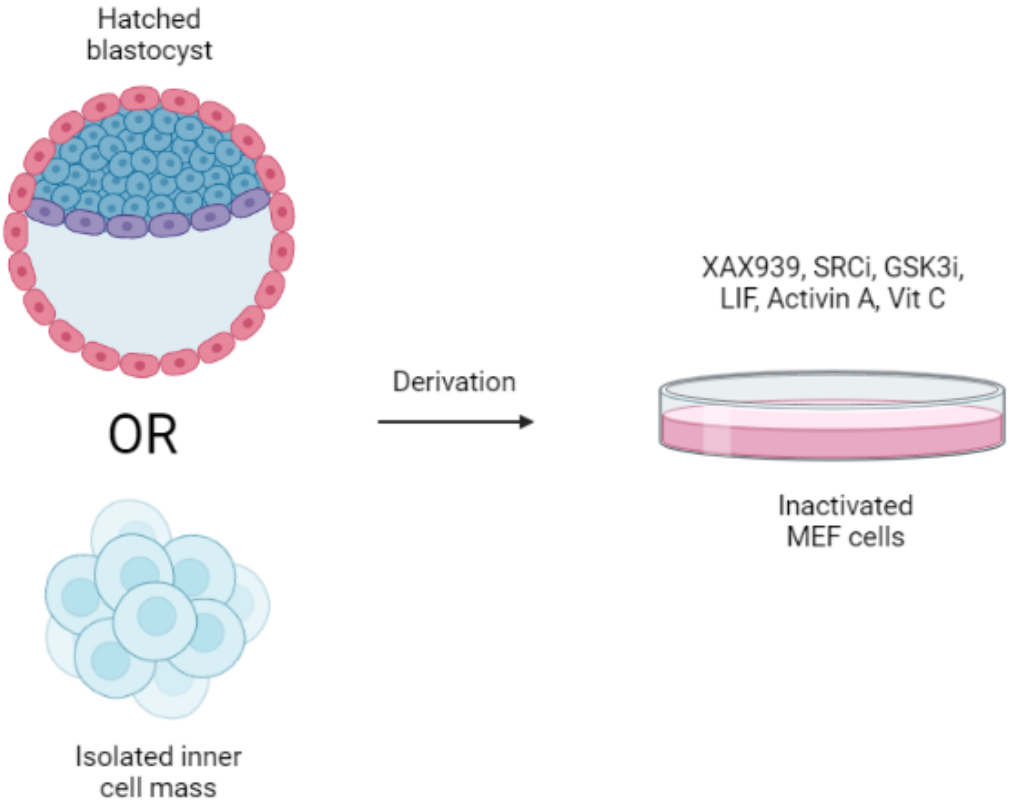

Figure 5. Methodology for derivation of embryonic stem cells

2.1 Cell Culture

All cell culture was carried out using aseptic technique, and where applicable, performed in a laminar flow hood. Cell cultures were incubated at 37°C in humidified air with 5% CO2 (SANYO Electric Company Ltd., Japan). General cell culture reagents were purchased from Gibco (Thermofisher Scientific, Auckland, NZ): heat-inactivated fetal bovine serum (FBS), Penicillin and Streptomycin (PenStrep), Dulbecco's Modified Eagle Medium (DMEM).

2.1.1 Thawing of Cells

To defrost cells, a vial was removed from liquid nitrogen and placed in a water bath at 37°C for approximately 90 seconds until only a small crystal of ice remained. Prewarmed cell culture media was added dropwise to the vial, and the cells were then transferred to a 15 ml centrifuge tube containing 10 ml of 37°C culture media, and pelleted at 200 RCF for 5 minutes at 25°C. Cells were resuspended in fresh culture medium and transferred into cell culture plates at the desired seeding density.

2.1.2 Trypsinisation and Passaging of Cells

Trypsinisation was carried out by aspirating the cell culture medium and twice rinsing the cells with phosphate buffered saline (PBS) (Gibco, Thermofisher Scientific, Auckland, NZ) followed by incubation with 3 mL of TrypLE (Gibco, Thermofisher Scientific, Auckland, NZ) at 37°C for 3-5 minutes. Manual agitation was used to assist the TrypLE in detaching the last of the cells from the plate and 7 ml pre-warmed culture medium was added to each plate. The cell suspension was pipetted up and down gently and transferred into a 15 ml falcon tube and centrifuged at 200 x g for 5 minutes. The supernatant was discarded, and the cell pellet was gently resuspended in 10 ml of culture medium.

100 µl of cell suspension was pipetted into a 500 µl micro centrifuge tube. An equal volume of trypan blue solution was added to the tube and mixed by gentle pipetting. Both chambers of a haemocytometer were filled with 10 µl of the cell/trypan blue solution and the number of viable cells was counted and averaged. From this known cell concentration, the appropriate dilution was calculated and the cells were seeded at the desired density.

2.1.3 Cryopreservation of cell lines

In preparation for cell cryopreservation, freezing media was prepared. This media consisted of 70% culture media, 20% FCS and 10% DMSO, and was chilled to 4°C. After trypsinisation, the cells were spun down and gently resuspended in freezing media at a concentration of 1 x 10^6 cells/ml. 1 mL aliquots were immediately transferred to cryovials and placed in a chamber known as a “Mr Frosty”

and then into the -80°C freezer. The freezing chamber ensured that the cells cooled at a standard rate so as to maximise the quality of the frozen stocks. The following day, the cryovials containing frozen cell stocks were transferred into the liquid nitrogen (-196°C) for long term storage.

2.2 Derivation of Mouse Embryonic Primary Fibroblasts

The initial derivation of mouse embryonic fibroblast cells (MEFs) was kindly carried out by my supervisor, Peter Pfeffer. Pregnant female mice were killed between 12.5-13.5 days post coitum. The uterine horns containing the embryos were dissected out and briefly rinsed in 70% ethanol. The horns were placed into a 100 mm diameter culture dish with 20 ml PBS. Using a dissection microscope, dissection forceps, and scissors, the walls of the uterine horn were cut, releasing the embryos and placenta. Each embryo was separated from the placenta and surrounding membranes.

The heads and internal abdominal and thoracic organs (lungs, heart, liver, intestine, stomach etc.) were removed from all embryos. The embryos were transferred into 35 mm dishes (two per plate) containing 1 ml of 0.25% trypsin/EDTA. The embryonal tissue was trypsinised for 2-4 minutes in a 37°C incubator until the tissues started to disintegrate. The tissue was then pipetted up and down with a 1 ml pipette to achieve efficient dissociation. The trypsin was inactivated by pipetting it into 10 ml of MEF culture medium (DMEM containing 10% FCS and 1% PenStrep). The embryos were pooled into 50ml falcon tube and left to allow the large pieces of tissue to sediment.

The supernatant was collected and used to seed out a homogenous suspension, keeping a ratio of two embryos per 100 mm plate. After 4-5 hours the culture medium was changed to remove unattached cells. The MEF cells were incubated for 2-3 days to grow to 90% confluence before being frozen down or typsinized and passaged as described above in sections 2.1.2 and 2.1.3.

2.3 Mitomycin C Treatment of Mouse Embryonic Fibroblasts

A frozen vial of MEF cells was thawed as described in 2.1.1 and cultured at 37°C. The cells were passaged as required until twenty 100 mm plates were grown to ~ 90% confluency. Mitomycin C (MMC) aliquots were made up just before use. Wearing protective gloves and working in a fume hood (as mitomycin C is very toxic) 2 ml of sterile water was pipetted into a vial containing 2 mg of mitomycin C (Sigma-Aldrich M0503). 1 ml of MMC was added per 99 ml of MEF culture medium, giving a final product of DMEM/10% FCS containing 10 µg/ml MMC.

The culture media was removed from the MEF plates, and the cells were rinsed twice with pre-warmed PBS (calcium and magnesium free). 8 ml media containing MMC was added to each 100 mm diameter plate, and the cells were incubated for 2 hours at 37°C. After 2 hours the medium was removed and the cells were trypsinised and pelleted as described above in 2.1.2. The cells were resuspended in MEF culture medium for counting. 1 x 10^6 cells were resuspended in 1 ml freezing media (DMEM/20% FCS/10% DMSO) and were transferred to cyrotubes for cryopreservation.

2.4 Media Preparation

DMEM Medium

A defined type of media was used for the growth of mouse embryonic fibroblast cells, namely Dulbecco's modified eagle medium (DMEM), with 10% heat-inactivated FCS (foetal calf serum), 1% weight/volume PS (penicillin-streptomycin). All media solutions were sterilised by filtering through a 0.22 µm filter (Millipore, Germany). Medium was prewarmed to 37°C in a heated water-bath prior to application.

CTFR Medium

CTFR medium was prepared by combining solutions as according to the appendix. The medium was then adjusted to an osmolarity of 340-350 mOsm and a pH of 7.4 before being sterilised using a 0.22 µm filter (Millipore, Germany).

EPSC Medium

Expanded Potential Stem Cell Medium (EPSCM) was prepared by combining solutions as according to the appendix.

2.5 In Vitro Bovine Embryo Production

2.5.1 Ovary Aspiration

Fresh bovine ovaries were obtained from a nearby abattoir in Johnsonville and transported back to the lab in a thermos filled with warm 0.9% saline. Upon arrival to the lab the ovaries were rinsed with fresh saline and strained until there was no blood present in the saline. Tubes containing 2ml of preheated aspiration media (see appendix) were placed into a warming tray (38.5°C), and an aspiration needle bung was inserted into the top of one of the tubes. An aspiration needle was inserted into the second hole on the aspiration bung, and the vacuum tube was attached to the bung.

Clear or yellow follicles between 3 and 10 mm in diameter were selected and aspirated by inserting the aspirating needle into the follicle, ensuring the pressure was 40-50 mm Hg, and moving the needle around inside the follicle to increase the chance of aspirating cumulus oocyte complexes (COCs). The needle was removed and reinserted into a second selected follicle, and the process was repeated until all of the ovaries had been aspirated.

2.5.2 In Vitro Maturation

The cumulus oocyte complexes (COCs) were left to settle in the bottom of the tube and were transferred into a sterile petri dish containing 3 ml aspiration media. Using a heated stage (38.5°C) and dissection microscope under 8-fold magnification, evenly granulated COCs were selected and transferred into a petri dish containing H199 + 10% FCS media (AgResearch, Hamilton). They were washed with pre-warmed and equilibrated B199 media (see appendix), before being placed into the drops of equilibrated maturation media (AgResearch, Hamilton) on a pre-prepared IVM dish at 38.5°C, 5% CO_2 and incubated for 22-26 hours.

2.5.3 In Vitro Fertilization

In preparation for IVF, 3.5 ml of IVF media (AgResearch) was defrosted and supplemented with 35 µl of penicillamine/hypotaurine, 3.5 µl heparin and 3.5 µl pyruvate (IVF + PPHH). 30 µl drops of IVF + PPHH media were pipetted onto 60 mm plates, 1 drop per 5 oocytes, overlaid with mineral oil (Sigma), and placed in the incubator to equilibrate for at least 2 hours. 80% and 40% BoviPure/BoviDilute (AgResearch) solutions were prepared as per Table 4, and left to warm in the incubator for a minimum of 30 minutes.

	40%	80%
Bovipure	200 µl	400 µl

Bovidilute	300 µl	100 µl

Table 4. Preparation of density gradient for sperm preparation

The 80% solution was carefully laid under the 40% solution with a sterile glass pipette. Semen (one straw per 150 oocytes) was removed from the liquid nitrogen tank, thawed in the air for 5-10 seconds and placed in a 35°C water bath for 40 seconds to defrost. The straw was wiped with 70% ethanol and cut with sterile scissors, releasing the straw contents into a sterile 35 mm dish. The sperm was gently mixed and then slowly laid on top of the BoviPure gradient. The sperm preparation was centrifuged at 300 rcf for 20-30 minutes at room temperature.

Cumulus oocyte complexes (COCs) were removed from the maturation media and taken through two washes of Hepes Synthetic Oviduct Fluid (HSOF, AgResearch). A 10 µl pipettor was used to remove the outer layers of cumulus cells by manual agitation in preparation for fertilization. The COCs were then transferred to a third well containing equilibrated IVF + PPHH media. From this well they were transferred into the IVF dish, 5 COCs per drop. The IVF plate was returned to the incubator.

As soon as the centrifuge stopped spinning the sperm pellet at the bottom of the tube was transferred to a tube containing 1 ml of HSOF at room temperature and gently mixed. This tube was centrifuged for 5 minutes at 300 rcf. When the centrifuge stopped, the sperm pellet was aspirated with a Pasteur pipette and resuspended in 200 µl equilibrated (38.5°C, 5% CO_2) IVF media. A 10 µl aliquot of sperm suspension was mixed with 190 µl of water for a sperm count. The remaining volume of sperm preparation was measured (A) and placed in a dark place while a sperm concentrated was calculated. 10 µl of the diluted sperm was added to each side of a haemocytometer. Sperm heads within the 25 large squares in the haemocytometer grid were counted, and the mean of the two was used to calculate the sperm dilution needed (B).

$$\frac{Volume\ measured\ (\boldsymbol{A}) \times Average\ number\ of\ sperm\ count\ (\boldsymbol{B})}{\mathbf{25}}$$

$$= Total\ Volume\ (\boldsymbol{C})$$

$$Total\ Volume\ (\boldsymbol{C}) - Volume\ measured\ (\boldsymbol{A})$$
$$= Volume\ of\ IVF\ media\ to\ add\ to\ sperm$$

Using the formula above the volume of IVF + PPHH media needed to dilute the sperm to a final concentration of 1 million/ml was calculated. The required volume of IVF media was added slowly to the sperm preparation and gently mixed. 10 µl of the final sperm preparation was added to each drop of IVF media containing the oocytes. The IVF plates were placed back in the 5% CO_2 incubator for 18-24 hours.

2.5.4 In Vitro Culture

The day after IVF, 18-24 hours after the sperm were introduced to the oocytes, the cumulus cells were removed and the embryos were cultured in a modulation chamber for 6 days.

Using a pipette and sterile pipette tips, 20 µl drops of early synthetic oviduct fluid (ESOF, AgResearch) were made inside a 35 mm petri dish with two central wash drops of 40 µl. The drops were overlaid with 3 ml of mineral oil (Sigma). The plates were placed into a flying saucer and gassed with 5% CO_2, 7% O_2, 88% N_2 gas mixture for 5 minutes and left to equilibrate for a minimum of 2 hours.

After 2 hours, the oocytes were removed from the IVF drops using a mouth pipette and transferred to a 4-well plate containing HSOF. After the second wash of HSOF the oocytes were transferred to an eppendorf tube containing 500 µl of HSOF and were vortexed for 2 minutes, before being centrifuged for 15 seconds at 1800 rpm. The embryos were removed from the eppendorf tube and placed into the third well containing HSOF. Upon transfer into the 4th well all remaining debris was left behind to make for an easy embryo transfer into the IVC plate. While gassing the flying saucer, the IVC plate was removed and the embryos were transferred into the ESOF drops (10 per droplet). The plate was returned to the flying saucer and re-gassed with 5% CO_2, 7% O_2, 88% N_2 gas mixture for 5 minutes.

2.5.5 Day 5 IVC

On Day 5 of IVC the media was changed to late-stage synthetic oviduct fluid (LSOF, AgResearch). A 35 mm petri dish containing 20 µl drops and 2 central 40 µl drops of LSOF medium was set up. The droplets were overlaid with 3 ml of Sigma mineral oil and the dish was placed into the flying saucer and gassed with 5% CO_2, 7% O_2, 88% N_2 gas mixture for 5 minutes and left to equilibrate for a minimum of 2 hours. The embryos were then transferred through the wash drops of the LSOF plate,

and 10 embryos were placed into each culture drop. The LSOF plate was returned to the flying saucer and gassed with 5% CO_2, 7% O_2, 88% N_2 gas mixture for 5 minutes.

