Inhibition of proteolytic degradation of the basement membrane during embryogenesis impacts collagen IV biosynthesis and cell differentiation

Nicola Stark
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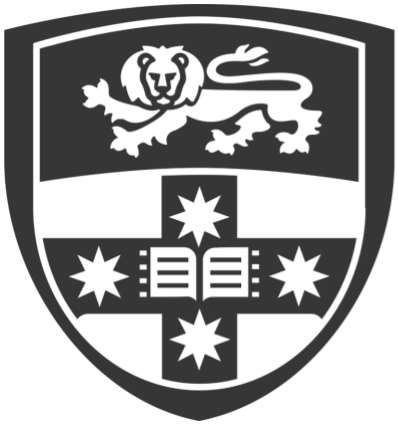

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Statement of Authentication

This thesis is submitted to the University of Sydney in fulfilment of the requirements for the degree of Doctor of Philosophy in Medicine.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. All assistance is acknowledged in the appropriate
location within the text.

I, hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Signed _____

Nicola Stark

13th June 2023

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My PhD has been one of the most challenging journeys I have ever managed to accomplish in my life.
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Abstract

Preimplantation embryo development involves a series of complex biological processes which involve cell communication, signalling pathways, cell migration and differentiation. Advancements in the field of Assisted Reproductive Technology (ART) have aimed at optimising preimplantation embryo culture environments and thereby improving embryo quality and success rates. Previous studies have described how quintessential amino acid L-proline is naturally found in hydrolysed collagen, specifically collagen type IV within the embryo's basement membrane, and has shown to play an important role in biological function and regulation in the developing pre-implantation embryo. During collagen biosynthesis, matrix metalloproteinases (MMPs) will break down the basement membrane, releasing L-proline facilitating the cell differentiation, predominantly within the embryo's developing inner cell mass. The secretion of L-proline into the microenvironment is driven by intracellular prolidase (PEPD) which has previously been shown to play a crucial role in the recycling of L-proline for collagen synthesis and extracellular matrix (ECM) remodelling as well as cell growth.

Our study aimed at observing how by inhibiting proteolytic degradation of the basement membrane during preimplantation development will potentially limit the natural release of L-proline from collagen IV, impacting essential biological functions associated with healthy embryo development.

As collagen IV is made up of approximately 25% of the amino acid residues and since collagen is one of the most abundant proteins in the human body, the degradation of collagen IV during collagen synthesis would potentially provide an excellent source since L-proline (Phang *et al.*, 2008). The collagen is initially broken down by specific MMPs, and the degradation continues as a result of protease activity. Lastly, the final. In humans, the homodimeric enzyme is found within the cytoplasm and functions by cleaving iminodipeptides containing C-terminal proline or hydroxyproline as the tertiary amide bond is unable to be identified by other peptidases (Palka, 1996). Through previous studies used to determine the role of enzymes required for ECM integrity, prolidase has been identified as a cytosolic enzyme that is required for sufficient metabolism and synthesis of proline-rich proteins (Insolia *et al*., 2020). Specific and non-specific MMP inhibitors will target enzymes responsible for the breakdown of the embryo's ECM, limiting or potentially preventing a sufficient concentration of L-proline.

By introducing a specific inhibitor that actively targets the enzymes responsible for the breakdown of the ECM, it can be hypothesized that this action will restrict the release of L-proline subsequently impacting cell differentiation. MMP inhibitors will have an imperative effect on specific pathways related to enzymatic activity, influencing cell differentiation, embryo development and viability. As two specific gelatinases have been identified in having a significant role in basement membrane (BM) degradation, they, along with their associated pathways will be the primary focus in our experiments and how obstructing their activity will impact the biological functions associated with healthy embryo development.

List of Abbreviations

ART	Assisted Reproductive Technology
B0AT1	Sodium-dependent neutral amino acid transporter
BM	Basement Membrane
BSA	Bovine Serum Albumin
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
ECM	Extracellular Matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2 pathway
ESC	Embryonic stem cells
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
Gly	Glycine
GLYT1	Glycine transporter 1
GM6001	Ilomastat
ICM	Inner cell mass
IGF	Insulin-like growth factor
IVF	In vitro fertilisation
JAK	Janus kinase signal transducer
KO	Knock out

L-Gln	L-Glutamine
L-Orn	L-Ornithine
L-Pro	L-Proline
L-Thr	L-Threonine
MAPK	Mitogen-activated protein kinases
MEK1/2	Mitogen-activated protein kinase
MMP	Matrix metalloproteinases
mOsm	Milliosmoles
mTOR	Mammalian target of rapamycin
NC1	Non-collagenous domain
P5C	Pyrroline-5-carboxylic acid
PAF	Platelet activating factor
PBS	Phosphate-buffered saline with Triton X
PE	Primitive endoderm
PEPD	Prolidase
PFA	Paraformaldehyde
PGC	Primordial germ cells
PI3K-AKT-mTOR	Phosphoinositide 3-kinase-AKT-mammalian target of Rapamycin pathway
PRODH/POX	Proline Dehydrogenase/Proline Oxidase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase pathway
SB-3CT	2-[[(4-phenoxyphenyl) sulfonyl]methyl]-thiirane
SHH	Sonic hedgehog pathway

SIT1	Signalling threshold-regulating transmembrane adapter 1
SNAT1/2	Sodium-coupled neutral amino acid transporter 1
TE	Trophectoderm
TGF	Transforming growth factor
TGFβ	Transforming growth factor-beta
TIMP	Tissue inhibitors of metalloproteinases
VEGF	Vascular endothelial growth factors
Wnt	Wingless-related integration site

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Chapter 1: Introduction

1.1 Introduction

In 2018, there were 84,064 Assisted Reproductive Technologies (ART) treatment cycles reported from fertility clinics throughout Australia and New Zealand. Of these treatment cycles, 83.5% resulted in either an embryo transfer or all oocytes and or embryos being cryopreserved. Of these initiated cycles, 23.2% resulted in a clinical pregnancy and 18.4% resulted in a live birth (Newman 2020). Throughout the past decade, data reported by the University of Technology in Australia and New Zealand (ANZARD), success rates for live births per frozen embryo have increased by approximately 50%, while success rates for live births following a fresh embryo transfer has increased approximately 25% (Macaldowie, 2012).

These improved success rates are due to advancements in laboratory techniques and developments leading to more optimal embryo culture. Research has shown that specific amino acids in the embryo's microenvironment can work as growth factors in the early stages of embryogenesis (Morris *et al*., 2020) (Lane and Gardner, 1997). By identifying specific amino acids that work in this way, it may be possible to further improve the early stages of embryo development *in vitro* (Li *et al*., 2009).

The extracellular matrix (ECM) is an intricate protein network comprised of collagens, proteoglycans, elastin, and cell-binding glycoproteins which are arranged in a cell/tissue-specific manner during early preimplantation development. In addition to being a reservoir for bioactive molecules and numerous growth factors and cytokines, the ECM exists as a highly dynamic structure that is both enzymatically

and non-enzymatically being constantly remodelled. Furthermore, the ECM serves as both structural support for the embryo and but additionally integrates fundamental cell functions such as differentiation, proliferation, and cell migration. As the multicellularity evolves independently in different embryonic cell lineages, the specific formation of the ECM will vary between these structures.

The basement membrane (BM) is a dense layer of ECM which provides support for the surrounding tissues, modulates cellular behaviour and promotes tissue repair. One of the major components of the BM is type IV collagen, a ubiquitous protein that is one of the most widely distributed molecules in the BM during preimplantation development (Poschl *et al*., 2004).

The flexibility and support of the lattice-like structure of collagen IV is made possible, predominantly due to the presence of L-proline, an amino acid that permits the sharp twisting of the collagen helix, while also increasing the stability (Shoulders and Raines, 2009). L-proline is a non-essential amino acid which has been identified as additionally being responsible for stimulating cell pluripotency and differentiation during the early stages of preimplantation embryo development (Washington *et al.*, 2010). L-proline can act in the same way as a growth factor when added to low density culture medium, improving embryo development, and helping to promote cell differentiation (Morris *et al*., 2020). Natural proteolytic degradation by metalloproteinases (MMPs) is an important physiological process that results in the breakdown of the BM. The breakdown of collagen IV within the BM has been hypothesised to cause the release of free L-proline from the collagen IV helix, which

in turn could potentially be one of the driving forces behind cell differentiation and maintaining cell pluripotency within the embryo's inner cell mass (ICM).
The present study was aimed to investigate the expression of collagen IV in the mouse preimplantation embryo and whether the inhibition of natural proteolytic degradation of collagen IV will have an impact on embryo development, blastocyst hatching and cell differentiation within the blastocyst.

1.2 Embryogenesis

Embryogenesis is the fundamental process of cell division and differentiation of all tissues from a fertilized oocyte, resulting in the formation and development of the embryo (Figure 1.1). The process incorporates reprogramming, progressive cleavage divisions and mitotic chromosome segregation followed by the activation of the embryonic genome (Ambartsumyan and Clark, 2008). Successful development of the embryo relies primarily on the transition from meiosis to mitosis and the mechanisms involved with this process (Courtois *et al*., 2012). It is important to understand the fundamentals of gamete and early embryo development to fully comprehend the mechanics and biological circuitry involved with embryogenesis.

1.2.2 Preimplantation embryo development

1.2.2.1 Fertilisation

The development of the mammalian embryo begins at fertilisation. A protective coat of non-cellular material named the zona pellucida surrounds the mature oocyte, which is a haploid cell that contains half the number of chromosomes. For fertilization to take place, a single haploid sperm cell must

penetrate the zona pellucida, enter the oocyte cytoplasm, and unite its pronucleus with the oocyte pronucleus.

The union of the sperm pronuclei and oocyte pronuclei restores the number of chromosomes that is representative of a given species. In humans, the mature sperm and oocyte carry 23 chromosomes each, and when fused together, the result will develop a diploid zygote, containing 46 chromosomes. After fertilization, the embryo begins to develop accordingly to its genetic programming. The morphological changes that occur following fertilization are well documented and have been elucidated thoroughly.

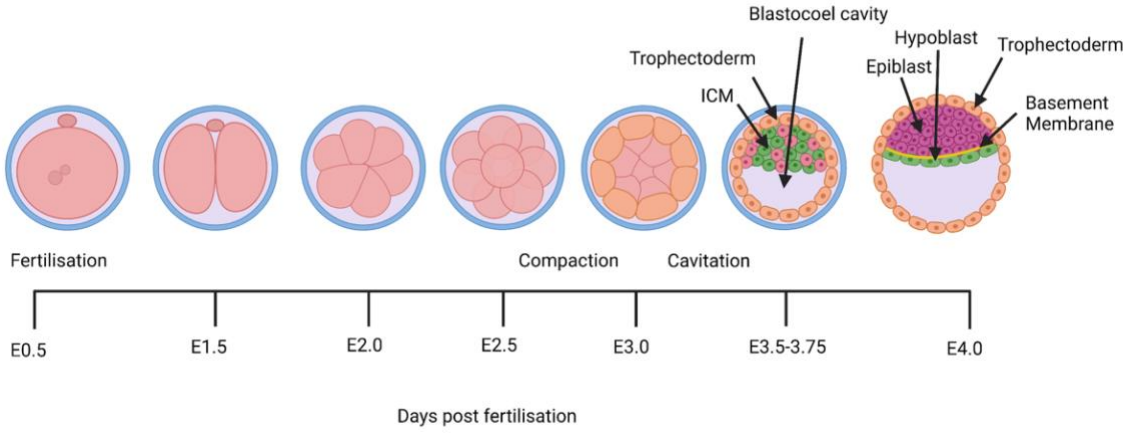


Figure 1.1 Stages in mouse preimplantation development
Timeline of the various stages of preimplantation development in mouse embryos from fertilisation to blastocyst formation. Sequential morphological changes in the developing mouse embryo will give rise to three distinct cell lineages, the trophectoderm, the epiblast and the hypoblast (or primitive endoderm). The basement membrane is deposited between the embryo's hypoblast and epiblast cells. This image was created with BioRender (https://biorender.com/)

1.2.2.2 Cleavage and compaction

Following successful fertilisation, the embryo will commence the cleavage phase of embryo development. During the cleavage stages, the embryo actively splits into blastomeres where they will exhibit correct morphokinetic cell division. Embryos that display either a somewhat lagged or abnormally rapid cleavage rate have been shown to have chromosomal and metabolic anomalies, often resulting in failed development (Prados *et al*., 2012). Symmetric division of blastomeres also directly correlates to normal mitosis. When cleavage rate is asymmetric, there is an increased rate of multinucleation in blastomeres, which is associated with genomic abnormalities and poor embryo development (Scott *et al*., 2007). Multinucleation is linked to aneuploidy and abnormalities in DNA synthesis (Van Royen *et al*., 2003) and is more prominent in embryos with uneven cell numbers, and display either delayed and rapid cleavage.

After the embryo has undergone the first three cleavages, the blastomeres will initiate cell compaction at approximately 2-3 days post fertilisation, where they will form tight intercellular junctions and subsequently form a morula (Prados *et al*., 2012). Compaction is a critical process in embryo morphological development, where the embryo's blastomeres increase their cohesion due to mechanical interactions of the blastomeres. The cell-to-cell adhesion molecule responsible for this process in known as E-cadherin, an epithelial cell adhesion protein which drives compaction (Turlier and Maitre, 2015). The adhesion molecules will additionally act as signalling molecules and help to regulate the activity of mitogen-activated protein kinase (MAPK), a key pathway in cell survival, proliferation and apoptosis, which are essential for normal cell functions (Pece and Gutkind, 2000).

1.2.2.3 Blastocyst formation and lineage allocation

Once the embryo has undergone compaction, cell differentiation is regulated through the expression of specialised gene families that will encode for ion transporter, cell junction and polarity products for the cell lineages to form.

Blastocyst formation involves two fundamental events; cavitation and cell differentiation, whereby the embryo begins to develop the blastocoel and the first two cell types begin to form: the trophectoderm (TE) and inner cell mass (ICM). Cavitation is driven by the expression of gene products which drive the acquisition of TE cell polarity. The permeability of tight junctions in the TE regulate blastocoel fluid outflow and the polarised distribution of Na^+/K^+-ATPase generates trans-trophectoderm Na^+ gradients which are responsible for osmotic accumulation of water within the blastocoelic cavity (Watson, 1992).

The cells in the TE are responsible for attachment and invasion of the embryo into the uterine endometrium, while the inner cell mass contains cells which give rise to the definitive structure of the developing foetus (Wiley, 1987). The three distinct cell groups that will arise in the mammalian blastocyst include the TE cells, the epiblast, which is derived from the ICM, and the primitive endoderm or hypoblast. Of these three lineages, only the epiblast will give rise to the embryo proper, consisting of the ectoderm, mesoderm, and definitive endoderm (Rossant and Tam, 2009).

1.3 Preimplantation embryo cell lineages

As mentioned above, three distinct cell lineages form within the blastocyst, namely the trophoblast, epiblast, and hypoblast (Figure 1.2).

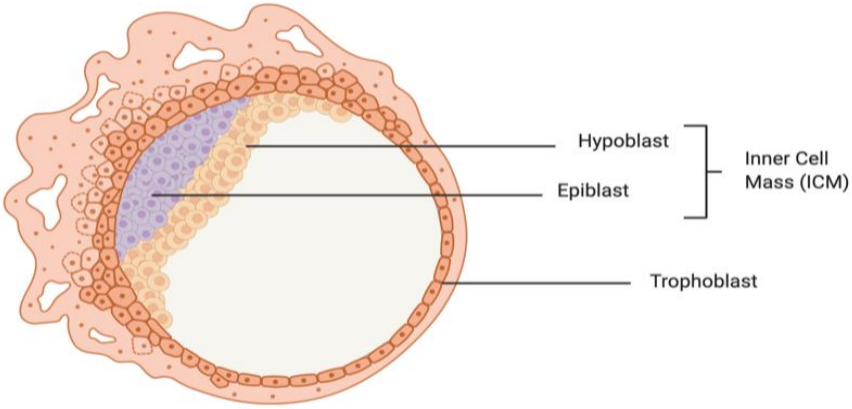


Figure 1.2 The preimplantation epiblast and hypoblast
The epiblast and hypoblast in the preimplantation embryo will arise from cell differentiation within the embryo's inner cell mass. The epiblast will later form the three primary germ layers of the embryo, while the hypoblast will give rise to the yolk sac and eventually the chorion. The trophoblast will form a thin layer of cells that will assist the embryo in implantation and will ultimately form the placenta. This image was created with BioRender (https://biorender.com/)

1.3.1 Epiblast

The epiblast is one of two distinct layers that forms the inner cell mass of the mammalian blastocyst (Figure 1.2). During early embryo development, the transcription factor Nanog is responsible for the formation of the ICM and maintaining pluripotency in the developing epiblast (Sun *et al*., 2014). Previous studies have shown that Nanog functions with other transcription factors, such as Oct4 and SOX2 in regulation of cell differentiation within the ICM (Kashyap *et al*., 2009). An interruption to Nanog expression in preimplantation embryos can impede

the development of the ICM and as a result, the formation of the epiblast is impacted (Mitsui *et al*., 2003).

1.3.2 Hypoblast

The hypoblast consists of small cuboidal cells that lay beneath the blastocyst's epiblast (Figure 1.2). Although the cells in the hypoblast do not appear to contribute in the formation of the embryo proper, they do help to facilitate the implantation of the embryo, and along with the trophoblast cells will also help to form the placenta and play a vital role in influencing the alignment of the embryonic axis (Bertocchini and Stern, 2002). Lineage marker transcription factors that help to regulate gene transcription in the embryo's hypoblast include Gata4, SOX17 and GATA6. Each factor has previously been described as a key marker of differentiated hypoblast during preimplantation embryo development (Wamaitha *et al*., 2015).

1.3.3 Trophectoderm

During early preimplantation, the trophectoderm (TE) is the first cell type to differentiate, forming an epithelial layer which surrounds the fluid filled cavity, known as the blastocoel. The development of the TE is regulated by transcription factors and signalling cascades, specifically, Hippo and Ras/mitogen-activated protein kinase (MAPK) signalling (Sasaki, 2010). The TE forms from the outer layer of cells of the morula, with transcription factor Sox2 inducing cell differentiation and helping to facilitate the establishment of TE lineage (Keramari *et al*., 2010).
Trophoblast cells are specialised embryonic cells that upon dynamic structural changes, differentiate into the developing placenta where they will play a fundamental role in implantation and maternal-foetal interface formation. Wingless

(WNT) and NOTCH signalling pathways are required for the expansion of trophoblast progenitors, differentiation and proliferation of extra-villous trophoblasts (Dietrich *et al*., 2022). The trophoblast cells essentially derive from the polar trophectoderm in mouse blastocysts and will continue to support placental development through until approximately E8.5

1.4 Growth factors involved in embryo development

The growth and development of the embryo greatly depends on external environmental influences, which may affect apoptosis, interaction between cells, cell differentiation and cell proliferation. Embryonic growth relies on mitosis and various stimulatory and inhibitory factors control the rate of cell division during this process.

Autocrine, paracrine, and endocrine factors are known to influence preimplantation development. During development *in vivo* these factors are readily available and being produced by the female reproductive tract. Research has shown previously that levels of apoptosis are threefold higher in embryos developed *in vitro* compared to *in vivo*, suggesting that factors responsible for normal embryo development are absent or supressed when embryos are cultured *in vitro* (Brison and Schultz, 1998). During *in vitro* development, embryos cultured in groups or under high density conditions (e.g., 1 embryo/10 µL medium) have a reduced rate of apoptosis, demonstrating that embryos produce autocrine growth factors that can improve development (O'Neill, 1997). In contrast, culturing embryos individually, in a low density environment (e.g. 1 embryo/100 µL medium), results in the dilution of these autocrine growth factors which has a detrimental effect on development (Dai *et al*.,

2012) . Consequently, supplementation of culture medium with growth factors can improve embryo development and quality (Yang *et al*., 2022).

1.4.1 Autocrine and paracrine factors

Several factors have been identified as acting in an autocrine manner on preimplantation embryos. Platelet activating factor (PAF) is a signalling phospholipid that is produced by the preimplantation embryo (O'Neill, 1997) where it will act as a survival factor by mediating intercellular functions by stimulating embryo metabolism, embryo viability, fertilisation, implantation and cell-cycle progression (O'Neill, 2005). Additionally, experimental studies have described the way embryo derived PAF can enhance developmental and implantation rates during preimplantation development due to stimulation of embryonic metabolism (Saxena *et al*., 2021).

The insulin-like growth factors (IGF's), IGF-I and IGF-II, increase the number of ICM and TE cells in preimplantation embryos (Byrne *et al*., 1999). Previous studies have described the way in which growth factors play fundamental roles in influencing cell behaviour during embryo development by increasing the number of ICM and TE cells in preimplantation embryos (Byrne *et al*., 1999). IGF-I is present in the basal lamina and the site of trophoblast invasion during the maternal-embryo interface in preimplantation embryos, largely functioning as an autocrine factor to promote cell survival and proliferation (Green and Day, 2013).

Epidermal growth factors (EGF) are mitogenic polypeptides that are responsible for regulating cell proliferation, maturation, and cell differentiation within the embryo. EGFs stimulate the proliferation of different cell types such as preantral follicular granulosa cells and activate receptors that initiate cell signalling pathways.

Furthermore, there is growing evidence to suggest that apoptosis is regulated by soluble peptide growth factors like EGF and IGF which will act as survival factors (Kamijo *et al*., 1998). During preimplantation development, all cellular activities are influenced by the growth factors in the culture environment, for instance, IGF may act as survival factors for embryos while other growth factors such as Tumour Necrosis Factor alpha (TNF α) will stimulate apoptosis, particularly in the ICM (Hardy and Spanos, 2002). *In vitro* studies have previously revealed a link between autocrine and paracrine pathways, and the important role growth factors have on preimplantation development. IGF-1 can potentially help to improve the proportion of embryos developing to blastocyst stage and increase the number of cells in the mammalian inner cell mass (Harvey et al., 1995). This is an excellent example of how paracrine signalling through maternal derived growth factors are important to cell survival at early morula and blastocyst stages of development and can improve blastocyst formation, most likely through inhibiting apoptosis (Spanos *et al*., 2000).

The receptor that EGF binds to is known as the epidermal growth factor receptor (EGFR) which activates signalling pathways MAPK/ERK and PI3K/Akt that are essential for regulating growth and embryonic development, cell survival, proliferation and differentiation (Oda *et al*., 2005).

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein and tyrosine kinase receptor that plays a role in helping to maintain the intracellular effects of ligands. Growth factors are essential for normal development and maintaining the homeostasis of multicellular organisms. EGFR binds to and is activated by specific ligands such as epidermal growth factor (EGF) and mitogenic

peptide, transforming growth factor-α (TGFα) which will permit receptor dimerization and autophosphorylation of specific tyrosine residues within the COOH-terminal receptor tail (Scaltriti and Baselga, 2006).

EGFR will primarily over activate downstream signalling pathways including RAS-RAF-MEK-ERK MAPK and AKT-PI3K-mTOR which are primarily responsible for their role in fundamental cellular functions including transcription, translation and cell growth, proliferation, and survival (Asati *et al*., 2016). Additionally, phosphorylated ERK (pERK) is an important downstream component that will regulate various transcription factors and modifications in gene expressions (Asati *et al*., 2016).
The EGFR family of receptor tyrosine kinases (ErbB-2, ErbB-3, ErbB-4) will play an integral role in mammalian development functions including the stimulation of proliferation of specific cells such as fibroblasts and epithelial cells, and will additionally initiate intracellular signalling (Wong and Guillaud, 2004). The receptors are involved with key cellular functions by signalling through phosphorylated tyrosine and will play a central role in the determination of cell lineages in tissues.

1.4.2 Transforming growth factors

Transforming Growth Factors (TGFs) are categorised into two classes of polypeptide growth factors, alpha and beta. TGFα is part of the EGF family and is primarily responsible for stimulating epithelial development. Furthermore, it has been involved with tumerogenesis as well as angiogenesis, playing an essential role in cell differentiation, tissue regeneration, embryonic development. TGFβ superfamily contains the three mammalian types TGFβ1, TGFβ2 and TGFβ3 in addition to activins, growth and differentiation factors and various morphogenetic factors.

Together, they combine to form a cohesive network that provides crucial cellular activates that are responsible for embryo development (Liu *et al*., 2018).

1.4.3 Fibroblast Growth Factors (FGFs)

Fibroblast Growth Factors (FGFs) will act as multifunctional proteins that function though the activation of tyrosine receptors. The principal role of the FGF families involve the regulation of processes involved with embryo development, some of the FGF receptors found to be isolated to the embryo's extracellular matrix (Thisse and Thisse, 2005). FGF additionally play a crucial role in cell survival, cell migration and differentiation during culture, while the signalling of FGF during embryogenesis is fundamental for the generation and maintenance of the embryo's mesoderm, cell fate and axis determination (Balasubramanian and Zhang, 2016)

1.5 Transcription factors involved in cell differentiation in the preimplantation embryo

Nanog is a transcription factor crucial for the modulation and regulation of pluripotency within the embryo's ICM, helping the embryonic stem cells maintain pluripotency through the suppression of cell determining factors. Nanog expression begins during the compaction stage and gradually relocates to the embryo's ICM (Figure 1.3) where it will take on a salt-and-pepper-like distribution in the blastocyst's epiblast (Messerschmidt and Kemler, 2010). Gata4 is regulated primarily by mitogen-activated protein kinase (MEK1/2) signalling cascade by means of signalling cascades via phosphorylation. Transcriptional activity is regulated post-translationally by enzymes MEK1/2 and extracellular signal-regulated kinases (ERK1/2) (Valimaki and Ruskoaho, 2020). Gata4 has additionally been used as an

effective lineage marker when identifying hypoblast cells (Figure 1.3) from an extraembryonic layer of cells in mouse blastocysts (Plusa *et al*., 2008).

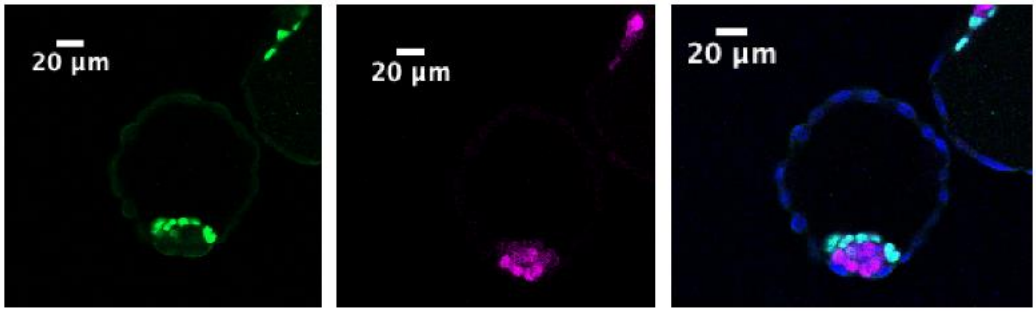


Figure 1.3 Gata4 and NANOG expression in the mouse preimplantation blastocyst
Expression of transcription factors Gata4 (green) and Nanog (pink) in an E4.5 mouse blastocyst representing the hypoblast and epiblast cells respectively. Dapi (blue) has been used for the nuclear counterstain. By E4.5 both just epiblast and hypoblast lineages are established, each arising from the inner cell mass in the early blastocyst. Image generated by N. Stark.

1.6 The extracellular matrix in the preimplantation embryo

The extracellular matrix (ECM) is a multidimensional structural network made up of extracellular multidomain macromolecules which are primarily organised into a cell/tissue specific arrangement. The macromolecules are made up of fibrous proteins including collagen, fibronectin and laminin, and complex polysaccharide chains that contributes to mechanical scaffolding of the cells and regulate aspects of growth and development (Figure 1.4). Some of the most fundamental functions of the ECM involve cell adhesion, cell differentiation and intercellular communication. The varying elasticity and rigidity of the ECM's structure can additionally contribute to gene expression and cell migration, as well as cell survival and proliferation (Provenzano *et al*., 2009).

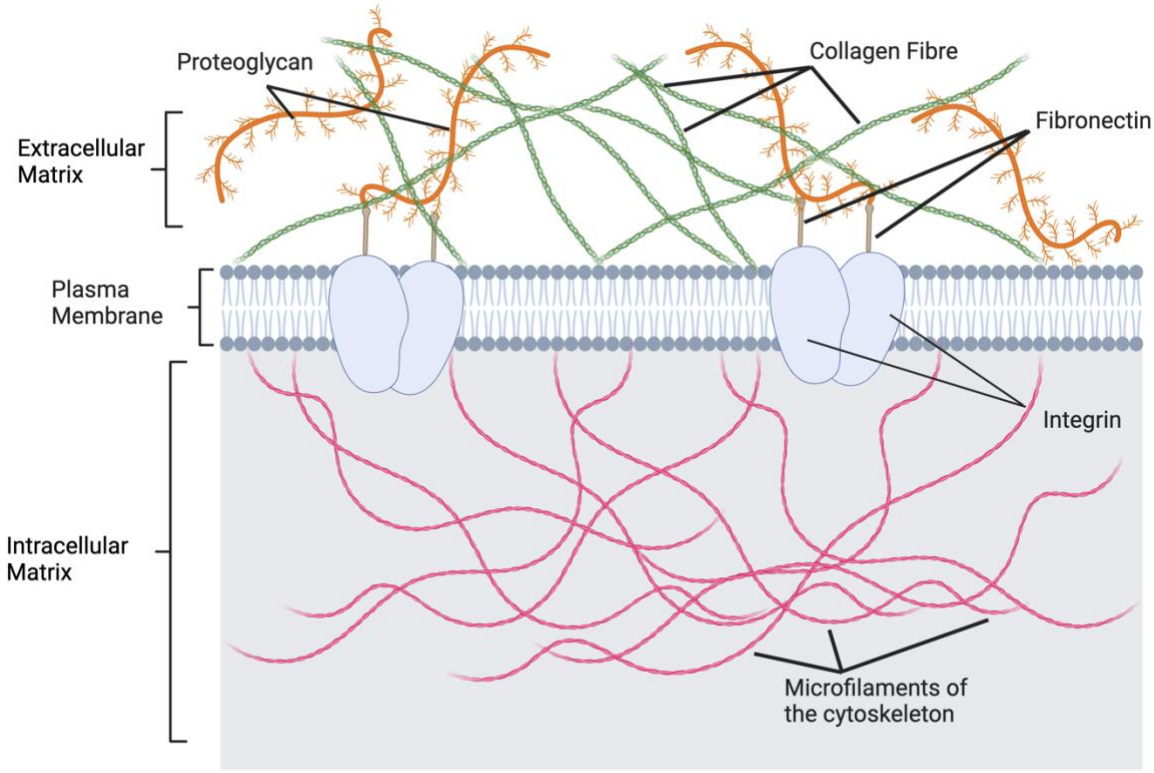


Figure 1.4 Illustration of the components of extracellular matrix
The extracellular matrix (ECM) is a specialised network of multidomain macromolecules, growth factors bioactive molecules and proteins that provide structural support to cells and tissues in the body. Proteoglycans, collagen fibres and fibronectin fill the greater part of the extracellular interstitial space and are responsible for using their unique binding properties to form large structural networks. (Schaefer and Schaefer, 2010). Integrins are proteins that help to mediate ECM-cell adhesion (Khadilkar *et al*., 2020). Actin microfilaments within the cytosol help cell motility, cytokinesis, endocytosis, and exocytosis as well as providing mechanical stability for the cell. This image was created with BioRender (https://biorender.com/)

1.6.1. The basement membrane

Basement membranes (BM) are thin, fibrous layers of specialised ECM that forms predominantly through self-assembly on cell surfaces and is initiated when laminin heterotrimers bind to cell surfaces (Yurchenco and Patton, 2009). The BM lines most mammalian tissues and plays a significant role in helping to promote cell signalling, regulating cell proliferation and facilitating cell adhesion while also providing crucial structural support for most epithelial tissue (Morrissey and Sherwood, 2015).

