The role of sphingosine 1-phosphate in mouse preimplantation embryo development

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Statement of originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

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Author attribution statement

This thesis contains material published in Rayhanna (2019). This is section 3.3.3 and Figure 3.6 of Chapter 3. This data was collected during my Honours year and appears in my Honours thesis. I performed the research experiments, analysed the data, and prepared the figure. Associate Professor Margot Day and I designed the research experiments.

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Margot Day
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Presentations and Publications

Journal articles

Green, CJ, Span, M, **Rayhanna, MH**, Perera, M & Day, ML 2022, 'Insulin-like Growth Factor Binding Protein 3 Increases Mouse Preimplantation Embryo Cleavage Rate by Activation of IGF1R and EGFR Independent of IGF1 Signalling', *Cells*, vol. 11, no. 23, pp. 3762.

Presentations

Investigating the role of sphingosine 1-phosphate in mouse preimplantation embryo development.
Oral Presentation, FMH HDR Oral Presentation Program, University of Sydney
June 2021

Mouse preimplantation embryo development in vitro requires endogenous sphingosine-1-phosphate (S1P)/S1PR signalling.
Oral presentation, ESA-SRB-APEG-NZSE Annual Scientific Meeting
November 2022

Posters

Endogenous sphingosine 1-phosphate (S1P) signalling via the $S1P_1R$ is essential for mouse preimplantation embryo development in vitro.
E-Poster, ESA-SRB-ANZBMS Annual Scientific Meeting
November 2021

Sphingosine kinases are required for mouse preimplantation embryo development in vitro
Poster, ESA-SRB Annual Scientific Meeting
November 2023

Abstract

In vitro embryos develop poorer than their *in vivo* counterparts, as characterised by slower developmental rate and increased arrest. Improving the viability and quality of cultured embryos would be valuable as the demand for assisted reproductive technologies, including the use of IVF, continues to rise. A contributing factor to this poor development is the media in which embryos are cultured, which typically lack constituents of the maternal reproductive tract such as growth factors. Sphingosine 1-phosphate (S1P) is one such growth factor that is present in follicular fluid, and exogenous S1P improves cleavage rate of mouse embryos when added to culture medium. However, the mechanisms by which S1P promotes cleavage are not known.

The first aim of this study was to identify the expression and function of S1P receptor (S1PR) 1 in the embryo. Expression of S1PR1 was identified across preimplantation embryo development, and inhibition of S1PR1, by 10 μM NIBR0213, reduced embryo cavitation and blastocyst formation during low-density embryo culture. S1P likely increases embryo cleavage rate via S1PR1, but neither S1P nor inhibition of S1PR1, by 1 μM NIBR0213, affected embryo cavitation or blastocyst attachment. These results suggest that not only does exogenous S1P promote embryo development potentially via S1PR1, but there is also embryo-derived S1P signalling via S1PR1 that supports development *in vitro*. Acute treatment of embryos with exogenous S1P (for 10 min) reduced the presence of S1PR1 in all cellular compartments, an effect also seen with inhibition of S1PR1, but the reasoning behind this is not fully understood.

Following this, the expression and function of S1PR2-5 in the embryo was examined. All four S1PRs were expressed throughout development, though the localisation showed differences. Selective antagonists of S1PR2 or S1PR4 impaired embryo cavitation, while antagonism of S1PR3 prevented development past the early cleavage stages. Inhibition of each of S1PR2, 3, and 4 by their respective antagonists during only early (prior to compaction) or only late preimplantation development (compaction onwards) revealed different roles for these receptors in development, with S1PR2 required for both early and late development, S1PR3 only for early development, and S1PR4 only for late development. Despite these differences, combined inhibition of S1PR1-4 using concentrations of inhibitors that had no effect on development alone resulted in arrest at the 2-4-cell stage, suggesting functional redundancy between S1PRs.