2.6 Removal of Zona Pellucida

2.6.1 Pronase Digestion of the Zona Pellucida

A 60 mm petri dish was set up with 4 x 50 µl drops of 5 mg/ml pronase containing 0.1 mg PVA, 4 x 50 µl drops of HSOF + 10% FCS and 4 x 50 µl drops of PBS containing 0.1 mg/ml PVA. Day 7 blastocysts were placed into the first wash drop of pronase (to remove protein) and then transferred into a new drop of pronase for 1 – 5 minutes. When the zona pellucida swelled or became out of focus the blastocyst was immediately transferred to a drop of HSOF + 10% FCS to stop digestion. The zona pellucida was removed completely by gently pipetting the blastocyst up and down in a glass pipette approximately the size of a blastocyst with an intact zona pellucida. The zona-free blastocysts were removed from the HSOF containing FCS and transferred from drop to drop of PBS containing 0.1 mg/ml PVA to remove excess FCS and pronase.

2.6.2 Extended Culture of Blastocysts

20 µl droplets of extended culture media were pipetted onto a 35 mm diameter plate, overlaid with 3 ml mineral oil, and left to equilibrate for a minimum of 2 hours in a humidified 38.5°C incubator with 5% CO_2. Blastocysts were taken through 2 wash drops of extended culture media, before being placed into a culture droplet and returned to the incubator. The following day, which marked 8 days of culture, the hatched blastocysts were removed from the culture droplet and mouth pipetted directly onto inactivated MEF cells.

2.6.3 Mechanical Isolation of the Inner Cell Mass

Using two sterile ophthalmological knives, the inner cell mass was cut out manually under a dissection microscope. One knife was used to hold the blastocyst in place while the other was used to cut out the inner cell mass.

2.7 Culture of Embryo Outgrowths

2.7.1 Plating of Bovine Blastocysts/ICM

Mitomycin C treated MEF cells were thawed, counted, and diluted to a concentration of $\sim 1 \times 10^5$ cells/ml. 500 µl cell suspension was added to each well of a 4-well plate to achieve a seeding density of 20-25,000 cells/cm^2. If using a 96-well plate, 200 µl of cell suspension was added to each well. The plates were placed in the incubator at 37°C in humidified air with 5% CO_2, and left to settle overnight.

After 24 hours the cells were washed twice with DMEM/F12 (Gibco) and the media was replaced with pre-warmed embryonic stem cell media. Zona free blastocysts or isolated inner cell masses, were mouth pipetted into the dish which was then placed back into the incubator.

2.7.2 Culture and First Passage of Blastocyst Outgrowths

The media was carefully replaced after 24 hours under a dissection scope to ensure that the blastocysts were not removed during the process. After 48 hours a sterile needle was used to facilitate attachment of blastocysts which had failed to attach to the feeder layer. Outgrowths were passaged after 7-9 days in culture using either trypsin, accutase, or mechanical dissociation.

Trypsin dissociation of cells was performed as described above in 2.1.2. Accutase dissociation was performed by aspirating the cell culture media from the wells, and rinsing the cells gently with PBS. Enough accutase to cover the bottom of the well was added to the wells. The plate was left at room temperature for 5-10 minutes. Once the cells had begun to detach, the plate was given a whack to fully dislodge them. The accutase solution containing the dissociated cells was aliquoted into new wells containing fresh MMC treated MEF cells. The wells were filled with cell culture media and returned to the incubator. Mechanical dissociation was performed under a dissection scope, using a pipette tip to dislodge the outgrowth from the bottom of the dish, and very gently pipetting up and down to break up the outgrowth into smaller chunks.

2.8 Immunohistochemistry

Cells were fixed and stained directly in the cell culture dish. All steps were carried out with 500 µl of reagent, and unless stated otherwise, were gently rocked for 5 minutes.

Cells were washed two times with PBS and were then dehydrated via 5 minute washes through 50%, 70% and 100% ethanol. The dish was stored overnight at -20°C in 100% ethanol. The following day the cells were rehydrated via transfer through 70% ethanol, followed by 50% ethanol for 5 minutes each. They were then washed twice in PBT (PBS + 0.1% Tween-20). The cells were permeabilized with PBS + 0.5% Triton X-100 for 10 minutes. 500 µl PBT containing 10% FCS + 3% BSA) was added to each well in the dish for 1 hour at room temperature. The primary antibody(s) were added, with the antibody being diluted in PBT containing 3% BSA (Table 5). The cells were incubated for either 2 hours at room temperature, or overnight at 4°C. The cells were washed two times with cold PBT, and then once in cold PBT containing 3% BSA. An appropriate fluorescence-conjugated secondary antibody was added and gently rocked in the dark at room temperature for 2 hours. The cells were washed in the dark for 15 minutes each with PBT containing 1% BSA. The cells were rocked in DAPI for 15 minutes to stain the nuclei. Following this, the cells were washed once more in PBT/1% BSA and then a drop of slowfade diamond was added on top of the cells before photographing the cells on a fluorescent microscope.

Primary Antibody Gene	**Dilution**	**Labels**	**Origin (species)**
OCT4	**1:100**	ICM; epiblast	**Rabbit**
GATA6	**4:200**	Hypoblast	**Rabbit**
GATA3	**1:100**	Trophoblast	**Mouse**
SOX2	**1:200**	Inner Cell Mass	**Goat**
NANOG	**1:500**	Epiblast	**Mouse**
SOX17	**1:100**	Hypoblast	**Goat**

Table 5. Primary antibodies used in this study, their dilutions, labels, and origin

2.9 Alkaline Phosphatase Staining

2.9.1 Cell Fixation

Cells were fixed in 4% paraformaldehyde for one hour, and washed two times for five minutes in PBT. The cells were taken into 70% ethanol via 30% ethanol/PBT and 50% ethanol/PBT and left at room temperature for a minimum of 3 hours.

2.9.2 Alkaline Phosphatase Detection

Cells were rehydrated by transfer through 50% and 30% ethanol. The cells were washed two times for 10 minutes each in 500 µl NTMT (see appendix). The cells were incubated in 500 µl NTMT containing 3.38 µl/ml NBT and 3.5 µl/ml BCIP. When the blue stain had developed to the desired extent the cells were rinsed two times with PBT and refixed in 4% paraformaldehyde for 10 minutes at room temperature. The wells were rinsed again in PBT before being photographed in 80% glycerol/PBT.

2.10 RT-PCR

2.10.1 RNA Isolation

The cells were washed with PBS and 100 µl of trizol was pipetted into each well. The cells were scraped off the bottom of the dish using a pipette tip and the cells were homogenised by pipetting up and down repeatedly. The cell slurry was pipetted into a microcentrifuge tube and MS2 phage RNA and chloroform were added. The sample was briefly vortexed at high speed before being centrifuged at 12,000 xg for 15 minutes at 4°C to separate the phases. After centrifugation, the top layer was removed using a gel-loading tip and placed into a new tube. An equal amount of ice-cold isopropanol was added, the solution was vortexed and left to stand for 10 minutes at room temperature. The RNA was pelleted in the microfuge for 30 minutes at room temperature, the supernatant was removed and the pellet was washed with 70% ethanol, air dried and resolubilized in DEPC-treated water.

2.10.2 DNAase treatment

To 8 µl of RNA from protocol 2.9.1, 1 µl of DNAse buffer and DNase I were added and left in a 37°C heat block for 60 minutes. To stop the reaction 25 mM EDTA was added and the solution was heated to 75°C for 10 minutes. Sodium acetate and 3 volumes of ethanol were added, the solution was left overnight at -20°C, spun 30 minutes at 12,000xg, ethanol washed and dried as previously and resuspended in 11 µl of DEPC-H_2O.

2.10.3 cDNA Synthesis

1 µl from each sample diluted with 9 µl T0.1E and placed in a -80°C freezer, these samples were later used as controls. The remaining 10 µl was mixed with oligo-dT primer and dNTP mix, incubated at 65°C for 5 minutes, chilled rapidly on ice for 2 minutes and then collected by brief spin.

For reverse transcription, the following reagents were added:

4 μl	5x 1st strand buffer (supplied with Superscript3, Invitrogen)
2 μl	0.1 M DTT (supplied with Superscript)
1 μl	RNAsin (Promega) or RNaseOUT (LifeTechnologies)
1 μl	Superscript III (LifeTechnologies)

The samples were subjected to the following incubations:

10 minutes at 40°C (use heated lid)
1 hour at 50°C
5 min at 85°C
4°C hold (or chill on ice)

2.10.4 RT-PCR

cDNA solution was diluted 1 in 5 with T0.1E (10 mM Tris, pH7.5, 0.1 mM EDTA). Technical triplicates with 2 original concentration and 1 half concentration of samples were set up of both the cDNA solution and the RT samples for each gene. TaKaRa mastermix containing taq DNA polymerase and SYBR green in appropriate buffer was added into the solution alongside primers for the genes of interest. The conditions for PCR was as follow: an initial denaturation at 95°C for 3 minutes then 40 cycles of 95°C for 10 seconds and 60°C for 35 seconds for DNA elongation and primer annealing. A dissociation curve was generated through 72°C for 90 seconds then from 72°C to 95°C at 1°C every 5 seconds. Each PCR run included a no template control and an RT control.

Chapter Three: Results

3. 1 Achieving Attachment

The first step in establishing an embryonic stem cell line is the production of embryos, to derive such cells from. Cumulus-oocyte-complexes were aspirated from slaughterhouse derived ovaries and fertilised with sperm at a concentration of 1 million/ml. Embryos were cultured for 7 days in synthetic oviduct fluid, those that reached the blastocyst stage of development were selected for embryonic stem cell derivation.

In vitro production of bovine embryos is a difficult technique to learn which takes a long time to master. The number of blastocysts produced was a major limitation for the production of embryonic stem cells. The numbers of oocytes and blastocysts produced from my first 12 runs are recorded in figure 6; figure 7 depicts the rate of blastocyst production from these same runs. The blastocyst rate is calculated by dividing the total number of blastocysts by the number of oocytes which were fertilised. By the end of my project (30 IVP runs) I was able to maintain an average blastocyst production rate of 35%.

However, the greatest source of uncontrollable variation in the production of blastocysts came from the number of ovaries available for each IVP run. The number of ovaries obtained directly influenced the number of oocytes which could be matured for fertilisation, and consequently the number of blastocysts which could be produced. Day 7 blastocysts are shown in Figure 8.

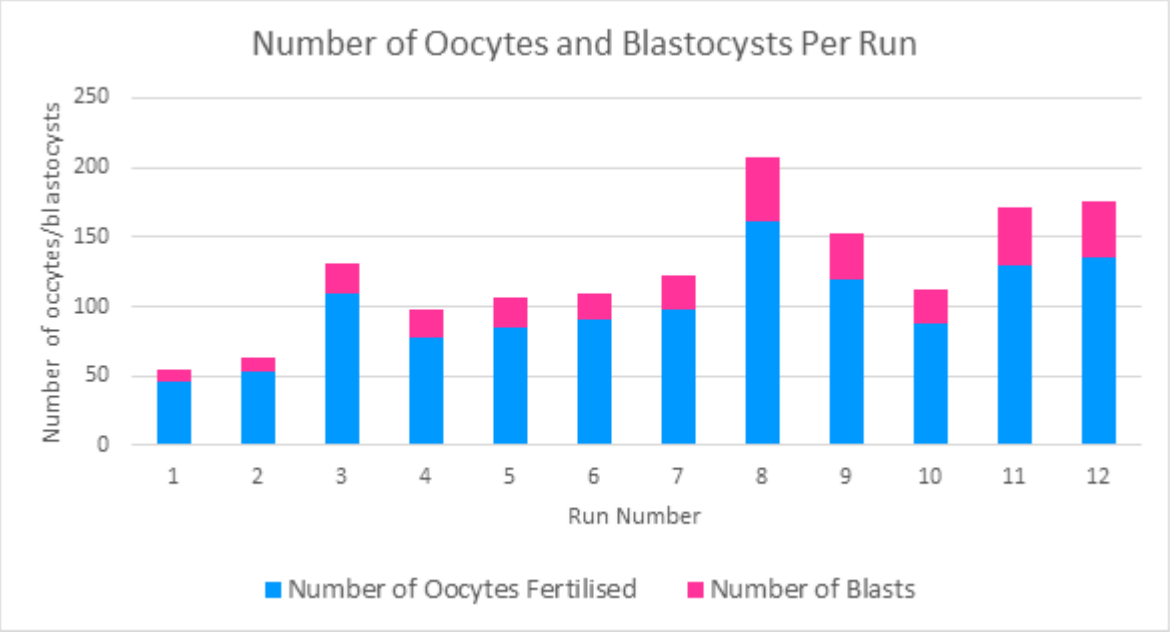

Figure 6. Number of oocytes and blastocysts per in vitro production run.

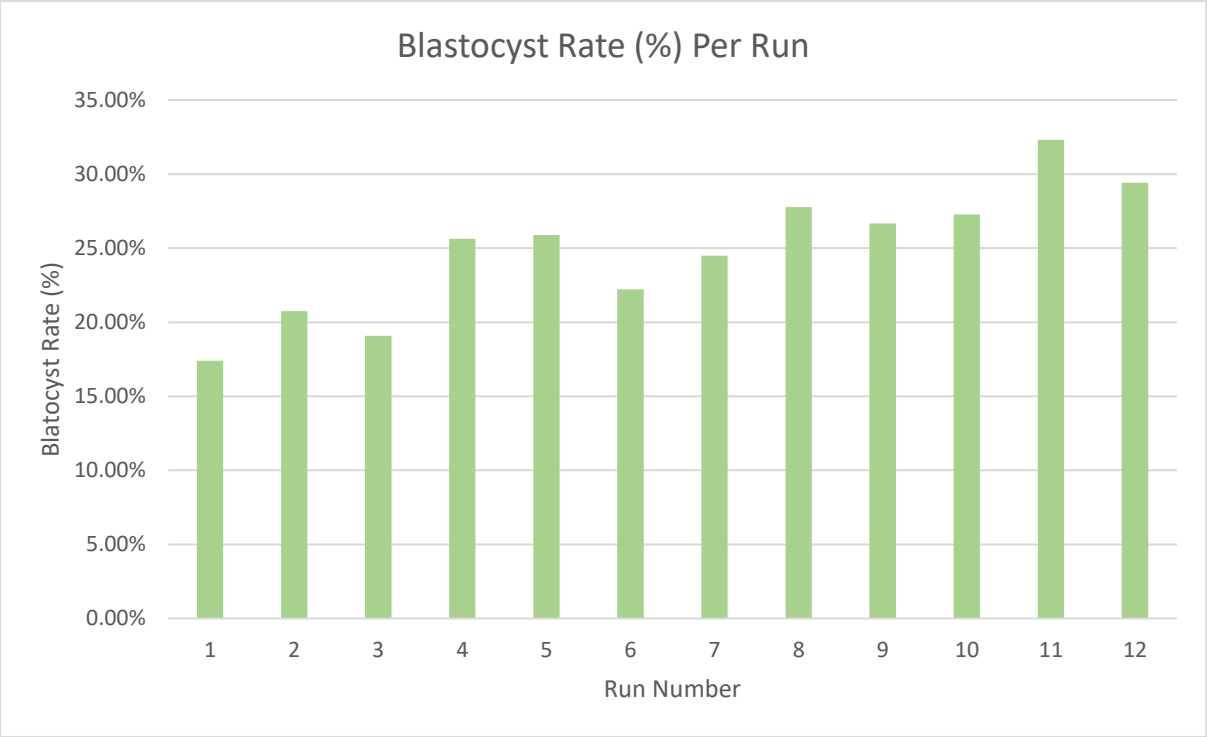

Figure 7. Blastocyst rate per run.