Most BMs are composed of a diverse assortment of proteins that help to serve multiple biological functions during embryo development and embryonic differentiation (Yurchenco, 2011). The first basement membrane is expressed between the epiblast and the primitive endoderm or hypoblast, of the preimplantation embryo. The remodelling of the BM assists in facilitating further growth and development of the embryo while nodal signalling regulates the expression of specific matrix metalloproteinases (MMPs) which supports further remodelling of the ECM (Birkedal-Hansen, 1995). During preimplantation development, direct adhesive interactions with the ECM have an impact on homeostatic turnover of basement membrane components and signalling activity. Specifically, proteins that make up the intricate structure of the basement membrane will function to regulate cell behaviours extending from cell migration, patterning, cell survival and cell differentiation (Kim *et al*., 2022).

1.6.2. Proteins of the extracellular matrix

The ECM of the preimplantation embryo is predominantly comprised of three principal proteins: collagen, fibronectin, and laminin. Collagen forms a lattice-like network which is separated into two primary classes: fibrillar (types I, II, III and V) and non-fibrillar (types IV and VI). Fibrillar types provide important structural properties to the ECM, which exist in shorter chains, while non-fibrillar types influence the shape and thickness of fibres in addition to helping to anchor fibres to one another and surrounding tissues (Frantz *et al.*, 2010). Type IV collagen (COLIV) is one of the major components of the BM, in preimplantation embryos and provides a scaffold with macromolecules such as laminins, heparan, proteoglycans and fibronectin (Gersdorff *et al*., 2005). Type IV collagen has been located primarily within the ICM cells in early mouse embryos as the first BMs form, gradually shifting towards the trophectoderm as the embryo begins to expand and eventually hatch (Thorsteinsdottir, 1992).

ECM proteins such as fibronectin bind to multiple integrins in blastocysts during preimplantation development (Green *et al*., 2015). Integrin affinity for the ECM proteins is regulated by signalling pathways such as PI3K/Akt pathways, which is activated by insulin-like growth factors which are required for mediating the assembly of the matrix (Somanath *et al*., 2007).

There are suggestions that fibronectin assembly during embryogenesis may occur as both paracrine and autocrine communication event between tissues, depending on the tissue, with emphasis on tissue morphogenesis (de Almeida *et al*., 2016). Glycoprotein fibronectin, along with collagen IV (Green and Day, 2013), is found

primarily within the cells of the ICM in the early embryo, and has been revealed to be an essential structural component in the embryo's developing BM (Figueiredo *et al.*, 2002).

Laminin is the first extracellular matrix protein to be synthesized during preimplantation development in mouse embryos (Shim *et al*., 1996). The protein plays an integral part of the internal structure of the extracellular matrix, while also contributing to essential biological processes such as cell differentiation, migration, and cell adhesion during early embryo development (Kim *et al.*, 2022). Laminins will independently bind to cell membranes when recognised by integrins, and thereby help to mediate signals from the ECM to the cell cytoskeleton (Dziadek and Timpl, 1985).

1.7 Structural properties of collagen IV

Collagen IV is composed of six genetically distinct alpha chains, alpha-1(IV) to alpha-6(IV), encoded by the genes COL4A1 to COL4A6 (Table 1.1, Figure 1.5). Each is comprised of a collagenous domain formed mainly of repeats of three amino acids Glycine-proline and Lysine (Oefner *et al.*, 2015).

After synthesis of the alpha-chain in the ER the protein undergoes post-translational modification, including the hydroxylation of the proline by prolyl-hydroxylase, and lysine by lysyl-hydroxylase (Gorres *et al*., 2008). These modifications considerably increase the stability of the collagen helix (Rappu *et al*., 2019).

1.7.1 Alpha chains of collagen IV

The six alpha chains combine to form three specific heterotrimers α1α1α2, α3α4α5 and α5α5α6, which are responsible for the specific networks formed in basement membranes (Parkin *et al.*, 2011) and exhibit tissue-specific patterns (Kolahi *et al.*, 2012) (Table 1.1, Figure 1.5). The sub-chains alpha-1(IV) and alpha-2(IV) are primarily expressed in all basement membranes of all tissues and their levels are decreased in tissues with higher expression of alpha-3(IV), alpha-4(IV) and alpha-5(IV) begin to activate (Zagris, 2001). Obtaining a better understanding of the physiological and structural roles of the alpha subunits that make up BM collagen IV will provide an insight into the unique biochemical properties during early embryo development (Kuhn, 1995).

Table 1.1 Distribution of collagen IV alpha chains with their corresponding MMPs

Alpha Chain	Gene	Chromosome	Function in adult tissues	Associated MMPs	TIMPs	Fragment
α1	COL4A1	13q34	Angiogenesis	2, 7, 9	1, 2, 3	Arrestin
α2	COL4A2	13q34	Anti-angiogenic activity. Involved with inhibition, proliferation, and migration of endothelial cells, induces apoptosis, and reduces mitochondrial membrane potential.	2, 9	1, 3	Canstatin
α3	COL4A3	2q36.3	Anti-angiogenic and anti-tumour cell activity.	9	1	Tumstatin
α4	COL4A4	2q36.3	Anti-angiogenic and anti-tumour cell activity.	2, 9	1	Tetrastatin
α5	COL4A5	Xq22.3	Collagen catabolic process; extracellular matrix disassembly, organisation, and biogenesis; neuromuscular junction development	19	1, 3	Pentastatin
α6	COL4A6	Xq22.3	Anti-angiogenic and anti-tumour cell activity.	2, 9	1	Hexastatin

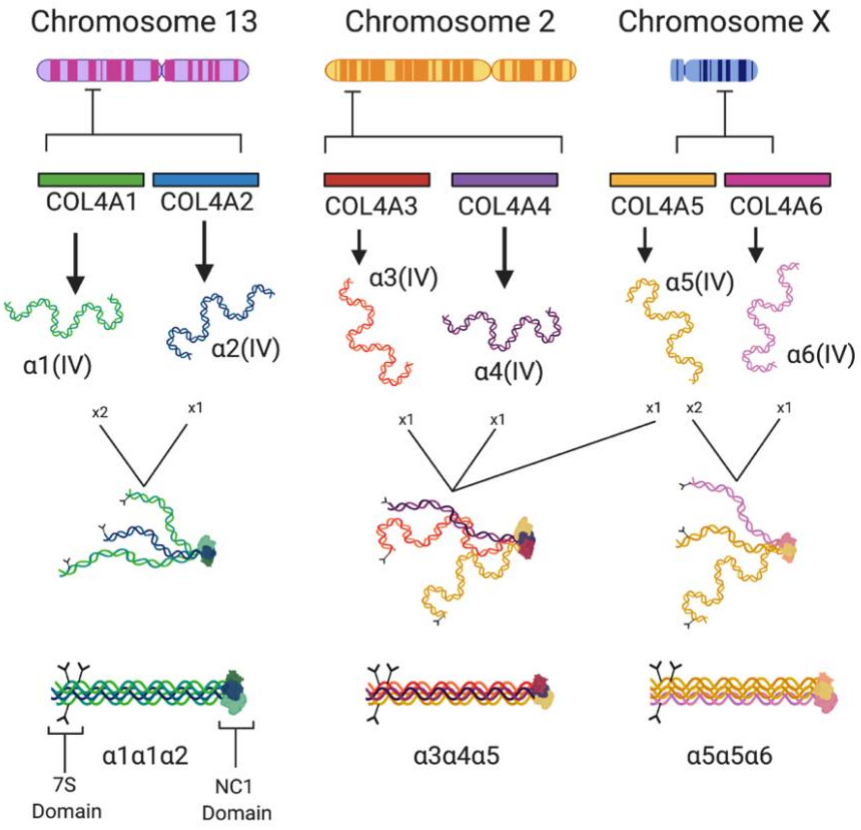


Fig 1.5 Collagen IV alpha chains
Six collagen IV genes have been identified (COL4A1 to COL4A6), each gene being expressed during different stages of embryogenesis. Each chain is comprised of three domains which are responsible for the way the heterotrimers are assembled to create the basement membrane (LeBleu *et al*., 2010). This image was created with BioRender (https://biorender.com/)

1.7.2 Structure of alpha chains

The alpha chains of Collagen IV are composed of three domains which are responsible for their assembly to form heterotrimers (Ortega and Werb, 2002). The alpha chains are comprised of an N-terminal, a triple helical domain, and a globular C-terminal (LeBleu *et al*., 2010). The globular C-terminal (NC1) domain is involved with the assembly of the alpha chains to form the collagen IV heterotrimers and is the site for molecular recognition during the triple-helical configuration. The amino

terminal domain (N-terminal 7S) which is rich in lysine and cysteine-rich is primarily responsible for the assembly of the heterotrimers formed by the NC1 (Ortega and Werb, 2002). Crosslinking through the lysine-hydroxylysine and disulphide bonds relies heavily on the presence of the lysine and cysteine-rich residues at the 7S domain (Figure 1.6).

i) C-Terminal domain (NC1)

The non-collagenous C-terminal domain of collagen IV has been the focus of several studies that investigate the specific role of the domain as a structural component of the basement membrane in addition to its morphogenetic processes and biological functions (Ortega and Werb, 2002). As the basement membrane begins to breakdown in early embryonic development, the degradation generates collagen fragments from the NC1 region, which generate specific physiological and pathological processes (Boutaud *et al*., 2000).

Fragments and alpha subunits released during the degradation process have previously been identified as angiogenesis inhibitors and are thought to play roles in other physiological processes (Ortega and Werb, 2002). The complex proteolytic pathways that are specifically involved with the creation of these NC1 fragments are still being investigated, however, studies have suggested that they may exist as physiological cleavage products because of matrix metalloproteinase activity (John *et al*., 1999).

ii) Central triple helical interrupted domain.

The triple helical domain located between the non-collagenous C-terminal domain and the N-terminal 7S domain is arranged in a specific tripeptide sequence. The sequence is comprised of glycine triplicate repeat Gly-X-Y and hydroxyproline residues that play an important stabilising role in the collagen's structure (Bhattacharjee and Bansal, 2005). A significant characteristic of the collagen IV molecule is the considerable amount of proline and hydroxyproline residues located within the triple helical structure (Motooka *et al.*, 2012). Type IV collagen has a greater number of inter-triple helix interactions and contain a higher content of hydroxyproline residues that assist in stabilising the triple helical structure in addition to facilitating intermolecular functions (Radmer and Klein, 2006). The hydroxyproline residues in the triple helical domain occur at the Y-position, providing an additional hydrogen bond in the hydroxyl group. This significant structural arrangement has supported previous hypotheses that the specific composition of the hydroxyproline in the triple helical composition is directly associated with the structural stability of the type of collagen in which it is found (Bhattacharjee and Bansal, 2005).

iii) N-Terminal 7S domain

The cysteine rich N-terminal 7S domain is primarily responsible for the covalent assembly and configuration of four heterotrimers that form the complex branching in the extracellular matrix (Boutaud *et al*., 2000). The cross linkages that are formed in the heterotrimer arrangement are heavily glycosylated, leaving the structures resistant to collagenase activity (Zagris, 2001).

Although each domain plays a significant structural and physiological role in the composition of Collagen IV, specific fragments produced by the breakdown of specific domains have become the subject for several studies.

Recent studies have identified fragments of NC1 domains in collagen IV and recognised their potential in affecting angiogenesis, a complex physiological process by which blood vessels develop from pre-existing blood vessels (Hamano *et al*., 2003). Although angiogenesis is an important and fundamental process during early growth and development, it plays a significant function during tumorigenesis in adults and has a crucial role in the transformation of benign to malignant tumours (Ogata *et al.,* 1992).

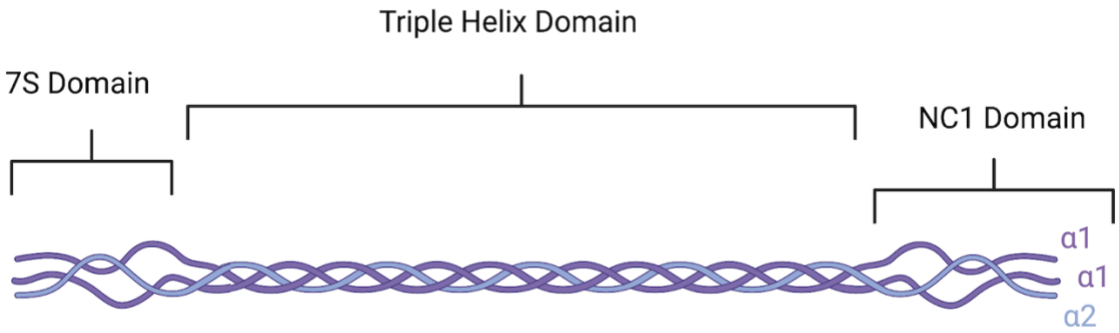


Figure 1.6 Schematic structure of type IV collagen
The three domains consist of a non-collagenous C-terminal (NC1), an amino acid terminal (N-terminal of 7S domain) and a central triple helical interrupted domain. The NC1 domain will be responsible for the site of molecular recognition during the configuration of the triple helix formation, and the N-terminal 7S plays a crucial role in the covalent assembly and organisation of the four heterotrimers that will subsequently form the complex branching in the extracellular matrix. The central triple helical interrupted domain is the sequence of glycine triplicate repeat Gly-X-Y and hydroxyproline residues that will function by promoting stability within the collagen IV structure (Bächinger *et al.*, 2010). This image was created with BioRender (https://biorender.com/)

1.7.3 Collagen Synthesis

The process of collagen synthesis occurs both extracellularly and intracellularly and primarily involves co-translational and post-translational modifications (Figure 1.7). The modifications are catalysed by several specific and non-specific enzymes which play an integral role in stabilising the collagen triple helices during physiological conditions (Pihlajaniemi *et al*., 1991). The availability of extracellular L-proline will essentially help to establish the rate at which the biosynthesis of collagen IV occurs (Karna *et al*., 2020).

Prolyl-4-hydroxylase is a specific enzyme that is responsible for catalysing 4-hydroxyproline in lumen of the rough endoplasmic reticulum (ER) and producing 4-hydroxyproline residues that play an imperative role in maintaining collagen stability (Pihlajaniemi *et al*., 1991). Together with the enzyme lysyl-hydroxylase, prolyl-4-hydroxylase is responsible for the post and co-translational hydroxylation of L-proline by modifying the precursor to mature collagen known as procollagen. Prolyl-4-hydroxylase is essential for the correct folding of the newly synthesized collagen polypeptide chains and previous studies have demonstrated that inhibition of this modification will prevent the correct formation of the collagen IV triple helix, thus leading to embryonic lethality and the cellular degradation of the unfolded chains (Winter and Page, 2000, Myllyharju, 2003).

The production of type IV collagen-degrading enzyme has been thought to potentially expedite the penetrability of the embryo, facilitating the effectiveness of implantation (Puistola *et al*., 1989). Further studies will be required to assess and understand the mechanisms behind subsequent breakdown of collagen IV and the

generation of L-proline during preimplantation development, however strong suggestions from previous research predict that the production of L-proline may lead to and influence ICM cell differentiation and migration.

Collagen synthesis occurs both intracellularly and extracellularly during six crucial steps (Wu *et al*., 2020). The first step occurs intracellularly and begins with mRNA transcription in the nucleus, activating the genes pro- alpha-1 and pro- alpha-2 which are directly associated with the establishment of the specific alpha peptides. Following transcription, the mRNA will migrate towards the cytoplasm and interact with ribosomes as part of the translation, resulting in the formation of the pre-pro-peptide. From this position, the chain will then subsequently relocate to the ER for post-translational modification. Post-translation modification occurs before the peptide becomes pro-collagen and involves three major conversions. The conversions involve the removal of the signal peptide on the N-terminal, the lysine and L-proline residues gaining an additional hydroxyl group because of the hydroxylase enzyme activity and is followed by the glycosylation of selected hydroxyl groups on lysine. The hydroxylated and glycosylated pro-a-chains then configure into distinctive triple helix formation where it begins to travel towards the Golgi apparatus where it will undergo the final modifications required for the pro-peptide cleavage stage in the extracellular space. Once the molecule moves into the Golgi apparatus, collagen peptidases assist in removing the ends of the procollagen molecule, transforming it into the triple helical monomer tropocollagen. The sixth and final step of the collagen synthesis pathway consists of the tropocollagen molecules forming a collagen fibril with the assistance of lysyl oxidase (LO), an enzyme which targets

hydroxylysines to help stabilize collagen by forming crosslinks and elastin fibres (James, 2017).

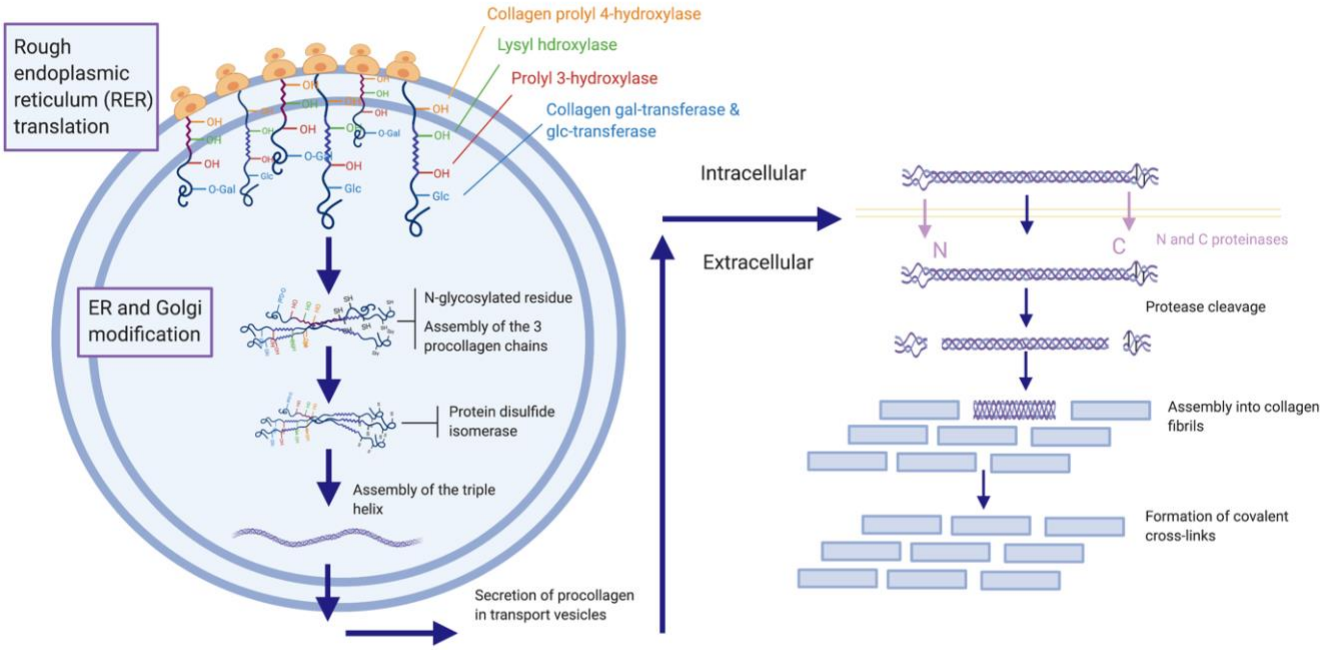


Figure 1.7 Collagen Biosynthesis
Specific co-translational and post-translational modifications are required both extracellularly and intracellularly, for the biosynthesis of type IV collagen. Initial steps occur intracellularly during mRNA transcription in the nucleus, which will activate pro-α1 and pro-α2. Following transcription, mRNA will migrate to the cytoplasm and interrelate with ribosomes as part of translation, which will relocate to the endoplasmic reticulum (ER) where the formation of the pre-pro-peptide takes place.
Prolyl-4-hydroxylase will naturally catalyse the 4-hydroxyproline in the lumen of the ER, which will produce 4-hydroxyproline residues. The residues are fundamental in providing stability to the lattice-like structure of the basement membrane (Pihlajaniemi *et al*., 1991). Post-translation will occur before the peptide becomes pro-collagen. The post-translation steps involve the removal of the N-terminal's signal peptide, the hydroxylation of the proline and lysine and the disulphide bind establishment (Wu *et al.*, 2020). The hydroxylated pro-a-chains will subsequently form the pro-collagen molecule by twisting and assembling into a triple helix-like structure. The pro-collagen molecule now proceeds to the Golgi apparatus where it will undergo the final modifications to enter the extracellular space. Once the molecule enters the extracellular space, collagen peptidases cleave and remove the ends of the molecule to form a primary structural unit of the collagen fibre known as tropocollagen. At this point specific copper dependent enzyme, lysyl oxidase (LOX) will initiate covalent crosslinking to form the collagen fibril (Herchenhan *et al.*, 2015). This image was created with BioRender (https://biorender.com/)

1.7.4 Breakdown of collagen IV

Proteolytic degradation of the ECM is a natural and essential process that occurs during normal embryo development. The process occurs in the presence of matrix metallopeptidases (MMPs), zinc-dependant peptidases that are capable of degrading most of the components of the ECM and as a result, break down the structure of the collagen IV (Bu *et al*., 2010). The MMP family can be separated into six distinctive groups: The collagenases, gelatinases, matrilysins, stromelysins and the membrane-type MMPs in addition to an assortment of non-classified MMPs. Each group is characterised by functionality and cellular localisation as well as substrate specificity. The collagenases are proteins which are capable of cleaving the peptide bonds in the collagen triple helix near the N-terminus (Holmbeck and Birkedal-Hansen, 2013). Stromelysins play a significant role in tissue remodelling and the degradation of collagen. This family are generally found to have a broad substrate specificity for proteoglycans, fibronectin, and laminin within the ECM, as well as having the ability to cleave and stimulate pro-collagenase, an enzyme which is responsible for activating collagen (Gravallese and Monach, 2015). Matrilysins are similar to Stromelysins in terms of the substrate specificity, however it differs structurally from the other MMP families as it lacks a carboxy-terminal protein domain. This family of MMPs have effectively demonstrated their ability to play important roles in ECM degradation, wound repair, cellular proliferation and the regulation of biochemical processes such as activation and shedding of non-ECM proteins (Klein and Bischoff, 2011).

Membrane-type MMPs form a specific subcategory of the MMP family and typically expressed as active enzymes on cell surfaces and have been thought to contribute

to cellular signalling and proteolysis. The gelatinase family are named for their ability to primarily degrade denatured collagen or gelatine. These matrix zinc-dependent MMPs additionally break down a variety of ECM proteins and are distinguished by an additional fibronectin type II domain which is part of the collagen-binding (Tuula and Jouko, 1985). MMP-2 and MMP-9 are the two gelatinases which contribute to extracellular degradation and play an active role in tissue remodelling within the ECM (Hoyhtya *et al.*, 1990).

MMPs will use their proteolytic activity to break down the extracellular matrix, and it can be assumed that the natural release L-proline from the hydrolysed collagen will subsequently influence specific physiological processes such as trophoblast implantation, blastocyst development and the cell differentiation.
The role of L-proline in preimplantation development has been described in studies as an amino acid that will improve development by stimulating the differentiation of mouse ES cells through a growth-factor-like response (Morris *et al*., 2020).
MMP inhibitors will restrict the release of L-proline through impeding basement membrane degradation. This is achieved by targeting the MMPs responsible for the breakdown of collagen IV and preventing the proteolysis of the ECM (Figure 1.7).

During normal physiological development, the homeostasis of ECM degradation needs to be tightly controlled and precisely regulated to ensure there is balance between activity and the inhibition of the MMPs (Yalcinkaya *et al*., 2014).

1.7.5 Matrix metalloproteinase inhibitors

Tissue inhibitors of matrix metalloproteinases (TIMPs) are proteins that specifically inhibit the activity of specific enzymes involved with the breakdown of connective tissue (Brew and Nagase, 2010). The role of TIMPs in medical science and physiology has been of particular interest, especially when considering their therapeutic potential (Baker *et al*., 2002). The proteolytic pathways involved with TIMPS, and ECM degradation have a significant influence on cell-to-cell and cell-to-ECM interactions and are responsible for the regulation of MMP activity during pathophysiological processes such as embryogenesis, angiogenesis and remodelling of the extracellular matrix. Four groups of TIMPs are known (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) and the primary function of each group is to inhibit specific MMP activity and the balance between the activity of the MMPs and the inhibition by the TIMPs (Table 1.2).

Although the TIMPs have been subdivided into four distinct groups, they all share a similar physical structure, effectively comprising of a signal peptide, an N-Terminal inhibitory domain and a C-Terminal inhibitor-binding domain. The disulfide bonds that help to form the structure of the N-terminal region are essential for contributing to the MMP-inhibitory activities (Hua *et al*., 2011). TIMPs have additionally been shown to exhibit biological activities that are independent of MMPs, including but not limited to cell growth, differentiation and migration and anti-angiogenic activities (Brew and Nagase, 2010). TIMPs may be subdivided into two categories: synthetic inhibitors and non-synthetic. The non-synthetic or endogenous inhibitors are important regulators of ECM degeneration and control the activities of proteinases in vivo (Okada, 2017). These inhibitors are predominantly derived from plasma or cells

in the surrounding tissues and are responsible for the remodelling of the ECM, their activity being dependent on the MMP being inhibited (Arpino *et al*., 2015).

The synthetic inhibitors or exogenous inhibitors which usually consist of a chelating agent that assists in the binding of the zinc atom to the MMP binding site. The design of these synthetic MMP inhibitors have been developed to specifically target MMP activities by obstructing their various functions and by also inhibiting their expression in specific diseases such as cancer (Acharya *et al*., 2004). Synthetic inhibitors of MMPs have been applied during clinical trials when observing tissue regeneration, cancer treatment and trauma healing. TIMPs can act both as a broad spectrum or a selective inhibitor which can fundamentally impact the ECM degradation and enzymatic activity (Schelter *et al*., 2010). For example, SB-3CT is a mechanism based inhibitor that is specifically selective for MMP-2 and MMP-9, two gelatinases that are responsible for the degradation of collagen IV in the basement membrane. The inhibitor will specifically coordinate the catalytic zinc ion and will influence a slow-binding, mechanism-based inhibition, which is a distinctive characteristic among synthetic inhibitors (Gu *et al*., 2005).

Previous research has described expression of the specific TIMPs responsible for ECM degradation in preimplantation embryos. MMP-2 and MMP-9 are the MMPs primarily responsible for the proteolytic degradation of the ECM in preimplantation embryos, the tissue inhibitors for these MMPs include TIMP-1,2,3,4. Each TIMP regulates MMP activity and will be expressed during different stages of preimplantation development (Kikuchi *et al*., 1996). TIMP-1 will target MMP-2 and is expressed during preimplantation development from the single cell stage to the

blastocyst stage, however TIMP-2, targeting MMP-9 is not detected in early stage preimplantation embryos (Wang *et al*., 2003). Moreover, TIMP-2 has been expressed primarily in later stage preimplantation embryos, similarly with TIMP-1 and TIMP-3, which have described as being expressed from the 8-cell stage through until the compaction and blastocyst stages (Kikuchi *et al*., 1996).

Table 1.2 Functional classification of metalloproteinases, their substrates, and their subgroups

Subgroup	MMP	Nomenclature	Substrate
Interstitial Collagenases (Contain hemopexin domain and peptide linking with catalytic domain)	MMP-1	Collagenase-1	Collagen type I, II, III, V, VII, VIII, X, gelatine, IL-1β, MMP-2, MMP-9, fibronectin
	MMP-8	Collagenase-2	
	MMP-13	Collagenase-3	
Gelatinases (Consist of high substrate specificity to denatured collagen and gelatin)	MMP-2	Gelatinase-A	Collagen type I, IV, V, VII, X, gelatine, elastine, laminin
	MMP-9	Gelatinase-B	
Stromelysins (metalloproteinases of stroma)	MMP-3	Stromelysin-1	Collagen type III, IV, V, proteoglycanes, fibronectins, laminin, elastine, gelatine, vitronectine, MMP-1, MMP-2, MMP-8, MMP-9, MMP-13
	MMP-10	Stromelysin-2	
	MMP-11	Stromelysin-3	
	MMP-12	Metalloelastase	
Matrilysins (Lack hemopexin, the smallest among MMPs)	MMP-7	Matrilysin-1	Collagen type IV, glycoproteins, gelatine
	MMP-26	Matrilysin-2, Endometase	

1.7.6 Protein fragments produced by proteolytic degradation of collagen

During proteolytic degradation of the basement membrane, small fragments are released. These fragments have physiological properties that may essentially act as endogenous inhibitors of angiogenesis during protein synthesis and help regulate cell migration, survival and proliferation in addition to releasing specific signalling molecules (Sand *et al*., 2016). Tumstatin is the cleavage fragment produced when the α3 chain of collagen IV breaks down due to the generation of human matrix

metalloproteinase 9 (MMP-9), which is activated by human matrix metalloproteinase 3 (MMP-3) (Vempati *et al.*, 2007). Endostatin is a naturally occurring protein fragment derived from the C-terminal of type XVIII collagen which has been shown to serve as an anti-angiogenic agent and broad-spectrum inhibitor. The inhibitory effects of endostatin will obstruct the pathways that influence cell viability, proliferation and will interfere with the MAPK pathway, ultimately inhibiting the proliferation and migration of endothelial cells (Ahmed and Lutty, 2017). Arrestin is derived from the α1 chain of collagen IV, which has been shown to effectively inhibit endothelial cell migration and proliferation (Assadian and Teodoro, 2008). The activity is mediated through the activation of the downstream MAPK pathway, and is essentially facilitated by specific mechanisms which involve cell surface proteoglycans (Colorado *et al*., 2000).