Finally, the expression and localisation of sphingosine kinase (SphK) 1 and 2, and their phosphorylated active forms was investigated. These isoenzymes catalyse the production of S1P from its precursor sphingosine. Both unphosphorylated and phosphorylated SphK1 and SphK2 were present throughout preimplantation development. Inhibition of SphK1, by the competitive substrate inhibitor, PF543, reduced the percentage of embryos that cavitated and formed blastocysts, while inhibition of SphK2, by the competitive substrate inhibitor, ABC294640, reduced embryo development to all stages. These results suggest that pSphK2 produces the S1P that acts at S1PRs in the embryo, rather than pSphK1 as in other cells. Combined inhibition of SphK1 and SphK2 using individually sublethal concentrations of the inhibitors impaired embryo cavitation and blastocyst formation, suggesting functional redundancy between the two isoenzymes.

Overall, this study established the presence of constituents of S1P signalling pathways in preimplantation embryos, as well as the requirement of embryo-derived S1P signalling via S1PRs for early development. This study provides the foundation for further investigation into S1P signalling in the embryo that will facilitate a better understanding of the molecular mechanisms that underpin development and inform improvements in the *in vitro* culture environment for assisted reproductive technologies.

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Abbreviations

ADAM	a disintegrin and metalloproteinase
ART	assisted reproductive technologies
AU	arbitrary units
BSA	bovine serum albumin
CCN2	cellular communication network factor 2
CerS	ceramide synthase
COC	cumulus-oocyte complex
COX2	cyclooxygenase 2
CTCF	corrected total cell fluorescence
DAPI	4′,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
EGA	embryonic genome activation
EGF	epithelial growth factor
EGFR	epithelial growth factor receptor
ER	endoplasmic reticulum
ERK1/2	extracellular signal-related kinase 1/2
EVT	extravillous trophoblast
Flk-1	foetal liver kinase 1
FSH	follicle stimulating hormone
gp130	glycoprotein 130
GPCR	G-protein coupled receptor
hCG	human chorionic gonadotrophin
HD	high density

HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	hypoxia inducible factor
hTERT	human telomerase reverse transcriptase
HUVECs	human umbilical vein endothelial cells
ICM	inner cell mass
IGF	insulin-like growth factor
IGF1R	insulin-like growth factor receptor 1
IGFBP3	insulin-like growth factor binding protein 3
IP3	inositol 1,4,5-trisphosphate
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
JNK	c-Jun N-terminal kinase
LD	low density
LIF	leukemia inhibitory factor
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
MMP	matrix metalloprotease
ModHTF	modified human tubal fluid
mTOR	mammalian target of rapamycin
OCLN	occludin
OCT4	octamer-binding transcription factor 4
P1P	phytosphingosine 1-phosphate
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PGE2	prostaglandin E2
PI3K	phosphatidylinositol-3-phosphate kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKR	protein kinase R
PLC	phospholipase C
PLK1	Polo-like kinase 1
PMS	pregnant mare's serum gonadotrophin
PPARγ	peroxisome proliferator-activated receptor-γ
pSphK1	phosphorylated SphK1
pSphK2	phosphorylated SphK2
PVA	polyvinyl alcohol
QS	Quackenbush Swiss
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
S1P	sphingosine 1-phosphate
S1PR	sphingosine 1-phosphate receptor
SGPP	sphingosine 1-phosphate phosphatases
SPGL	sphingosine 1-phosphate lyase
SphK	sphingosine kinase
Spns2	spinster 2
TEAD	TEA domain transcription factor

TERT	telomerase reverse transcriptase
TFAP2C	transcription factor AP-2 gamma
TNFα	tumour necrosis factor α
TRAF2	TNF receptor-associated factor 2
VEGF	vascular endothelial growth factor
YAP	yes-associated protein
ZO-1	zonula occludens-1

CHAPTER 1

Introduction

1.1 Embryo development *in vivo*

1.1.1 Folliculogenesis and oocyte development

The mammalian ovary contains tens of thousands of follicles, each comprised of an oocyte and its supporting follicular somatic cells, including granulosa cells, thecal cells, immune cells, and endothelial cells (Figure 1.1) (Grive, 2020). While the follicular pool is established during foetal development, follicular maturation - or folliculogenesis - only occurs after the onset of puberty. This allows follicles to progress from immature primordial follicles through the primary, secondary, early antral, and antral stages, characterised by the proliferation of supporting cells and formation of a fluid-filled antrum. Expansion of this antrum and remodelling of the follicular wall results in ovulation, in which the follicle ruptures and the oocyte is expelled into the oviduct. Folliculogenesis is a tightly controlled process regulated by a complex network of signalling pathways, and the vast majority of follicles undergo degeneration prior to ovulation of a single or small number of oocytes (Zhang & Liu, 2015).