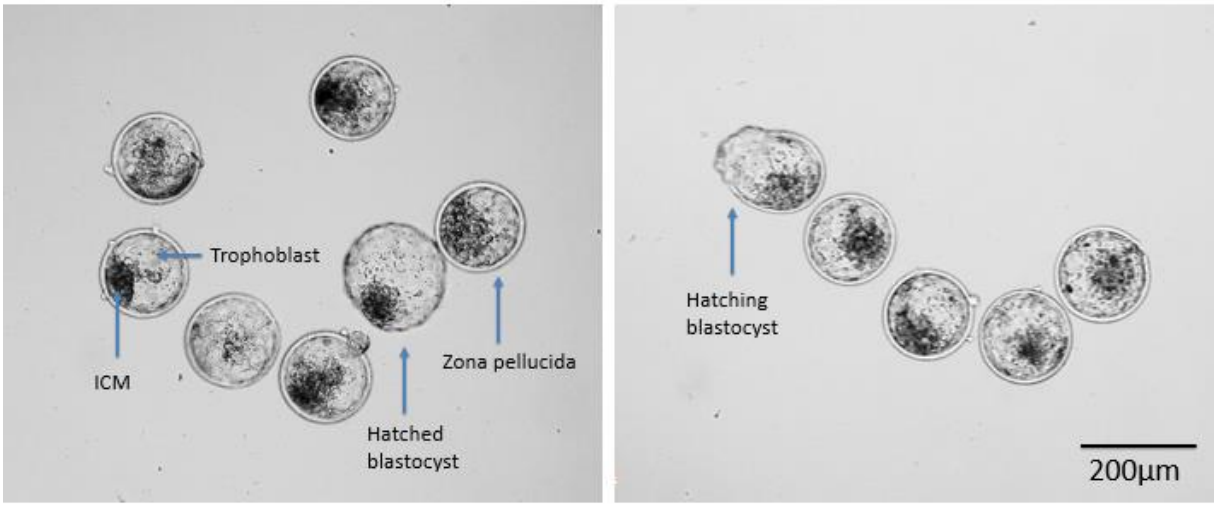

Figure 8. Day 7 blastocysts.

3.1.1 Protease Digestion of Zona Pellucida

Once embryos had been cultured for seven days, the zona pellucida had to be removed before the blastocysts could attach to the feeder layer. The primary strategy used to achieve this was to use a protease to digest the zona pellucida. Blastocysts were treated with 0.2 mg/ml pronase for a variable length of time, ranging from 1-5 minutes (**Error! Reference source not found.**). It was difficult to get the timing right, as each embryo needed to be digested for a different length of time. Consequently, each blastocyst had to be individually monitored, meaning that this was a very time-consuming process which often resulted in the blastocyst being overdigested (Figure 10a). On average blastocysts would take 2-3 minutes before the zona started to thin and become distorted. It was at this stage that I would remove the blastocysts from the pronase, and begin trying to manually remove the expanded and weakened zona pellucida by aspirating it up and down a glass pipette. If the zona pellucida could not be removed, often the blastocyst would hatch overnight after being left in the stem cell media on the feeder cells. This was the preferred alternative to the blastocyst being over-digested by the pronase, as this would render the blastocyst unviable. The majority of hatched blastocysts settled down onto the feeder layer within 48 hours (**Error! Reference source not found.**). If the blastocyst did not attach within 48 hours, attachment could be facilitated using a sterile 22-gauge needle.

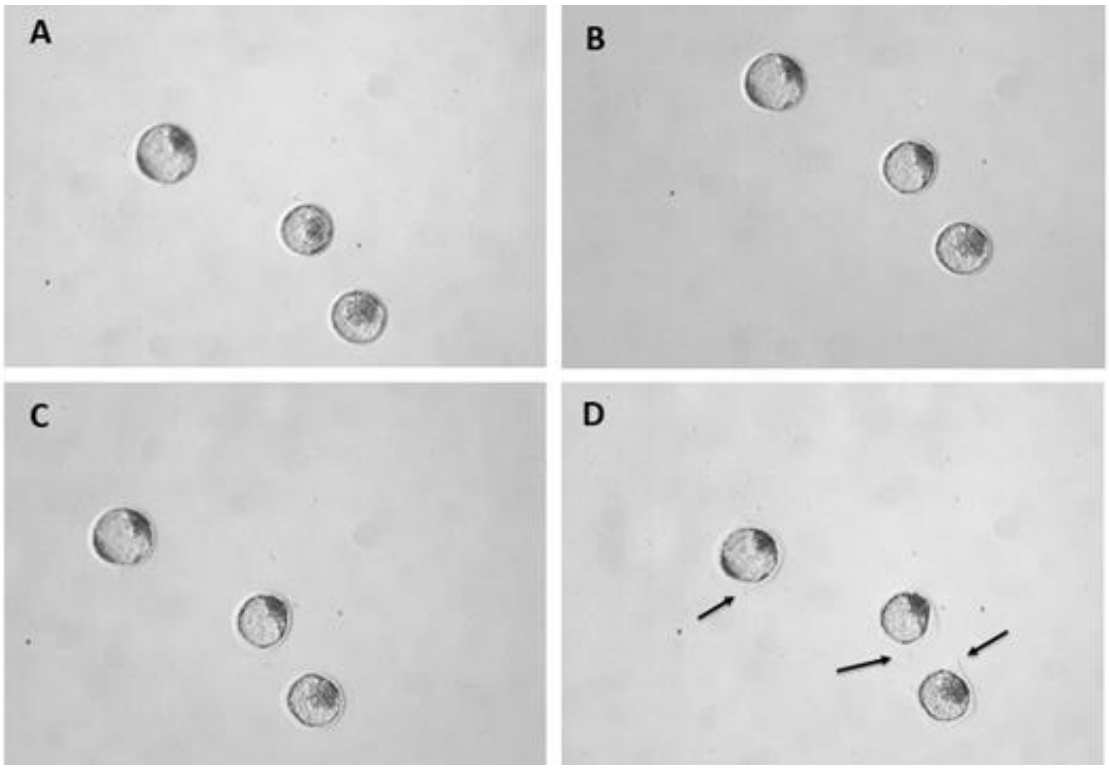

Figure 9. Pronase digestion of zona pellucida. A. 1 minute in 0.2 mg/ml pronase. B. Two minutes in pronase. C. Three minutes in pronase. D. Four minutes in pronase. Arrows depict zona pellucida thinning and expansion.

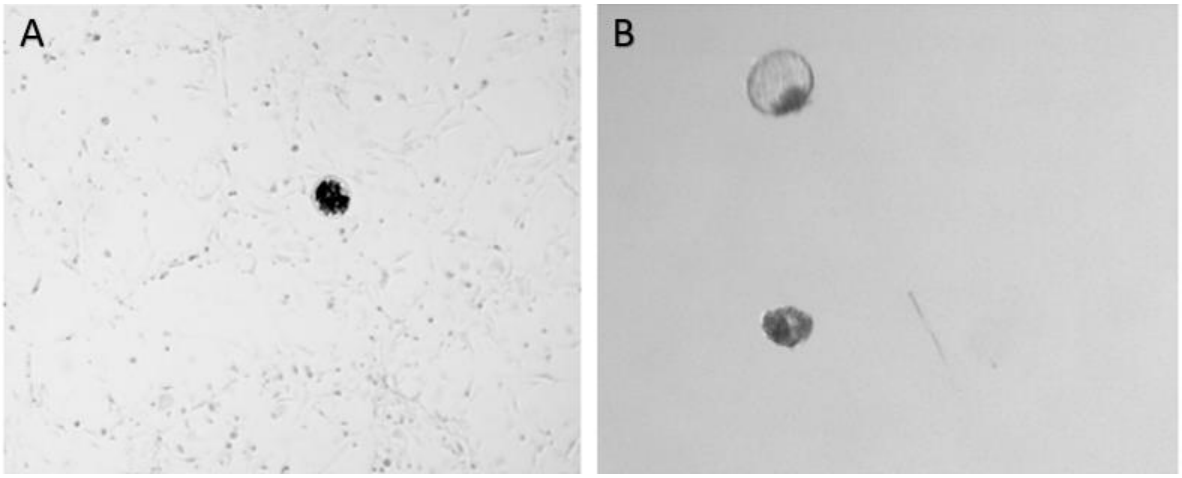

Figure 10*.* Embryos following pronase digestion. A. Unhatched embryo which has failed to attach to feeder layer and subsequently died. B. Top embryo is now zona free, bottom embryo has been overdigested.

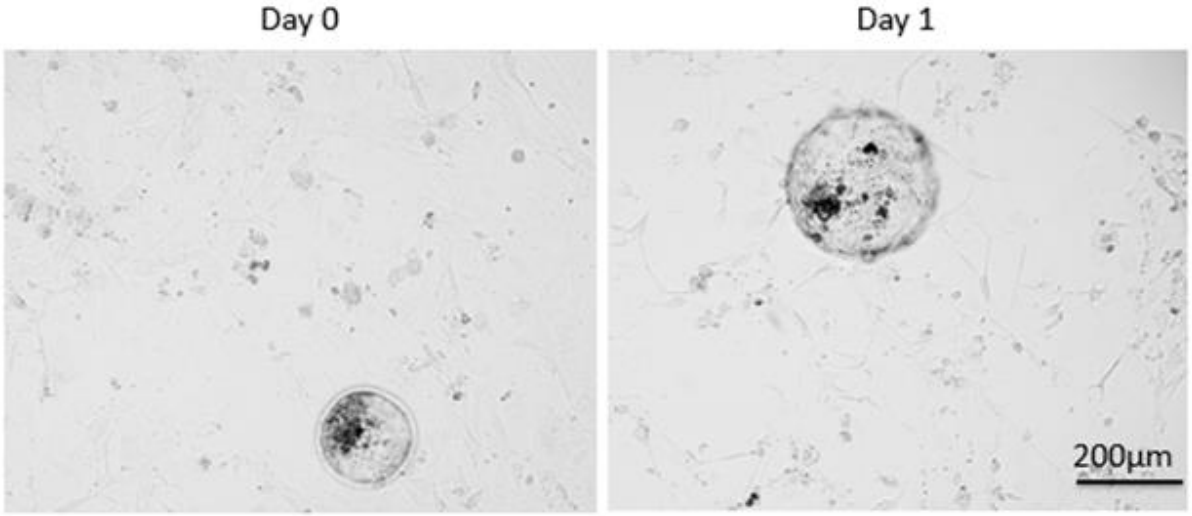

Figure 11. Blastocyst hatching overnight after being placed onto the feeder layer.

3.1.2 Mechanical Isolation

Mechanical isolation of the inner cell mass was another method of removing the zona pellucida. Isolating the ICM also provided the benefit of ensuring that any cell line which was established, came from the inner cell mass, rather than the trophectoderm, thus avoiding the creation of trophoblast stem cells. However, dissection of the blastocysts was time consuming and difficult to perform, creating yet another bottleneck. Two ophthalmologic knives were used for dissection; one to hold the blastocyst in place, and the other to excise the inner cell mass. Isolated ICM's generally attached more quickly to the feeder layer than whole blastocysts.

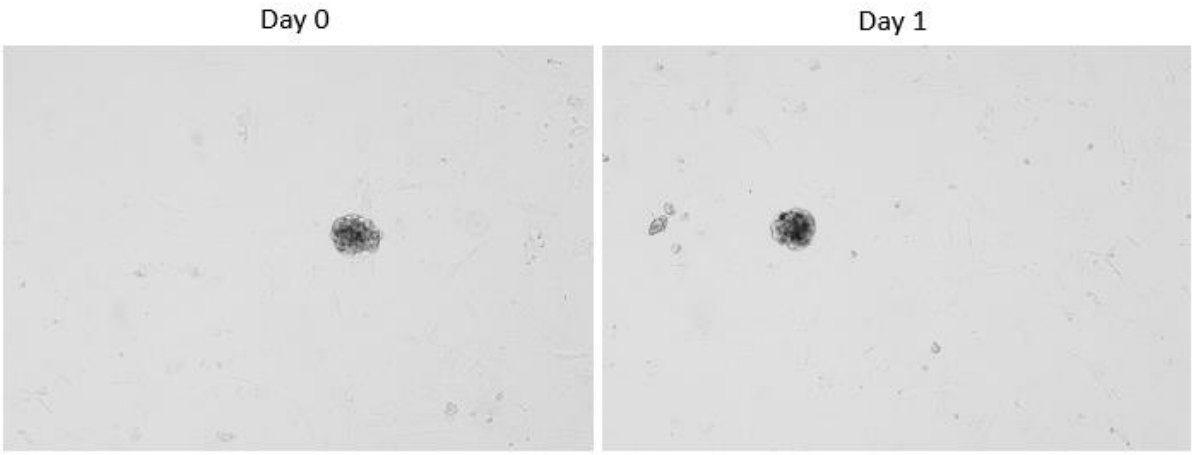

Figure 12. Two examples of isolated ICMs.

3.1.3 Extended Culture

The final method used to remove the zona pellucida was to extend the culture of the embryos, so that they naturally hatched out of the zona pellucida. This was achieved by culturing embryos until day 8 using a culture medium established by Kate Isaac, a PhD student in our lab (Isaac and Pfeffer 2021). At this stage around 90% of the blastocysts had hatched and could be plated as a whole blastocyst onto the feeder layer. Cells derived from these blastocysts are coming from a later stage in development, and consequently are more likely to be primed cell type.

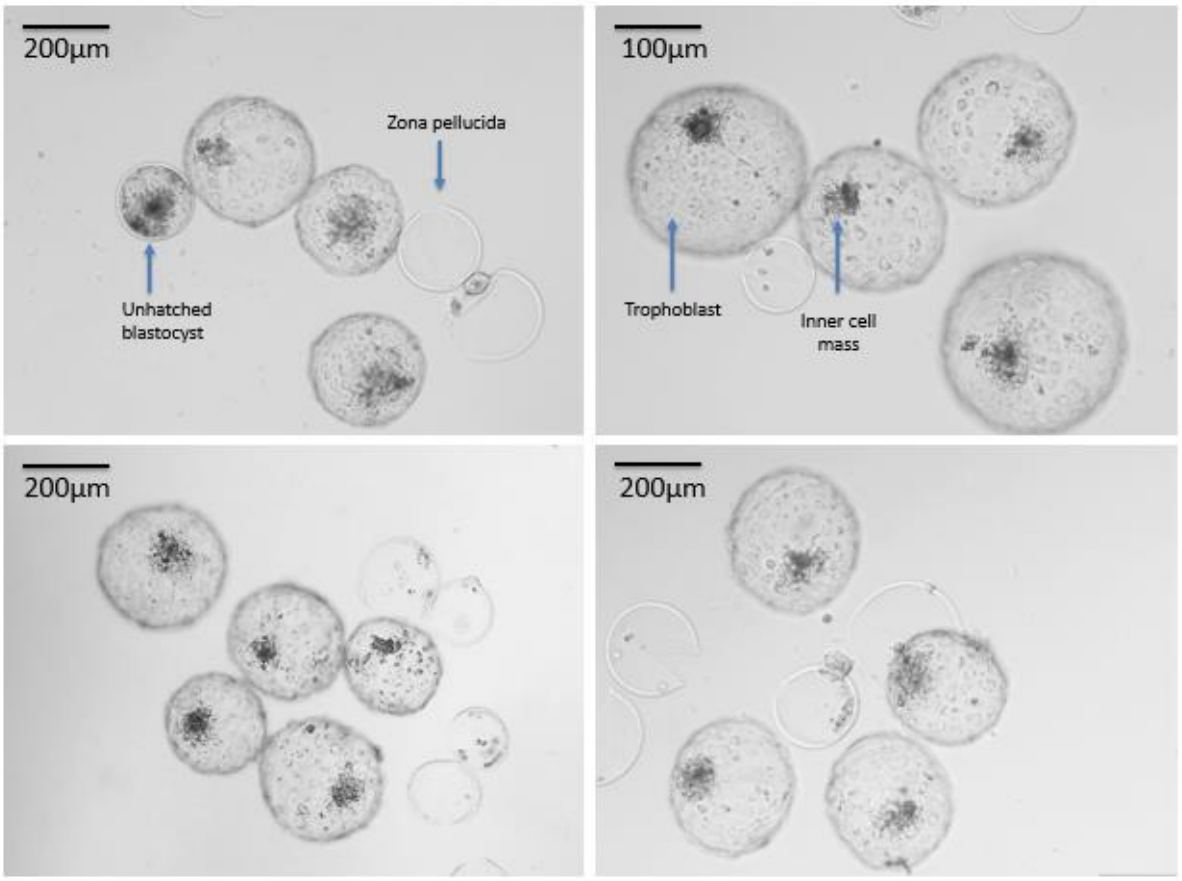

Figure 13. Day 8 blastocysts.