1.8 The role of amino acids during preimplantation development

L-proline has been identified as an amino acid that regulates cell pluripotency and differentiation during early embryo development (Glover *et al*., 2022). When added to low density culture media containing embryos, L-proline acts as a growth factor, stimulating embryo development when autocrine support is unavailable (Morris *et al*., 2020).

Effects of L-proline on signalling pathways such as mTOR and ERK1/2 during embryogenesis have indicated that cell differentiation, proliferation and renewal are stimulated, thus potentially improving embryo health and development (Liu *et al.*, 2021). Data from previous experiments has described the significant changes in differentiation kinetics, gene expression and morphology when proline is added to

low density culture media containing embryos (Washington *et al*., 2010), strongly supporting the notion that L-proline will play a significant role in healthier blastocyst formation.

The mRNAs that encode proteins have been directly correlated to the alteration of activities involving sodium dependent transport systems. The systems that are associated with taurine, glycine an anionic amino acid such as Gly and GLYT1 are expressed throughout the cleavage stage of development (Ho *et al*., 1995)

Essential amino acids will have a beneficial effect from approximately day three onwards (8 cells) and will ultimately provide developmental support for embryos when they reach the blastocyst stage (Gardner and Lane, 1993). The transport processes involved, help to influence the concentration of the essential amino acids and the developmental regulation, in addition to sub sequentially relying on the expression of the accessory protein mRNAs and the relevant transporter (Lane and Gardner, 2003).

Addition of specific amino acids to embryo culture media in vitro has been studied and shown to influence the quality of development in the embryo. L-glutamine metabolism is responsible for the release of ammonium that ultimately impedes growth and development in the preimplantation embryo (Lane *et al*., 2001). If the ammonium is removed from the culture media, the L- glutamine will promote growth over the day 2-3 stage of development (Lane and Gardner, 1997, Lane *et al.*, 2001).

Specific amino acids L-proline and L-glutamine can act in a growth factor-like way when added to low density culture media, resulting in improved embryo development and the increased likelihood of the embryo forming a blastocyst (Morris *et al.,* 2020,

O'Neill *et al.*, 1990). In addition to improving the rate of blastocyst formation, L-proline and L-glutamine are also effective in stimulating blastocyst hatching in both low and high-density culture media. L-proline also has a significant influence on embryo development when presented alone to the embryo's culture environment (Treleaven *et al*., 2021). Additionally, the metabolism of L-proline has been identified as a critical component that influences the way specific mechanisms stimulate cellular functions, namely the way L-proline is metabolised through the proline metabolic pathway. Studies have shown strong indications that when introduced to the embryo's fertilization media, and then essentially removed the rate of blastocyst formation almost doubles in number. In addition to considerably improving preimplantation development while in the cleavage stage, L-proline also appears to be beneficial over a specific period of time for the embryo (Casalino *et al*., 2011).

The comprehensive effects of amino acid mediated signalling have been observed in mouse embryos and the molecular circuitry surrounding L-proline and L-glutamine. L-proline is involved with the mTOR pathway, an intracellular signalling pathway that plays a principal role in apoptosis and is activated by IGF-1. The addition of L-proline to low density culture media will ultimately support normal development in preimplantation embryos (Fremeau *et al.*, 1992).

Further research has shown that the cellular uptake of L-proline by mouse embryos occurs by means of the amino acid transporter B^0AT1. While the transporter is expressed throughout the various phases of preimplantation embryo development, it does not become effective until approximately the later stages of the cleavage phase. During fertilisation, the primary amino acid transporter for L-proline

in oocytes appears to be different from the B^0AT1 transporter active during cleavage stage. The transporter associated with L-proline uptake during fertilisation has not been identified, however, through extensive research using the oocytes and embryos from knockout (KO) mice, we will be able to identify the amino acid transporter responsible.

Along with L-proline and L-glutamine, glycine (Gly) and betaine can also have a significant impact on the growth on development of preimplantation embryos. Both Gly and betaine can act as osmolytes and regulate cell volume, thus contributing to a positive effect on embryo's development. When betaine is added to embryo culture media, it requires a transporter that will also actively accept proline. The transporter associated with betaine and L-proline uptake will remain active for a short period of time during the first two days as the embryo starts to enter the cleavage stage.
The presence of L-proline and L-glutamine in embryos has proven to be of great long-term benefit, assisting in the development of healthy embryos by means of activating specific mechanisms that ultimately effect pregnancy outcomes and the embryo's gene expression (Morris *et al*., 2020).

It has been well established that L-proline is required to form the collagen helix within the basement membrane. It has also been described throughout this chapter that extracellular L-proline is one of the driving forces behind improved development in preimplantation embryos as well as blastocyst hatching rates (Ozsoy, 2011). Additionally, L-proline is responsible for regulating embryogenesis and activating pathways such as mammalian target of rapamycin complex 1 which contributes to the stimulation of trophectoderm cell proliferation (Liu *et al*., 2021).

Furthermore, the regulation of L-proline is essential for collagen biosynthesis. Sources of intracellular L-proline are available by means of glutamine metabolism or by where imidodipeptides are hydrolysed by prolidase during collagen biosynthesis (1.6.3) (Karna *et al.*, 2020). As L-proline is required to form the collagen molecules that make up the basement membrane in the preimplantation embryo, during collagen biosynthesis, L-proline is released through proteolytic degradation and will support normal embryo development from fertilisation to implantation (Harris *et al*., 2005).

1.8.1 The effect of L-proline on preimplantation development and embryonic stem cell differentiation

Select amino acids may function as osmolytes for preimplantation mouse embryos and improve embryo development (Morris *et al*., 2020). L-proline has been shown to significantly improve the rate of development in preimplantation embryos, increasing the proportion of blastocysts and hatching rates by acting as a signalling molecule pathways associated with the regulation of cellular processes (Ozsoy, 2011). The Akt, ERK1/2 and mTORC1 signalling pathways are fundamental in the regulation of cell growth and proliferation (Winter *et al*., 2011). The mTORC1 pathway is activated by L-proline and is essential for the regulation of embryonic stem cells (ESCs), including growth, proliferation, and differentiation. Previous studies have described how the addition of L-proline to ESCs at concentrations of 100 μM or greater will result in changes to gene expression, cell morphology and stem cell differentiation (Tan *et al*., 2011). Furthermore, it is known that sodium coupled neutral amino acid transporter 2 (SNAT2) is involved with proline-induced

differentiation with mouse embryonic stem cells. L-proline and/or SNAT2 will activate multiple intracellular pathways to activate mTOR, consequentially regulating ESC gene expression (Washington *et al*., 2010).

1.8.2 The L-proline metabolic pathway

Specific amino acids have been identified as regulators that are involved with various cellular processes such as cell differentiation and migration, L-proline is an important secondary amino acid that is involved with integrating these processes (Figure 1.8).

L-proline is the only secondary amino acid that is not only capable of producing proteins but it is also involved with production of physical cellular properties that contribute to tissue composition (Phang *et al*., 2008). Furthermore, L-proline has been recognised for its ability to function as a signalling molecule (Washington *et al*., 2010) and influence cellular processes, such as activating the mTOR pathway to regulate cell pluripotency *in vitro* (Patriarca *et al*., 2021).

Metabolism of L-proline can modulate specific carcinogenic pathways and is made available as a stress substrate by the degradation of the microenvironmental ECM. L-proline is a proteogenic secondary amino acid and has its α-amino group within a pyrrolidine ring. Due to these unique properties, it has its own metabolic pathway and will act as a stress substrate in microenvironments (Phang, 2019).

Proline dehydrogenase/proline oxidase (PRODH/POX) is a mitochondrial enzyme degrading proline that initiates the first step in the L-proline cycle and is upregulated

in response to stress signalling. PRODH/POX is tightly bound to mitochondrial inner membranes and will catalyse the initial stage involved with L-proline degradation, consequently using proline to generate superoxide radicals or reactive oxygen species (ROS) for cell apoptosis (Palka *et al.*, 2021).

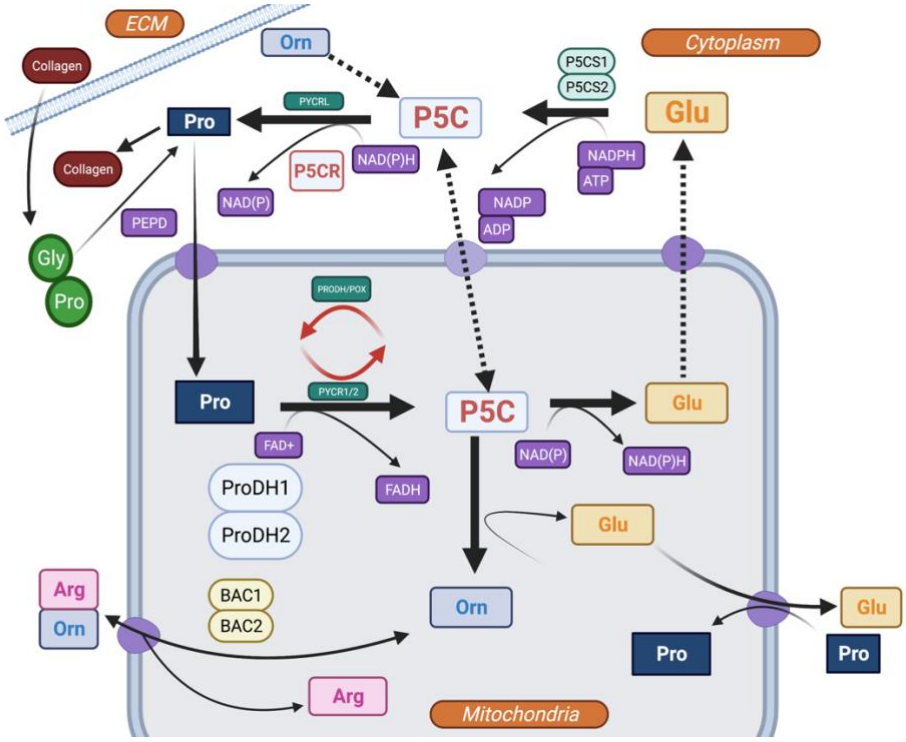


Figure 1.8 Proline-Prolidase pathway
Prolidase (PEPD) is a cytosolic metalloproteinase generally responsible for catalysing and degrading imidodipeptides where a proline or hydroxyproline residue is situated on the C-terminal (Eni-Aganga *et al*., 2021). In addition to the degradation of proline residues, PEPD also plays a role in regulating activation of β-integrin receptors, IGF-1 and TFG- β1. The activation of these mechanisms is directly associated to the availability of proline in the proline cycle which in turn generates proline as a substrate for the resynthesis of collagen type IV. As the receptors are stimulated by their ligands, PEPD enzymatic activity will be affected as will the downstream processes that regulate cell growth and proliferation. The breakdown of the embryo's extracellular matrix is dependent on PEPD activity, which will catalyse the final step in collagen degradation, resulting in the release of free proline which will drive cell differentiation (Karna *et al*., 2020). Proline is also synthesized from glutamine (Gln) and glutamate (Glu) or from arginine (Ar) through conversion to ornithine (Orn). Glu can also be converted to proline through Δ1-pyrroline-5-carboxylate (P5C) by P5C synthase (P5CS) and P5C reductase (PYCR). Alternatively, proline can be converted to Glu through the catabolic pathway where it may be catalysed by proline oxidase/dehydrogenase (POX/PRODH) and P5C dehydrogenase (P5CDH) (Palka *et al.*, 2021). This image was created with BioRender (https://biorender.com/)

1.8.3 PRODH/POX

L-proline metabolism plays a significant role in metabolic programming in reproductive development, cellular division, and deterioration and in cancer pathways. L-proline will additionally inhibit the degradation of Hypoxia-inducible factor 1-alpha, also known as HIF-1-alpha, which in turn activates pro-angiogenic and neoplastic genes, specifically Vascular endothelial growth factor (VEGF), Transforming growth factor (TGF) and Tumour necrosis factor (TNF) (Phang *et al*., 2012). Furthermore, there are mechanisms that utilise L-proline such as collagen synthesis and L-proline conversion to 1-Pyrroline-5-carboxylic acid (P5C) during proline degradation. The process of L-proline degradation is initiated by Proline dehydrogenase/proline oxidase (PRODH/POX), a mitochondrial inner membrane enzyme which, when activated, will induce ATP production (Zareba *et al*., 2017). Conversion of mitochondrial proline into P5C by PRODH/POX generates reactive oxygen species (ROS) dependent intrinsic and extrinsic apoptotic pathways (Figure 1.9). (Zareba *et al*., 2017, Kononczuk *et al*., 2015). ROS will be generated through a selection of biological activities mediated through the mitochondrial electron transport chain (ETC) (Hardy *et al*., 2021). The importance of ROS is demonstrated by their ability to act as signalling molecules and assist in facilitating cell proliferation, cell fate determination through a highly regulated homeostasis and redox regulation during cell signalling (Ray *et al*., 2012). When embryos are cultured *in vitro*, the generation of ROS will be intensified due to embryo culture conditions, therefore upsetting the physiological balance required for normal cellular function (Schafer and Buettner, 2001).

Overexpression of PRODH/POX will occur in the presence of L-proline and has been shown that the upregulation of PRODH/POX will contribute to the activation of the specific caspase proteins which play central roles in cell apoptosis (Phang *et al*., 2008).

PRODH/POX expression has also been shown to significantly decrease the phosphorylation of MEK and ERK signalling pathways, strongly indicating that the inhibition of the MEK/ERK signalling is related to POX-induced apoptosis or autophagy. Moreover, ERK plays a principal role in suppressing PRODH/POX-induced apoptosis or autophagy by inhibiting caspase activation (Liu *et al*., 2006). AMP-activated protein kinase (AMPK)-dependent pathways and tumour suppressor protein p53 and directly linked as potent stimulators to PRODH/POX and its role in apoptosis and autophagy. As the availability of L-proline is crucial to the regulation of PRODH/POX, collagen synthesis and prolidase activity become fundamental to biological processes (Huynh *et al*., 2020).

1.8.4 Prolidase

Prolidase is a peptidase that cleaves dipeptides that comprise of L-proline or hydroxyproline at the c-terminal, it is one of the only enzymes that will recover and recycle L-proline from imidodipeptides resulting from degradation of collagen (Karna *et al*., 2020). The activity of prolidase is regulated by stimulation of the Integrin beta-1 receptor (ITGB1) and is important in helping to regulate collagen biosynthesis by acting as a step-limiting factor (Surazynski *et al*., 2008). Prolidase in encoded by the Peptidase D (PEPD) gene and is located on chromosome 19 on the short arm region (Tanoue *et al*., 1990).

Prolidase has been identified as a step-limiting factor in the biochemical pathway of collagen degradation, the breakdown of the ECM being initiated by MMP activation and biochemical signalling. As the collagen in the basement membrane is broken down, this will result in the assembly of imidodipeptides which will only be degraded by prolidase and prolinase (Zanaboni *et al*., 1994, Phang *et al*., 2010). The process will involve cytokine-mediated activation of specific MMPs which will result in an increase in prolidase activity, which will consequentially influence the controlled degradation of collagen IV in the embryo basement membrane and therefore release free proline (Phang *et al*., 2008). While it is known prolidase will play a pivotal role in the regulation of collagen biosynthesis and the remodelling of the ECM, the interaction between EGFR and prolidase has additionally be shown to function as an effective activator of EGFR signalling. As EGFR signalling is one of the most important pathways relevant to mammalian cell survival, growth and differentiation (Oda *et al*., 2005). Prolidase will activate EGFR signalling, which in turn will induce cell proliferation and migration through the upregulation of pathways PI3K/Akt/mTOR, JAK/STAT and Ras/Raf/ERK (Yang *et al*., 2013).

1.8.5 Prolidase Inhibitors

Transforming growth factor (TGF-β1) can promote collagen IV biosynthesis and fibronectin production in the ECM (Kim *et al.*, 2018). As a product of collagen IV biosynthesis, L-proline will increase the expression of TGF-β1 receptors along with the phospho-AKT (Ser473) and phospho-mTOR (Ser2448) in the Akt/PI3K signalling pathway (Surazynski *et al.*, 2010). Stimulated TGF receptors will induce kinases Akt, PI3K and mTOR, with mTOR being a key molecule involved with the regulatory

pathway of L-proline metabolism (Reiling and Sabatini, 2006). Downregulation of the mTOR pathway and prolidase activity will ultimately have a counteractive result on the regulation of collagen biosynthesis, including the production of L-proline (Surazynski *et al*., 2010)

Prolidase inhibitors will decrease the expression of TGF-β1 receptors, induce down-regulation of Ser473 and decrease Ser2448 expression (Misiura *et al*., 2020) Specifically, acetylsalicylic acid (ASA) has been reported to impede the phosphorylation of prolidase distinctively on the threonine residue at concentrations of 5 mM, consequently inhibiting the biosynthesis of collagen IV in the BM (Surazynski *et al*., 2010).

1.9 Transport Systems

The mechanisms that are associated with positive effects on embryogenesis are yet to be investigated and defined. The functional importance of the transportation systems of amino acids has in the past, been approached by systematically studying the mechanisms associated with the control of the process at a subcellular level. This has been studied by looking at the effects of complications arising within the surroundings in which the amino acid transportation naturally transpires (Kilberg *et al*., 1993).

Amino acids are important biological molecules that comprise of an amino group, at least one carboxyl group, bound to the same carbon atom and a side chain, specific to each individual amino acid. The transportation of the amino acid across the membrane is considered an important step in amino acid metabolism and will include

three events that are essential to the mediated transfer of a solute across the membrane.

The transport systems will also rely heavily on various operational characteristics, boundary conditions and responses to control mechanisms to mediate the transport of amino acids across a membrane. (Benz, 1994, Babasola *et al*., 2013). The mediated transport of amino acids can be understood as a similar method developed for the catalysis of enzymes from a kinetic perspective, specifically, Michaelis–Menten kinetics. Transporters that are participating in the acceptance and transference of substrates across a membrane create the transport producing systems (Longuet-Higgins and Austin, 1966).

L-proline is a selected amino acid which has been shown to function as an osmolyte for preimplantation mouse embryos and is responsible for improving embryo development (Morris *et al*., 2020). In addition to being dependent on transporter uptake L-proline is also involved in the stimulation and regulation of the Akt, ERK1/2 and mTORC1 signalling pathways, which are fundamental for cell survival and growth promoting signals. The transport of L-proline into cells is mediated through specific amino acid transporters which subsequently help to regulate the uptake of amino acids and induce cell differentiation (Tan *et al.*, 2011). It can be suggested that if the source of free L-proline is to be limited or restricted, the pathways responsible for supporting normal development in preimplantation embryos will be inhibited. It is also important to mention that studies have shown that amino acids such as L-proline and L-glutamine may act in a trophic factor-like way to regulate the pathways that are responsible for preimplantation development. Previous studies

have presented several factors that may regulate homeostatic balance of amino acids, like L-proline in cells. Amino acid biosynthesis, the entry and exit of amino acids into cells through amino acid transporters and through protein biosynthesis and degradation (Broer and Broer, 2017). Additionally, recent experiments have shown that L-proline uptake is capable of being saturated due to specific amino acid transporters expressed in preimplantation mouse embryos (Treleaven *et al*., 2021). Earlier research primarily based on cell viability involving the exogenous addition of L-proline to porcine trophoblast cells, revealed that the critical threshold concentration was 1.0 mmol/L (Liu *et al*., 2021). Given this evidence, it can be ascertained that the optimal concentration of L-proline needed for future experiments appears to be approximately 0.5 mmol/L. In our experiments, we used concentrations of 0.4 mmol/L.

1.10 Summary and hypothesis

There is an abundance of amino acids found naturally within the natural reproductive tract as well as in the embryo's extracellular matrix. The amino acids facilitate the biological functions of cells involved with early embryo development and provide substantial structural support for the embryo's developing extracellular matrix. Collagen IV provides the structural support which forms the foundation of the embryo's basement membrane. The lattice-like assembly of the collagen IV fibrils is abundant in L-proline, which contributes to its ability to provide site-specific flexibility.

As specific matrix metalloproteinases initiate the natural breakdown of the embryo's basement membrane, L-proline may be released from the ECM which could help to drive cell differentiation. We hypothesize that if the natural degradation of collagen IV

is inhibited, this will result in limited differentiation of cells in the blastocyst and poor quality of development.

1.11 Aims

The main aims of this study are:

1. To determine the expression pattern of Collagen IV alpha sub-chains during preimplantation development.
2. To examine the role of Collagen IV in the differentiation of cells in the blastocyst by the use of inhibitors of MMPs, by preventing the production of free L-proline this will help to determine the underlying molecular mechanisms that are associated with various stages of embryogenesis, specifically during the natural breakdown of the embryo's ECM.

Chapter 2: Materials and Methods

2.1 Culture media and Reagents

The medium used for zygote isolation was HEPES-buffered modified human tubal fluid (HEPES-modHTF) + 3 mg/ml bovine serum albumin (BSA) with an osmolality of 270 mOsm/kg and a pH of 7.4. The medium used for embryo culture was modHTF + 3 mg/ml BSA (270 mOsm / kg, pH of 7.4) (Tables 2.1 and 2.2) (Morris *et al*., 2020). All stock solutions and media were prepared the day before oocyte isolation by weighing the appropriate compounds and tissue-culture grade reagents (Sigma-Aldrich) out individually and reconstituting them with Milli-Q grade water.

The pH was adjusted to 7.4 using 2 M NaOH of 1 M HCl and the media were filtered using a 0.22 μm filter (Millipore). Media and stock solutions were stored at 4°C for no more than 2 weeks.

Table 2.1 Stock solutions for preparing embryo isolations and culture medium.

All stock solutions were prepared using Milli-Q water

Concentration	**Component**	**MW**	**Amount/Volume H_20**
1 M	NaCl	58.44	2.92 g / 50 ml
0.1 M	KCl	74.55	0.37 g / 50 ml
0.1 M	KH_2PO_4	136.09	0.1361 g /10 ml
0.1 M	$MgSO_4 \cdot 7H_2O$	246.47	0.2465 g /10 ml
1 M	$NaHCO_3$ *	84.01	0.4201 g / 5ml
0.001 M	Na_2EDTA	372.24	0.0186 g / 50 ml
0.1 M	Na Pyruvate *	110.00	0.055 g / 5 ml
0.1 M	Glucose *	180.16	0.901 g / 5 ml
6 mg / ml	Penicillin	-	60 mg / 10 ml
0.17 M	$CaCl_2 \cdot 2H_2O$	147.02	0.2514 g / 10 ml
25 mg / ml	Phenol Red	-	250 mg / 10 ml
0.154 M	HEPES **	238.3	1.835 g / 50 ml

* Freshly prepared for one week only
** pH of HEPES adjusted to 7.4 by addition of 2 M NaOH

Table 2.2 Preparation of modHTF medium and HEPES-modHTF from stock solutions

Component	modHTF		HEPES-modHTF	
	Final Conc (mM)	Volume of stock (ml / 50 ml)	Final Conc (mM)	Volume of stock (ml / 50 ml)
Lactic Acid	21.4	0.20	21.4	0.20
NaCl	85	4.25	85	4.25
KCl	4.6	2.30	4.6	2.30
KH_2PO_4	0.4	0.20	0.4	0.20
$MgSO_4$	0.2	0.10	0.2	0.10
$NaHCO_3$	25	1.25	4	0.20
Na_2EDTA	0.11	5.50	-	-
Na Pyruvate	0.4	0.20	0.4	0.20
Glucose	2.8	1.4	2.8	1.4
Phenol Red	0.001 %	0.02	0.001 %	0.02
Penicillin	0.06 mg /ml	0.50	0.06 mg / ml	0.50
$CaCl•2H_2O$	2.04	0.60	2.04	0.60
HEPES	-	-	21	6.82
H_2O	-	to 50 mL	-	to 50 mL
pH adjusted to 7.4 with 2 M NaOH for HEPES-modHTF pH adjusted to 7.4 with 1 M HCl for modHTF 150 mg/50 ml of BSA added following the adjustment of pH. The media was then filtered with 0.22 µm syringe filter				

2.1.1 L-proline

The 0.1 M L-proline (Sigma) was prepared with Milli-Q water and diluted in culture media to 400 μM on the day of embryo isolation. A final concentration of 400 μM was used in this study since this is has been shown previously to improve embryo development (Morris *et al.*, 2020) and is the concentration present in uterine fluid (Day Lab, unpublished).

2.1.2 Matrix metalloproteinase inhibitor GM6001

GM6001 (Cayman Chemical Company) was supplied as a crystalline solid and reconstituted with DMSO to create a 20 mM stock as per the recommendations from Cayman Chemical for maximum solubility. The stock was then stored in 5 μl aliquots at -20°C and diluted in modHTF to 100 μM on the day of embryo isolation (Martin-Martin *et al*., 2011).

2.1.3 Matrix metalloproteinase inhibitor SB3CT

SB3CT (Cayman Chemical Company) was reconstituted in DMSO to create a 10 mM stock solution, stored in aliquots at -20°C for maximum solubility as per the recommendations from Cayman Chemical. The stock was then diluted in modHTF to 10 μM on the day of embryo isolation (Theodore *et al*., 2017).

2.1.4 Acetylsalicylic Acid

Acetylsalicylic Acid (ASA) (Sigma-Aldrich) was reconstituted in DMSO to create a 10 mM stock solution as per the recommendations from Sigma-Aldrich and diluted to 0.5 mM, 2 mM, or 5 mM in modHTF on the day of embryo isolation for culture (Chrzanowski *et al*., 2004).

2.1.5 Hyaluronidase

Hyaluronidase (Sigma-Aldrich) hydrolyses the hyaluronan matrix surrounding cumulus-oocyte complexes, allowing the separation of cumulus cells from zygotes (Ishizuka *et al*., 2014). Hyaluronidase was prepared as a stock 1 mg/ml in HEPES modHTF + 0.3 mg/ml BSA, filtered and stored at -20°C until use.

2.2 Animal models

The use of mice was in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose and was approved by the Institutional Animal Care and Ethics Committee. Outbred Quackenbush Swiss (QS) mice (Animal Resource Centre, Perth, WA, Australia and Lab Animal Services, The University of Sydney, NSW, Australia) were housed under a 12 h light,12 h dark cycle.

All experiments were conducted within the University of Sydney's facilities and complied with the NSW Animal Research Act of 1985, the Australian Code for the care and use of animals for scientific purposes 8th edition 2013 in accordance with the University's Animal Ethics Committees (AECs).

2.2.1 Superovulation of female mice

Female QS mice (>3 weeks of age) were super ovulated by means of intraperitoneal (IP) injection of 10 IU of pregnant mares' serum (PMS; Intervet, Sydney Australia), followed by 10 IU of human chorionic gonadotropin (HCG; Intervet, Sydney Australia) 48 h later. Females were then paired for 24 h with male

studs (2-6 months of age, housed individually) of proven fertility. Evidence of coitus was observed the following day by the presence of the copulatory plug.

2.2.2. Embryo collection

No more than 16 h after hCG and pairing with male studs, the female mice were euthanised by cervical dislocation and placed in the supine position before dissection. A neat incision was made along the abdominal skin and peritoneum area to expose the viscera and the oviduct along with each intact uterine horn. Separation and removal of the oviducts containing the embryos was then carried out using fine forceps and surgical scissors and the separated oviducts, along with a portion of the uterus was place in a prewarmed 35 mm Petri dish containing phosphate buffered saline (PBS). The oviducts were then transferred to a 55 mm Petri dish containing HEPES modHTF + 0.3 mg/ml BSA and microdissection of the oviducts was then performed on a heated stage. A fine gauge insulin needle was prefilled with warm HEPES modHTF + 0.3 mg/ml BSA and gently inserted into the ampulla where the oviduct was then flushed, releasing the cumulus mass containing the embryos. ~ 100 μl warmed hyaluronidase was added to the dish to assist in separation of the cumulus cells from the embryos.

The zygotes were transferred into a new 55 mm Petri dish containing prewarmed 50 μl droplets of modHTF + 0.3 mg/ml BSA under oil (Sigma) where they were washed thoroughly and sorted to exclude unfertilised oocytes and abnormally fertilised zygotes. From here, the zygotes with two pronuclei were moved to 55 mm Petri dishes containing either control droplets of modHTF + 0.3 mg/ml BSA or treatment groups for culture at 37°C and at 5% CO_2.

2.3 Embryo culture ± MMP inhibitors

A protocol was developed to observe the effect of MMP inhibitors on preimplantation embryos. Embryos were cultured from the day of isolation on day one, through until day six. Embryos were then divided into high and low density groups and cultured in either a 35 mm embryo culture dish in evenly spaced 50 µl droplets under oil or 10 µl droplets under oil in a 96-well plate. The high density groups were split into control groups and treatment groups, with a maximum of 10 embryos/50 µL droplet, while the low density group had no more than 1 embryo/10 µl droplet in the 96-well plate.

Embryos were cultured at high density with no exposure to MMP inhibitors (control group), exposure from day of isolation and exposure from day three of culture and exposure from day four of culture.

2.4 Immunofluorescence staining of embryos

The immunofluorescent staining protocol was that of (Falini *et al*., 1986) and further developed in our laboratory to complement my project. Prior to staining, the embryos were fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature and then washed three times in PBS + polyvinyl alcohol (PVA; 0.1% 1 mg/ml) and stored in PBS + PVA until ready to stain. Once the embryos were ready for immunostaining, they were permeabilised in PBS + PVA + 0.7% BSA + 0.3% Triton X 100 for 1 h. The embryos were then washed in PBS + PVA three to four times and blocked for 1 h at room temperature in PBS + PVA + BSA + 0.1% Tween-20 (Sigma-Aldrich).