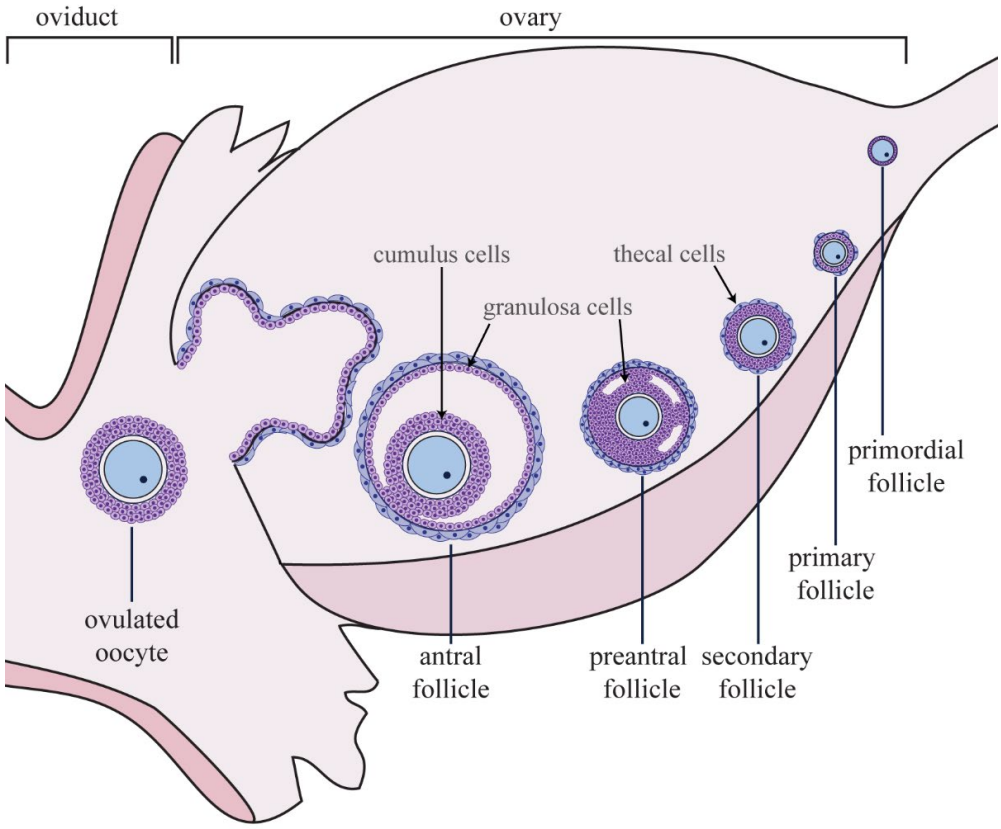


Figure 1.1. Ovarian follicular development in mammals. Oocytes within the ovary are contained within follicles, of which there is a large but finite pool. Folliculogenesis involves development of primordial follicles into primary, secondary, preantral, and antral stage follicles. This is characterised by oocyte growth and proliferation of supporting granulosa and thecal cells. At the preantral stage, the follicle draws in fluid to form an antral cavity, after which the oocyte undergoes maturation and ovulation into the oviduct.

1.1.2 Preimplantation embryo development

Following ovulation, the oocyte can be fertilised by sperm to produce a diploid zygote (Figure 1.2) (Clift & Schuh, 2013). The zygote undergoes a series of symmetrical reductive mitotic cleavages to form a 2-cell, 4-cell, and 8-cell embryo. At the 8-cell stage, the embryo compacts to form the morula, in which the outer blastomeres flatten and form tight junctions (White et al., 2016). The morula then cavitates to form the blastocyst with a fluid-filled blastocoel. At the blastocyst stage, there are two distinct cell types – the inner cell mass (ICM) and the trophectoderm. While the trophectoderm goes on to form the placenta, some cells of the ICM differentiate to form a single layer of primitive endoderm cells, the hypoblast, which ultimately go on to form the extraembryonic yolk sac (Beddington & Robertson, 1999). The remaining cells of the ICM, the epiblast, go on to form the embryo proper and the remaining extra-embryonic tissues. At this point, expansion of the blastocoel and secretion of proteases facilitates blastocyst hatching from the surrounding zona pellucida (Leonavicius et al., 2018; O'Sullivan et al., 2001).