3.2 Preparation of Feeder Layer

Cells can be grown as a suspended or adherent culture. Adherent cell cultures are grown attached to a cell culture dish, however embryonic stem cells are unique in the way that they are generally co-cultured on a monolayer of inactivated cells. This layer of cells consists of adherent growth-arrested but viable cells. These cells secrete factors important for the growth of ES cells and provide an extracellular matrix for attachment of the cells.

Mouse embryonic fibroblast cells were grown to 90% confluence before treatment with mitomycin C (MMC). The first treatment performed with MMC resulted in the death of most of the cells as the MMC had expired, and had consequently become toxic to the cells. Sigma states that if the MMC forms a precipitate it can be toxic to cells, this is the problem I encountered (Sigma). This resulted in a very sparse feeder layer which was inadequate at supporting the expansion of the cells (Figure 14b). To combat this problem, new mitomycin C was ordered. In the meantime, STO cells were treated with MMC to be used as an alternative feeder layer. However, unfortunately these cells were found to have a bacterial contamination and consequently could not be used for this project.

Cells treated with the new solution were successfully mitotically inactivated and were plated at a density of 20,000 cells/cm^2 (Figure 14a).

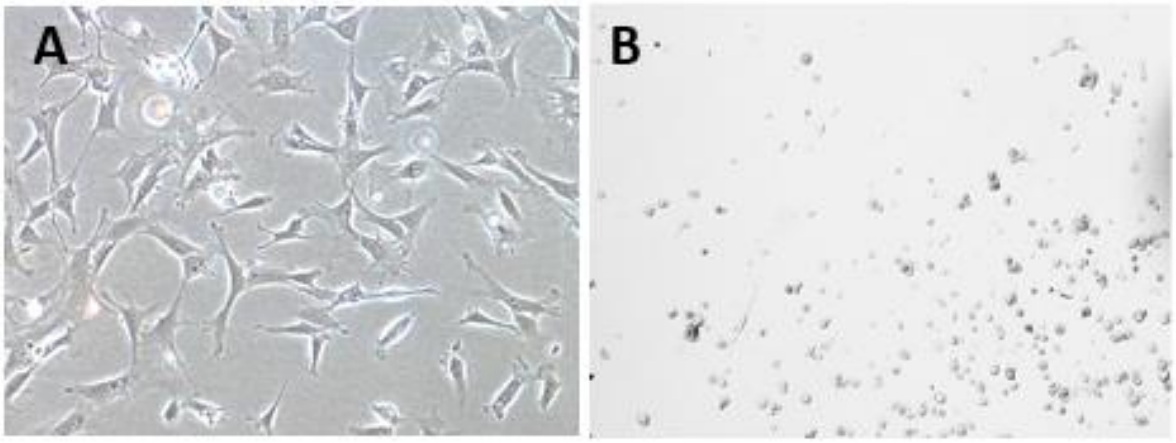

Figure 14. Mouse embryonic fibroblasts. A. Mouse embryonic fibroblasts at a suitable density for ES cell derivation B. MEF cells treated with expired MMC. The morphology of the cells is different than what it should be. The cells are displaying the signs of stress: they are not attaching and spreading.

3.3 Passage Zero

Whole blastocysts attached to the feeder layer, usually within 48 hours. Around 2 days after attachment, initial outgrowths from the blastocyst became visible. If the feeder layer was sufficiently dense and the zona was removed from the blastocysts, outgrowths formed with a high efficiency (>90%). However, conditions were not always ideal and therefore the average efficiency of outgrowth formation was 67%. These outgrowths expanded, the growth of 4 individual outgrowth is shown in Figure 16. After 7-11 days in culture with daily refreshment of the culture medium most outgrowths had expanded to cover a significant portion of the culture well and were ready to be passaged onto fresh feeder cells.

The first approach to passaging was to use standard trypsin methods. The first attempts at passaging were unsuccessful and did not result in the formation of colonies. Therefore, these cells did not make it to passage one.

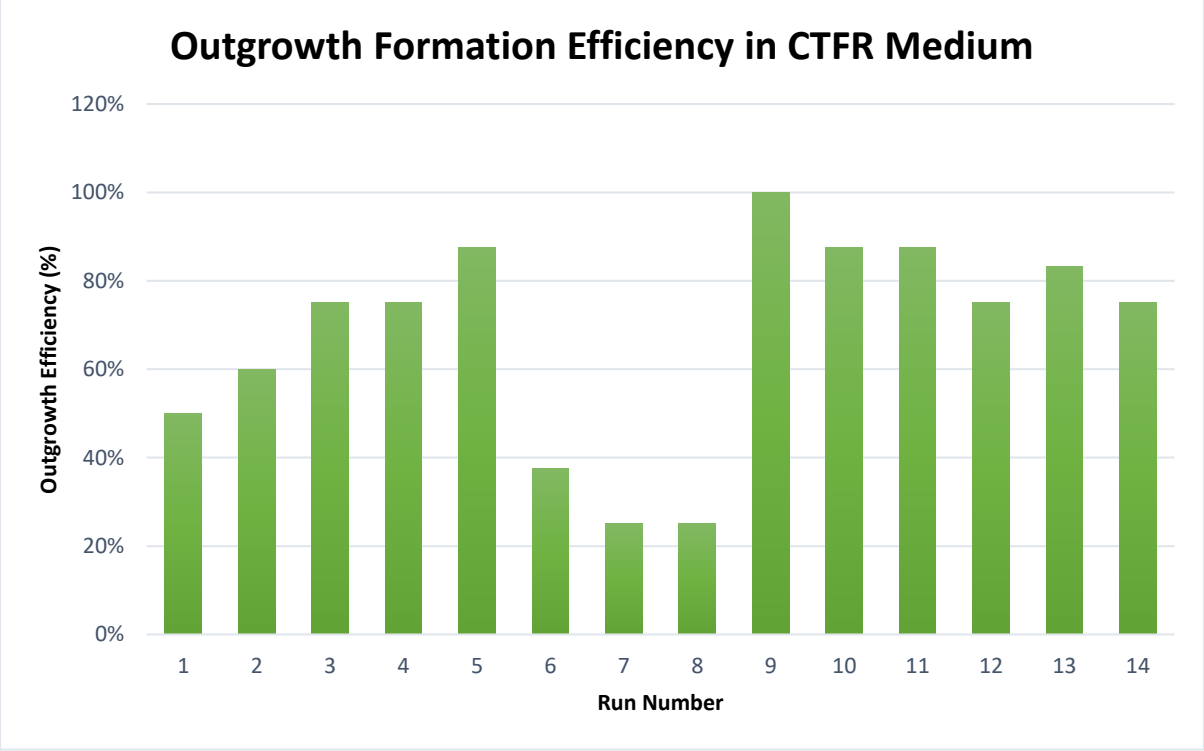

Figure 15. Efficiency of outgrowth formation. Note the low efficiency for runs 6, 7, and 8 where the feeder layer was barely present.

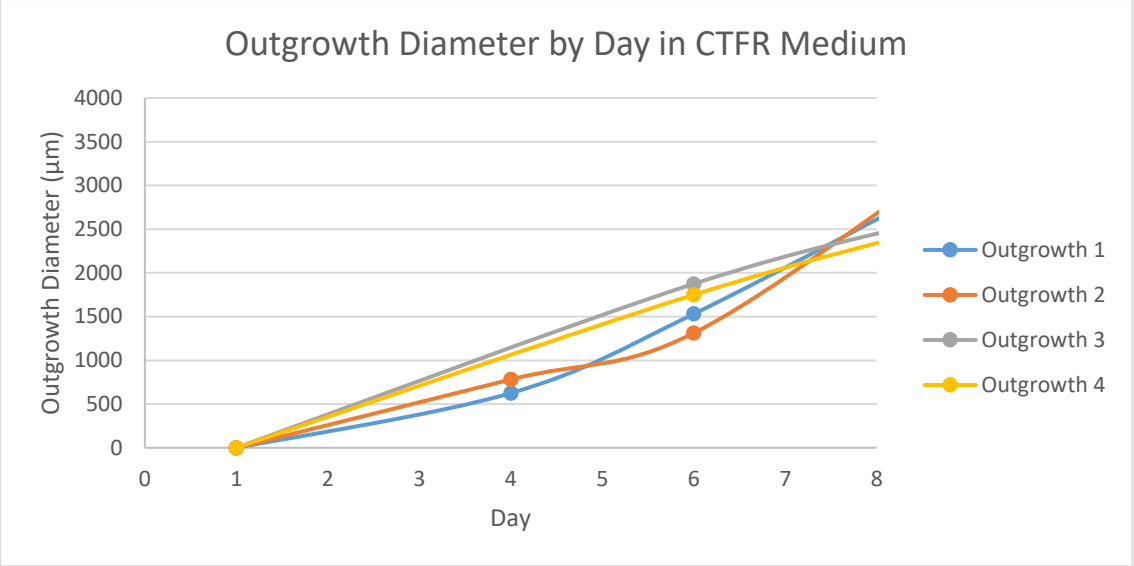

Figure 16. Outgrowth diameter by day. Day zero represents the day of blastocyst or ICM addition.

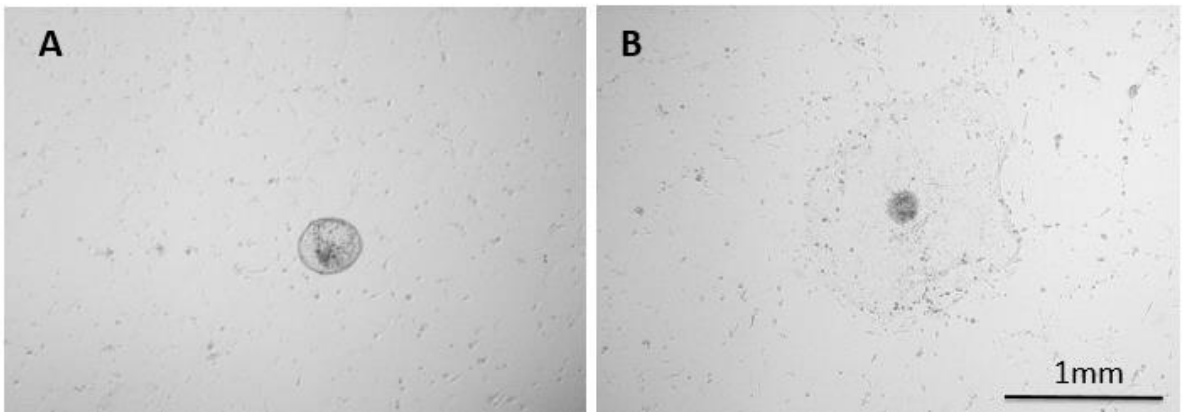

Figure 17. Outgrowth expansion in CTFR medium. A. Day of plating. B. 4 days after plating.

3.4 Passage Zero Characterisation

Once I had several outgrowths expanding, characterisation of these cells was performed to confirm that these outgrowths were originating from the inner cell mass of the blastocyst, rather than the trophectoderm.

3.4.1 Immunofluorescence

Staining of initial outgrowths of cells grown in CTFR medium show that the cells stained positive for the epiblast marker Sox2, and negative for Gata3 and Gata 6, which mark the trophectoderm and hypoblast respectively (Figure 18). A separate outgrowth (stemming from a separate embryo) showed a strong positive signal for the hypoblast marker Sox17. It also stained positive for Oct4 (ICM marker), however the signal for Nanog is weak (Figure 19).

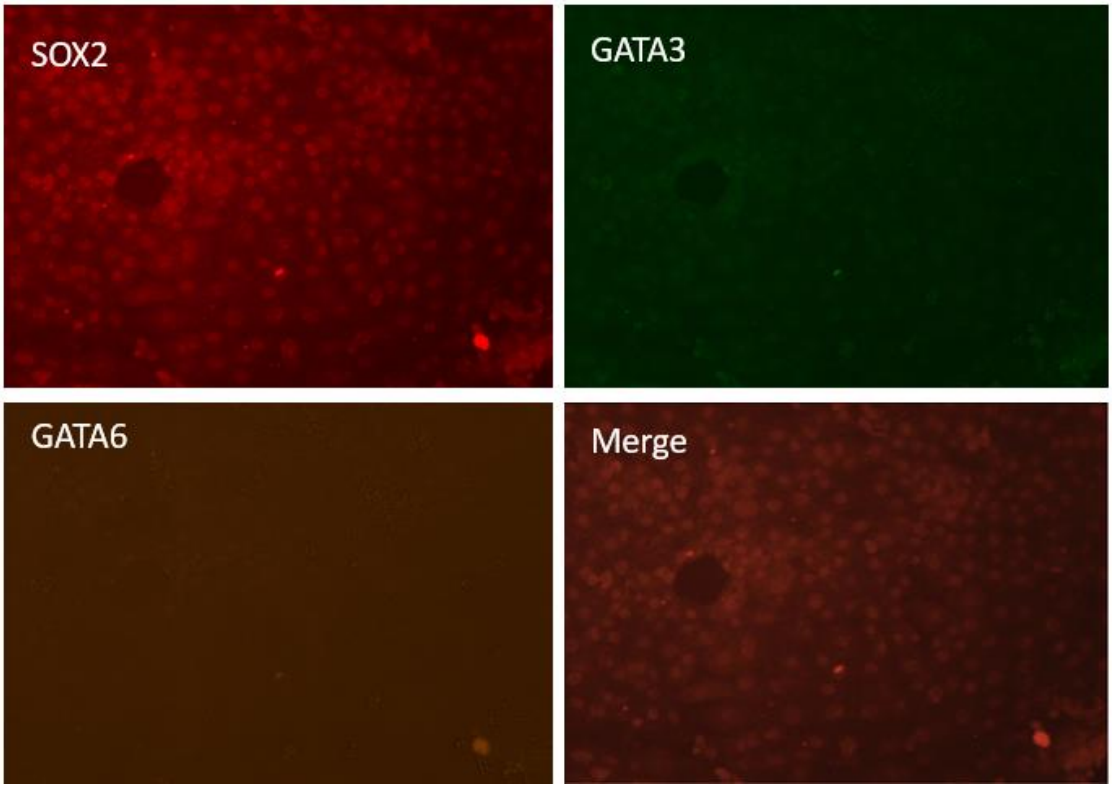

Figure 18. Staining of one outgrowth of passage zero cells.