Following blocking, the embryos were incubated in primary antibody diluted with PBS + PVA + BSA + Tween-20 (Table 2.3) and incubated either for 2 h at room temperature or overnight at 4ºC. The embryos were washed three to four times in PBS + PVA + BSA + Tween-20 and left in the wash for 30 min before being incubated at in the dark at room temperature for 1 h in secondary antibody diluted with PBS + PVA + BSA + Tween-20 (Table 2.3). This process of antibody staining was repeated depending on the number of primary antibodies used in the experiment. Once the staining was complete, the embryos were mounted onto coverslips for the confocal microscopy in 2-3 µl of Vectashield containing 1.5 µg/mL DAPI (Vector Laboratories, CA, USA). The coverslips were sealed onto slides using Vaseline to maintain the 3D structure of the blastocysts and stored in the dark at 4ºC before imaging in the confocal microscope.

2.5 Confocal microscopy and cell counting

Embryos were imaged using both the Zeiss confocal Meta 510 and the Zeiss LSM 800 laser confocal microscopes with a 20x objective. The images were viewed and analysed using the Fiji imaging software application.

Following sequential staining (section 2.4) and imaging, the number of cells in each embryo was counted. Each of the cell counts was recorded and tabulated to illustrate the impact the exposure of each MMP inhibitor had on Nanog and Gata4 distribution.

2.6 Statistical analysis

Each experiment was performed independently at least three times for reliability and to ensure that variability of the results was reduced. Statistical analyses were performed using GraphPad Prism version 8.0.0 statistical software using a one-way ANOVA with Tukeys post hoc test for multiple comparisons. A P-value less than 0.05 was considered significant.

Table 2.3 Primary and secondary antibodies used for immunofluorecence

Target	Primary antibody	Source (Cat #)	Conc used	Secondary antibody	Source Company (Cat #)	Conc used	Emission (nm)
Nanog	Rabbit Monoclonal IgG	Cell Signalling (8822)	2.58 µg/ml	Goat anti-rabbit IgG-CFL-647	Santa Cruz (SC-362292)	4 µg/ml	647
Gata4	Goat Polyclonal, IgG	Santa Cruz (SC-1237)	2 µg/ml	Donkey anti-goat IgG CFL 488	Santa Cruz (SC-362255)	4 µg/ml	488
Collagen IV $\alpha 1$	Rat Monoclonal, IgG2a	Chondrex (7070)	10 µg/ml	Goat anti-rat IgG(H+L) AlexaFlour 546	Life Technologies (A11081)	20 µg/ml	546
Collagen IV $\alpha 2$	Rat Monoclonal, IgG2a	Chondrex (7071)	10 µg/ml	Goat anti-rat IgG(H+L) AlexaFlour 546	Life Technologies (A11081)	20 µg/ml	546
Collagen IV $\alpha 3$	Rat Monoclonal, IgG2a	Chondrex (7076)	10 µg/ml	Goat anti-rat IgG(H+L) AlexaFlour 546	Life Technologies (A11081)	20 µg/ml	546
Peptidase D (PEPD)	Polyclonal Rabbit IgG	Proteintech 12218-1-AP	1.5 µg/ml	Goat anti-rabbit	Life Technologies (A11037)	20 µg/ml	594

Chapter 3:

Expression of Collagen IV alpha chains during preimplantation development

3.1 Introduction

Formation of the basement membrane consists largely of self-assembly of ECM proteins, integrin-mediated adhesion, and receptor kinases. Basement membranes are comprised of specific ECM components, each of which makes a contribution to structural integrity and continued development of the embryo (Gersdorff *et al*., 2005). There are three types of specific matrix components: (i) Viscous proteoglycans which play a role in providing structural support to cells, (ii) insoluble collagen fibres, which provide structural integrity and resilience to the ECM, and (iii) adhesive proteins such as fibronectin and laminin whose primary role is to help localise proteoglycans and collagen fibres close to cell-surface receptors (Kruegel and Miosge, 2010).

During preimplantation development, basement membrane assembly initiates when laminin heterotrimers connect to cell surfaces, assembling into a lattice-like scaffold which provides structural support for the embryo (Morrissey and Sherwood, 2015). In addition to structural support, individual components of the basement membrane will regulate biological activities such as cell proliferation, differentiation, cell migration and growth (Erickson and Couchman, 2000). This is accomplished primarily through interactions with cell surface receptors and mediated by transmembrane molecules like integrins (Aumailley and Gayraud, 1998).

Laminins are a family of glycoproteins which are major components of the basement membrane. They are primarily responsible for influencing cell differentiation and coordinating the assembly of the embryo's basement membrane (Poschl *et al*., 2004). In addition to laminins, perlecan, a large multi-domain heparan-sulfate proteoglycan, helps maintain cell adhesion to the ECM and enhances the stability of the basement membrane by maintaining biochemical properties such as modifying and regulating specific bioavailability of growth factors which activate various signalling pathways required for normal embryo development (Gubbiotti *et al.*, 2017). More specifically, perlecan links the laminin and collagen IV networks by binding multiple growth factors and at the same time promoting cell survival and differentiation (Sekiguchi and Yamada, 2018).

A strong association has previously been described between the role of autocrine growth factors during early embryo development and the quality of the embryos that are developing during in-vitro culture. A number of embryotrophic factors such as IGF1 and PAF have been identified (Morris *et al.*, 2020) which help to regulate embryo development by facilitating the autocrine support required for preimplantation embryos cultured in high density (O'Neill, 1997). During low-density culture, where autocrine support is minimised, the addition of amino acids, such as L-proline and L-glutamine (Lane *et al.*, 2001), under isosmotic conditions serves to restore the rate of growth and development of preimplantation embryos to that seen in high density (Green and Day, 2013).

Experiments from our laboratory and previous studies carried out by Professor O'Neill has revealed the significant correlation between the role of platelet-activating

factor (PAF) and cellular signalling during embryo development in-vitro (Prescott et *al.*, 2000, Wydooghe *et al.*, 2017). These studies have demonstrated that PAF will act as a survival-like factor that will ultimately stimulate the metabolism in the developing embryo and influence development (O'Neill, 1997). PAF has been described as an important mediator involved with intracellular signalling (Gay, 1993) and can induce MMP-9 expression through phosphorylation of PI3K and ERK signalling (Sato *et al.*, 1992) and induce MMP-2 secretion (Ko *et al*., 2005). Development of mouse embryos cultured in high destiny will secrete PAF, which acts in a receptor-mediated manner to stimulate the rate of development (Stoddart *et al*., 1996), similarly with preimplantation embryos cultured in low density environments with the addition of PAF conditioned medium (Lu *et al.*, 2004).

The lattice-like structure of the basement membrane predominantly contains type IV collagen (Mak and Mei, 2017). In addition to contributing to the basic structural support, collagen IV is largely responsible for influencing cell migration and differentiation, and contributes to cell proliferation, all of which contribute to normal development (Poschl *et al.*, 2004). Approximately 23% of collagen IV is comprised of L-proline and hydroxyproline combined (Albaugh *et al*., 2017). L-proline allows the collagen helices to twist and bend more sharply and make function sites more accessible for the interacting proteins and cell receptors (Brinckmann, 2005). During turnover of the basement membrane components, including especially collagen IV, L-proline is released with the assistance of specialised enzymes, matrix metalloproteinases (MMPs). Since L-proline can drive differentiation of embryonic stem cells as well as promote improved development of pre-implantation embryos cultured *in vitro* (Ozsoy, 2011), we hypothesise that the turnover of collagen IV in the

ECM that separates the hypoblast from the epiblast in the peri-implantation mouse embryo (Kay *et al.*, 2021) releases sufficient L-proline to nearby inner cell mass cells to trigger their transition to pluripotent primitive ectoderm cells. We have used two specific transcription factors, Nanog and Gata4, as markers to aid in identifying the epiblast and hypoblast in the preimplantation embryo. We have additionally used Nanog and Gata4 to observe how the restriction of L-proline to the embryo, will impact the differentiation and proliferation of cells in the inner cell mass.

MMPs are grouped according to their function and substrate specificity and their domain organisation, each group being responsible for different degradation activity (Klatte-Schulz *et al*., 2015). Specifically, MMPs 2 and 9, also referred to as gelatinases, degrade collagen IV and gelatin. Gelatinases can also cleave different targets such as cytokines and growth factors (Bauvois, 2012), and so influence the regulation of key signalling pathways involved with cell growth, inflammation, and angiogenesis (Cabral-Pacheco *et al*., 2020).

Aberrant activity of issue inhibitors of metalloproteinases (TIMPs) are responsible for several pathophysiological processes during embryogenesis, angiogenesis and remodelling of the extracellular matrix (Arpino *et al*., 2015). Four groups of TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) and inhibit the activity of specific MMPs (Murphy, 2011). The appropriate balance between the activity of the MMPs and their inhibition by TIMPs is important for normal preimplantation embryo development (Wang *et al*., 2003).

Synthetic inhibitors of MMPs have been used in clinical trials for tissue regeneration, cancer treatment and trauma healing (Agren *et al*., 2001). One broad spectrum inhibitor, GM6001 (Ilomastat) is a potent reversible inhibitor which has antitumor and anti-angiogenic properties (Devy *et al*., 2009). GM6001's hydroxamate group binds the active-site Zn^{2+} ion, and together with a backbone that resembles the configuration of collagen, results in its broad spectrum inhibitor properties (Overall and Kleifeld, 2006). GM6001 functions by inhibiting activation of extracellular signal-regulated kinase and decreasing collagen synthesis *in vivo* (Garcia-Alloza *et al.*, 2009). The broad spectrum inhibitor will inhibit zinc-containing thermolysin and members of a disintegrin and metalloproteinase proteins which have roles in cell signaling and the development of epithelial tissues, fertilization, and cell migration (Edwards *et al.*, 2008).

Synthetic inhibitors like GM6001 will effectively inhibit the natural breakdown of the embryo's basement membrane, and with an absence of free amino acids such as proline present to help drive normal cellular functions, the growth and development of the preimplantation embryo will be impaired significantly. Hypothetically, the addition of L-proline under these conditions would counteract the effect of the broad spectrum inhibitor and biological functions including cell differentiation would continue.

We hypothesise that exposing embryos to GM6001 during different stages of pre-implantation development will result in significant impact on their growth and development including cell differentiation and blastocyst formation. We further hypothesize that the impact on growth and development will be due to the inhibitory

effects of GM6001 on collagen IV degradation, preventing the release of free L-proline.

3.2 Materials and methods

3.2.1 Immunofluorescence staining of embryos

The embryos were fixed after six days in culture as described in 2.4 and immunostained for Nanog and Gata4 to identify the hypoblast and epiblast of the preimplantation embryo while also helping to establish the expression of the collagen IV alpha chains in the basement membrane after exposure to GM6001.

3.2.2 High and low density culture in broad spectrum inhibitor GM6001

Embryos were isolated on day one, as described in Chapter 2, and cultured in either high density in evenly spaced 50 μl droplets of modHTF under oil in a 35 mm dish, with no more than 10 embryos/50 μl or at low density in 10 μl droplets in a 96-well plate, with 1 embryo/10 μl. Embryos were cultured until day 6.
Embryos were cultured in modHTF in the absence or presence of 100 μM GM6001.

3.2.3 Exposure to 100 μM GM6001 at different stages of development

Following isolation, mouse embryos were split into groups for staggered exposure; a control group and treatment group from day one, a treatment group from day three and a treatment group from day four. The control groups were cultured in high density in modHTF + 3 mg/ml BSA (270 mOsm/kg, pH of 7.4) (Tables 2.1 and 2.2) (Morris *et al*., 2020) and the treatment groups were cultured in GM6001 diluted in modHTF to 100 μM. Embryos were moved to treatment groups on day three and

day four of culture to observe the impact of GM6001 during fundamental stages of preimplantation development.

3.2.4 Exposure to 100 μM GM6001 with the addition of 400 μM L-proline

All embryos were isolated on day one where they were split into control groups and three different treatment groups and cultured to day six. Each group was cultured in low density, which contained a maximum of 1 embryo/10 μL droplet in each well of a 96-well plate. The treatment groups consisted of GM6001 diluted in modHTF to 100 μM, L-proline diluted with modHTF to 400 μM, and GM6001 100 μM + 400 μM in modHTF.

3.2.5 Immunostaining of Nanog and Gata4

Following the sequential staining of the blastocysts (as described in 2.4), images were taken using the Zeiss LSM 510-META Confocal microscope (2.5). These images were then used to individually count every cell in each embryo across groups that had been exposed to GM6001 and the control groups by using Fiji imaging software (Schindelin *et al*., 2012). The number of cells in each embryo were counted by using transparency film and non-permanent overhead projector markers and recorded in Microsoft Excel.

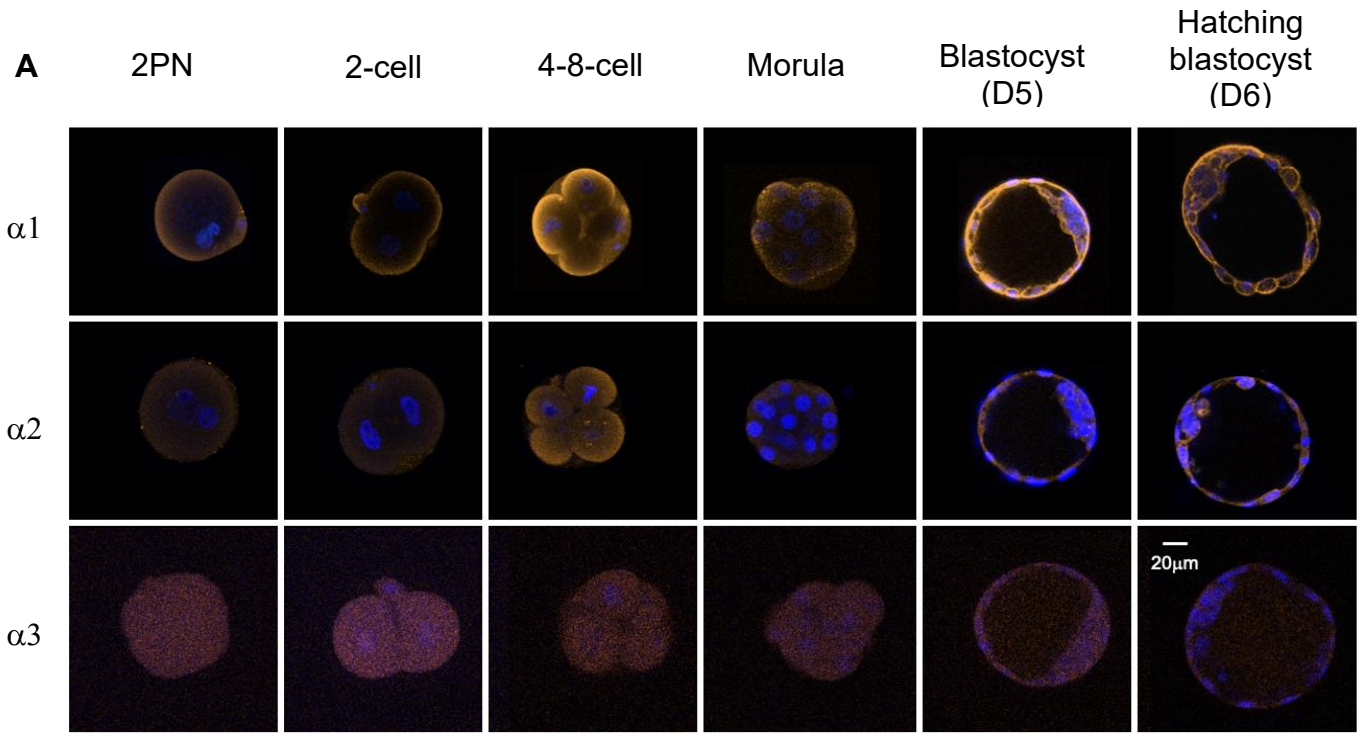
3.2.6 Statistical analysis

Data was analysed in GraphPad Prism version 8.0.0 statistical software using a one-way ANOVA with Tukeys post hoc test for multiple comparisons.
$P < 0.05$ was considered statistically significant and the data was expressed as mean ± SEM. Experiments were repeated at least three times.

3.3 Results

3.3.1 Expression of alpha sub-chains of collagen IV

Embryos were cultured from the day of isolation to day six. During each stage, embryos were fixed and stained as described in 2.4 to detect the most prevalent collagen IV alpha chain present in the preimplantation embryo. From these results (Figure 3.1) we were able to establish that collagen IV alpha-1 was expressed ubiquitously during each stage of preimplantation development, compared to alpha-2 and alpha-3 and we could identify the alpha 1 chain in the putative basement membrane in the blastocyst (Figure 3.2).



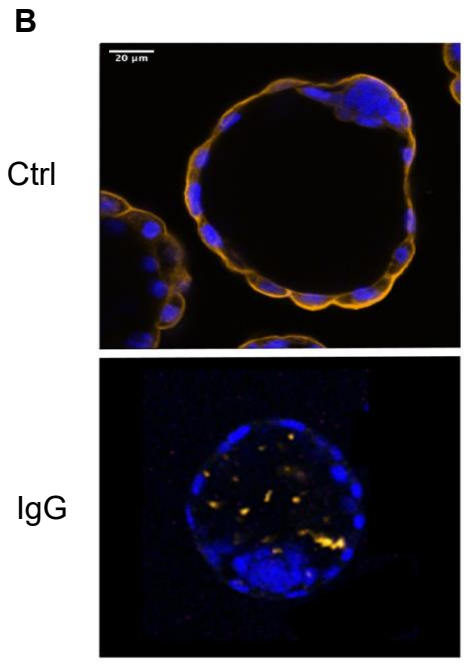


Fig 3.1 Collagen IV alpha chain distribution
Expression of collagen IV sub-chains during mouse preimplantation embryo development. Embryos were stained with DAPI to identify nuclei within the cells (blue) at 405nM. Expression of collagen IV alpha sub chain 1, 2 and 3 (orange) at 546nM. A) Collagen IV α1, α2, and α3 sub chain expression from E1.0 to E6.0, B) Collagen IV (α1chain) control with rat IgG only staining to serve as a negative control. Bars = 20μm
Images generated by N. Stark

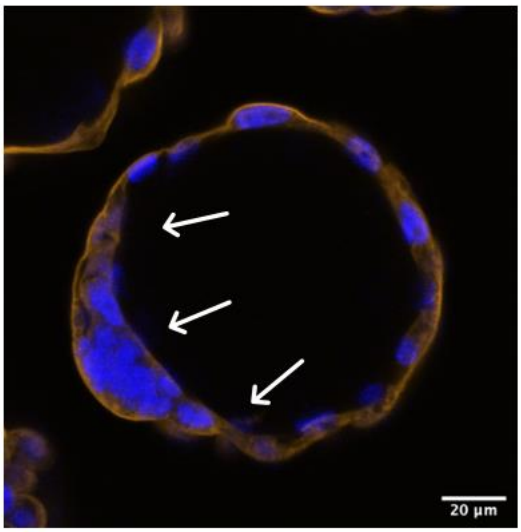


Fig 3.2 Collagen IV alpha chain 1 expression (orange) in a mouse blastocyst, illustrating the presence of α1 chain in the putative basement membrane (indicated by arrows) between the epiblast and hypoblast cells,
Bars = 20μm
Image generated by N. Stark

3.3.2 Culture in high density environments when exposed to GM6001

This study examined the effect inhibitors of matrix metalloproteinases have on embryo development and compared the impact of high and low density culture environments. The inhibitory effects of 100 μM GM6001 on development of zygotes over the course of six days to the hatching blastocyst stage in both exposure of embryos to GM6001, regardless of the density of culture, there was no effect on embryo development at any developmental stage (Figure 3.3)

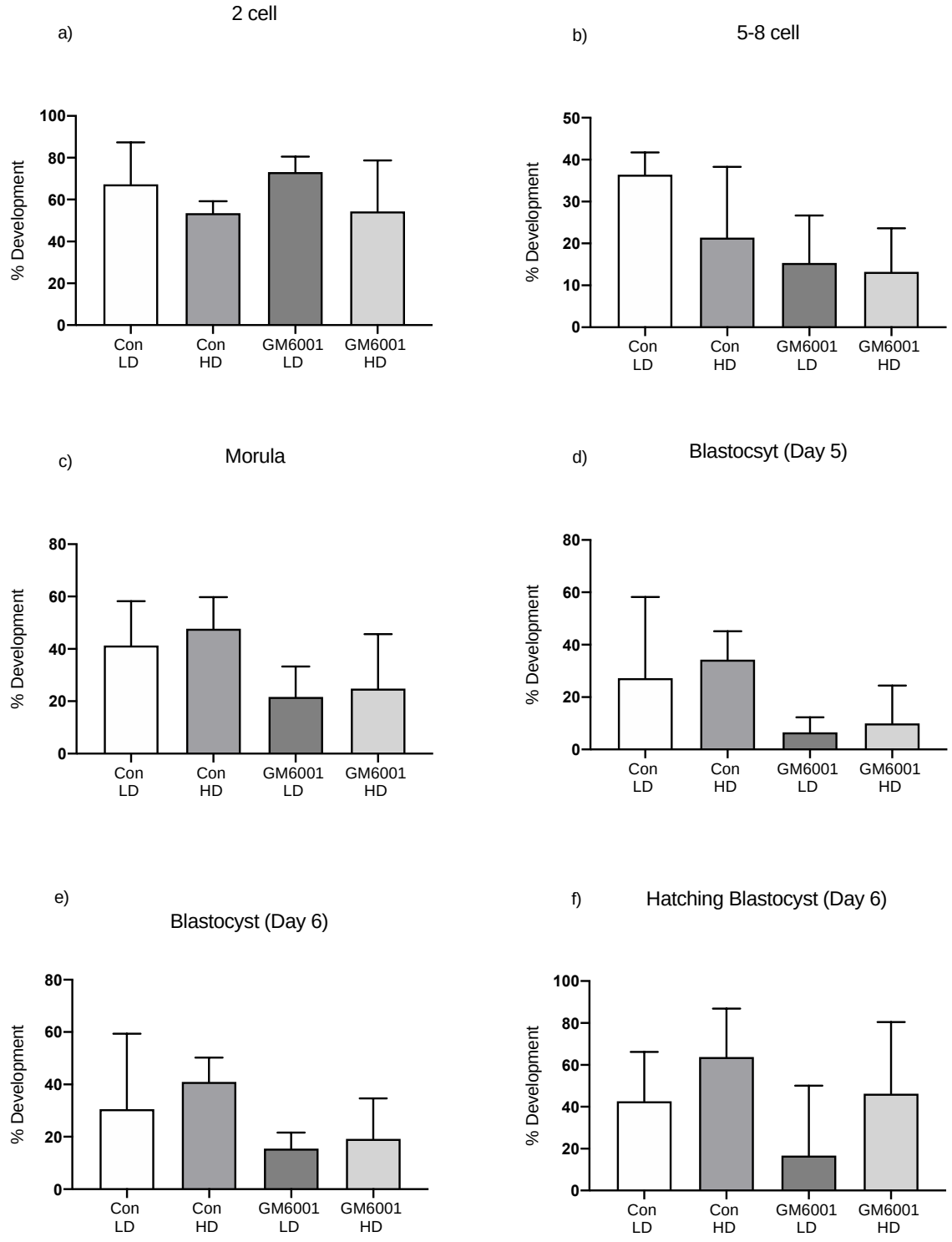


Fig 3.3 Effect of low density and high-density culture of mouse embryos ± 100 μM GM6001

Freshly isolated zygotes were cultured for six days to the hatching blastocyst stage at low - (1 embryo/10 μl) or high-density (no more than 10 embryos/50 μl) culture in the absence or presence of 100 μM GM6001 from day one. Development to (a) 2-cell (day two of culture), (b) 5-8 cell (day 3 of culture), (c) morula (day 4 of culture), (d) blastocyst on day 5 of culture, (e) blastocyst on day 6 of culture and (f) hatching blastocysts on day 6 of culture. Results analysed using an ordinary one-way ANOVA and Tukey's post-hoc test. Error bars represent SEM and results were obtained from at least 3 different experiments. No significant differences were observed.

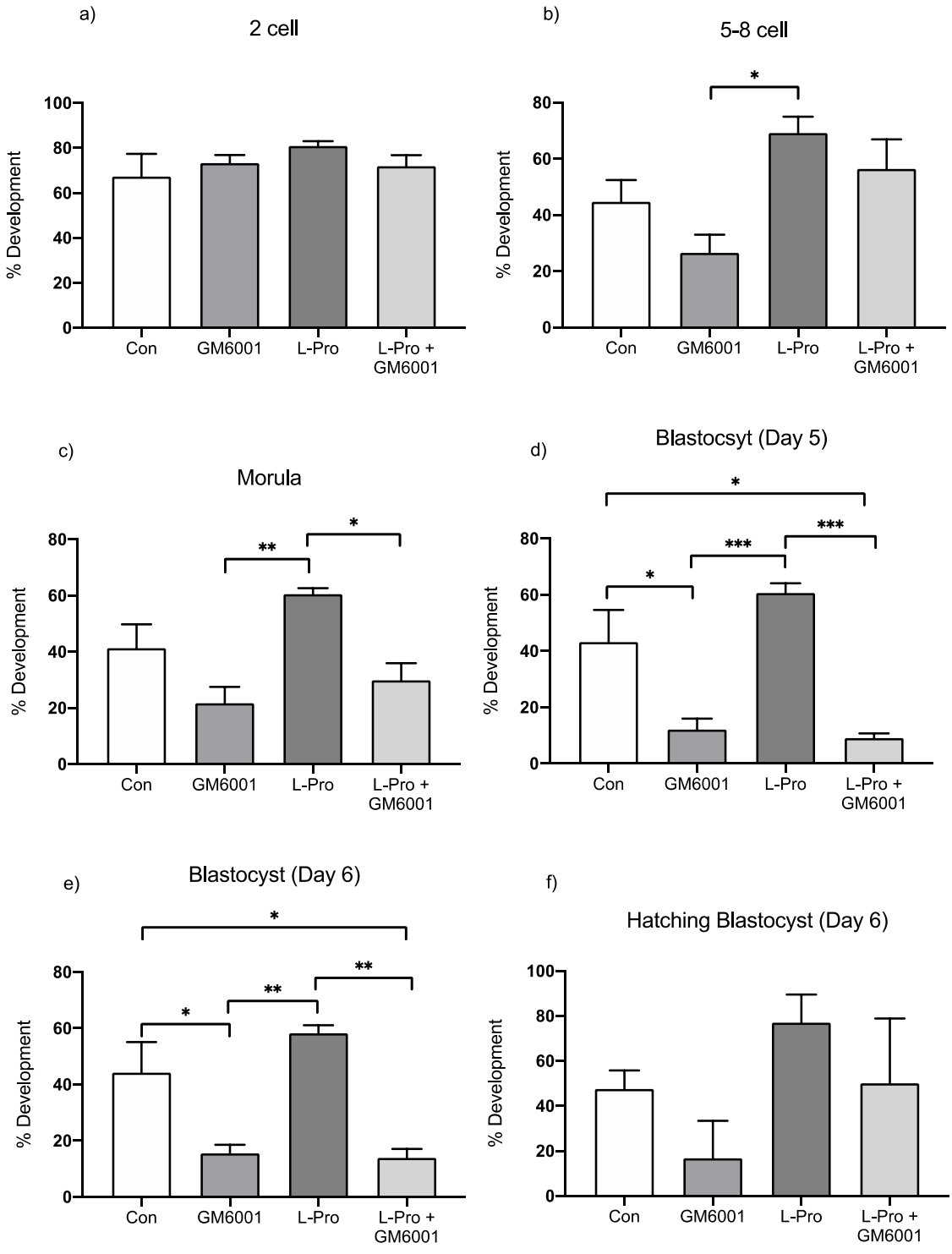


Fig 3.4 Effect of GM6001 and 400 μM L-proline in low density culture of mouse embryos. Freshly isolated zygotes were cultured for six days to the hatching blastocyst stage at low density (1 embryo/10 μl) for six days. Development to (a) 2-cell (day two of culture), (b) 5-8 cell (day 3 of culture), (c) morula (day 4 of culture), (d) blastocyst on day 5 of culture, (e) blastocyst on day 6 of culture and (f) hatching blastocysts on day 6 of culture. Results analysed using an ordinary one-way ANOVA and Tukey's post-hoc test. Results were obtained from at least 3 different experiments. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

3.3.3 Exposure to 100 μM GM6001 during low density with addition of L-proline

The effect of L-proline on embryo development during low density culture with the addition of 100 μM of GM6001 for zygotes cultured over six days to the hatching blastocyst stage was also examined. Figure 3.4 shows significant improvement in embryo development, with the addition of 400 μM L-proline improving the rate of development from the 5-8 cell stage ($P < 0.05$) and showing a significant improvement in the rate of blastocyst development on day 5 ($P < 0.001$). When exposed to 100 μM GM6001, the inhibitory effects were only evident from the 5-8 cell stage of development in comparison with the control groups. A greater margin of error was due to notably smaller embryo sample sizes, which were due to isolation and breeding difficulties. Nevertheless, our findings did reveal that the addition of GM6001 100 μM to the L-proline group appeared to reverse the improvement we had observed previously.

After six days in culture, the embryos were stained for Gata4 and Nanog, the transcription factors that are expressed in the hypoblast and epiblast, respectively (Figure 3.5). There was noticeable disparity between the intensity of the transcription factor staining between the groups cultured in GM6001 and the groups cultured in L-proline.