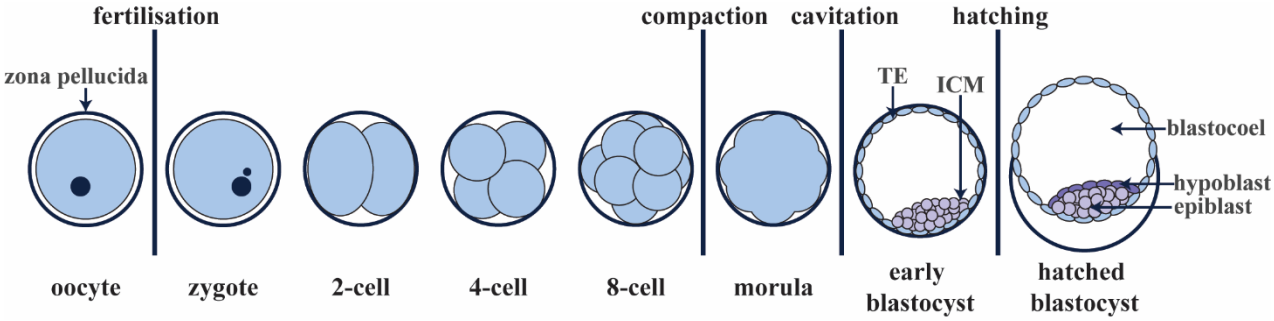


Figure 1.2. Mammalian preimplantation embryo development. Embryo development begins with fertilisation of an oocyte to form a diploid zygote. The zygote undergoes a series of reductive mitotic cleavages, forming the 2-cell, 4-cell, and 8-cell embryo. At the 8-cell stage, the embryo compacts to form the morula and then cavitates to form the blastocyst, which can hatch from the zona pellucida to implant in the uterine wall.

1.1.3 Blastocyst implantation

Following hatching, the blastocyst implants into the uterine wall. During implantation, there is bidirectional crosstalk between an implantation-competent blastocyst and the receptive uterus. This occurs in a narrow window known as the "window of implantation" and is regulated by estrogen and progesterone (Sun & Yeh, 2022). There are three main components of blastocyst implantation: (i) apposition, (ii) adhesion, and (iii) invasion.

The blastocyst comes into apposition with the uterine wall as a result of an oestrogen spike triggering stromal oedema and closure of the uterine lumen (Kennedy et al., 2007; Rockwell et al., 2002). This is followed by attachment of the trophectoderm cells of the blastocyst to the epithelial cells of the uterus. Attachment of trophectoderm stimulates a decidualisation of uterine stromal cells surrounding the blastocyst, characterised by rapid proliferation and differentiation of these cells into decidual cells (Matsumoto, 2017). Blastocyst attachment is also accompanied by a localised increase in stromal vascular permeability, enabling trophectoderm cells to invade and establish a blood supply to the embryo (Kennedy et al., 2007).

1.2 Preimplantation embryo development *in vitro*

Preimplantation embryo development is impaired by *in vitro* culture conditions, with embryos exhibiting slower development (Harlow & Quinn, 1982), decreased viability, higher incidence of apoptosis and developmental arrest (Giritharan et al., 2007; Hardy et al., 2001; Rizos et al., 2002), chromosomal abnormalities (Maurer et al., 2015), and altered gene expression (Giritharan et al., 2007). This is attributable, in part, to *in vitro* culture conditions not adequately reflecting the maternal reproductive fluids that embryos develop in *in vivo*. Notably, simple culture media lack molecules such as amino acids, antioxidants, and growth factors. The addition of these constituents to culture media improves the quality of embryos cultured *in vitro* but, nevertheless, these cultured embryos are still under stress (Hardy et al., 2021; Llobat, 2021; Morris et al., 2020).

Improving the quality of cultured embryos is important as the demand for assisted reproductive technologies (ART) continues to rise. In Australia, 6% of babies born in 2021 were a result of ART treatment (Newman et al., 2023). However, the success rates of ART are still low. Of the over 111,000 ART cycles initiated in 2021, only 23.1% resulted in a clinical pregnancy and 18.3% in live birth (Newman et al., 2023), and these percentages have changed little, if at all, over the last 20 years.