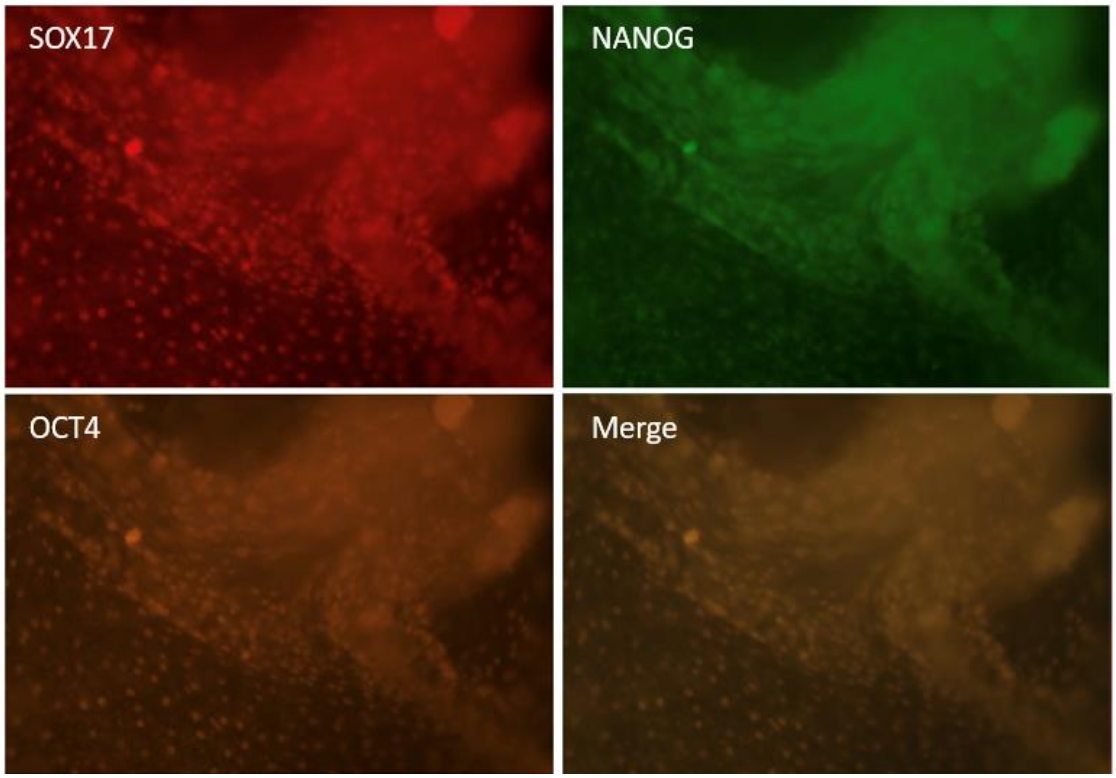

Figure 19. Immunofluorescent staining of an ICM outgrowth in CTFR medium.

3.4.2 Alkaline Phosphatase Staining

The initial outgrowths of cells were subjected to alkaline phosphatase staining. Cells stained positive for AP activity, indicating that the initial outgrowths of the cells were pluripotent. More extended outgrowths did begin to show a patchy expression of AP, with some individual cells not staining positive for AP.

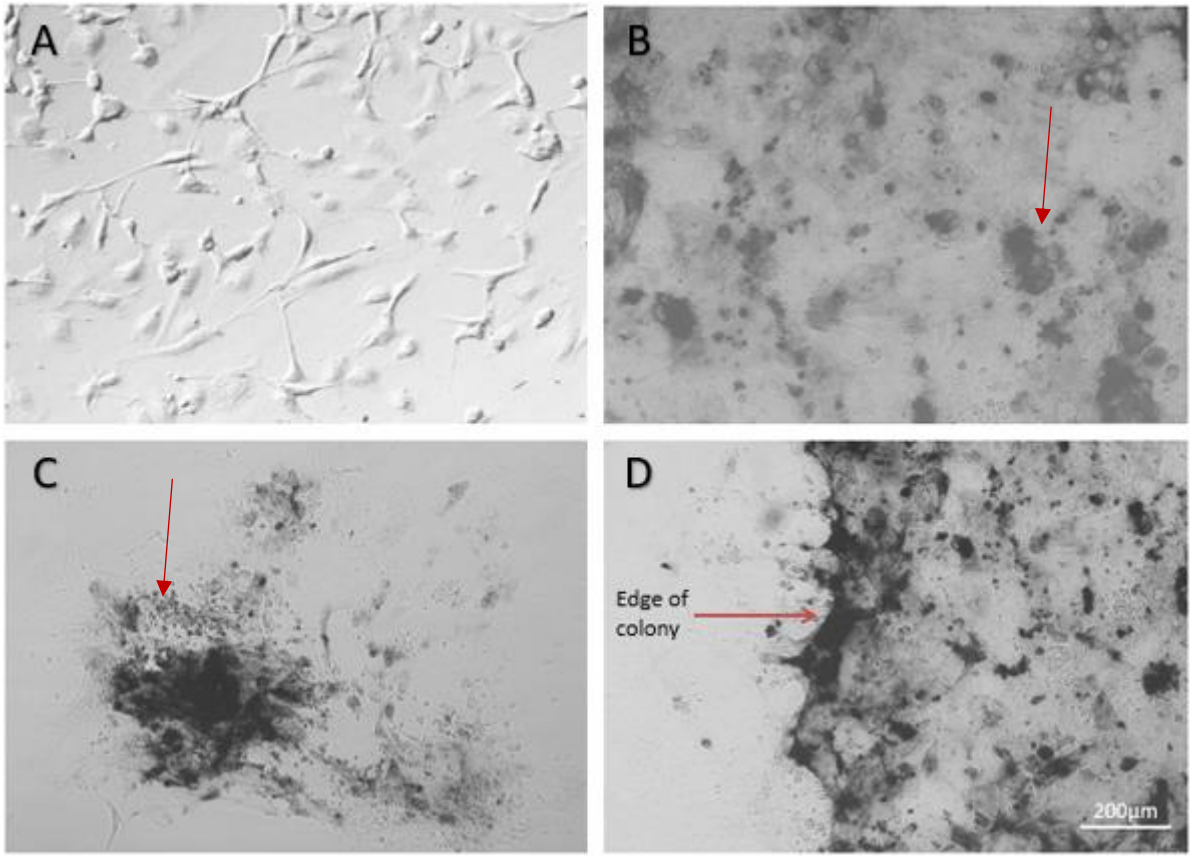

Figure 20. Alkaline phosphatase staining. A. Mitomycin C treated MEF cells showing a negative result for alkaline phosphatase staining. B, C, D. Arrows point to positive AP staining of CTFR outgrowths. Note that these photographs were captured in greyscale, however this staining is a navy-blue colour.

3.5 Cell Maintenance

During cell culture a pH indicator, such as phenol red, is often added to culture mediums so that the pH of the culture can be easily monitored. Fresh cell culture media will be orange-red in colour. As the cells begin to use up nutrients, metabolic waste products such as lactic acid begin to accumulate, and the media will become more acidic. This decrease in pH of the media can be observed as the media becoming more orange-yellow. Embryonic stem cells require a higher level of maintenance than other cell cultures, and need daily refreshment of their growth medium to ensure that they do not undergo differentiation. The first medium I established (CTFR) was derived from the Bogliotti paper; it turned an orange-yellow colour overnight, indicating that the medium was either unable to buffer pH efficiently, or that it did not contain enough energy and nutrition that the cells needed for proliferation. Consequently, this medium was unable to support the extended growth of embryonic stem cells. I attempted to combat this issue by performing more frequent changes of the medium. However, this was difficult to maintain as I was unable to be in the lab every 12 hours to change the medium. The eventual conclusion made was that this medium was not ideal for the derivation of embryonic stem cells. I began to troubleshoot the methods and based on literature which had been recently published, decided to formulate a different medium. This new medium, termed EPSC medium, was based on (Gao et al. 2019) and had been used to successfully isolate stem cells from pigs and humans. Additionally, I decided to try different approaches to passaging the cells, as the trypsin passaging of the initial outgrowths was the method which had consistently failed. I hoped that the combination of these two changes would result in a more successful derivation of cells.

3.6 Media Optimisation

The newly derived medium (termed EPSCM) was better able to support the growth of the cells. The efficiency of outgrowth formation was slightly higher in this medium, although this was may also have been influenced by my increasing skill at tending to the outgrowths. When outgrowths in CTFR and EPSCM were grown in parallel, EPSCM outgrowths proliferated at a greater rate than those in CTFR media (Figure 23). The EPSC media stayed a healthy red colour overnight, indicating that the pH of the culture was able to be maintained in the physiological range.

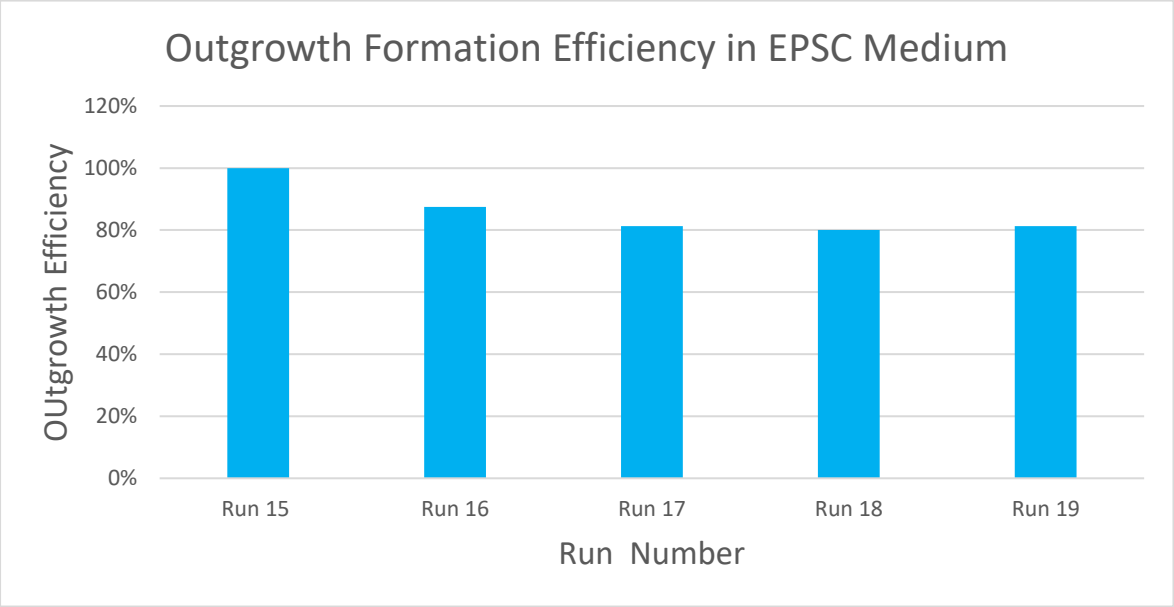

Figure 21. Outgrowth formation efficiency in EPSC medium.

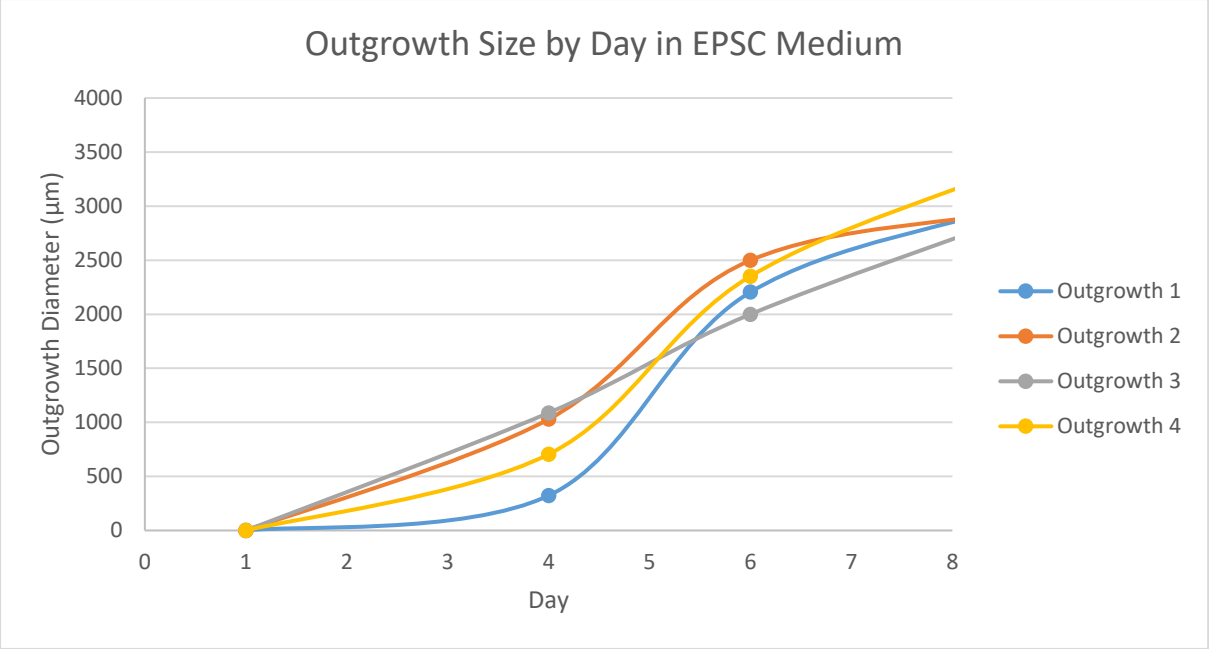

Figure 22. Diameter outgrowth by day in EPSC medium.

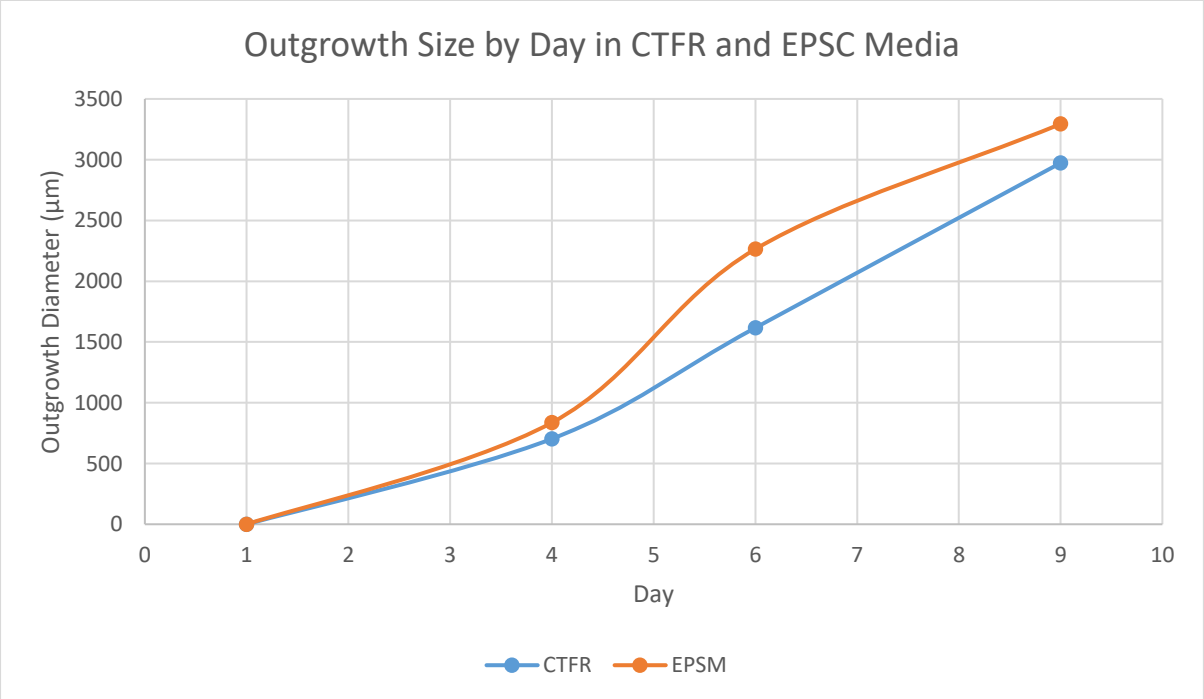

Figure 23. Comparison of outgrowth diameter in CTFR and EPSC Media.