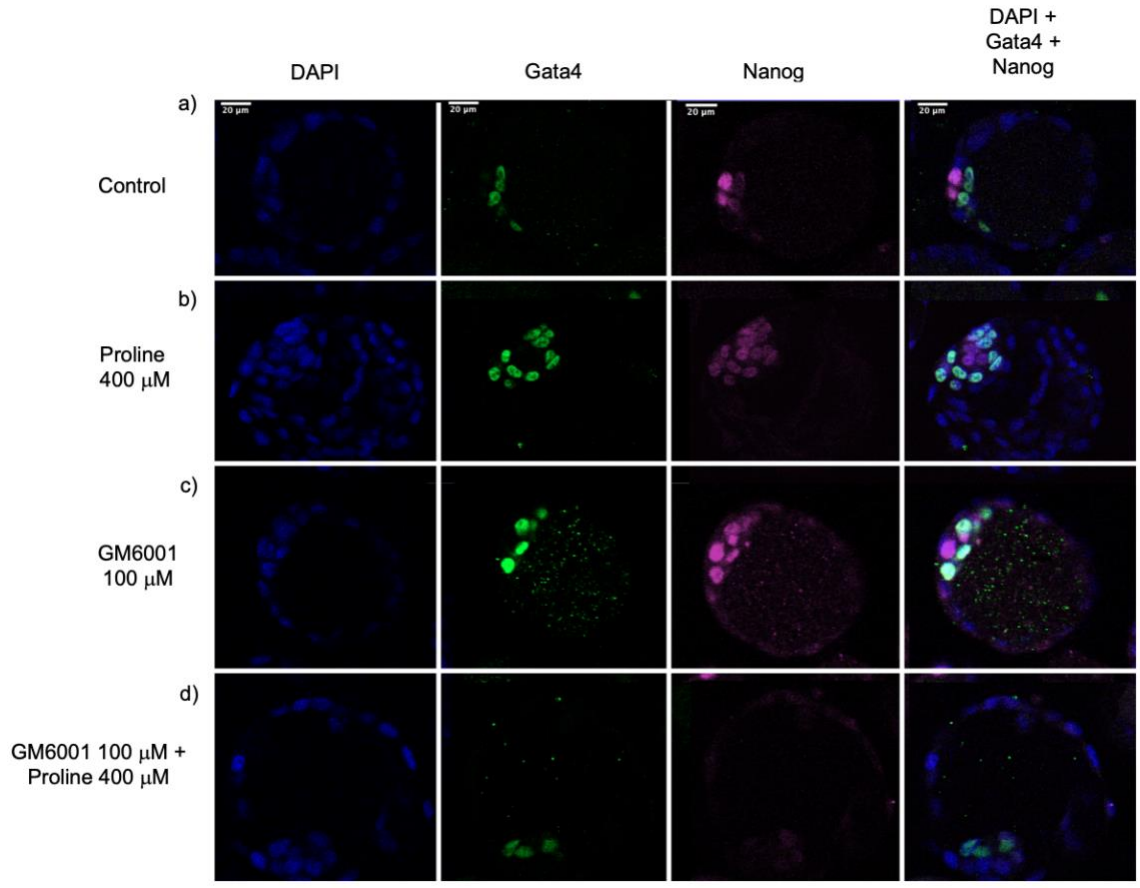


Figure 3.5 Nanog and Gata4 staining in blastocysts after culture at low density culture in the presence and absence of GM6001 and/or L-proline. Blastocysts were fixed on day 6 and stained with DAPI (blue) to identify nuclei, Gata4 (green) and Nanog (pink) representing the hypoblast and epiblast respectively. Embryos were cultured in (a) control medium, (b) medium containing 400 μM L-proline, (c) medium containing 100 μM GM6001, and (d) medium containing 100 μM GM6001 and 400 μM L-proline. Results were obtained from at least 3 different experiments. Images generated by N. Stark

3.3.4 Effect of 100 μM GM6001 on embryo development at high density

Embryos were exposed to 100 μM GM6001 in high density culture from different stages of development (Figure 3.6). GM6001 was introduced to high density culture from Day 1, Day 3 after the activation of the embryonic genome, and Day 4 during the compaction stage. All embryos were cultured in their respective treatment groups at high density until day 6. After culture, embryos were fixed and stained in

accordance with the protocol designed by our lab and described in 2.4. The embryos that were cultured in the group with the longest exposure to 100 µM GM6001 (i.e., from Day 1) had the lowest rate of development compared to all other groups, particularly on day 5 ($P < 0.001$). Likewise, in groups exposed to GM6001 from Day 3 and Day 4 in culture, there was a significant difference in rates of development compared to the control group which had no exposure.

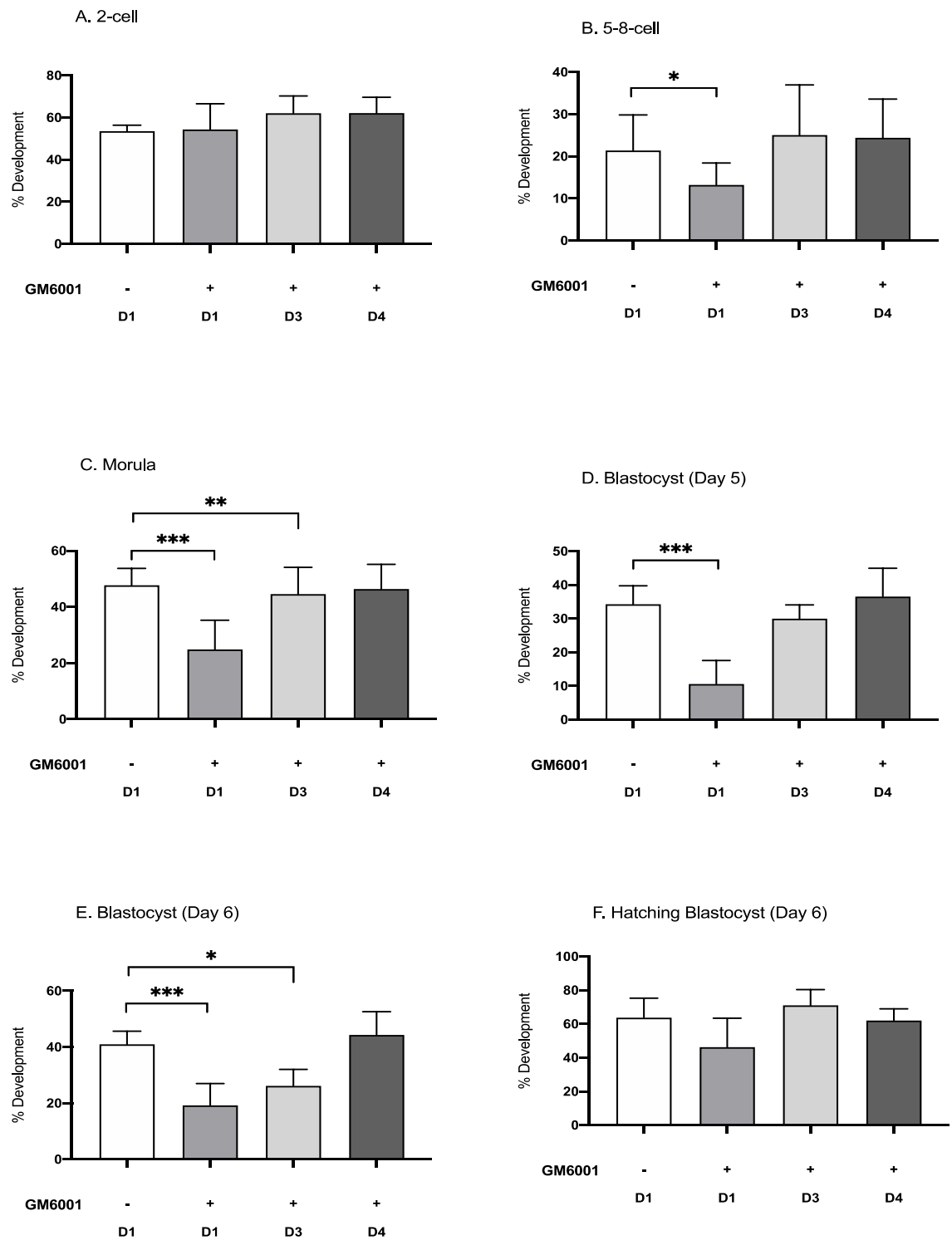


Fig 3.6 Effect of culture of embryos in GM6001 from different stages of development.
Freshly isolated zygotes were cultured for six days to the hatching blastocyst stage at high density (no more than 10 embryos/50 μl droplet) in the absence of GM6001 and the presence of 100 μM GM6001 from Day 1 (D1), Day 3 (D3) and Day 4 (D4). Development to the (A) 2-cell, (B) 5-8-cell, (C) morula, (D) blastocyst on Day 5, (E) blastocyst on Day 6 and (F) hatching blastocysts on Day 6. Results analysed using an ordinary one-way ANOVA and Tukey's post-hoc test. Results were obtained from at least 3 different experiments.
* indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$

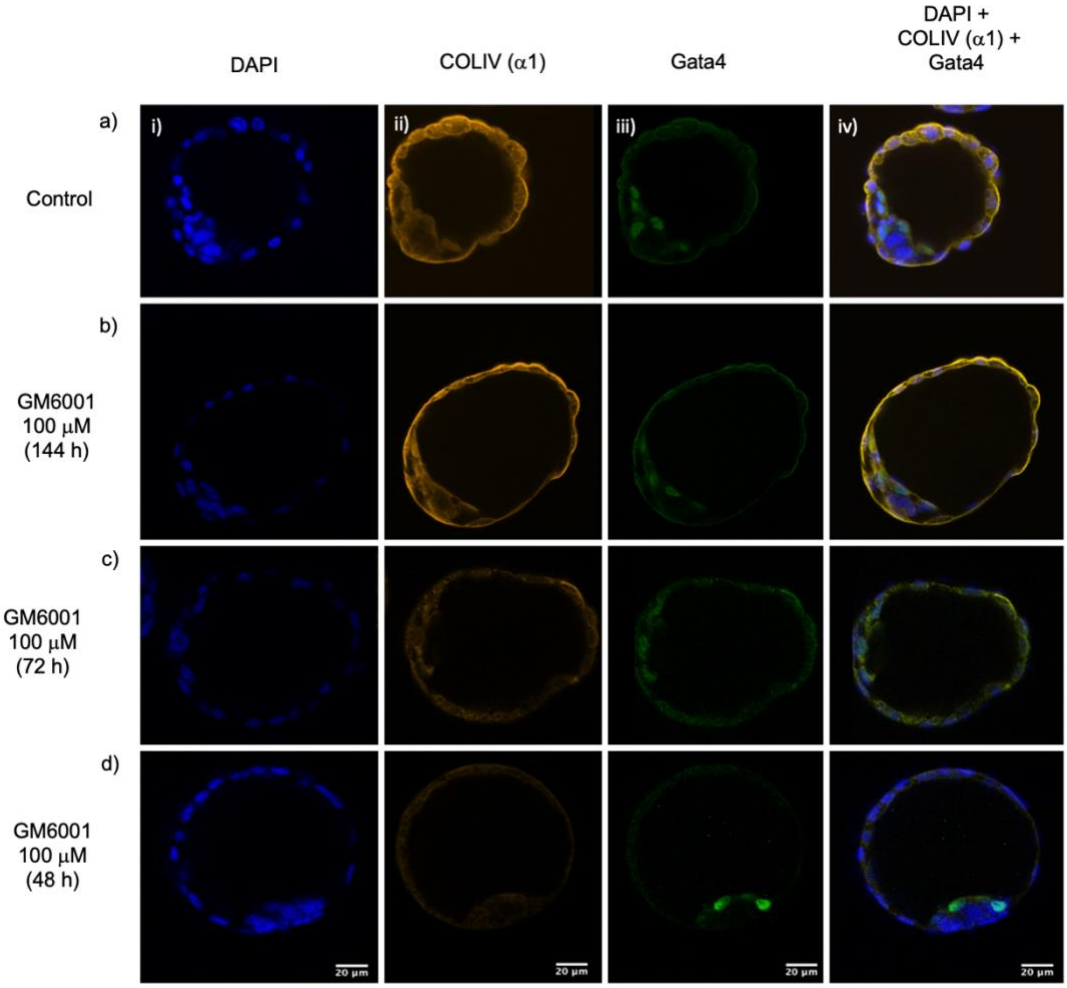


Fig 3.7 Collagen IV α1 and Gata4 staining on embryos after exposure to GM6001 at different stages of development

Mouse blastocysts after six days in high density culture (no more than 10 embryos/50 μl droplet in modHTF under oil) after exposure to 100 μM GM6001 at different stages of development. Embryos were stained with DAPI (i) to clearly identify nuclear staining within the cells (blue), expression of collagen IV alpha 1 sub chain (ii) present in the embryo's extracellular matrix (orange), expression of Gata4 (iii) representing the embryo's hypoblast cells (green), the combined staining (iv).

a) Control group b) 100 μM GM6001 from day of zygote isolation, (total exposure time 144 h), c) 100 μM GM6001 from day three of culture, (exposure time 72 h), d) 100 μM GM6001 from fourth day of culture, (exposure time 48 h). Results were obtained from at least 3 different experiments. Images generated by N. Stark

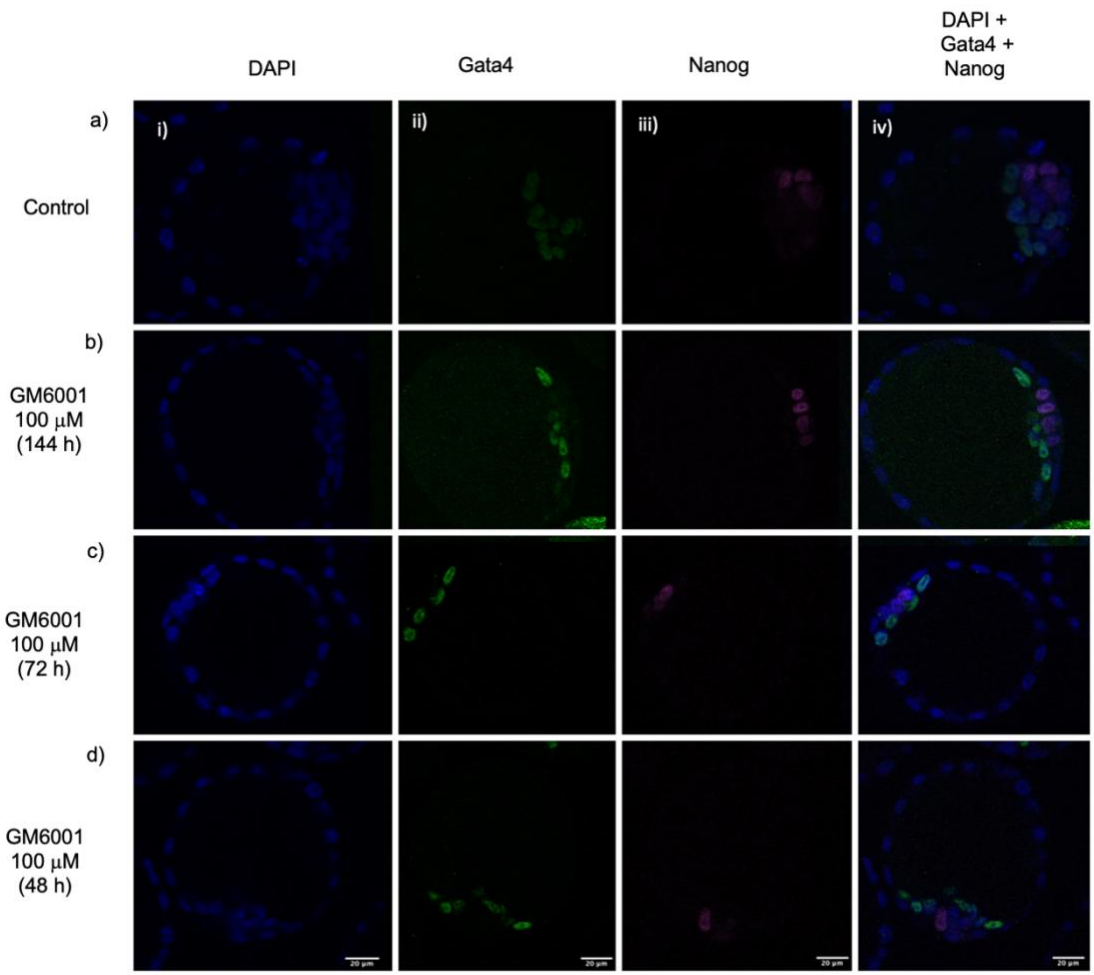
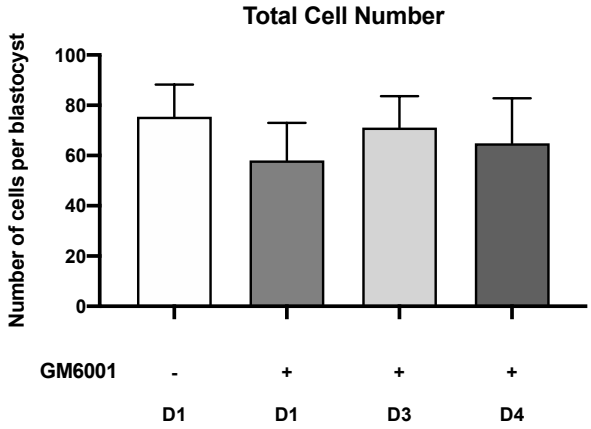


Fig 3.8 Expression of NANOG and Gata4 during exposure to GM6001 at different stages of development.
Mouse blastocysts after six days cultured at high density (no more than 10 embryos/50 μl droplet in modHTF under oil) after exposure to 100 μM GM6001 during different stages of development. Blastocysts stained with DAPI (blue) (i) to identify nuclear staining, (ii) expression of Gata4 (green) to identify hypoblast (iii) expression of Nanog (pink) to identify epiblast and (iv) combined staining.
a) Control group with no exposure to inhibitor b) 100 μM GM6001 from day of zygote isolation, (total exposure 144 h), c) 100 μM GM6001 from day three of culture, (exposure time 72 h), d) 100 μM GM6001 from fourth day of culture, (exposure time 48 h). Results were obtained from at least 3 different experiments.
Images generated by N. Stark

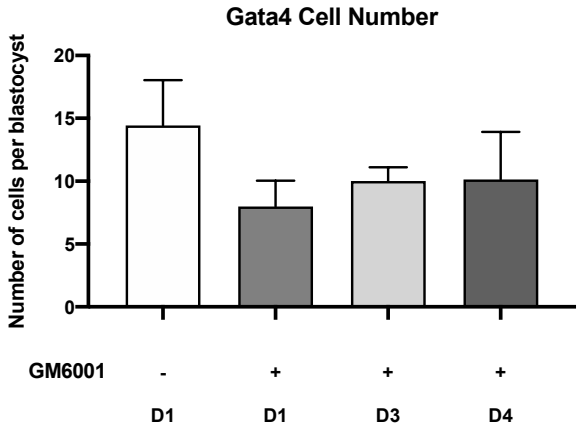
3.3.5 The effect of GM6001 on cell differentiation in blastocysts

Blastocysts that developed after culture in GM6001 from different stages were fixed and underwent sequential staining (described in 2.3), and the number of cells counted (Figure 3.9). Although all of the various GM6001 exposure times did not have a significant impact on the total cell number in each group, GM6001 did appear to reduce the number of cells expressing Nanog and Gata4 in the epiblast and hypoblast (Figure 3.7 and Figure 3.8).

a)



b)



c)

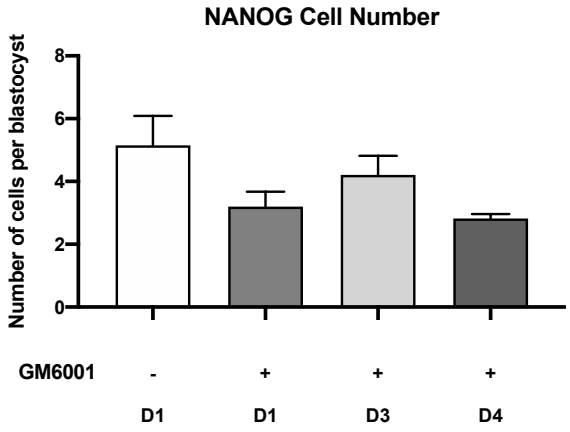


Fig 3.9 Cell numbers in mouse blastocysts after exposure to 100 μM GM6001 at different stages of development

Graph depicts the average a) total cell number per blastocyst during high density culture (no more than 10 embryos/50 μl droplet) b) the average total number of cells expressing Gata4, c) the average number of cells expressing Nanog after exposure of 100 μM GM6001 at different stages of development. Results analysed using an ordinary one-way ANOVA and Tukey's post-hoc test. Results were obtained from at least 3 different experiments.

3.4 Discussion

Proteolytic degradation of the basement membrane has been described as one of the fundamental and most complex mechanisms in during embryo development (Veidal *et al*., 2011). Our data has shown that there is potentially a layer of collagen IV layered between the blastocyst's epiblast and hypoblast. Collagen IV contains a high percentage of L-proline residues, an amino acid which plays a dynamic role in cell differentiation and pluripotency during embryo development (Kyprianou *et al.*, 2020).

We can hypothesize that L-proline is released from the embryo's basement membrane as degradation takes place and is a natural process during embryo development and is regulated by the proteolytic activity of MMPs. Just before implantation, the ICM cells that line the blastocoelic cavity will differentiate into the primitive endoderm, or hypoblast, which will leave a thin layer of basement membrane between the cells (Bedzhov and Zernicka-Goetz, 2014). We hypothesized that the ECM turnover, specifically within this portion of basement membrane would result in the release of L-proline and drive cell differentiation. In these experiments, we wanted to investigate the effect of inhibiting the proteolytic activity by the MMPs by a broad spectrum MMP inhibitor, GM6001 and measure the impact it had on the embryos' growth and development.

Preliminary data in our lab has revealed that nonessential amino acid, L-proline will act as a significant growth factor during low density culture, when autocrine support is unavailable, consequently promoting healthy embryo development (Morris *et al*., 2020). The results in figure 3.3 revealed that the level of MMP activity inhibited by

GM6001 during low density culture was enough to impact the normal growth of the embryos. Following the addition of L-proline to the culture media, we observed no impact on the embryos being grown in the presence of GM6001, which was surprising as we would have expected the addition of L-proline to counter the effect of the inhibitor and support normal growth and development. When L-proline was added to the culture media without GM6001, as expected, the growth and development of the embryos appear to match if not surpass what we were observing in our control groups (Figure 3.4). From these figures, we can see that exposure to L-proline will improve development from four to six days in culture at low density. The group that exhibited the poorest performance was the low density group that had been exposed to the inhibitor.

The effects of the length of exposure of GM6001 to various cell types have shown how the impact the inhibitor has on specific mechanisms and pathways involved with development (Santiskulvong and Rozengurt, 2003). There are several stages in development that are determining factors for the future of the embryo. The initial stages include the fusion of the sperm and the oocyte, the migration and confluence of the germ cell pronuclei and the embryo will undergo preliminary stages of epigenetic reprogramming followed by a series of cell division which will ultimately lead to the activation of the embryonic genome at approximately the 2-cell stage in mouse embryos (Aoki, 2022).

We have used transcription factors Nanog and Gata4 as markers of the embryo's hypoblast and epiblast to help define ECM formation. Previously, Nanog and Gata4 have also been described as playing a pivotal role during embryo development. The

expression of Nanog and Gata4 will generally take place on approximately after four days of in vitro culture as the embryo undergoes compaction and has previously been recognized as being initiated by signalling pathways that involved fibroblast growth factor-2 (FGF-2). The FGF/Erk pathway will play an essential role in pluripotency and lineage specification during early embryo development, and the formation of the embryo's primitive endoderm (Lanner and Rossant, 2010).

The embryos exposed at varying times to the GM6001 were stained with goat Gata4 polyclonal Ab and rabbit Nanog monoclonal Ab after six days of culture to help locate the hypoblast and epiblast and to help identify the effects that the GM6001 would potentially have on cell differentiation. Addition of GM6001 *in vitro* caused inhibition of proteolytic activity during the natural breakdown of collagen IV in the embryo's basement membrane, therefore restricting the release of L-proline. The natural release of L-proline during collagen biosynthesis in one of the driving forces behind cell differentiation. When GM6001 was added during high density culture *in vitro*; the total number of cells in each blastocyst on day six weren't disparate from the control groups, however the number of cells expressing Gata4 and Nanog were reduced, indicating that although minimal, the effects of the inhibitor had an impact on the release of L-proline during collagen IV degradation.

Between two to four days of culture, the embryo will undergo the first morphological break in radial symmetry, and it has been shown previously that the mitogen-activated or extracellular signal–regulated protein kinase pathway (MAPK/ERK) is principally required for cell division during early embryonic compaction. MEK/ERK and PI3K-Akt pathways can upregulate the MMPs that play a role during the

proteolytic degradation of collagen IV (Cohen *et al*., 1999, Xiao *et al*., 2012). The ERK/MAP kinase function is essentially required for the early cell division in preimplantation embryos during the stage before compaction and cell differentiation.

3.5 Conclusion

Broad spectrum synthetic MMP inhibitor GM6001 has been designed to impede the activity of the MMPs-1, -2, -3, -7, -8, -9, 12, -14 and -26, which will impact the degradation of the basement membrane in the preimplantation embryo. As L-proline plays a principal role in maintaining cell pluripotency and facilitating cell differentiation during embryo development, and is released during the natural proteolytic degradation process, the addition of GM6001 theoretically would hinder the effectiveness of the MMP activity, therefore impacting the normal growth and development of the embryo and affecting cell pluripotency. Although GM6001 appeared to impact the growth and development in embryos during low density culture, the effect of the inhibitor didn't have a significant effect on the embryos being cultured in high density regardless of the length of exposure. The cell numbers weren't directly impacted by the addition of GM6001, indicating that the effectiveness the inhibitor had on the pathways associated with cell differentiation was minimal. Addition of L-proline appeared to somewhat restore the function of the signalling pathways after exposure to GM6001, however the effect was minimal. The experiment was initially designed to observe the effect of a broad spectrum MMP inhibitor on the proteolytic degradation of the basement membrane in preimplantation mouse embryos with the hope that by adding the inhibitor there will be a significant restriction of the release of L-proline, which will have a significantly negative impact normal embryo growth. It was hypothesized that from the addition of

L-proline at 400 µM *in vitro*, the effects of the amino acid would counteract the actions of the inhibitor, supporting the theory that the free L-proline naturally released during proteolytic degradation would help support normal preimplantation development while GMN6001 would restrict this process leading to impaired preimplantation development. The experiments showed that despite the addition of L-proline, embryos exposed to GM6001 *in vitro* had little improvement in development. It is believed this may potentially be due to other factors affecting the activity of GM6001 therefore L-proline would not be affecting the outcome of the inhibitor.

Future experiments carried out will likely apply a more specific MMP inhibitor such as SB-3CT, aimed at influencing the specialised gelatinases that are primarily associated with the breakdown of the embryo's extracellular matrix. The aim will be to target the gelatinases specifically responsible for basement membrane degradation. In doing so, we hope to observe the impact this inhibitor will have on the release of L-proline, which we hypothesize will have a negative effect on cell differentiation and healthy development in preimplantation embryos.

Chapter 4:

The effect of the MMP-2 and MMP-9 inhibitor SB-3CT on embryo development and collagen IV distribution in mouse blastocysts

4.1 Introduction

Collagen type IV is one of the major structural elements that makes up the basement membrane (BM) and is responsible for the various functions which are required for normal growth and development (Poschl *et al*., 2004). In BM, collagen IV provides the scaffolding with other molecules such as fibronectin, laminins and proteoglycans (Sudhakar and Kalluri, 2010). Collagen IV is made up of six distinct alpha chains that are expressed by six specific genes (Sand *et al*., 2013). Preimplantation collagen IV distribution was previously identified by Leivo *et al* in 1980 within the blastocysts inner cell mass and along the inner aspect of the embryo's trophectoderm (Leivo *et al*., 1980).

We know that each distinct alpha sub-chain of collagen IV will combine to form three specific heterotrimers which are primarily responsible for forming networks in the basement membrane, with each different sub-chain being expressed during specific stages of development (Jeanne *et al*., 2015).

L-proline constitutes approximately 10% of the total amino acids found in collagen, and is required for the process of collagen biosynthesis (Karna *et al*., 2020). L-proline has been identified as one of the most abundant proteinogenic amino acids in hydrolysed collagen. Additionally, L-proline has also been identified as an amino acid

that is thought to make up a significant portion of the BM (Shoulders and Raines, 2009) and more recent studies have identified the presence of the BM in preimplantation embryos (Biggers *et al.*, 2000). L-proline and hydroxyproline play key roles that are vital to the stability of the structure of collagen, facilitating the flexibility and durable twisting of the collagen helix. The hydroxylation of the L-proline by prolyl-hydroxylase, following protein synthesis, results in a significant modification of L-proline residue (Gorres and Raines, 2010) such a modification considerably increasing the stability and permanence of the collagen helix (Rappu *et al*., 2019). When added to low density culture media, L-proline acts as a growth factor, stimulating embryo development when autocrine support is unavailable (Morris *et al.*, 2020). It is not widely understood how embryos manage to obtain levels of L-proline that are sufficient to provide the embryo with the support required to help regulate growth and pluripotency. One possibility, which will be explored further is the potential for the embryo to maintain its own interminable source of L-proline through collagen biosynthesis in the ECM between the hypoblast and epiblast.

Synthetic MMP inhibitors can either be broad spectrum or selective, targeting either a broad range of MMPs or a specific MMP. Ilomastat, otherwise known as GM6001 is a hydroxamate-based, broad spectrum inhibitor which will target a wide range of zinc-containing proteases (Overall and Kleifeld, 2006). In contrast, covalent mechanism-based inhibitor, SB-3CT will specifically target MMPs by interfering with the extracellular factors and impeding signalling pathways that will inhibit MMP activity at a transcription, activation, and inhibition level (Jablonska-Trypuc *et al*., 2016). SB-3CT specifically targets gelatinases (MMP-2 and MMP-9) which can degrade collagen IV. PI3K/Akt signalling pathway is one of the downstream effectors

that can upregulate expression of MMP-2 and MMP-9 through a non-receptor protein tyrosine kinase, focal adhesion kinase (FAK) (Reif *et al*., 2003). FAK is regulated by sonic hedgehog (SHH) signalling pathway (Chen *et al*., 2012) which induces the production and activation of gelatinases.

In the presence of SB-3CT, specific MMPs responsible for the natural breakdown of the basement membrane would prevent the release of free proline, leading to poor embryo development and disrupting cell differentiation. We would hypothesize that the addition of L-proline would compensate for the restricted release of free proline that would otherwise be occurring during proteolytic degradation of collagen IV.