1.3 Role of growth factors in preimplantation embryo development

The oviductal and uterine fluids contains nutrients, growth factors, and cytokines that support the developing embryo (Gardner et al., 2015; Kelleher et al., 2016; Llobat, 2021). Maternal cells secrete growth factors into these fluids which then act via factor-specific receptors on and in maternal and embryonic cells. Similarly, the embryo secretes

factors, which act within the embryo or are secreted into the reproductive fluids to act back on the embryo or on maternal cells (Gardner et al., 2015; Llobat, 2021).

While *in vitro* culture of embryos removes paracrine signaling from maternally-derived factors, the importance of autocrine, or embryo-derived, growth factor signaling is highlighted by the effect of culture density on embryo development. High density (HD) culture involves the culture of a group of embryos in a small volume of media (e.g. 10 embryos/10 μL media) and is associated with better developmental outcomes due to accumulation of embryo-derived growth factors and activation of pro-developmental downstream signaling pathways (Dai et al., 2012; Green & Day, 2013; Lane & Gardner, 1992; Morris et al., 2020). In contrast, low density (LD) culture, in which embryos are cultured individually in a large volume of media (e.g. 1 embryo/100 μL media), produces poorer developmental outcomes due to dilution of these embryo-derived factors (Dai et al., 2012; Green & Day, 2013; Lane & Gardner, 1992; Morris et al., 2020).

Growth factors identified as important in preimplantation embryo development include sphingosine 1-phosphate (S1P), epithelial growth factor (EGF), and proteins in the insulin-like growth factor (IGF) family including IGF1 and IGF binding protein 3 (IGFBP3) (Chia et al., 1995; Green & Day, 2013; Span, 2015). These growth factors have a number of beneficial effects on embryo development, including increasing cleavage rates and blastocyst formation, and protecting oocytes and embryos from apoptosis.

1.4 Sphingosine 1-phosphate (S1P) signalling

1.4.1 S1P synthesis

Sphingolipids are a class of lipids containing a sphingoid base backbone and are essential structural constituents of the cell membrane. Sphingolipids are bioactive signalling molecules and play an important role in the regulation of cell function through the generation of metabolites such as ceramide, sphingosine, and S1P. Synthesis of these metabolites requires production of ceramide through either *de novo* synthesis or through cleavage of sphingomyelin by sphingomyelinase (Figure 1.3) (Wollny et al., 2017).