3.7 Passaging of Cells

With the newly formulated EPSM medium a combination of accutase and mechanical dissociation was used to passage the initial outgrowths, as I had previously found trypsin passaging to be ineffective. Accutase is a cell detachment solution which has been implicated at performing particularly well at detaching primary cells and stem cells. Accutase treatment resulted in the colonies peeling off the culture plates but did not dissociate the cells into a single-cell aggregate, instead leaving them as one large mass of cells. Plating of the whole mass did not result in successful passaging of cells. When manual agitation, aspirating up and down the cell pellet, was used to assist the accutase in breaking down the mass into smaller pieces, passaging was successful, with a 75% success rate.

3.7.1 Passage One

One day after accutase/mechanical passaging some small colonies were visible under the microscope. Over the next 3-4 days in culture, more colonies emerged. I believe that the colonies which took longer to show up stemmed from individual cells, as opposed to small masses of cells starting the faster appearing colonies. This is supported by the evidence that the faster appearing colonies had a significant mass of cells in the centre of the colony, whereas the slower colonies did not have a distinct point of origin. The cells were small and circular, and grew in colonies with clearly defined boundaries from the MEF cells. The size of individual colonies was tracked and recorded (Figure 24).

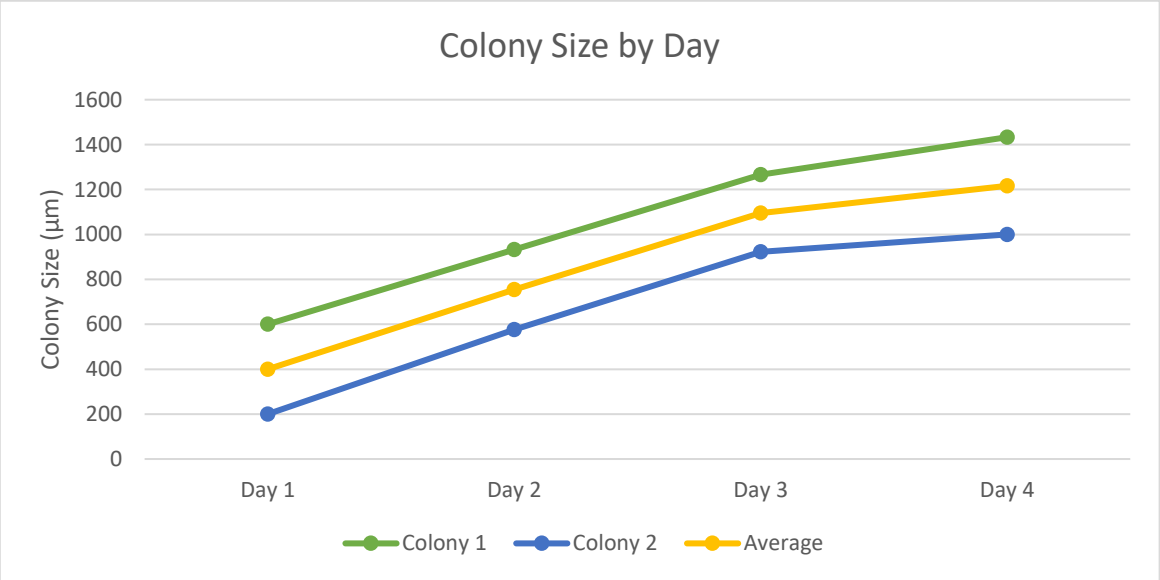

Figure 24. Colony expansion by day.

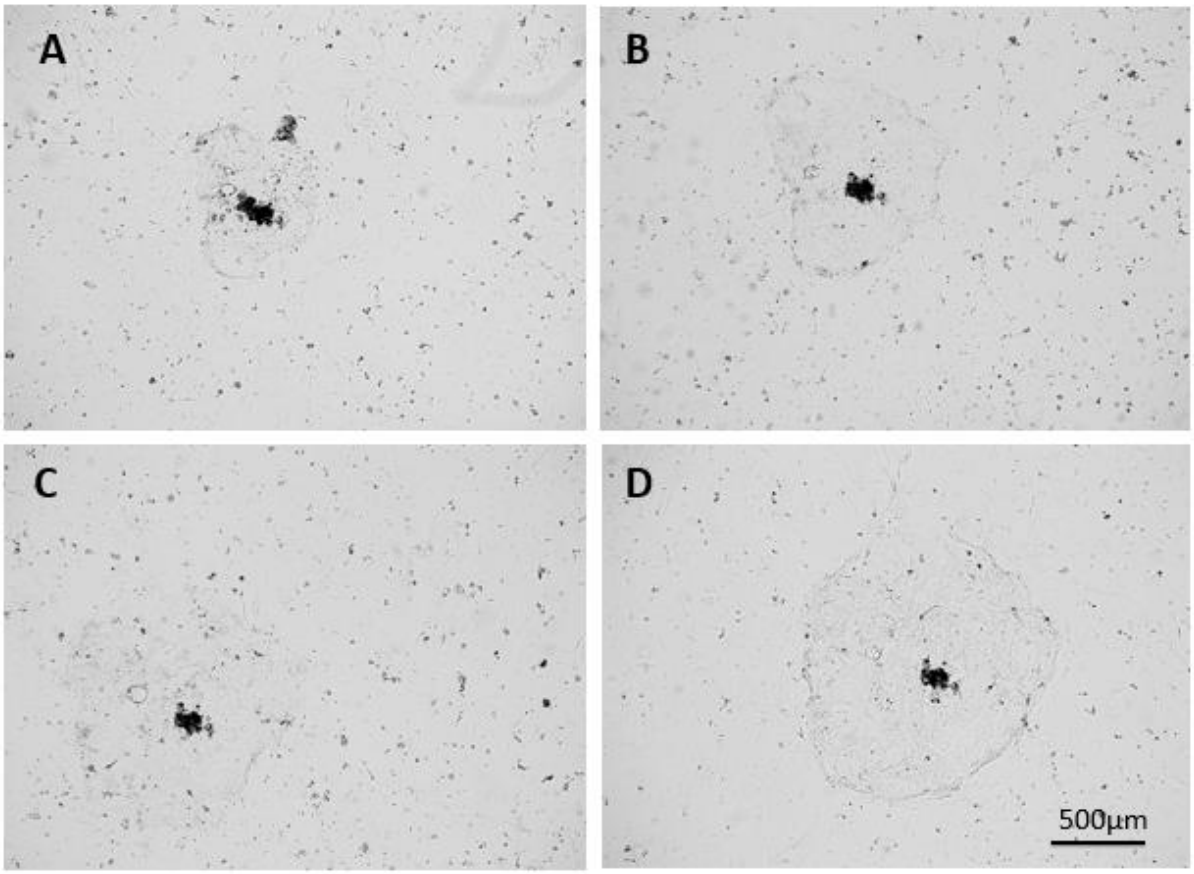

Figure 25. Passage one cells. A. 48 hours after passaging. B. 3 days after passaging. C. 4 days after passaging. D. 5 days after passaging.

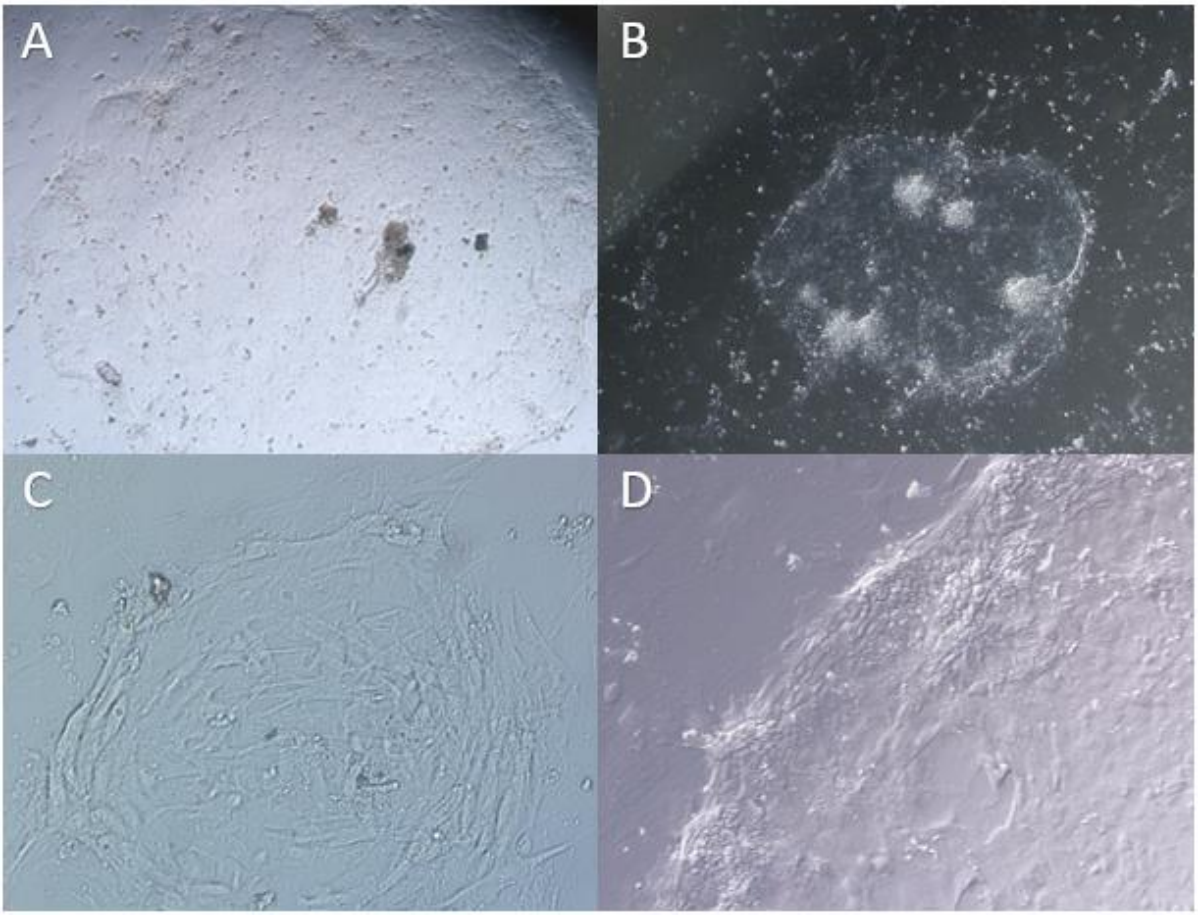

Figure 26. Various shots of passage one cells.

3.7.2 Passage Two

Passage one cells were grown in culture for 7 days before passaging. The cells were split 1:2 to give them the best chance of surviving the passage as possible. The passaging success rate was 57% (8/14). 48 hours after passaging, colonies were visible.

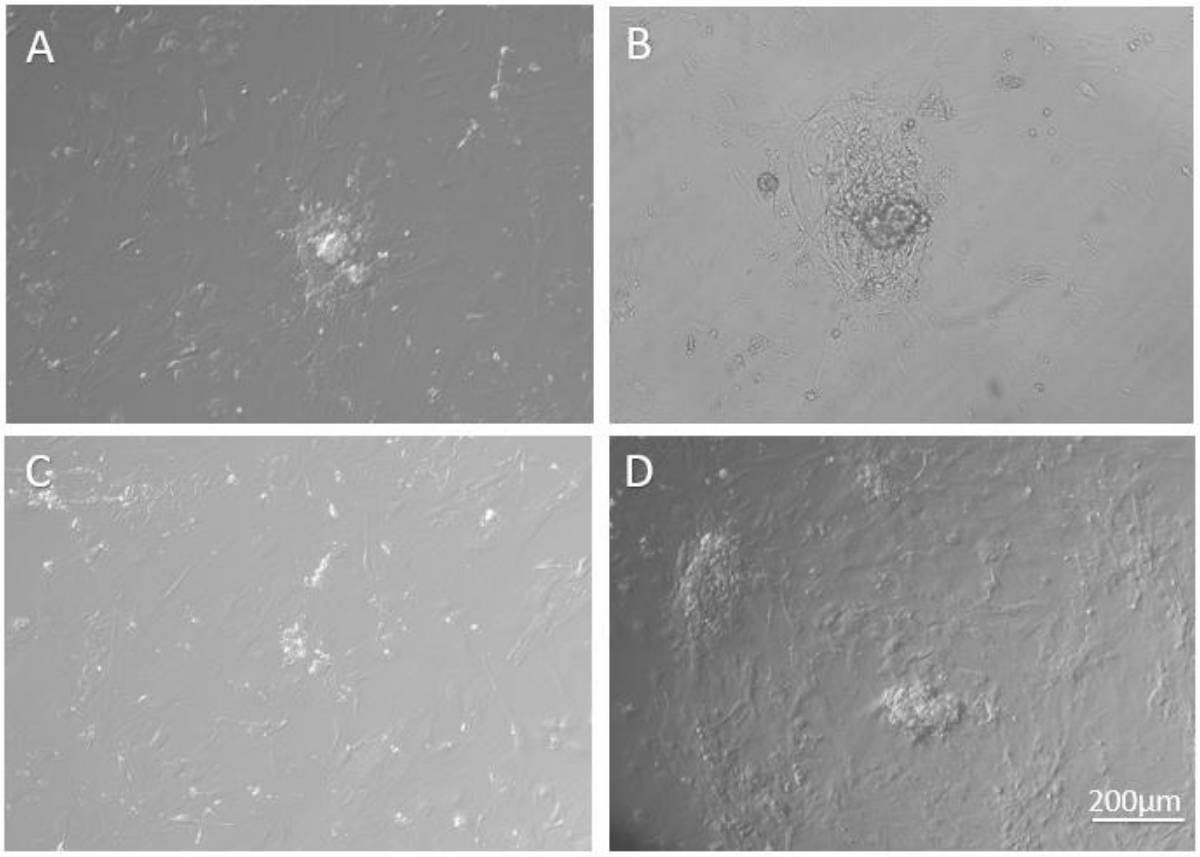

Figure 27. Passage two cells.

3.8 Characterisation of Passage Two Cells

3.8.1 Immunohistochemistry

Immunohistochemistry analysis showed that EPSCM-bESCs expressed the pluripotency transcription factors Sox2 and Oct4, but not Gata3, a marker of the trophectoderm. This indicates that EPSCM culture favoured the proliferation of the ICM over the trophectoderm.