4.2 Materials and Methods

4.2.1 Embryo culture

Zygotes were cultured in low density (no more than one embryo/10 μl droplet under oil) and high density (no more than 10 embryos/50 μl droplet under oil) conditions as described in 2.3.1. Control groups were cultured in mod-HTF, 270 mOsm/kg and a treatment group with a 10 μM concentration of SB-3CT. Zygotes in each group were scored for their developmental stages.

4.2.2 Exposure to SB-3CT with the addition of L-proline

L-proline was added at a concentration of 400 μM to high density and low density SB-3CT exposure groups and cultured for six days, each embryo scored for their developmental stages.

4.2.3 Exposure to SB-3CT at different stages of preimplantation development

The high density groups were divided into a further three culture groups; one exposed to SB-3CT at 72 ± 2 h, another exposed at 96 h, and the final exposed to SB-3CT 22 ± 2 h. Each group was then scored for their developmental stages as above. The time periods in the treatment groups described were chosen due to significance in preimplantation development. Embryos exposed to the inhibitor from the day of isolation were exposed for the maximum length of time to observe the impact. Additionally, 72 ± 2 h exposure time was chosen as this is the stage that the zygomatic genome activates and will denote the initiation of gene expression after fertilisation, which is a significant stage of development. And finally, inhibitor exposure at 22 ± 2 h for the final culture group was chosen as this is the stage of compaction for the embryo and a crucial point of development when cell differentiation begins to take place (Morris *et al*., 2020).

4.2.4 Immunofluorescent staining of blastocysts

The embryos were fixed after six days in culture as per 2.4 to identify the hypoblast and epiblast of the preimplantation embryo while also helping to establish the expression of collagen IV after exposure to SB-3CT.

4.3 Results

4.3.1 Culture in high density vs low density when exposed to SB-3CT

Embryos were cultured *in vitro* at either high density or low density and exposed in groups to 10 μM SB-3CT 22 ± 2 h to look at the impact a selective MMP inhibitor would have on normal development in mammalian embryos. Transcription factors, Nanog and Gata4 were used as markers to identify the epiblast and

hypoblast respectively in the embryos and to help determine whether cell differentiation is affected when exposed to SB-3CT.

Figure 4.1 shows embryos that were cultured at high density with exposure to 10 μM SB-3CT significantly decreased blastocyst development ($P < 0.0001$) and hatching ($P < 0.0001$) in comparison with the control group, while the addition of 400 μM of L-proline to 10 μM SB-3CT countered the inhibitory effect and improved the rate of development.

Embryo development at low density in the presence of 10 μM SB-3CT had the lowest rate of development, the addition of 400 μM L-proline showing a relative improvement at the 4-8 cell stage, relative to the group exposed to 10 μM SB-3CT. Addition of 400 μM of L-proline had the greatest impact on embryo growth, with the highest rate of development from the 4-8 cell stage to blastocyst formation.

During exposure to SB-3CT, the number of cells expressing Nanog within the embryo's inner cell mass (ICM) were counted and compared with our control group. The results showed there was no difference in cell number (Figure 4.3). For the duration of culture *in vivo* at high density, the total cell number was greater in the control group compared to the embryos being cultured in low density. The addition of SB-3CT saw the total cell number reduce further, especially in the treatment group being cultured in low density.

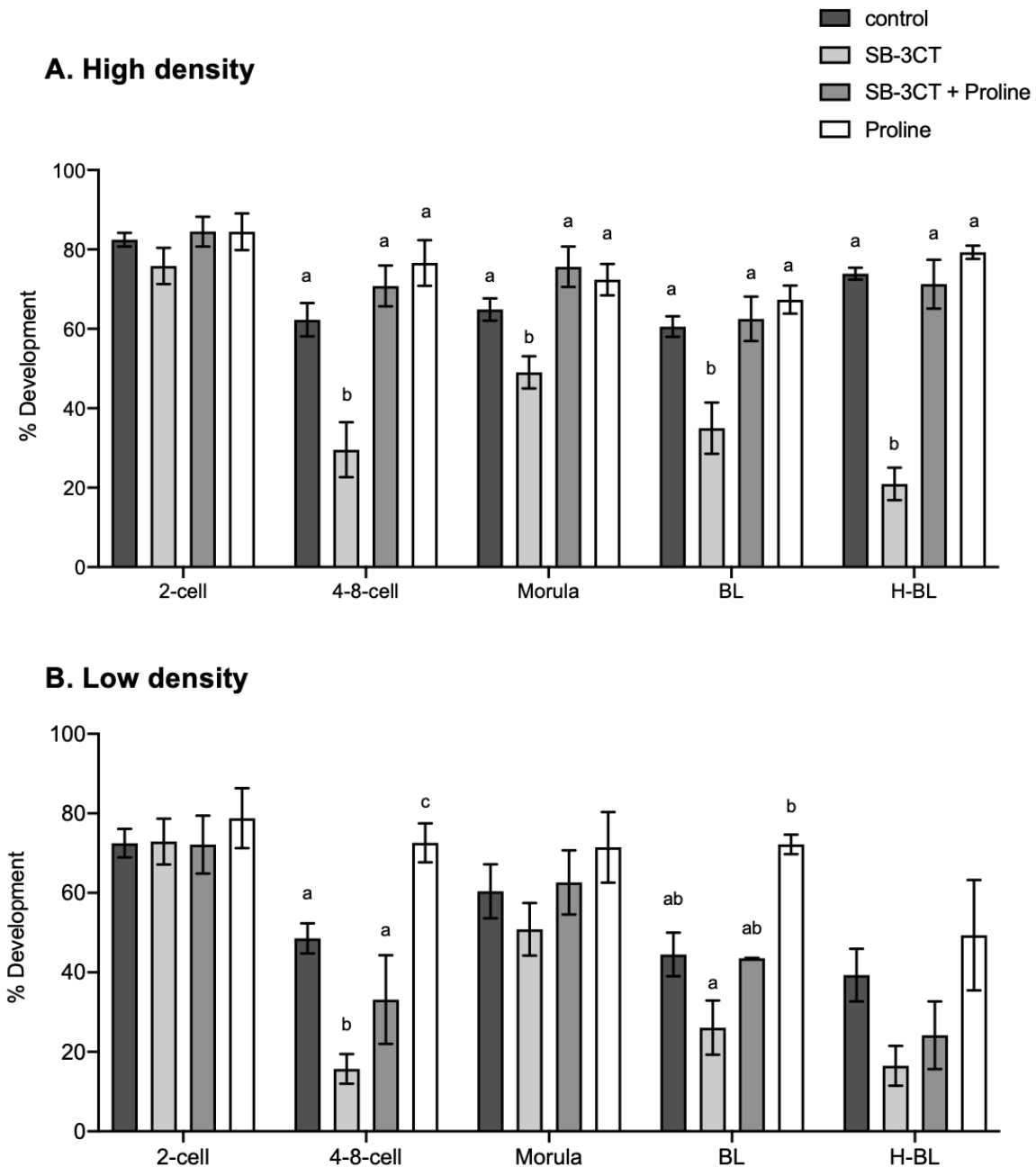


Figure 4.1. % Development in high vs low density in mouse blastocysts after exposure to 10 μM SB-3CT

Graphs depicting the normal rate of development in mouse blastocysts in low density (LD) and high density (HD) culture when exposed to specific MMP inhibitor SB-3CT and L-proline. Groups were cultured in low density environments with one embryo/10 μl droplet of mod HTF and high-density culture consisted of no more than 10 embryos/50 μl droplet of mod HTF. Control groups consisted of mod HTF culture media, Inhibitor groups were exposed to SB-3CT at 10 μM and L-proline at 400 μM. Results analysed using an ordinary one-way ANOVA and Tukey's post-hoc test. Results were obtained from at least 3 different experiments.

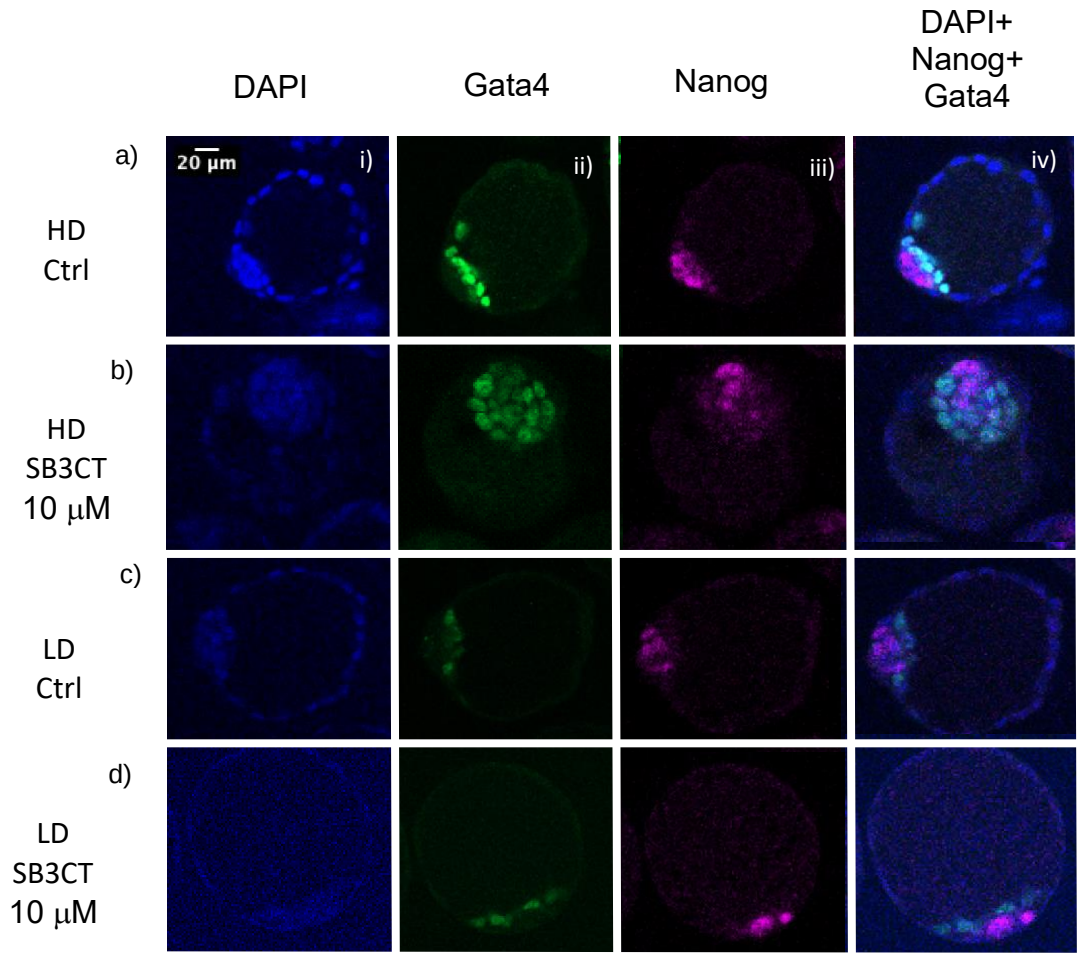


Fig 4.2 Nanog and Gata4 staining during high density vs low density culture with the addition of 10 µM SB-3CT
Immunofluorescent staining of mouse blastocysts after six days in high density vs low density culture when exposed to 10 µM SB-3CT stained with i) DAPI to identify nuclear staining (blue), ii) expression of Gata4 transcription factors (green) iii) cells expressing Nanog (pink) and iv) combined staining.
a) High density control group, b) high density SB-3CT group c) low density control group and d) low density SB-3CT group.
Results were obtained from at least 3 different experiments.
Images generated by N. Stark

4.3.2 Effect of L-proline during preimplantation development in embryos when exposed to 10 μM SB-3CT

As previously described in chapter 3, the inhibitory effect of 100 μM GM6001 on the gelatinases responsible for the degradation of collagen IV was minimal. It was hypothesized that the addition of L-proline at 400 μM would counteract the effect of the inhibitor to help restore the biological functions that would otherwise be impeded due to the limited release of the free proline from the basement membrane. SB-3CT was used as a more specific MMP inhibitor, to focus on the MMPs entirely responsible for the inhibition of gelatinases.

SB-3CT was added to both high and low density culture media groups at concentrations of 10 μM. Figure 4.1 shows that the embryos that exhibited the poorest development were exclusively exposed to the SB-3CT inhibitor without the addition of L-proline. Addition of 400 μM L-proline to the low density SB-3CT group improved the rate of development only marginally in comparison to that observed in the high density groups.

4.3.3 The effect of 10μM SB-3CT on cell differentiation in blastocysts in low density and high density with the addition of L-proline

Figure 4.2 shows that in the absence of growth factors, cell differentiation is reduced. The addition of 10 μM SB-3CT caused a significant reduction in total cell number and the number of cells expressing Gata4 ($P < 0.0001$). After the addition of 400 μM L-proline, cell number and expression of both Gata4 and Nanog was significantly increased ($P < 0.0001$).

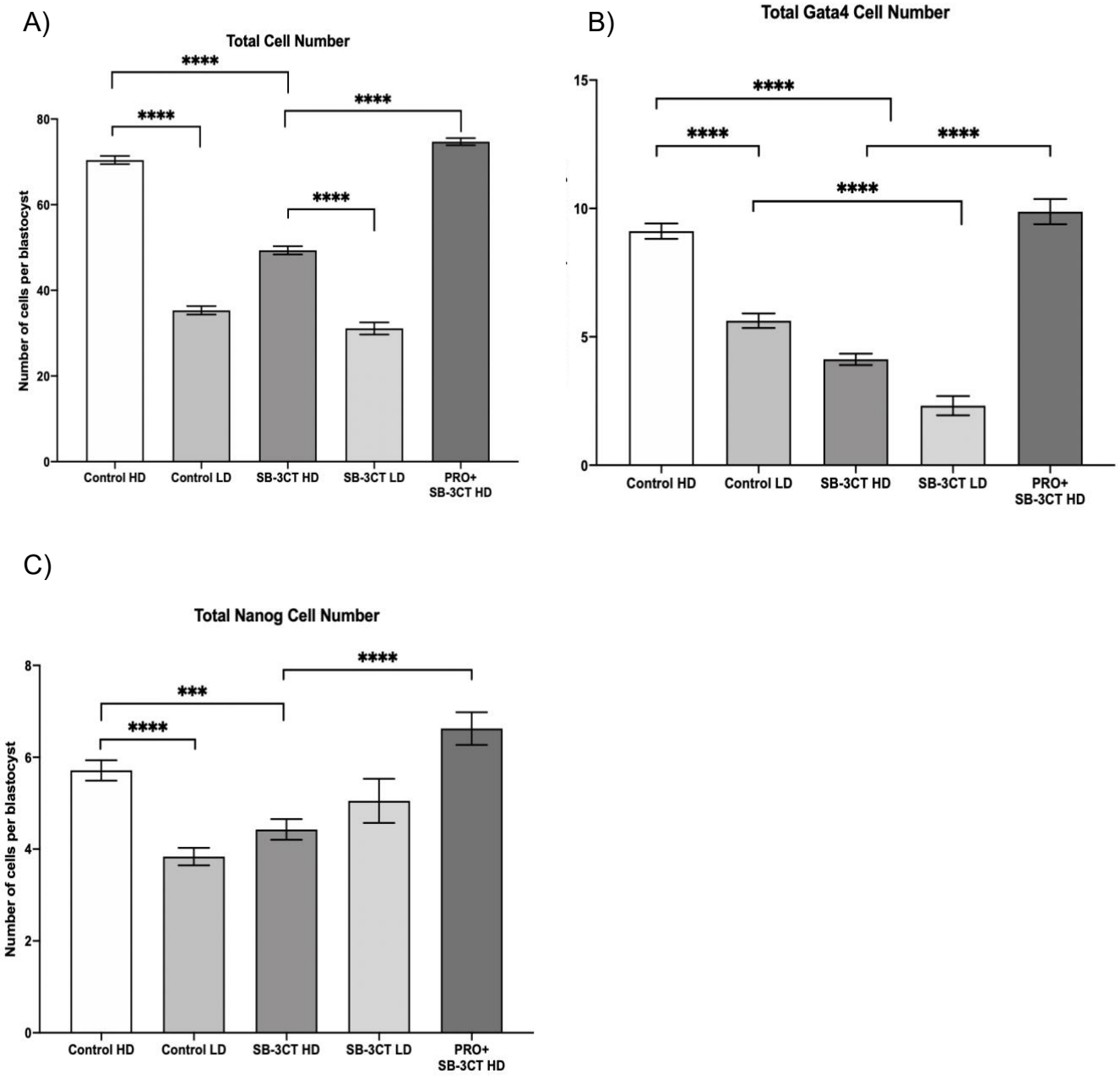


Fig 4.3 Cell numbers during high vs low density culture after exposure to 10 μM SB-3CT and 10 μM SB-3CT + 400 μM L-proline

Graph depicts A) average total cell number per blastocyst in high density vs low density when exposed to MMP inhibitor SB3CT at 10 μM and exposure to 10 μM SB-3CT + 400 μM L-proline, B) average total number of cells expressing transcription factor Gata4, C) average number of cells per blastocyst expressing Nanog

Data were analysed in GraphPad Prism using an ordinary one-way ANOVA and Tukey's post-hoc test. Bars represent mean ± SEM obtained from at least 3 independent experiments.

*** indicates $P < 0.0007$ and **** indicates $P < 0.0001$

4.3.4 Exposure to 10 μM SB-3CT in high density at different stages of development

Gene expression activation or zygotic genome activation (ZGA) in mouse embryos occurs from approximately the 2-cell stage of embryo development in mice and represents the initiation of the embryo's gene expression after fertilisation. During this highly complex transition period, initial transcriptional and post-translational reprogramming occurs, contributing to the development of the embryo (Svoboda, 2018).

The results depicted in figures 4.3 and 4.6 represent the effect 10 μM SB-3CT will have during different stages of preimplantation development, showing how the embryo's cellular differentiation will be negatively impacted by the exposure of a specific MMP inhibitor. To observe the affect the same MMP inhibitor would have during some of the crucial stages of early embryo development, the high density culture groups were exposed to 10 μM SB-3CT at varying lengths of time, each stage being representative of an essential point of time in a preimplantation embryo's development (Figure 4.3). Each high density group were exposed to the specific MMP inhibitor at 24 h or during time of zygote isolation, 48-72 h or during the window of ZGA, and between 72-96 h or during the stage of compaction (Pratt *et al*., 1982). Normal development was reduced across all groups cultured in 10 μM SB-3CT at high density, regardless of the length of exposure, including reduced hatching rates. Upon observation, the group that was shown to have the lowest rate of development was the high density group that had the longest period of culture following exposure to the inhibitor, from day of isolation (Figure 4.4).

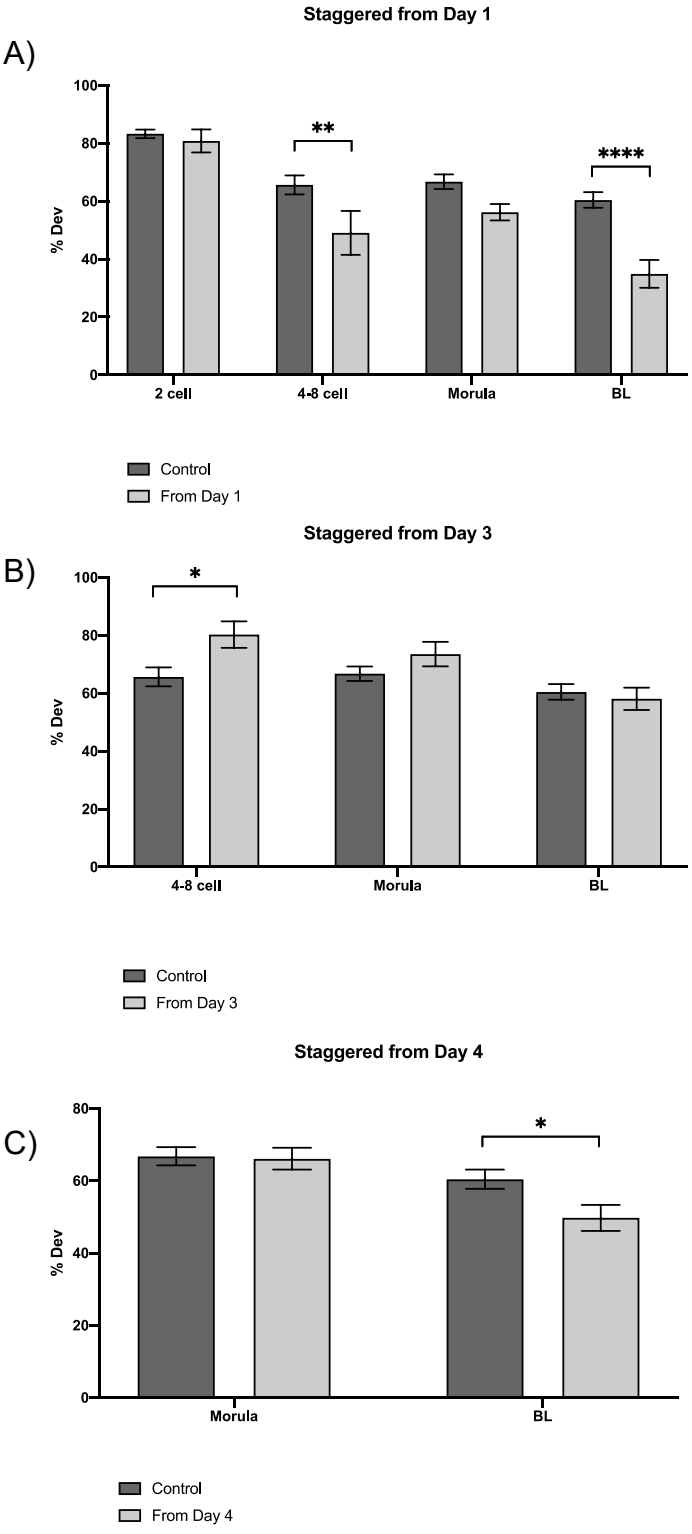


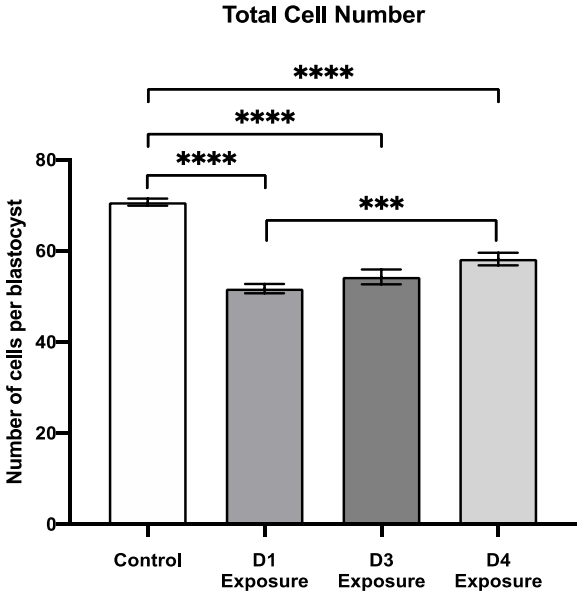
Fig 4.4 % Development in mouse blastocysts after exposure to 10 μM SB-3CT during different stages of development

Graphs displaying the % of development in embryos at high density culture (no more than 10 embryos/50 μl droplet) after exposure to specific 10 μM SB-3CT during different stages of development A) exposure from day of isolation, B) from day three of culture and C) from day four of culture. Data were analysed in GraphPad Prism using an ordinary one-way ANOVA and Tukey's post-hoc test. Bars represent mean ± SEM obtained from at least 3 independent experiments. * Indicates $P < 0.02$, ** indicates $P < 0.009$ and **** indicates $P < 0.0001$

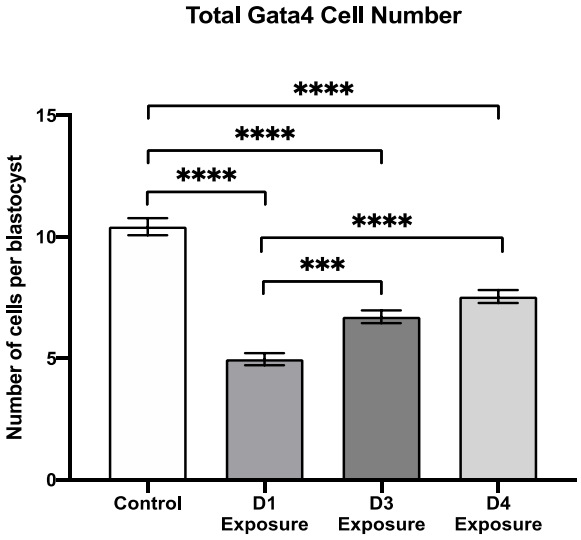
4.3.5 The impact of 10 μM on cell differentiation in high density culture during different stages of development

When looking at the impact 10 μM SB-3CT had on cell distribution and differentiation in the embryos, total cell numbers in all groups did not differ from each other, however when compared to control group, there was a significant decrease in the number of cells across all groups ($P < 0.0001$). Our results showed that groups with exposure to 10 μM SB-3CT had lower cell numbers expressing Gata4, especially in the groups that had a longer exposure time to the inhibitor compared to the control group ($P < 0.0001$) and shorter exposure times (Figure 4.5). Conversely, when looking at the distribution of cells expressing Nanog, there was not a significant difference between the control group and short exposure group when compared to the embryos that had been exposed to 10 μM SB-3CT for the longest length of time. Embryos cultured in the inhibitor from the day of isolation had fewer number of cells expressing transcription factor Nanog compared to the other groups.

A)



B)



C)

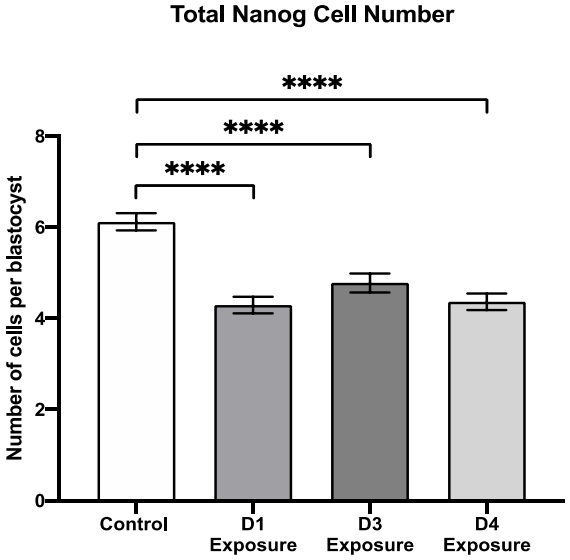


Fig 4.5 Cell numbers in mouse blastocysts after exposure to 10 μM SB-3CT in high density culture during different stages of development

The average cell number per E6.0 blastocyst in embryos after staggered exposure to 10 μM SB-3CT. Total number of blastocysts in control group, group exposed to 10 μM SB-3CT from E1.0, group exposed to 10 μM SB-3CT from E3.0, group exposed to 10 μM SB-3CT from E4.0.

Graph a) represents the average total cell number of the whole embryo, b) the average total number of cells expressing transcription factor Gata4 found in the hypoblast of preimplantation embryos and c) the average total number of cells expressing transcription factor Nanog located in the embryos' epiblast.

Results analysed using an ordinary one-way ANOVA and Tukey's post-hoc test. Results were obtained from at least 3 different experiments.

*** indicates $P < 0.0005$, **** indicates $P < 0.0001$.

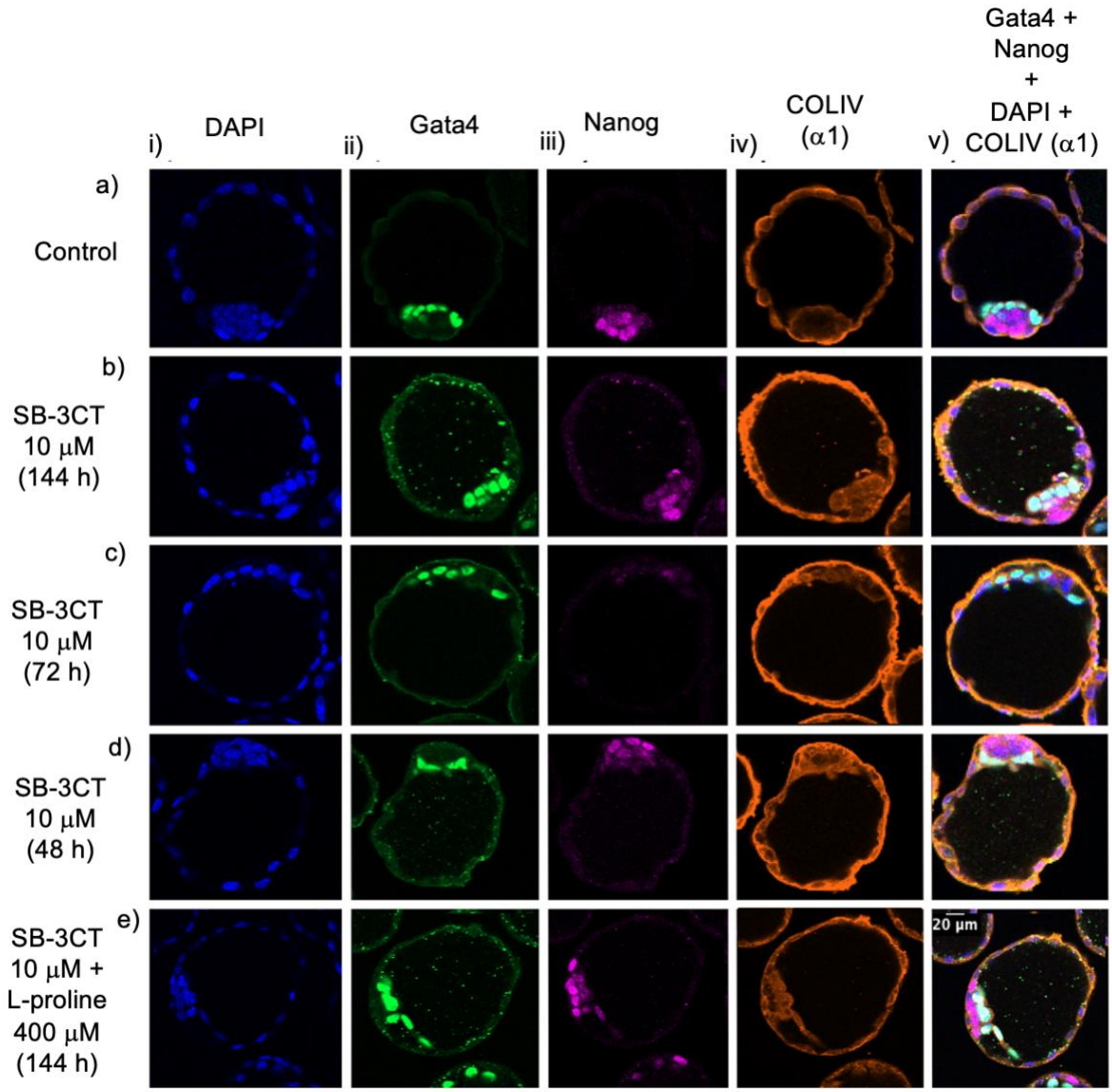


Fig 4.6 COLIV alpha 1, Nanog, and Gata4 staining during exposure of 10 μM SB-3CT and L-proline 400 μM in high density culture

Mouse blastocysts after six days of culture in high density culture stained with DAPI (blue) (i) Gata4 (green) (ii) Nanog (pink) (iii) expression of collagen IV alpha 1 sub chain in the embryo's extracellular matrix (iv) and combined staining (v).

a) Control, b) exposure to 10 μM SB-3CT from day of isolation, c) exposure to 10 μM SB-3CT from day three of culture, d) exposure to 10 μM SB-3CT from day four of culture, e) exposed to 400 μM L-proline + 10 μM SB-3CT from the day of zygote isolation. Results were obtained from at least 3 different experiments. Images generated by N. Stark

4.4 Discussion

To analyse any potential inhibitory actions on MMP-2 and MMP-9 activity, a competitive mechanism-based gelatinase inhibitor, 10 μM SB-3CT was used to impede basement membrane degradation. SB-3CT is the first mechanism-based inhibitor that specifically targets MMP-2 and MMP-9. Figures 4.3 and 4.5 show that by inhibiting MMP2 and MMP9, cell differentiation is affected.