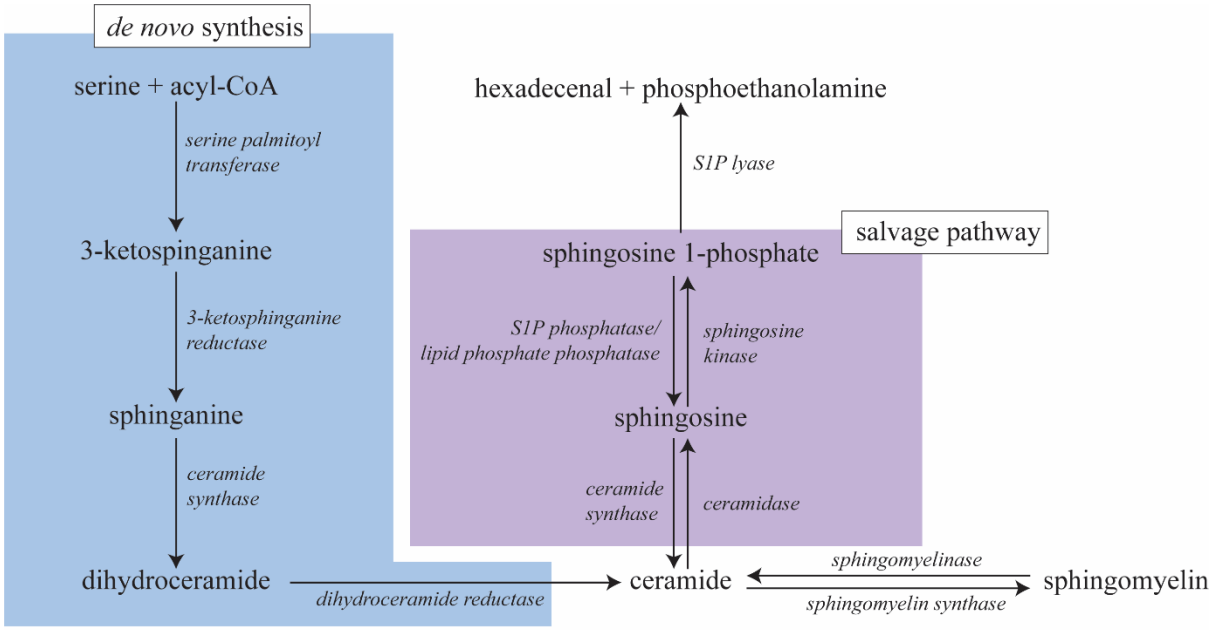


Figure 1.3. Sphingolipid metabolism for S1P synthesis. Production of signalling sphingolipids centres around ceramide, which is produced through either *de novo* synthesis (blue box) or cleavage of sphingomyelin. Ceramide can be hydrolysed to produce sphingosine, which is phosphorylated by sphingosine kinases (SphK1 and SphK2) to form S1P. This process is reversible, as ceramide can be regenerated from S1P through the actions of S1P phosphatases (SGPP1 and SGPP2), non-specific lipid-phosphate phosphatases, and ceramide synthases (CerS) (purple box). S1P can also be irreversibly degraded by S1P lyases (SPGLs), which break S1P down into hexadecenal and phosphoethanolamine.

1.4.2 The sphingolipid rheostat

S1P signalling promotes survival while ceramide promotes apoptosis, and the balance between the two is termed the sphingolipid rheostat (Bektas et al., 2005; Taha et al., 2006). Interconversion between S1P and ceramide is tightly regulated, and cells can be directed towards either an apoptotic or survival fate by altering this balance.

High levels of ceramide within the cell induce cellular arrest and apoptosis. Ceramide production in the endoplasmic reticulum is elevated in response to stress stimuli, including radiation (Mesicek et al., 2010), chemotherapeutic drugs (Alphonse et al., 2004), and cytokines such as tumour necrosis factor α (TNFα) (Garcia-Ruiz et al., 2003; Podbielska et al., 2016). Mitochondrial translocation of ceramide triggers Bax-dependent apoptosis through formation of ceramide channels in the mitochondrial outer membrane, release of cytochrome *c*, and subsequent activation of caspase cascades (Jain et al., 2020). Ceramide can also trigger apoptosis by binding to and stabilising the p53 tumour suppressor, allowing nuclear accumulation of p53 and transcription of genes relating to apoptosis and cell cycle arrest (Fekry et al., 2018; Fischer, 2017).

In contrast, high levels of S1P drive cell fate towards proliferation and survival. Basal levels of S1P are produced by intrinsic sphingosine kinase (SphK) activity but SphK activity can be upregulated by growth factors, hormones, angiogenic factors and cytokines, including endothelin-1 (Giusto & Ashby, 2018), TNF-α (Mastrandrea et al., 2005; Xia et al., 1998), interleukin-1β (Mastrandrea et al., 2005), EGF (Yuan et al., 2022), IGF1 (Wu et al., 2019), vascular endothelial growth factor (VEGF) (Lee et al., 2014) and IGFBP3 (Martin et al., 2009). SphK1 is activated by phosphorylation at Ser225, resulting in translocation of phosphorylated SphK1 (pSphK1) to the plasma membrane and increased production of S1P (Pitson et al., 2003). A number of cellular kinases have been implicated in SphK1 activation, including extracellular signal-related kinase 1/2 (ERK1/2) (Pitson et al., 2003), protein kinase C (PKC) (Johnson et al., 2002), and protein kinase R (PKR) (Qiao et al., 2021). Similarly, phosphorylation of SphK2 by ERK1 (Hait et al., 2007), protein kinase D (Ding et al., 2007) or PKC (Ebenezer et al., 2019) induces translocation of phosphorylated SphK2 (pSphK2) from intracellular compartments such as the nucleus into the cytoplasm.