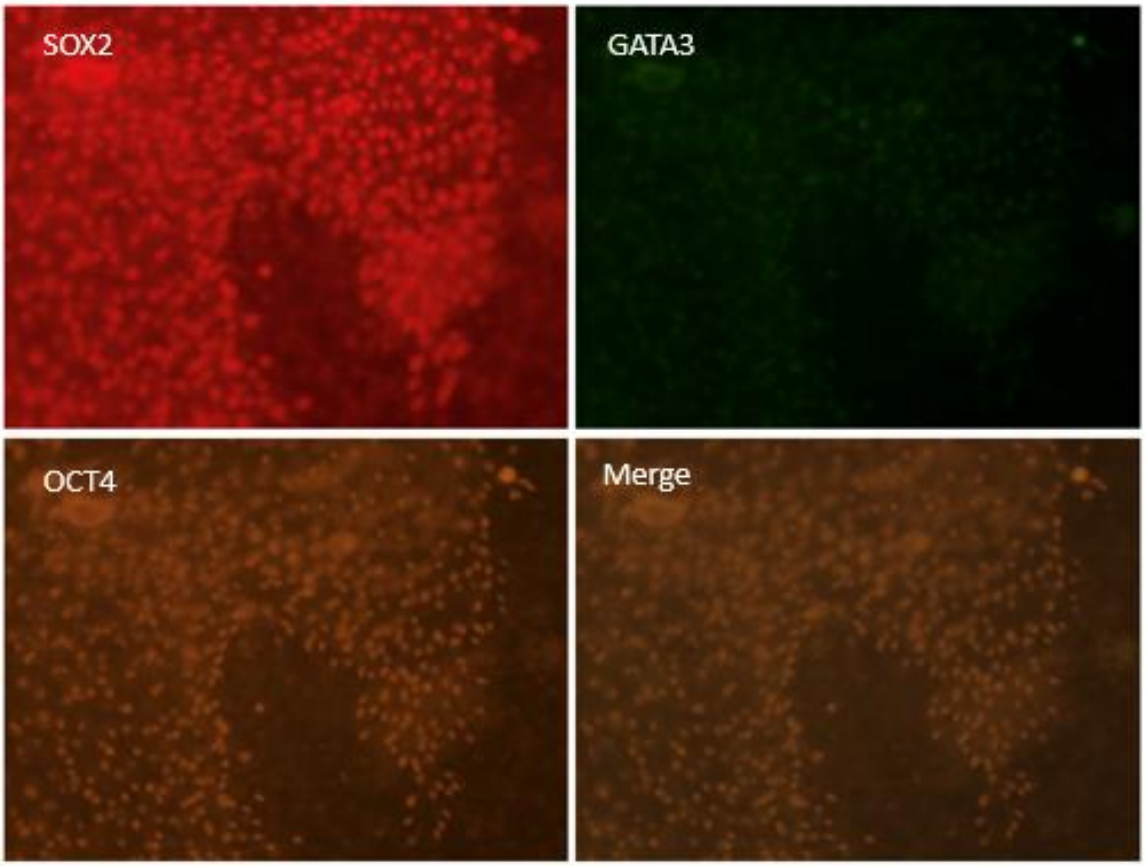

***Figure 28*.** Immunohistochemistry analysis of passage two cells.

3.8.2 PCR Analysis

PCR analysis passage two cells was performed to see whether they were expressing genes associated with pluripotency. Expression of Oct4 and Nanog was normalised to the expression of the housekeeper gene GAPDH. For each gene, no template controls and RT- controls were included. Most samples showed very minimal expression of Oct4 and Nanog. Only one sample showed significant expression of the pluripotency related transcription factors (Figure 29).

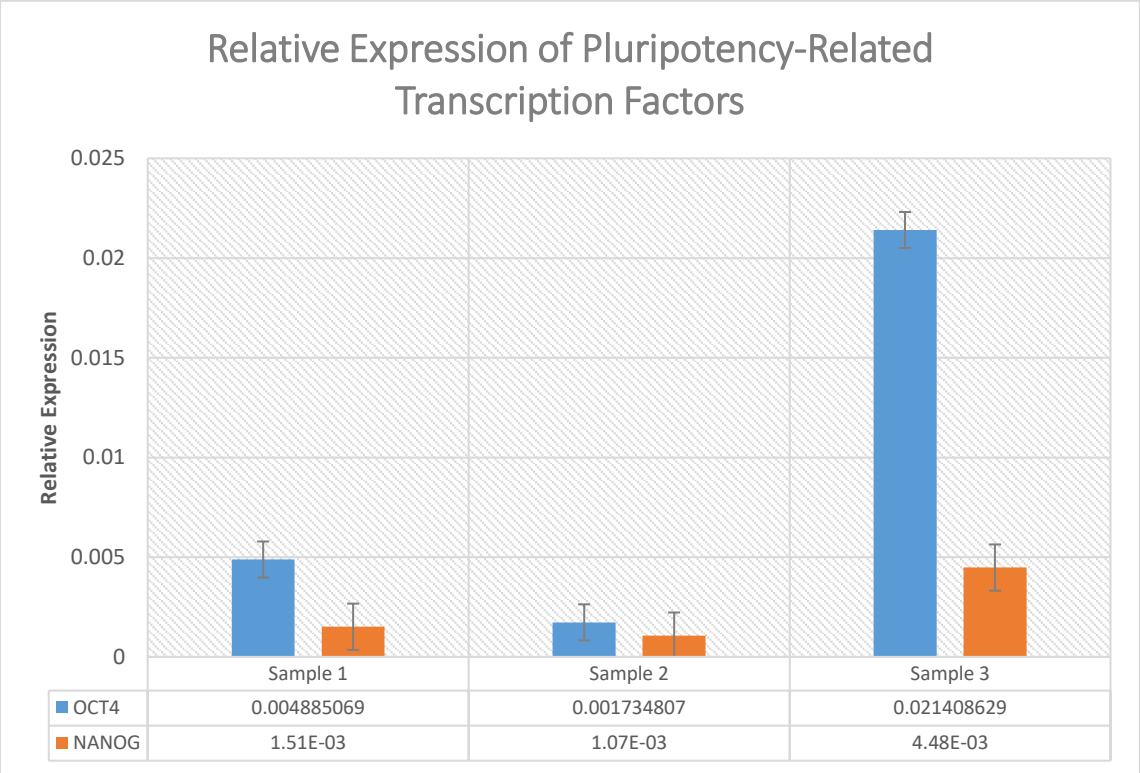

Figure 29. PCR Analysis of passage 2 cells.

Chapter Four: Discussion

4.1 Importance of an Adequate Feeder Layer and Defined Culture Conditions

The growth factors which sustain the growth of ES cells are provided by feeder cells and exogenously though the culture medium. For this research, inactivated mouse embryonic fibroblast cells were used as a feeder layer. Feeder cells provide active signals and factors to support the growth of embryonic stem cells, as well as an adherent surface for the cells to attach to (Llames et al. 2015). I found that an insufficient feeder layer was often the factor that limited the proliferation of the stem cells in this project. For feeder-dependent ES cells, having a feeder layer at the appropriate density is essential for the proliferation of ES cells. Too low a seeding density results in an insufficient amount of nutrients being secreted by the cells, and not enough extracellular matrix present to allow for the proliferation of the cells (Heng, Liu and Cao 2004). This problem was more evident with an increasing number of cells/colonies growing, suggesting that it becomes more of a limitation with a greater number of cells.

For this project mitomycin C was used to mitotically inactivate the feeder cells. In the future, gamma irradiation may be a better way to inactivate the cells. Studies which have compared gamma irradiation and mitomycin C treatment have shown that mitomycin C treated cells are metabolically altered, and are less efficient at supporting proliferation of the co-plated stem cell (Llames et al. 2015). I believe improving the quality of the feeder layer will improve the growth of the stem cells.

In ideal circumstances, bovine embryonic stem cells would be grown under feeder-free conditions. Feeder cells pose the risk of transferring animal viruses to the ES cells through their co-culture, and subsequently the ES cells cannot be used therapeutically. In order to achieve successful growth of ES cells without feeder cells, all nutrients and growth factors needed for the cells' survival and proliferation need to be supplied through the culture medium. Research has advanced enough that both mouse and human embryonic stem cells are able to be maintained in feeder free conditions. In an appropriate medium, these cells can be grown on a matrix rather than a layer of inactivated cells, therefore meeting the cells' requirement for both nutrients and an adherent layer for attachment.

Even when appropriate medium conditions have been established, variability of culture medium remains an issue. Small variations in culture components are extremely difficult to avoid, and can cause stochastic differentiation of cells (Moore 2006). This is particularly an issue with serum or protein supplements, which frequently contain undefined animal products which have been extracted from serum or blood. The components of these substances can vary from batch to batch,

resulting in issues when trying to reproduce the culture media (Yao and Asayama 2017). These xenogeneic components pose the same problem as feeder cells, in the way that they pose a risk of xenogeneic contamination, rendering the cells useless for any clinical application. Culture conditions for embryonic stem cells have improved immensely since their initial derivation in 1981. However, these issues emphasise the importance of having defined culture conditions which are both feeder-free and xeno-free. The medium conditions I grew my cells in does contain a small amount of serum (0.3%). Consequently, there is still room for culture conditions to be improved as the ideal medium would be completely defined, and animal product-free.

4.2 Passaging of Embryonic Stem Cells

During culture of cells, cells undergo logarithmic proliferation and need to be routinely passaged or 'split' into fresh culture wells as they approach confluence. This is usually carried out by dissociating the cells off the plate enzymatically and reseeding them at a lower density, allowing room for growth (Roach et al. 2006). However, human embryonic stem cells and epiblast-primed mouse embryonic stem cells have been shown to be intolerant to chemical dissociation, and passaging as single cells (Miki, Yasuda, and Kahn 2011). Consequently, care must be taken to avoid dissociation into a single cell suspension upon passaging of these cell types, as most will not survive typical passaging using dissociative enzymes. Because of this human ES cells are dissociated mechanically by hand, or by using a protease which does not damage the cell membrane, such as dispase (Bryja, Bonilla, and Arenas 2006).

The cells grown for this project were similar to human ES cells in the way that they were sensitive to chemical dissociation, and did not survive classical trypsin passaging. In order to mimic passaging of human embryonic stem cells, mechanical dissociation was used to assist accutase passaging of the cells. Accutase is a trypsin replacement which has been shown to be effective for the dissociation of more sensitive cells, including human embryonic stem cells. Using accutase the cells were able to be passaged, however they were not amenable to passaging as single cells like naïve mESCs are. During the initial derivation of cell outgrowths, and for the first 24 hours after passaging a Rho-kinase inhibitor, Y-27632, was added to the medium. This inhibitor was included because of its implication in the Bogliotti, Gao and Guo papers as being useful at increasing cell viability following passaging (Bogliotti et al. 2018, Gao et al. 2019, Guo et al. 2021). The mechanism by which the Rho-kinase inhibitor works to increase rates of cell survival is not fully understood, but an association with an inhibition of apoptosis and cell detachment has been made, mediated by the remodelling of the actin cytoskeleton (Llames et al. 2015).

To improve passaging conditions in the future, the cells could be grown on temperature sensitive slides. These slides promote dissociation of cells attached to their surface upon cooling of the slides to room temperature. This is a very gentle method of dissociating cells, and therefore likely to work well with sensitive cells. However, it comes with the drawback of expense, as these materials are very expensive to purchase.

4.3 Cellular Characterisation

The first cells derived in this project were grown in CTFR conditions (a custom mTeSR1 base medium, supplemented with FGF2 and IWR1). While these cells did show some expression of pluripotency factors (Oct4, Nanog) they also expressed Sox17, a marker of the hypoblast, therefore not expressing a completely ubiquitous pluripotent profile. Additionally, the cells also exhibited a capacity for limited proliferation, and were unable to withstand trypsin passaging. Consequently, a cell line was never able to be established using these culture conditions. CTFR conditions could be revisited in the future, as it is possible that these cells may be able to withstand passaging if another method is attempted.

The cells derived in the EPSC medium, based on the conditions published in the Gao et al. 2019 paper, show similar a morphology to epiblast primed stem cells, growing in large monolayer colonies. Immunohistochemistry analysis indicated that cell outgrowths originated from the inner cell mass, and showed expression of factors associated with pluripotency (Oct4, Sox2). RNA analysis did show expression of the pluripotency factors Oct4 and Nanog, however one could argue that this expression was minimal. Therefore, it is difficult to discern whether the expression of these pluripotency factors was due to the culture conditions being able to sustain growth in the pluripotent state, or rather the transient pluripotency state seen in the inner cell mass from which these cells were derived from. In order to confirm that these cells are pluripotent stem cells, more cellular material needs to be tested at a later passage, when the gene expression of cells becomes more homogenous. Unfortunately, the cells derived in this project cannot be considered an established cell line, as they have not been shown to be able to withstand extended passaging, freezing, and thawing. Once this milestone has been achieved and the RNA profile of the cells has been confirmed, karyotyping analysis needs to be performed to ensure that the cells have a normal karyotype. From here, a range of options can be explored in which the cells can be utilised in further research.

As a whole, the results of this research are indicative that EPSCM culture conditions established for porcine and human species, can be extended for the culture of bovine cells; however the work published here does not confirm that pluripotency is able to be maintained long term.

4.4 The Derivation of Bovine Expanded Potential Stem Cells

During the write up of this thesis the Liu lab published a report of the derivation of bovine expanded potential stem cells (Zhao et al. 2021). The Liu lab initially tried to culture bEPSCs in the culture conditions that they had established for pEPSCs. This medium was able to accommodate for the undifferentiated growth of the cells initially, however after several passages the cells differentiated, and expressed reduced levels of core pluripotency genes. This emphasises the fact that long-term culture of stem cells is required before a definitive characterisation of the cells can be made. The recent results published by the Liu lab suggest that the culture conditions I used in this research would initially be able to support the pluripotent growth of bovine stem cells, however continued passaged would lead to their eventual differentiation. This would perhaps explain the low expression levels of pluripotency-related genes shown by the RT q-PCR analysis.

The Liu lab made tweaks to the pEPSC media to accommodate for bovine stem cells. They used a mTeSR base supplemented with XAV939/IWR1, CHIR99021, WH-4-023/A419259, vitamin C, Activin A, and LIF. Therefore, the only difference between the culture conditions used in this research, and those established by the Liu lab for bEPSCs is the use of mTeSR base medium, rather than N2B27 supplementation. Remarkably, these culture conditions were able to support the growth of bEPSCs in feeder-free conditions for upwards of 30 passages (Zhao et al. 2021).

The results reported by the Liu lab show that the bEPSCs have a similar transcriptional signature to early pre-implantation bovine embryos, the ability to form cells of all three germ layers, and can contribute to both the embryonic and extra embryonic lineages in chimeras. The cells were able to be passaged successfully in a single-cell suspension, and could be used as donors in SCNT and cloning; all of these results suggest that bovine stem cells with an expanded potential have been successfully established. However, these results should be validated, as previous claims of establishing EPSCs were later contradicted by further analysis of the cells (Posfai et al. 2021, Guo et al. 2021). Therefore, it may be that these bEPSCs are also more similar to primed state stem cells.