When embryos were exposed to 10 μM SB-3CT during high density culture, the inhibitor impeded normal embryo development and reduced the hatching rate as the embryos reached day six. Although the rate of development was lower, the most notable impact the inhibitor had was on the group cultured in low density. As autocrine factors have been shown to facilitate growth and development (Lonergan, 2011), this suggests that in their absence, the inhibitor has greater impact.

Gata4 and Nanog are two antagonistic transcription factors that have previously been described as having principal functions in early embryo development by influencing cell differentiation (Messerschmidt and Kemler, 2010). Nanog expression is initiated during the fourth day of development when the embryo undergoes compaction and forms a morula, becoming confined exclusively to the embryo's inner cell mass while Gata4 is expressed in the embryo's hypoblast within the primitive endoderm. Evidence from previous research has suggested that Nanog expression, which is necessary for pluripotent epiblast formation, is facilitated by FGF/Erk signalling and plays an essential role in primitive endoderm formation (Messerschmidt and Kemler, 2010). Fibroblast growth factor (FGF),

specifically fibroblast growth factor-2 (FGF-2) is known for its role in biological activities such as cell differentiation and the FGF/Erk pathway is implicated in the control of pluripotency and lineage specification during embryo development (Lanner and Rossant, 2010).

Results shown in figures 4.3 and 4.6 reveal that exposure of 10 μM SB-3CT during different times of development could have a negative impact on the embryo's cellular differentiation. With the aim of observing the impact of 10 μM SB-3CT during fundamental stages of embryo development, embryos were exposed to the MMP inhibitor at varying lengths of time; 24 h or during time of zygote isolation, 48-72 h or during the window of ZGA, and between 72-96 h or during the stage of compaction (Pratt *et al*., 1981). The significant difference between groups and exposure times to 10 μM SB-3CT was observed between the group that was cultured in the MMP inhibitor from the day one of culture and the group that was transferred to the inhibitor at 96 h post isolation (Figure 4.3). Groups that were exposed to 10 μM SB-3CT regardless of the length of time all showed slightly reduced rates of normal development, including a lower hatching rate. The group that exhibited the lowest rate of development, including blastocyst development and hatching rates was the group exposed to 10 μM SB-3CT from the day of embryo isolation, with maximum exposure to the inhibitor (Figure 4.4). Once the embryos had been exposed to 10 μM SB-3CT for 12 h, there was a significant difference between groups. The rate of normal development was reduced in the groups that had a longer exposure time to 10 μM SB-3CT.

There are several pathways involved with MMP-2 and MMP-9 signalling during basement membrane degradation. Erk1/2, p38 kinase and protein kinase C (PKC) are three specific pathways that are directly associated with collagen IV breakdown, and it has previously been hypothesized that all three pathways could possibly amalgamate. Inhibition of a single pathway involved with basement membrane breakdown been to affect the expression and activity of MMP-9 (Yao *et al*., 2001).

4.5 Conclusion

Results from these experiments show that when MMP-2 and MMP-9 activity was inhibited by 10 μM SB-3CT there was significant impact on the rate of development and cell differentiation.

As these two specific gelatinases are the primarily responsible for the breakdown of the basement membrane, the natural process of releasing L-proline will be prevented. We found that in low density culture, where autocrine support is mitigated, embryo development is already negatively affected. With the addition of SB-3CT, there is a greater impact on the embryo's growth and development, including cell differentiation. While the activity of 10 μM SB-3CT appeared to have an inhibitory effect on growth, development, and cell differentiation; the addition of L-proline appeared to counteract this effect, suggesting that L-proline is a driving force behind normal embryo development and that its release during turnover of collagen in BM between the hypoblast and epiblast is an important source. Stages of development were more sensitive to the exposure of SB-3CT, particularly at low density when autocrine support was unavailable. Earlier studies have reported specific matrix metalloproteinase (MMP-2 and MMP-9) activity as early as during follicular development *in vivo* (Atabakhsh *et al*., 2018) where the gelatinases

modulate cellular functions through integrins (Smith *et al*., 2002). These experiments suggest that the gelatinases responsible for ECM remodelling are present and functional from the earliest stages of preimplantation development, which subsequentially will initiate the release of L-proline to drive cell differentiation and support healthy mammalian embryo development (Rifkin, 1997). The results indicate that the effect of long exposure to SB-3CT and prolonged inhibition of the gelatinases responsible for the controlled turnover of the ECM will have a greater effect on cell differentiation and normal embryo development. When SB-3CT was added to low density culture media, the autocrine support required for healthy mammalian embryo development was already absent and by restricting the proteolytic release of L-proline, there was a greater impact on cellular proliferation and differentiation. Furthermore, the exogenous addition of L-proline under isosmotic conditions was shown to act in a manner similar to growth factors to improve development.

This research has provided valuable insight into some of the pathways that contribute to early embryo development and outlines some of the mechanisms involved with collagen biosynthesis during embryogenesis.

Chapter 5:

The impact of Acetylsalicylic acid (ASA) as an inhibitor of prolidase on basement membrane degradation in mouse blastocysts

5.1 Introduction

Prolidase is a multifunctional cytosolic immunopeptide which will break down the terminal bond in peptide chains and is primarily involved with the recycling of L-proline for collagen biosynthesis and matrix remodelling. Prolidase was initially discovered in 1937 during the study of digestion in the intestinal tract, where they found that protein fragments located within the digestive tract had the ability to cleave dipeptides containing a C-terminal L-proline (Bergmann and Fruton, 1937). Prolidase is encoded by the PEPD gene at the long arm of chromosome 13, position 13.11 (Tanoue *et al*., 1990) and is primarily the only enzyme that is capable of breaking down imidodipeptides that contain C-terminal L-proline or hydroxyproline. The ubiquitously expressed dipeptidase is significant in recycling free proline from imidodipeptides for the resynthesis of collagen in the embryo's extracellular matrix (Cechowska-Pasko *et al*., 2006). In eukaryotes, prolidase will catalyse the final stages of degradation of intracellular collagen to release free proline into the cytoplasm (Phang *et al*., 2010).

Enzymatic activity of prolidase and its role during the catabolism of endogenous proteins is fundamental to the understanding of collagen biosynthesis during extracellular matrix breakdown. As the collagen in the embryo's ECM is naturally abundant in L-proline and hydroxyproline, the breakdown will contribute to the

release of higher quantities of dipeptides containing amino acids (McAnulty and Laurent, 1987). Furthermore, at the cellular level, PEPD will function as a regulator of epidermal growth factor receptors ErB1/EGFR and ErB2/HER2, help to activate the signalling pathways associated with growth factors and will act as a ligand of EGFR which will induce growth promoting signalling (Baszanowska *et al*., 2021).

Collagen biosynthesis will occur both intracellularly and extracellularly, involving both post-translational and co-translational modifications. These modifications will be catalysed by enzymes that are both specific and non-specific and will additionally function by facilitating the stability of the collagen triple helices (Pihlajaniemi *et al*., 1991). Prolidase will directly bind to and activate epidermal growth factor (EGFR) receptor which will lead to the stimulation of signalling proteins (Yang *et al*., 2013). EGFR is a cell surface receptor which is closely related to tyrosine kinase families ErbB-1, ErbB-2, ErbB-3, and ErbB-4. The activation of EGFR will stimulate the key signalling pathways downstream PI3K/AKT/mTOR, Ras/Raf/ERK and JAK/STAT, which contribute to the activation of DNA synthesis, cell growth and proliferation, and cell differentiation (Baselga, 2002). In addition to operating as a regulator for EGFR, prolidase will contribute as a step limiting factor in collagen biosynthesis by catalysing the final step of proline-degradation, cleaving the dipeptides containing L-proline and thereby producing free amino acids to be used in collagen resynthesis (Baszanowska *et al*., 2021). The process of dimerization of receptor subunits for EGFR leads to an induction of cell differentiation and proliferation through the phosphorylation of proteins involved with the downstream pathways. Prolidase will bind directly to EGFR to regulate cellular metabolism and manage the subcellular activation of protein p53 (Misiura and Miltyk, 2020).

The rate-limiting step during collagen biosynthesis is catalysed by prolidase. The actual biosynthesis of collagen is largely dependent on the availability of L-proline, which can either be synthesized from glutamine, glutamate, or arginine, or by being provided by prolidase through hydrolysis of imidodipeptides that are produced by the breakdown of collagen (Surazynski *et al*., 2008, Phang *et al*., 2010). The cytoplasmic localisation of prolidase is of great significance to other metabolic responses, however, L-proline may also be manipulated in the mitochondria in a process catalysed by proline oxidase (POX). The role of POX is to ultimately catalyse the conversion of L-proline into ∆1-pyrroline-5-carboxylate (P5C) to help maintain the reduction-oxidation equilibrium between the cytoplasm and the mitochondria as previously described in 1.7.3 (Oscilowska *et al*., 2021). In the cytoplasm, P5C is converted to proline by the enzyme pyrroline-5-carboxylate reductase during the proline cycle, and P5C reductase will act to convert mitochondrial proline-P5C by POX and P5C-proline within the cytosol (Liu *et al*., 2015).

Acetylsalicylic acid (ASA) is an effective prolidase inhibitor which has previously been shown to act as a regulator of prolidase-activated drugs. Cells exposed to ASA have previously been shown to have reduced prolidase activity as well as prolidase phosphorylation, and a decrease in collagen biosynthesis. The decreased activity suggests that the function of prolidase may possibly provide insight into both intracellular and extracellular biosynthesis of proteins that contain L-proline such as collagen IV (Misiura and Miltyk, 2020). By exposing embryos to ASA, prolidase will be restricted from recovering L-proline from collagen degradation and therefore will limit the availability of free proline required for the regulation of biological functions.

The mechanisms behind the inhibitory actions of the ASA involve prostaglandin-endoperoxide synthases (PTGS) or cyclooxygenases (COX), which are enzymes that catalyse the rate limiting step in the production of prostaglandins *(Surazynski et al*., 2004).

The prolidase-dependent regulation (Figure 5.1) of collagen biosynthesis is thought to take place at a transcriptional level, where stimulation of IGF-IR and β1-integrin receptor will contribute to signalling pathway activation (Prokop *et al*., 2013). Earlier research using skin fibroblasts and MDA-MB 231 cells has shown that ASA has a significant impact on prolidase activity (Chrzanowski *et al*., 2004). The study revealed that upon exposure to ASA, prolidase activity was significantly reduced, which would lead to a decrease in collagen biosynthesis. Prolidase activity is inhibited by ASA through a cascade of integrin-induced intracellular processes, that involve β1-integrin receptor signalling, the inhibitory effect of prolidase phosphorylation being directly associated with disruption of collagen biosynthesis (Surazynski *et al.*, 2004).

Phosphorylation of prolidase is stimulated through β1-integrin receptor at the threonine residue, which initiates the signalling cascade which encompasses MAP kinases, ERK1, ERK2, Raf and Raf. These pathways are involved with the phosphorylation of prolidase and other intracellular proteins. ASA will inhibit the signalling pathway cascade that is typically induced by the β1-integrin receptor, preventing prolidase phosphorylation at the threonine residue and which will result in a reduction in collagen biosynthesis. Additionally, the reduction in collagen biosynthesis will also lead to a significant reduction in L-proline concentration in the

cytoplasm, which as previously described will have a negative impact on embryonic cell regulation, differentiation, and development (Liu *et al*., 2008).

COX1 and COX2 contribute to the increase in prostaglandin synthesis, and it has been demonstrated that PEPD will lead to an induction of COX2, but only when it is present outside the cells (Yang *et al.*, 2013). As previously described, the prolidase activity in fibroblasts is primarily activated through pathways involved with a signalling cascade known to be involved with phosphorylation. The introduction of ASA during early embryo development will impact the biosynthesis of collagen by manipulating the signalling pathway cascade, the prolidase activity and signalling proteins in addition to the expression of prolidase (Surazyński *et al*., 2001). Prolidase activity is stimulated by cell surface receptors which activate the proteins involved with the initiation of the signalling pathway cascade. The pathways include Ras, Raf, MAP kinases, and ERK1 and ERK which are involved with the phosphorylation of intracellular proteins such as prolidase (Eblen, 2018).

Proficient degradation of the embryo's ECM and the biosynthesis of collagen is essentially dependent on the enzymatic activity of prolidase (Eni-Aganga *et al*., 2021). The molecular effect acetylsalicylic acid (ASA) and other non-steroidal anti-inflammatory drugs has in terms of substrate-assisted inhibition mechanisms been described as a covalent modification of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) through acetylation of Ser530 (Blobaum and Marnett, 2007). The mechanism as such will result in an irreversible inhibition by means of two specific steps beginning with rapid reversible non-covalent binding followed by the irreversible first order reaction (GIERSE *et al*., 1999). Previous research has

found that the biological function of prolidase is primarily involved with metabolism of protein degradation products containing proline (Chamson *et al*., 1989).

Further research has revealed that drugs conjugated with proline (pro-drugs) that are activated by prolidase like melphalan (Mel-pro), when specifically treated with concentrations of 5 mM of ASA, have been shown to exhibit decreased conversion of Mel-pro to Mel and inhibited prolidase activity, specifically in fibroblasts and breast cancer MDA-MB 231 cells (Chrzanowski *et al*., 2004). Invasiveness of the MDA-MB 231 cells has been observed to be mediated by the proteolytic degradation of the extracellular matrix and additionally, ASA has been determined to be an effective inhibitor of prolidase activity (Surazyński *et al*., 2004).

Furthermore, these experiments propose that with the addition of ASA *in vitro* cell differentiation and proliferation will be influenced through the inhibition of proteolytic activity of prolidase, specifically within the developing inner cell mass of the embryo.

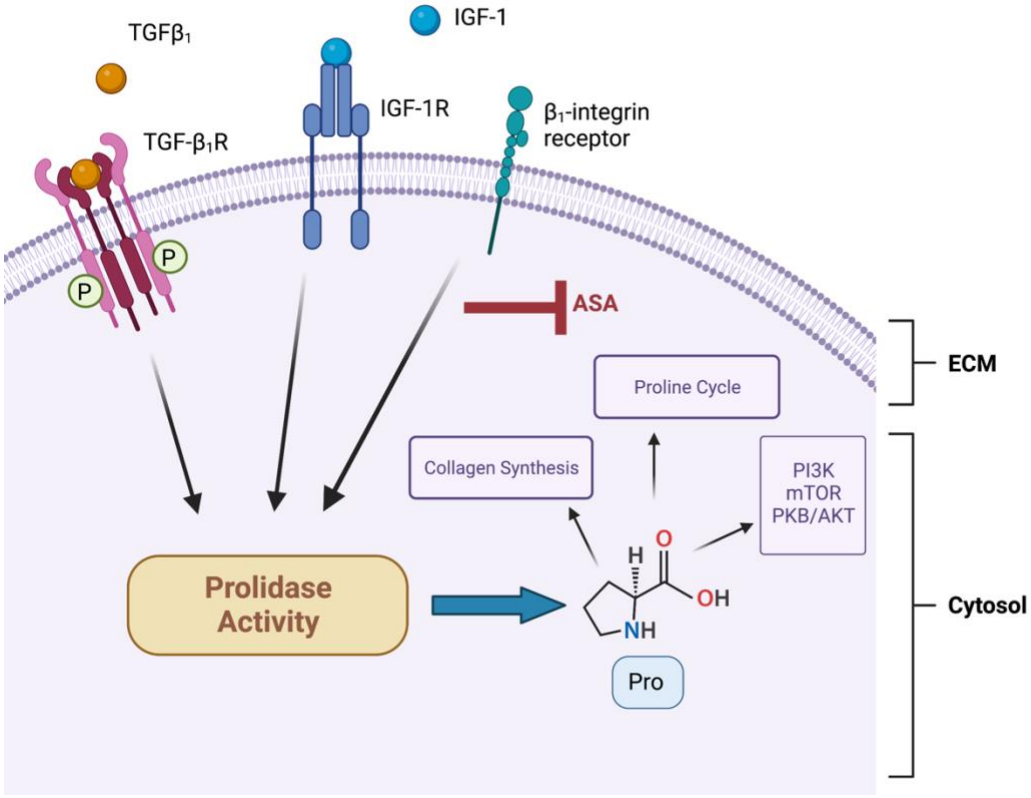


Figure 5.1 Mechanism of prolidase pathway
Prolidase enzymatic pathway is responsible for collagen biosynthesis, remodelling of the embryo's extracellular matrix (ECM). Prolidase activity is regulated by β_1-integrin receptor, insulin-like growth factor 1 receptor (IGF-1R) and transforming growth factor receptor (TGF). Products of prolidase activity, proline and hydroxyproline will stimulate signalling pathways associated with cellular functions. The inhibitory effect of ASA will impede prolidase phosphorylation and disrupt the physiological processes. This image was created with BioRender (https://biorender.com/)

5.2 Materials and Methods

5.2.1 Exposure of preimplantation embryos to ASA at different concentrations during high density culture.

Embryos were isolated and cultured for six days at high density. Following isolation, the embryos were then evenly distributed into droplets of 10 embryos/50 μl and cultured in modHTF, 5 mM, 2 mM and 0.5 mM of ASA in modHTF. The embryos were scored for their developmental stages every 24 h. Once the embryos had been cultured for six days, they were fixed, stained, and mounted onto slides for confocal imaging (described in 2.4) and the cell number and distribution of specific transcription factors were analysed as described in 2.5.

5.2.2 ASA exposure to preimplantation embryos in low density culture.

Embryos were collected 22± 2 h, cultured at low density, no more than one embryo per 100 μl droplet in the 96-well plate and grown for six days. During low density culture, the embryos were divided into control groups and treatment groups, each treatment groups. Treatment groups consisted of 5 mM, 2 mM and 0.5 mM of ASA in modHTF, and the experiment was repeated at least three times before embryos were fixed and stained as described in 2.4.

5.2.3 Distribution of transcription factors at different ASA concentrations during high and low density culture.

Following sequential staining (described in 2.4) each embryo was imaged on the Confocal microscope, and the number of cells counted as described in 2.5.

5.3 Results

5.3.1 Exposure of preimplantation embryos to ASA at different concentrations during high density culture

We observed a significant decrease ($P < 0.0001$) in rate of embryo development when exposed to 5 mM ASA (Figure 5.2). There was no significant difference in the rate of normal embryo development across the other treatment groups compared to the controls, except the treatment group at 2 mM ($P < 0.015$) on day three of culture, which has significantly decreased.

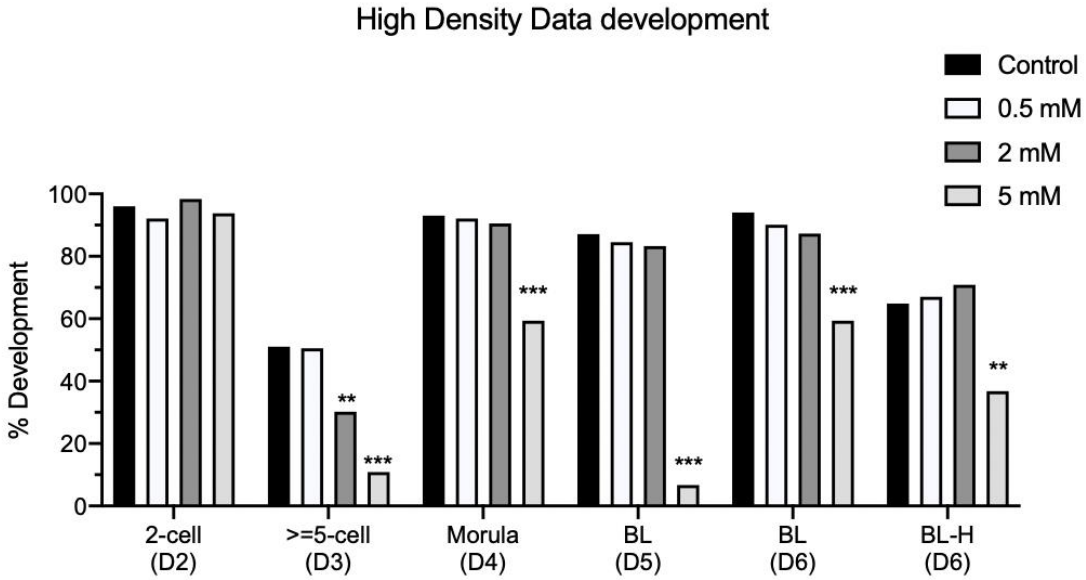


Figure 5.2 % Development of preimplantation embryos during ASA at different concentrations during high density culture
Graph depicting the % of development of preimplantation embryos at high density (no more than 10 embryos/50 μl). Embryos in control groups cultured in modHTF with treatment groups being cultured in 5 mM, 2 mM and 0.5 mM of ASA diluted in modHTF from day of isolation and scored for development every 24 h.
(** indicates $P < 0.015$ and *** indicates $P < 0.0001$). Data were analysed in GraphPad Prism using 1-way ANOVA and Tukey's post-hoc test. Data was obtained from at least 3 independent experiments.

5.3.2 Exposure of preimplantation embryos to ASA at different concentrations during low density culture

Figure 5.3 shows that the 5 mM treatment group had the most significant impact on normal embryo development . Results showed that there was a significant delay in blastocyst formation ($P < 0.0003$) in the 5 mM treatment groups on day five in culture. This trend continued when observing the hatching rate across all treatment groups ($P < 0.0041$). The difference in normal embryo development across the other treatment groups compared to the controls was not significant at low density culture.

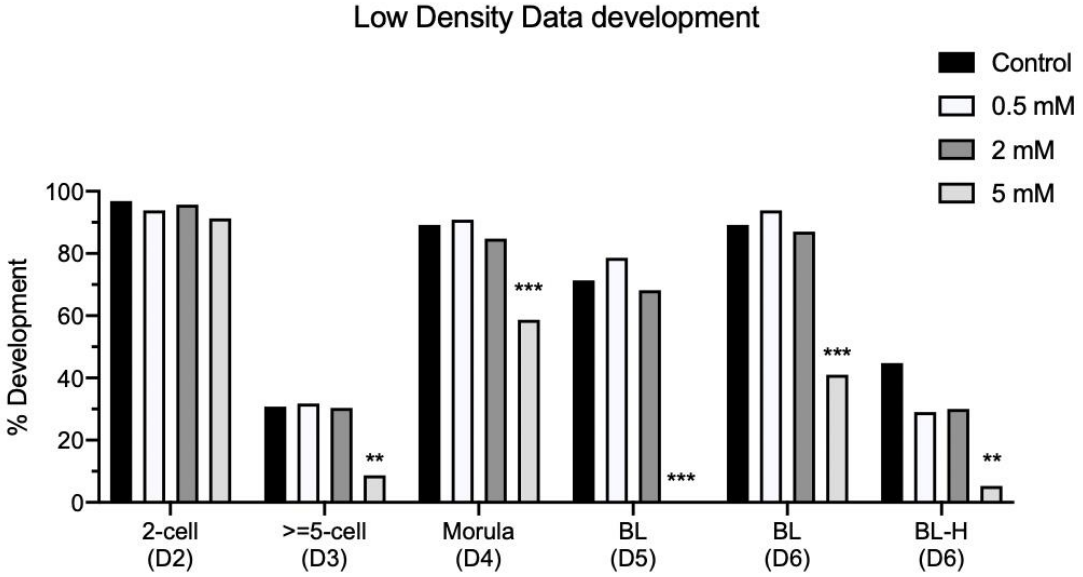


Figure 5.3 % Development of preimplantation embryos cultured in different concentrations of ASA at low density.
Graph depicting the % of development in preimplantation embryos from day of isolation to day six of culture at low density (1 embryo/10 μl). Embryos in control groups were cultured in modHTF in treatment groups being cultured in 5 mM, 2 mM and 0.5 mM of ASA diluted in modHTF from day of isolation until day six and scored for development every 24 h. Each experiment was performed at least three times.
(** indicates $p < 0.0041$, *** indicates $P < 0.0003$). Data were analysed in GraphPad Prism using 1-way ANOVA and Tukey's post-hoc test. Data was obtained from at least 3 independent experiments.

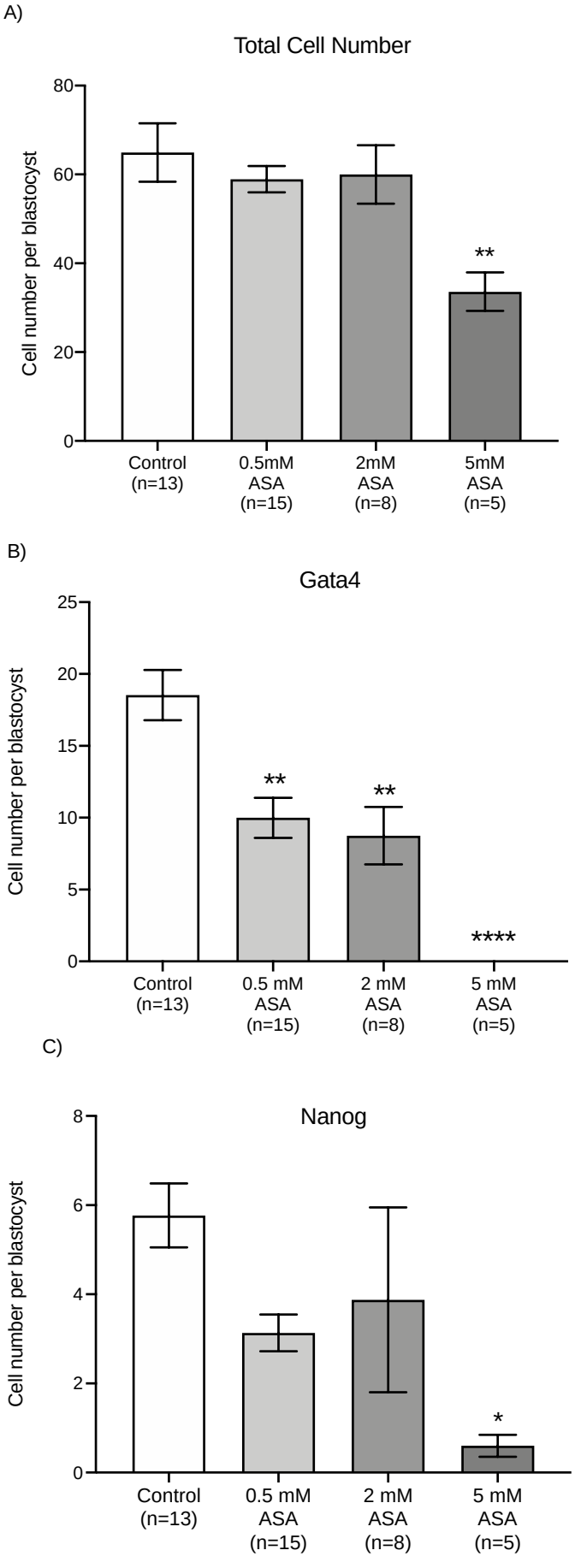
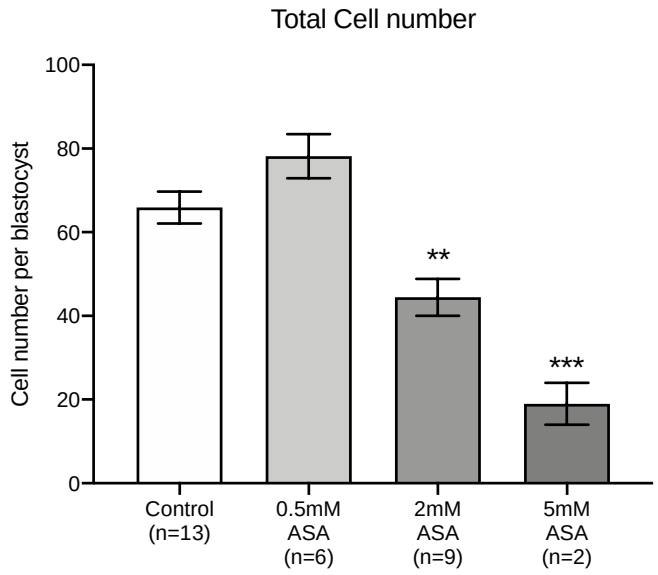


Figure 5.4 Blastocyst cell numbers in after culture in high density culture after exposure to different ASA concentrations

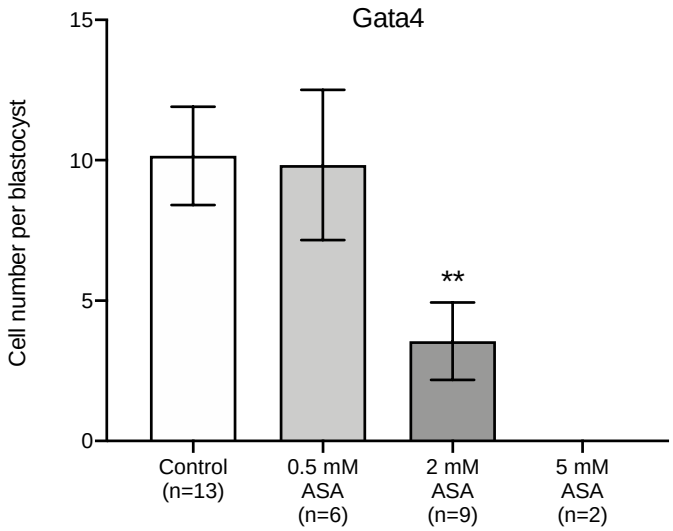
Graph depicts the average A) total cell number per blastocyst during high density culture (no more than 10 embryos/50 μl droplet) B) cells expressing Gata4, C) cells expressing Nanog after exposure to prolidase inhibitor, ASA diluted in modHTF at different concentrations, 0.5 mM, 2 mM and 5 mM

(* indicates $P < 0.04$, ** indicates $P < 0.0086$, **** indicates $P < 0.0001$). Data were analysed using GraphPad Prism using 1-way ANOVA and Tukey's post-hoc test.
Bars represent mean ± SEM obtained from at least 3 independent experiments.