1.4.3 **S1P signalling via S1P receptors**

S1P produced in the cytoplasm can be translocated out of the cell via sphingolipid-specific transporters such as spinster 2 (Spns2) (Spiegel et al., 2019), where it can act at a family of G-protein coupled receptors (GPCRs; S1PR1-5). Each of the S1PRs has a different expression pattern, with S1PR1-3 being widely expressed and S1PR4 and S1PR5 restricted to the lymph and to the brain and spleen, respectively (Takuwa et al., 2002). This diversity in tissue localisation along with differences in G-protein coupling of the S1PR family enables S1P to trigger different signalling pathways and therefore regulate many cellular processes (Figure 1.4) (Mendelson et al., 2014).

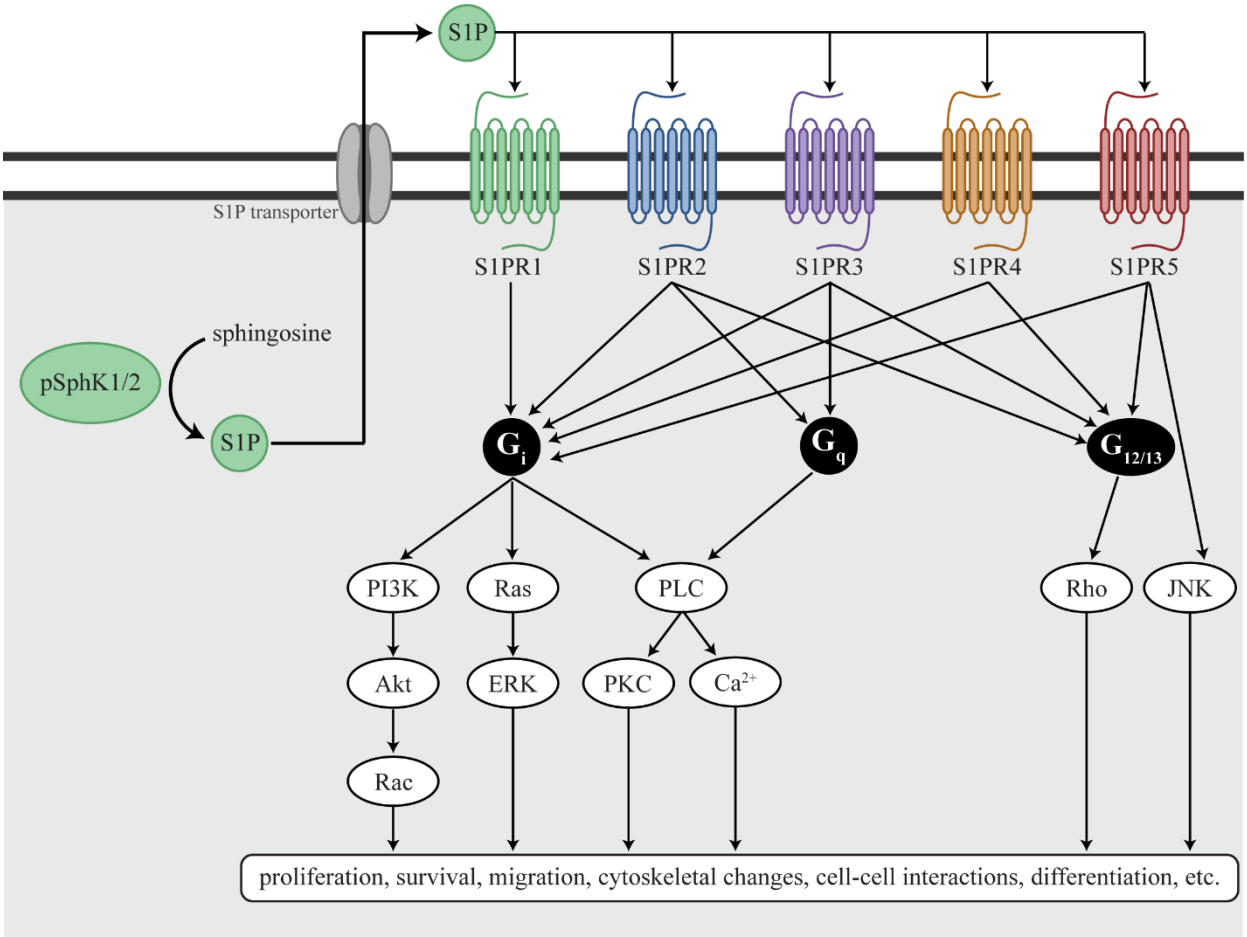


Figure 1.4. Signalling pathways activated by S1P receptors. S1P is produced in the cell by pSphK1 and 2, which catalyse the phosphorylation of sphingosine to S1P. S1P can then be translocated out of the cell by several transporters, including Spns2 and ABC transporters. Extracellularly, it acts as a ligand for the S1P receptors, a family of five GPCRs (S1PR1-5). While all five S1PRs couple to G_i, S1PR2 and S1PR3 also couple to G_q, and S1PR2-5 couple to $G_{12/13}$. This diversity in G-protein coupling allow the S1PR family to activate a wide range of downstream signalling pathways and thus regulate many different cellular functions, including cell proliferation, survival, migration, and differentiation.

Once activated by S1P, S1PRs can signal via transactivation of receptor tyrosine kinases (RTKs) in a ligand-dependent or independent manner. Ligand-dependent RTK transactivation occurs via activation of matrix metalloproteases (MMPs) or a disintegrin and metalloproteinases (ADAMs) to increase cleavage of pro-ligands into their respective ligands (Kilpatrick & Hill, 2021; Wang et al., 2016). Ligand-independent transactivation relies on GPCR-mediated activation of intracellular protein kinases, which then directly phosphorylate the intracellular tyrosine kinase domains of RTKs (Kilpatrick & Hill, 2021). S1PRs are implicated in the transactivation of a number of receptor tyrosine kinases, including the IGF1 receptor (IGF1R) and EGF receptor (EGFR) (Martin et al., 2009), glycoprotein 130 (gp130) (Alshaker et al., 2015) in breast cancer cells, foetal liver kinase 1