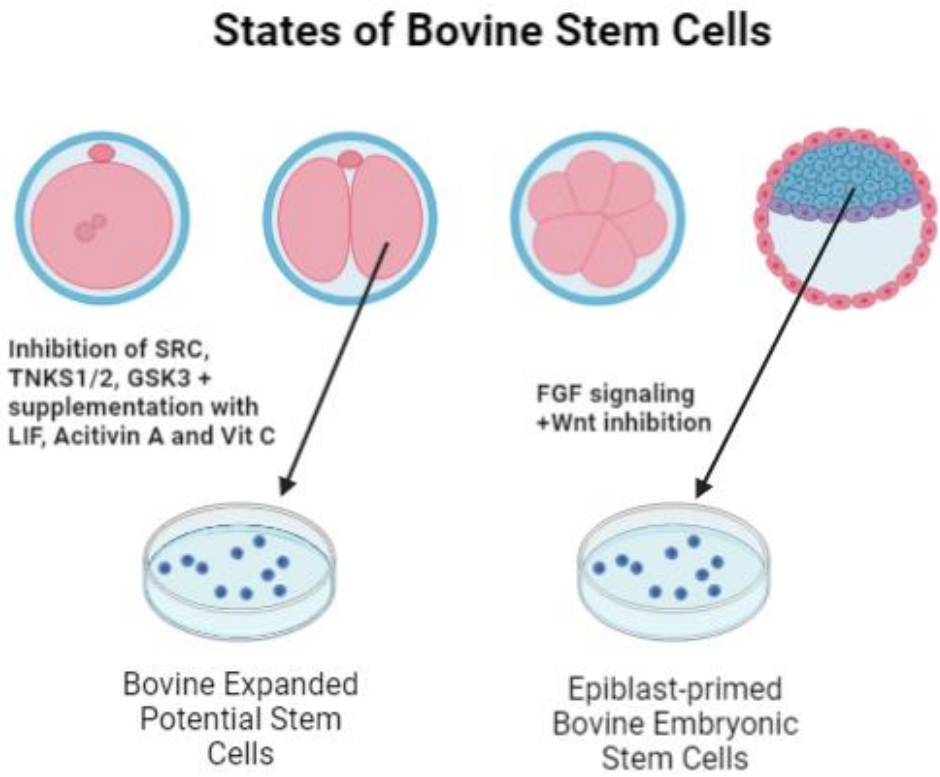

Figure 30. States of Bovine Stem Cells.

4.5 Discussion of Culture Components

The culture conditions used in this research were designed to mimic the conditions used to establish 'expanded potential' stem cells by the Liu lab in porcine and human species (Gao et al. 2019). Discussed below are the pathways thought to be involved in maintaining expression of pluripotency related genes, and how they tie into my results of this research, as well as the research recently published regarding the derivation of bovine EPSCs.

SRC Signaling

The Src family kinases, a family of non-receptor tyrosine kinases, play a key role in influencing cellular differentiation and proliferation. A Src inhibitor, A419259 was included in the EPSC medium due to results from the Liu lab. Their results stressed the importance of Src inhibition, demonstrating that the removal of a SRC inhibitor from the culture medium resulted in the reduction of the expression of pluripotency factors. Src inhibition has been shown to negatively impact the formation of the trophectoderm and the primitive endoderm, and consequently leads to partial arrest of the compact morula (Yang et al. 2017a). Therefore, it's inclusion in the medium acts to prevent differentiation into trophectoderm or primitive endoderm.

The Role of LIF

In 1998 LIF was identified as a factor which is excreted from feeder cells which helped prevent differentiation of ESCs. Consequently, culture conditions were able to be improved so that mESCs could be grown without feeders, given that the medium was supplemented with LIF. As mentioned previously, LIF promotes self-renewal of mESCs via it's activation of STAT3; however it also induces the PI3K pathway and the ERK pathway (Graf et al. 2011). This suggests that several LIF induced pathways are necessary for the maintenance of ESCs. The Liu lab has included LIF in their medium upon the basis of LIF promoting the expression of totipotent cells (Yang et al. 2017a). The inclusion of LIF may also be the reason that bEPSCs were able to be grown in feeder-free conditions.

Rho Kinase Inhibition

The inhibition of Rho kinase has been shown to increase the rates of cell survival for many cell lines during passaging. Consequently, it is routinely added to dissociated cell suspensions to increase viability of cells (Bogliotti et al. 2018, Gao et al. 2019). Interestingly, bEPSCs were able to be routinely passaged without the addition of a ROCK inhibitor, suggesting that they were not sensitive to passaging as single cells.

FGF/ERK/MEK Signaling

FGFs control a set of diverse cellular processes, including growth, cell survival, migration, and differentiation. FGF signaling activates a range of signals downstream, including MEK and ERK. The different states of pluripotent stem cells have different responses to FGF signaling (Zhang et al. 2019). Epiblast-primed hESCs and mESCs operate through FGF/ERK signaling, therefore FGF signaling is required to maintain these cells in a pluripotent state. However, naïve pluripotent cells will undergo differentiation upon exposure to FGF/ERK signaling. Consequently, naïve mESCS are cultured in conditions containing a MEK inhibitor. The Liu lab found that the MEK inhibitor PD-0325901 caused the differentiation or death of both pEPSCs and bEPSCs (Gao et al. 2019, Zhao et al. 2021). Therefore, it can be concluded that porcine and bovine stem cells require higher levels of MEK signaling than murine and human stem cells.

WNT Signaling

Wnt signaling has been implicated as having a role in many important biological processes, including embryogenesis (Yang et al. 2017a). Wnt signaling is activated through the binding of a WNT-protein ligand to a Frizzled family receptor, which passes the signal on to the cytoplasmic protein Dishevelled (Xu et al. 2016). Upon the binding of Dishevelled, the Wnt signal can branch off into multiple pathways, which have distinct effects. Wnt signaling pathways belong to one of two categories: canonical or non-canonical. Activation of the canonical Wnt pathway leads to the accumulation of b-catenin in the cytoplasm, which then translocates to the nucleus and initiates the transcription of transcription factors belonging to the TCF/LEF family. The effect of Wnt signaling in maintaining pluripotency is not fully understood. However, it is clear that the Wnt pathway has a significant role in influencing cell fate. Understanding the role of Wnt signaling in maintaining pluripotency may be the key to being able to better manipulate and control pluripotency across a range of organisms.

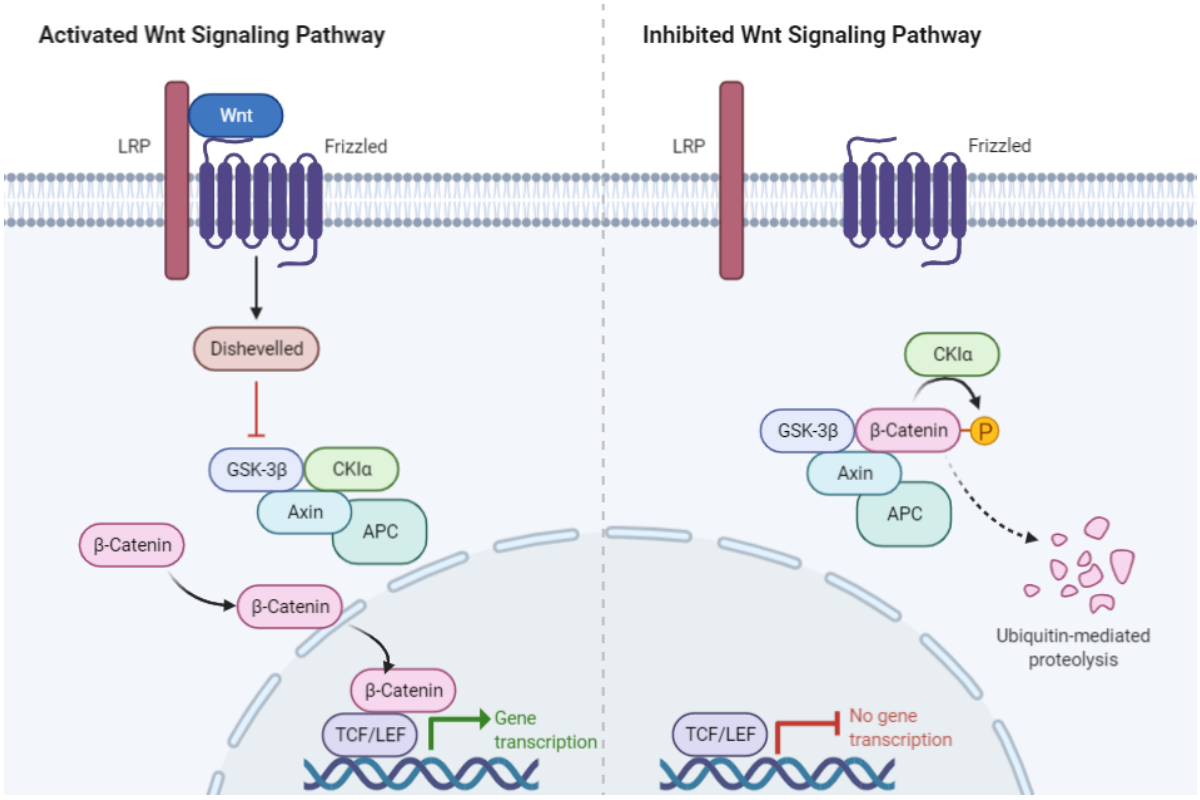

Figure 31. Active vs inactive Wnt signaling.

Wnt has been reported to be a positive regulator of pluripotency in both mESCs and hESCs; both types of cells were initially derived, and are still commonly maintained in conditions which promote Wnt signaling (Sokol 2011, Xu et al. 2016). In these cell types, Wnt signaling is activated by the inhibition of GSK3, an inhibitor of Wnt. The exact mechanism by which Wnt functions to maintain pluripotency is unknown. However, one possibility is that Wnt stimulation functions by its upregulation of STAT3, a downstream effector of LIF (Niwa 2011). This theory is supported by the evidence which shows that culturing murine ES cells in the presence of LIF replaces the need for b-catenin in the culture medium. Additionally, when GSK3 is added the requirement for LIF reduces, further emphasising that Wnt may operate to maintain pluripotency via it's activation of STAT3.

On the contrary, some research suggests that Wnt signaling is unnecessary for the derivation and establishment of ES cells. Although Wnt has been reported to be a positive regulator of pluripotency in mouse ES cells, its role has been debated in more recent years. B-catenin null mESCs have been generated successfully in naïve conditions (Lyashenko et al. 2011, Wray et al. 2011). It has also been demonstrated that when the secretion of WNT proteins is blocked in mESCs by deletion of the porcupine gene, the mESCs retain pluripotency (Biechele et al. 2013). Both bovine ES cells (Bogliotti et al. 2018) and porcine expanded potential stem (EPS) cells (Gao et al. 2019) employ a dual inhibition of GSK3 and Wnt in their culture systems, thereby inhibiting and activating Wnt simultaneously. Both cell types have not been successfully derived in conditions which do not contain a Wnt inhibitor, suggesting that Wnt inhibition may be necessary for the successful derivation of bovine and porcine ES cells. It is possible that this could be a species specific culture condition, or it may be an indication that the effect of WNT signaling on pluripotency is pleiotropic and not completely understood.

Bovine ES cells derived by Bogliotti and colleagues employed the use of the WNT inhibitor IWR1, while the tankyrase inhibitor XAV939 was used for the establishment of porcine embryonic stem cells (Bogliotti et al. 2018, Gao et al. 2019). The withdrawal of these inhibitors from the culture medium resulted in loss of pluripotency factors and caused differentiation of the cells. However, both culture conditions also contain a Wnt activator, either lithium chloride or CHIR99021. Therefore, the Wnt pathway may still be active to an extent within the cells and the Wnt inhibitors may be operating through a different pathway. For example, XAV939 also supresses Yap1, which potentially modulates targets relevant to the first lineage segregation into the TE/ICM.

4.5 Research Limitations

While some elements of this research were successful, several major obstacles were faced. Time was a major limitation for this research. Each experimental run took a minimum of 2 weeks, with the culturing of bovine embryos to the blastocyst stage alone taking 9 days. Following this, outgrowths of the ICM took 3-4 days to start growing and took 7-9 days to expand enough to be ready for passaging. This long experimental time meant that if something went wrong at any point during the run, it set progress back significantly. Additionally, ovaries were obtained from a local abattoir, and it was not always possible to obtain them, especially with lockdown restrictions.

4.6 Future Directions

This research saw the establishment of a culture medium which was able to support the proliferation of ICM-derived cells, as well as the milestone of getting them past their first few passages. The next steps for this research would be to try the bEPSC conditions for growth, to see whether they can be replicated. Once a pluripotent or totipotent bovine stem cell line has been established, the applications of such cells can finally be exploited. Bovine embryonic stem cells provide a wealth of opportunities for advancement of biomedical and agricultural research.

One exciting potential use of bovine stem cells is their use to create organoids. Organoids are 3D tissue culture systems that are derived from the self-organisation of stem cells as they differentiate in vitro. Organoids contain most relevant cell types of the in vivo tissue and consequently offer broad applications for in vitro research studying development, toxicology, cancer, and infectious diseases (Kar et al. 2021). Most organoid studies so far have been carried out in human and mouse models, very little organoid research has been conducted in agricultural species (Pain 2021). Organoid research in agricultural models have the potential to limit animal testing, as organoid models could be used as an alternative, providing the benefit of less ethical concerns.

Embryonic stem cells have been extensively researched in mice, however, the molecular regulation of cattle pluripotency remains less addressed. Cattle research is particularly relevant in New Zealand as we have such a large agricultural industry, with cattle exports alone generating around $250 million dollars for the country (Central 2021). As a result, there is great interest in increasing the rate of cattle reproduction efficiency, as well as the quality and quantity of beef and dairy. Bovine embryonic stem cells have the potential to improve breeding of cattle, in turn leading towards cattle which produce more milk, more tender meat, and face less complications in giving birth (Servick 2018).

The utilization of bovine induced pluripotent stem cells would be a huge next step in the field of bovine pluripotent cells. This would cut out the need for generation of bovine embryos, and lead to the same result of the generation of bovine pluripotent stem cells. Until the recent report by the Liu lab, attempts to derive induced pluripotent stem cells have resulted in a poor derivation cells with the inability to maintain pluripotency long-term, and limited developmental potential in both in vitro and ex vivo assays (Zhao et al. 2021). Therefore, this is another avenue to be explored for the generation of bovine stem cells.

Coming into this project I had 4 aims; to isolate stem cells, establish conditions in which they could proliferate in an undifferentiated state, find a sustainable way of passaging them, and characterise the cells. I was successful in isolating cells from the inner cell mass of bovine embryos, and tested

different culture conditions, managing to find one which supported the short-term growth of these cells. I found that accutase was the most viable method of passaging, resulting in the highest survival rate of the cells. The early passages of these cells were characterised in terms of gene expression and protein translation. However, without characterisation of later passage cells, I cannot say that I was successful at establishing an embryonic stem cell line. Having a well-established and characterised bovine embryonic cell line, and an understanding of the signaling mechanisms, which control the state of these cells, can be expected to substantially advance biotechnological and agricultural research.

Appendix

CTFR Medium

DMEM/F12	1x
Low FA BSA	1x
Thiamine	0.025 mM
Reduced glutathione	6.5 mM
L-ascorbic acid	0.375 mM
Lipid Concentrate	1x
Glutamax	1x
Non-essential amino acids	1x
Lithium chloride	80 µM
GABA	1 µM
Pipecolic acid	15.5 µM
Trace elements B	1x
Trace elements C	1x
ITS-X	1x
FGF2	20 ng/ml
IWR1	2.5 µM

EPSC Medium

DMEM/F12	1x
N2 supplement	1x
B27 with RA	1x
Glutamax supplement	1x
Pen/Strep stock	1x
Non-essential amino acids	1x
Beta/2-mercaptoethanol 14.3M	0.1 mM
CHIR99021	0.2 µM
A419259	0.1 µM
XAV939	2.5 µM
L-Ascorbic acid	200 µM
FBS	0.3%
LIF	10 ng/ml
Activin-A	20 ng/ml
Y27632	10 µM

Primers used for RT-qPCR:

GAPDH f: 5’ CTCCCAACGTGTCTGTTGTG

GAPDH r: 5’ TGAGCTTGACAAAGTGGTCG

NANOG f: 5’ CCAGTGTCCCTGAGCAGTTCAA

NANOG r: 5’ CAGGTTTCCAGGAGAGTTCCAC

OCT4 f: 5’ CCAGGACATCAAAGCTCTTCA

OCT4 r: 5’ AAAACCACACTCGGACCA

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