A)



B)



C)

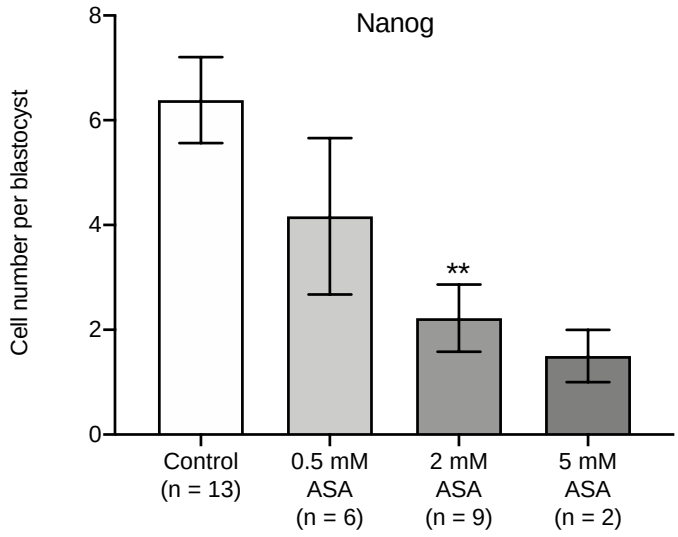


Figure 5.5 Cell numbers in mouse blastocysts cultured in low density prolidase inhibitor ASA at different concentrations

Graph depicts the average a) total cell number per blastocyst during low density culture (1 embryos/10 μl droplet) b) cells expressing transcription factor Gata4, c) cells expressing Nanog after exposure to prolidase inhibitor, ASA diluted in modHTF at different concentrations, 0.5 mM, 2 mM and 5 mM

(** indicates $P < 0.003$, *** indicates $P < 0.0003$). Data were analysed using GraphPad Prism using 1-way ANOVA. Bars represent mean ± SEM obtained from at least 3 independent experiments.

5.3.3 Inhibition of prolidase by ASA and the impact on transcription factor distribution during preimplantation development

The distribution of transcription factors Nanog and Gata4, was significantly reduced in the 5 mM treatment group in both low and high density culture (Figure 5.4 and Figure 5.5). Exposure to higher concentrations of inhibitor exhibited a significant decrease on cell numbers. The most significant effect the prolidase inhibitor had was on the transcription factors in both the inner cell mass and the hypoblast, Gata4 cells being significantly impacted the most in both the high and low density groups (Figure 5.4 and Figure 5.5).

Exposure to ASA had a significant reduction in the total number of cells in the blastocysts, particularly in the groups cultured at low density ($P < 0.0003$). Nanog distribution was also noticeably affected, with cell numbers significantly reduced, however to not the capacity as the Gata4. The results showed that the inhibitory effect of ASA was dose dependant, with embryos in the 5 mM ASA treatment groups exhibiting the most significant difference development, and with expression of transcription factors.

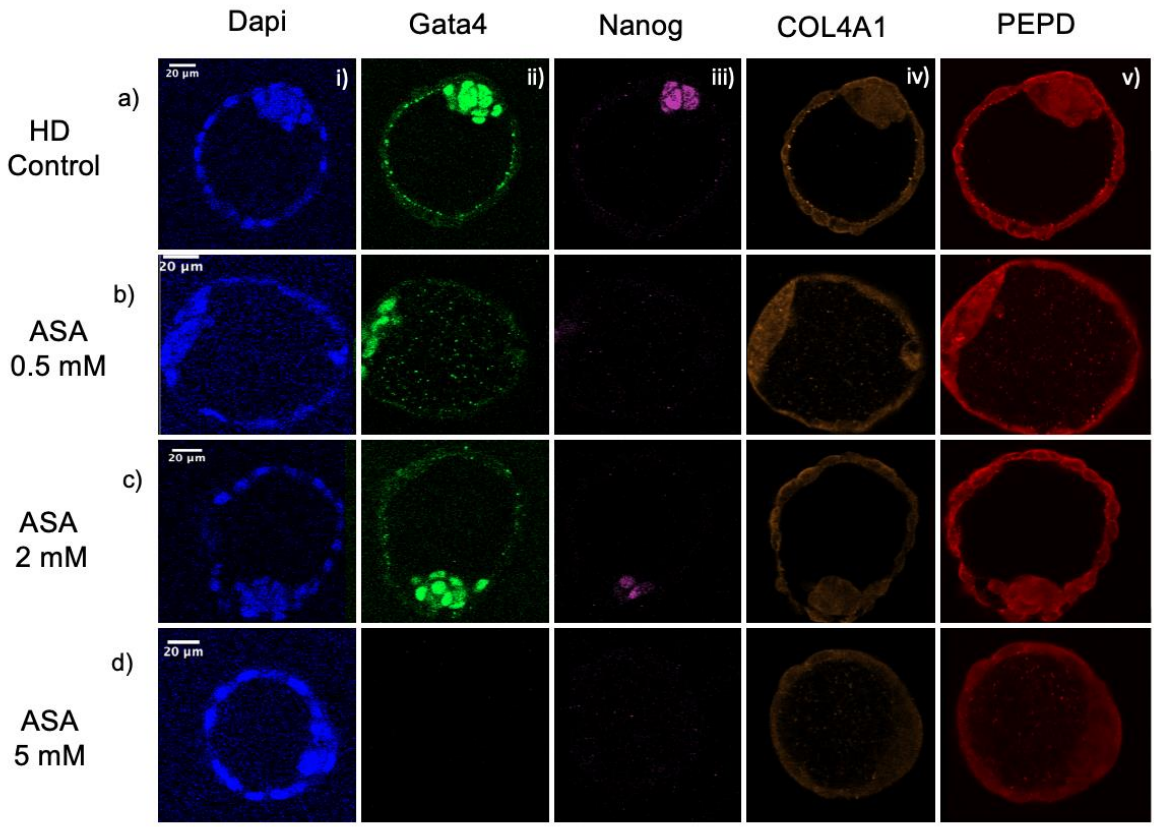


Figure 5.6 Immunofluorescent staining of preimplantation mouse embryos at high density culture after exposure to different ASA concentrations
Mouse blastocysts after six days of culture in high density culture stained with DAPI to identify cell nuclei (blue) (i) Gata4 transcription factors (green) (ii) Nanog transcription factors (pink) (iii) expression of collagen IV alpha 1 sub chain in the embryo's extracellular matrix (iv) and prolidase (PEPD) antibody staining (red) (v). a) Control at high density, b) exposure to 0.5 mM prolidase inhibitor ASA from day of isolation, c) exposure 2 mM ASA, d) 5 mM ASA.
Images are representative of at least three independent experiments.
Scale bar = 20 µm
Images generated by N. Stark

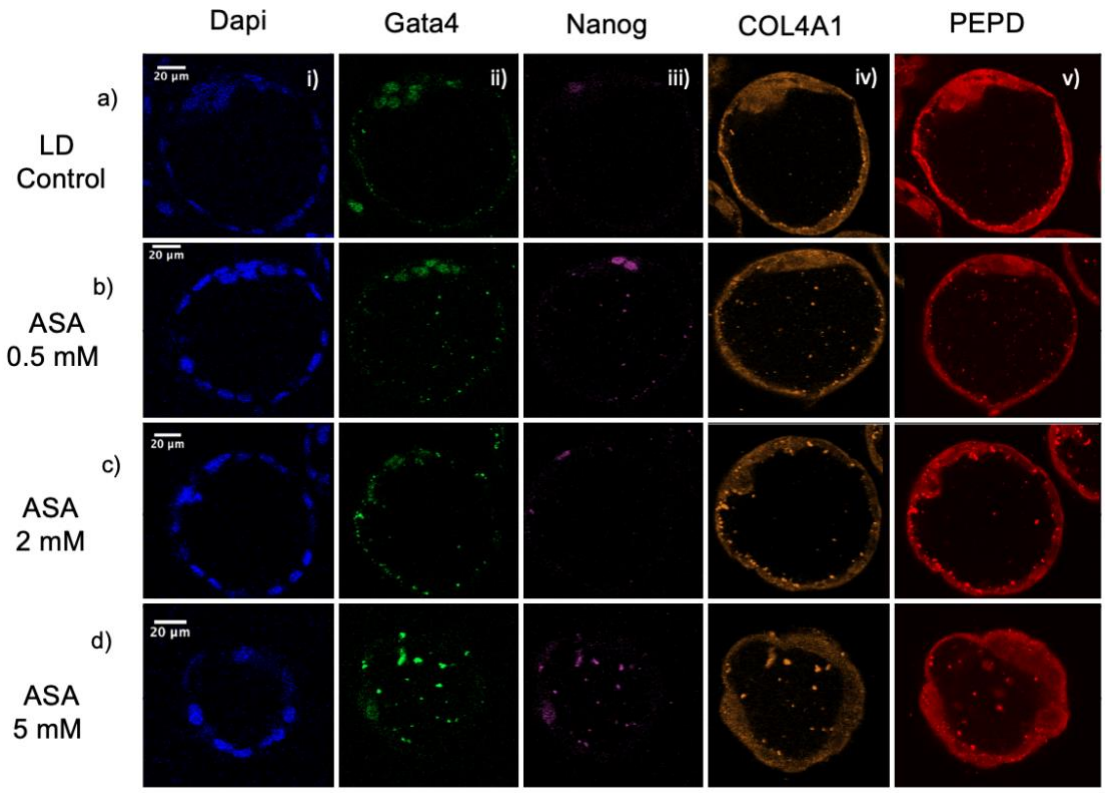


Figure 5.7 Immunofluorescent staining mouse blastocysts after exposure to different ASA concentrations at low density
Mouse blastocysts after six days of culture in low density culture stained with DAPI (blue) (i) Gata4 (green) (ii) Nanog (pink) (iii) expression of collagen IV alpha 1 sub chain (iv) and PEPD antibody staining (red) (v). a) Low density control, b) exposure to 0.5 mM ASA from day of isolation, c) 2 mM ASA, d) 5 mM ASA. Images are representative of at least three independent experiments.
Scale bar = 20 µm
Images generated by N. Stark

5.4 Discussion

The experiments have shown how prolidase will exhibit dual biological activity whereby acting as both a ligand for epidermal growth factor receptor (EGFR) and an enzyme what will split imidodipeptides at the c-terminal to release L-proline for collagen biosynthesis.

Previous studies have reported that once bound to the EGFR extracellular domain, prolidase will initiate a downstream cascade through dimerization, which will signal to PI3K/akt/mTOR pathways (Misiura and Miltyk, 2020). Ras/Raf/ERK and JAK/STAT3; the outcome activating proteins which will stimulate cellular processes such as proliferation, cell differentiation and growth (Tong and Wang, 2017, Yang *et al.*, 2014). Moreover, prolidase is additionally responsible for the upregulation of β_1-integrin and IGF-1 receptors which will initiate a cascade of phosphorylation of signalling proteins involved with cell proliferation, cell differentiation, migration and cell growth through PI3K/akt/mTOR pathways (Misiura *et al.*, 2020). The absence of Insulin-like growth factor-binding protein 1 (IGFBP-1), which binds to IGFs will result in a significant decrease in collagen biosynthesis and changes in phosphorylation of several kinases has been reported to alter prolidase activity (Miltyk *et al*., 1998).

Prolidase will activate EGFR downstream signalling through PI3K/Akt/mTOR and Ras/Raf/ERK pathways and additionally activate expressions of β_1-integrin and IGF-1R receptors (Baszanowska *et al*., 2021). This process will induce autophosphorylation of FAK, subsequently interacting with two MAP kinases, ERK1 and ERK2, thereby stimulating collagen biosynthesis (Misiura *et al*., 2020). Prolidase activity and the biosynthesis of collagen responds to signal mediated MAP kinases, which will add phosphate groups to neighbouring proteins, thereby acting as an

on/off switch. The signalling cascade will result in initiation of transcription factors which aid in the regulation of biological processes such as differentiation and cell growth (Labat-Robert and Robert, 2000). Obstruction or interrupting the natural flow of these complex signalling cascades by means of an inhibitor will impede any establishment of transcription factors, essential for regulating normal development in preimplantation embryos.

We hypothesized that addition of ASA would likely impede this further and furthermore have a direct impact on the distribution of transcription factors, specifically in the cells located in the embryo's developing hypoblast. The cells in the hypoblast and the epiblast are separated by a layer of extracellular basement membrane, the main component being collagen type IV. We determined that during proteolytic degradation of this layer of basement membrane, the release of L-proline would normally drive cell differentiation; however, upon exposure to ASA, its subsequent inhibitory effect on prolidase activity obstructed collagen biosynthesis from occurring, thereby restricting or in these circumstances preventing the distribution of transcription factors, specifically Gata4 (Figures 5.5 - 5.7).

5.5 Conclusion

Prolidase activity makes an important contribution to the biosynthesis of collagen IV and the degradation process of imidodipeptides by facilitating natural proteolytic breakdown of the embryo's basement membrane, thereby releasing amino acids such as L-proline and hydroxyproline. Chapter 1.7 describes the importance of L-proline and hydroxyproline during preimplantation development, the physiological mechanisms involved with their release and their correlation between

recycling L-proline for collagen synthesis. Our experiments aimed to reveal the impact prolidase inhibition would have on preimplantation development and transcription factor distribution in blastocysts. We found that the length of exposure to the prolidase inhibitor appeared to have less of an impact on embryo development and transcription factor distribution than the concentration of the inhibitor itself. We compared control groups from high and low density culture environments with groups that were exposed to the prolidase inhibitor, ASA from the time of embryo collection. From here, we compared the impact different concentrations of ASA had (0.5 mM, 2 mM and 5 mM) on embryo growth, blastocyst development, hatching rates and distribution of transcription factors. We previously described in 1.3 how lack of autocrine factors would impact normal preimplantation development.
Our results showed that the addition of ASA had a greater impact on embryo development and cell differentiation when exposed to both high density and low density environments at higher concentrations (5 mM). Blastocyst formation, hatching rate and the rate of development was negatively affected by higher concentrations (5 mM) of ASA.

Embryos that were cultured in low density will already lack the autocrine support required to maximise the quality of their growth and development. Addition of ASA was found to result in disruption to enzymatic activity required for proteolytic degradation of the embryo's ECM, thereby decreasing the quality of growth and development further and disrupting pathways that are involved with cell differentiation. Proficient degradation of the embryo's ECM, release of L-proline and subsequent biosynthesis of collagen is essentially dependent on the enzymatic activity of prolidase. The mechanism as such will result in an irreversible inhibition by

means of two specific steps beginning with rapid reversible non-covalent binding followed by the irreversible first order reaction (GIERSE *et al*., 1999). Previous research has found that the biological function of prolidase is primarily involved with metabolism of protein degradation products containing proline (Chamson *et al*., 1989).

Our results observed that as the concentration of ASA increased, the rate of normal growth and development significantly decreased, including hatching rates in blastocysts after six days in both high density and low density culture. Exposure to higher concentrations of inhibitor exhibited a significant decrease in cell numbers in blastocysts, specifically the prevalence of Gata4, which is predominantly found expressed in cells within the preimplantation embryo's hypoblast.

Exposing embryos to ASA at high concentrations, resulted in restricted prolidase activity, therefore the recovery of L-proline from collagen IV degradation would be limited for the regulation of essential biological functions.

Chapter 6 Overall discussion

6.1 Collagen IV alpha chain expression is affected by exposure to broad spectrum matrix metalloproteinase inhibitor GM6001

Proteolytic degradation of the basement membrane is a fundamental yet complex process that occurs naturally in preimplantation development. The configuration of the basement membrane is principally made up of a combination of laminins, entactin/nidogens, proteoglycans and collagen, predominantly type IV collagen. Collagen IV contains alpha chains which contain a significant portion of L-proline, an amino acid that has been known for its dynamic role in pathways associated with cell pluripotency, cell differentiation and embryo quality. Gelatinase activity is responsible for natural breakdown of the alpha chains that make up the structure of collagen IV. The result of this breakdown will produce a reliable source of free proline that will be released into cytoplasm and facilitate cellular functions such as cell differentiation by acting as signalling molecules. We sought to investigate whether restricting the natural degradation of the basement membrane would result in a series of cascading events that would ultimately impair embryo quality and affect differentiation of the cells in the preimplantation embryo.

Previous research has revealed that synthetic inhibitors of matrix metalloproteinases (MMPs) are effective at impeding proteolytic activity associated with the breakdown of the basement membrane. GM6001 or Ilomastat was chosen due as it was a broad spectrum inhibitor and capable of obstructing collagenase activity. As the role of collagenases is essentially to break down native collagen, we felt its application was appropriate.

The results demonstrated that when applied to low density culture environments, GM6001 was capable of negatively impacting the normal rate of growth and development in embryos from E2.0 onwards, more so than the embryos that were being cultured in high density. Cell numbers did not appear to be significantly impacted by GM6001, leading us to believe that although rate of development was affected, the overall impact of the inhibitor was less effective in relation to the signalling pathways associated with cell differentiation. It is known that GM6001 will function by inhibiting members of a disintegrin and metalloproteinase (ADAM) proteins (Edwards *et al*., 2008) which play crucial roles in generating active forms of epidermal growth factor receptor (EGFR), and cell signaling. EGFR proteins are tyrosine kinase receptors that are involved with pathways such as mitogen-activated or extracellular signal–regulated protein kinase signalling pathway (MAPK/ERK) that regulate cellular functions like cell division and survival. The impact GM6001 had on preimplantation development was likely due to the inhibition of the MMPs responsible for proteolytic degradation of collagen IV in the embryo's basement membrane. By impairing natural enzymatic breakdown of the basement membrane, this restricted the release of free proline into the cytoplasm, thereby interfering with the regulation of pathways that moderate normal cellular function during preimplantation development (Cohen *et al*., 1999). GM6001 appeared to have a greater impact on the growth and development in embryos that were being cultured in low density as opposed to high density, regardless of the length of exposure. We can assume that the high density groups obtained a level of protection from the effects of GM6001, potentially due to the presence of autocrine factors (Morris *et al.*, 2020).

Furthermore, addition of GM6001 to both high and low density culture environments had minimal impact on the rate of cell differentiation. These results were unexpected because we knew that the mechanism of the inhibitor was designed to target collagenases which are responsible for basement membrane degradation. Moreover, the addition of L-proline to the culture medium in the treatment groups to counteract the absence of free proline improved the rate of normal growth and development only marginally, with the effect on the distribution of transcription factors being relatively inconsequential.

From what we know, GM6001 is a broad spectrum, non-zinc binding MMP inhibitor that will target a wide range of collagenases, gelatinases, matrilysins and stromelysins that are capable of degrading triple helical fibrillar collagens. Our data suggests that the effect broad spectrum inhibitors have on proteolytic degradation may not be as significant as we initially postulated. As such, further research is aimed to observe the effects a selective inhibitor would have on MMP activity, one which specifically targets the MMPs responsible for collagen degradation in the extracellular matrix. Gaining a better understanding of how natural proteolytic activity of collagenases facilitates the release of L-proline, and how this process regulates normal cellular development in preimplantation embryos, it is possible to apply this research to ART and subsequently improve embryo quality *in vitro*.

6.2 Exposure to inhibitor SB-3CT will impede natural proteolytic degradation of the preimplantation embryo ECM by targeting pathways associated with MMP-2 and MMP-9 activity.

MMP-2 and MMP-9 are two metalloproteinases that are primarily responsible for the enzymatic breakdown of the extracellular matrix. As we know, L-proline is a proteinogenic amino acid that plays a fundamental role in cellular functions such as differentiation and pluripotency. During natural proteolytic degradation of the basement membrane, L-proline is released from hydrolysed collagen IV. From here, it can act as a growth factor to help drive cell differentiation regulate pluripotency of embryonic stem cells. Previously, we studied the effects of GM6001, a broad spectrum synthetic inhibitor which was capable of obstructing collagenase activity, thereby restricting the release of L-proline by impeding basement membrane breakdown. The results were able to show that the impact of the broad spectrum inhibitor was not as effective as we had anticipated, therefore we opted for SB-3CT, a more selective inhibitor that specifically targeted the MMPs primarily responsible for ECM degradation.

Embryos were exposed at both high density and low density *in vitro* to SB-3CT at a concentration of 10 μM and noticed a significant reduction on normal development and hatching rate, as well as a noticeable inhibitory effect on cell differentiation within the inner cell mass. While SB-3CT had a much more profound impact on all groups in comparison to the inhibitory effects of GM6001, the most noticeable difference was observed in the low density groups, where autocrine support and growth factors were unavailable. Furthermore, the results revealed that following the addition of 400 μM L-proline to the high density SB-3CT groups to counteract the

inhibitory effect, the rate of normal development was matched, if not exceeded the control groups. Interestingly, addition of L-proline to the low density groups that had been exposed to SB-3CT only improved the rate of development marginally in comparison to the embryos being cultured in high density SB-3CT exposure. As anticipated, the groups that exhibited the lowest rate of development were entirely exposed to SB-3CT without the addition of L-proline.

It could be deduced from these results that although the addition of L-proline would normally act as a growth factor and improve embryo development within both high and low density culture environments *in vitro*, the inhibitory effects of SB-3CT may have superseded the abilities L-proline would generally have on the regulation of cellular processes.

We explored how the inhibitory effect of SB-3CT would impact crucial stages of preimplantation development by varying the length of exposure time to high density groups, targeting fundamental stages that included time of embryo isolation, zygomatic genome activation and the stage of development during compaction when the embryonic cells begin to differentiate. We found that in all groups that were exposed to SB-3CT, regardless of the length of time, the rates of normal development, including hatching at blastocyst stage, were reduced. As anticipated, longer exposure to the inhibitor resulted in a more prominent change in the rate of development, the embryos cultured at high density with SB-3CT from day of isolation at maximum exposure having the most significant reduction in hatching blastocysts compared to those with minimal exposure.

When looking at total cell number, all groups exposed to the selective gelatinase inhibitor didn't appear to differ significantly from one another. Cells expressing Gata4 in embryos from the inhibitor groups, however appeared to be significantly decreased, especially during longer exposure compared to embryos exposed from day four in culture. In contrast, there appeared to be very little difference between the number of cells expressing Nanog when comparing control groups to the short SB-3CT exposure groups, especially when compared to embryos that had been exposed for longer periods of time.

We know that Nanog expression is necessary in the formation of the epiblast and is facilitated by the fibroblast growth factor/extracellular signal-regulated kinase pathway (FGF/Erk) and Gata4 has been thought to help regulate genes involved with embryogenesis. Expression of Nanog will initiate during E4.0 while Gata4 is expressed within the embryo's hypoblast within the primitive endoderm. Additionally, the FGF/Erk pathway has been associated with cell pluripotency and lineage specification. Moreover, research has demonstrated that FGF will stimulate MMP activity during collagen IV biosynthesis, specifically MMP-2 and MMP-9.

From experiments using SB-3CT, we have observed effective inhibition of MMP-2 and MMP-9 activity responsible for influencing cell differentiation through degradation of the basement membrane. MMP-2 and MMP-9 activity is responsible for the breakdown of collagen IV in the basement membrane during preimplantation development, allowing for the release of L-proline. Inhibition of these specific MMPs will prevent the release of L-proline and particularly in the absence of autocrine support and additional growth factors, embryo development will be impaired and

there will be a greater impact on the embryo's ability to effectively manipulate the natural cell differentiation process. Addition of L-proline to counteract this process appeared to improve normal growth and development and helped improve differentiation. As we know, the presence of proline during embryo development will activate intracellular signalling pathways such as mTOR, thereby regulating cell differentiation and proliferation (Washington *et al*., 2010). The availability of proline will help to influence the activity of other intracellular pathways involved with cell proliferation and differentiation. This has led us to believe that L-proline is essentially a driving force behind the normal biological processes involved with preimplantation development, and that through normal basement membrane degradation, the embryo is responsible for its own source of free proline.

6.3 Acetylsalicylic acid (ASA) will obstruct collagen IV biosynthesis by inhibiting prolidase activity

Prolidase is an enzyme that will catalyse the rate limiting step during collagen biosynthesis by splitting he imidodipeptides at the c-terminal to release proline. Additionally, we know that prolidase will also act as a ligand for EGFR and will initiate a downstream cascade of signalling pathways, primarily effecting PI3K/Akt/mTOR and Ras/Raf/ERK to stimulate cellular processes such as proliferation, cell differentiation and growth. Prolidase activation of EGFR will also induce β_1-integrin and IGF-1R receptor expression which will stimulate collagen IV biosynthesis through mediated MAP kinases, ERK1 and ERK2. The signalling cascade will initiate the transcription factors that will consequently influence biological processes that are essential for normal embryo development such as cell growth and differentiation.

From our previous experiments, we can affirm that by inhibiting the activity of specific MMPs with broad spectrum and selective inhibitors, the degradation of the basement membrane during preimplantation development will be affected, essentially obstructing, or limiting the natural release of free proline. As prolidase makes a significant contribution to the biosynthesis of collagen IV by facilitating natural proteolytic degradation, we wanted to understand how by impeding this enzyme, normal preimplantation development would be altered.

We found that by preventing prolidase activity using acetylsalicylic acid (ASA), it would inhibit the downstream cascade of signalling pathways by suppressing the enzymatic activation of EGFR. By inhibiting this complex network of signalling cascades, we discovered that not only was the rate of development affected, but

additionally there was a negative impact on establishment of transcription factors required for normal development. Interestingly, the transcription factors that presented the greatest change in expression in response to the lack of prolidase activity appeared to be Gata4. Gata4 was used primarily to identify the developing hypoblast in the blastocysts, while Nanog was used to identify the epiblast. It was observed that as the hypoblast and epiblast are separated by a thin layer of extracellular basement membrane, largely comprised of collagen IV, the cells that were mostly impacted were the ones expressing transcription factor Gata4.

Moreover, we found that as we increased the concentration of ASA, there was a greater difference in cell number and transcription factor distribution. From these observations, we can determine that the enzymatic activity of prolidase on the basement membrane separating the epiblast from the hypoblast, will normally release concentrations of free proline, which will drive cell differentiation. Upon exposure to ASA, which we know will inhibit prolidase activity, there will be an inhibitory effect on the natural biosynthesis of collagen IV, preventing the release of proline and hydroxyproline. We found that the inhibitory effect of the ASA will have a greater impact at higher concentrations, observing a dramatic decrease distribution of transcription factors, blastocyst development and hatching rates.

During our earlier experiments involving GM6001 and SB-3CT, we described how a lack of autocrine support and growth factors in low density culture environments can have a greater impact on the hatching rates when compared to high density culture. Remarkably, introducing the embryos to ASA at both low and high density culture *in vitro*had similar results. Regardless of the presence of growth factors and autocrine

support required for normal development, both groups exhibited the same trend, that being when exposed to higher concentrations of ASA (5 mM) compared to the controls and lower concentrations (0.5 mM, 2 mM), the blastocyst and hatching rates declined significantly. From what we have observed, it can be suggested that as we increased the concentration of ASA, we effectively restricted the prolidase activity. Furthermore, what we currently know about L-proline and the fundamental role it plays in embryo development, inhibiting a cytosolic imidodipeptidase such as prolidase will obstruct its ability to contribute to the recovery and recycling of L-proline, and resynthesis of collagen IV.

6.4 Conclusions and summary

The aim of my study was to demonstrate how the inhibition of collagen IV breakdown will impact preimplantation development and the differentiation of cells in the blastocyst. To study this, we used both a broad spectrum inhibitor and a selective inhibitor to target the matrix metalloproteinases (MMPs) responsible for the degradation of the basement membrane and an inhibitor that would target prolidase, a cytosolic imidodipeptidase that contributes to the resynthesis of collagen resynthesis. We observed the way these inhibitors affected development during different culture environments, during various stages of preimplantation development, the way they impacted cell differentiation and transcription factor distribution and examined how the addition of L-proline would potentially counter these effects.

We know that basement membrane degradation is a natural physiological process that occurs during preimplantation development. Furthermore, a substantial portion of the basement membrane is comprised of collagen IV, which contains the embryo's natural source of free proline. During proteolytic degradation of the basement membrane, proline is released from the collagen IV where it will support the biosynthesis of collagen, drive cell differentiation and help to regulate growth in preimplantation embryos.

We focused on the enzymes that contribute to the natural degradation process of the basement membrane, and the enzyme that plays a fundamental role in catalysing dipeptides that contain a C-terminal of L-proline. Each enzyme plays an essential role in collagen biosynthesis and turnover by breaking down the embryo's

extracellular matrix and enabling the embryo to support its own constant supply of free proline. In conclusion, this study found that by obstructing proteolytic degradation of the basement membrane, we have effectively inhibited the natural supply of L-proline for the preimplantation embryo, leading to adverse effects on blastocyst development, hatching rates and expression of transcription factors. Our data has provided valuable insight into the complex mechanisms involved with collagen IV degradation, and the crucial function of L-proline on preimplantation development and cell differentiation, which thereby will be beneficial to future advancements in the field of assisted reproductive technology.

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