(Flk-1) receptor in mouse embryonic stem cells (Ryu et al., 2014), and VEGF receptors in corneal ocular cells (Yasuda et al., 2021).

1.4.4 Receptor-independent intracellular S1P signalling

S1P can signal in the cytoplasm, mitochondria, and nucleus in a range of cell types independently of GPCRs. In the cytoplasm, S1P interacts with targets such as TNF receptor-associated factor 2 (TRAF2) (Alvarez et al., 2010; Harikumar et al., 2014; Park et al., 2016), peroxisome proliferator-activated receptor-γ (PPARγ) (Parham et al., 2015), and atypical PKC (Kajimoto et al., 2019). These interactions activate transcription factors and pro-survival signalling pathways, thus enabling S1P to regulate gene expression to promote normal cellular functioning and protect cells from stress and apoptosis.

S1P produced within the mitochondria by pSphK2 binds homomeric prohibitin 2 and facilitates assembly of the respiratory complex IV in the electron transport chain (Strub et al., 2011). S1P is also linked to the mitochondrial pathway of apoptosis, with S1P produced by pSphK2 promoting cell apoptosis through BAK/Bax-dependent cytochrome c release from the mitochondria (Chipuk et al., 2012).

Nuclear S1P associates with and inhibits histone deacetylase 1 and 2 (HDAC1/2), resulting in increased H3 and H4 histone acetylation and epigenetic regulation (Ebenezer et al., 2019; Hait et al., 2010). Additionally, S1P produced in the nucleus by pSphK2 can directly bind to and stabilise human telomerase reverse transcriptase (hTERT), thus promoting telomere stability and cell proliferation (Selvam et al., 2015).

1.5 S1P is a growth factor present in the ovary, reproductive tract and embryo

1.5.1 Role of S1P in folliculogenesis

In ovarian cells, S1P signalling is associated with follicular health and production of sex steroids such as oestradiol and anti-Mullerian hormone (Guzel et al., 2018; Tan et al., 2014; Tsai et al., 2014). S1PRs are present in the ovary, with S1PR1, 2, 3, and 5 expressed in human granulosa lutein cells (Becker et al., 2011). Within the follicle, S1P is bound to high density lipoprotein, the sole lipoprotein and main cholesterol carrier in follicular fluid (Becker et al., 2011; Browne et al., 2008). High density lipoprotein-associated S1P in follicular fluid is correlated with improved survival of the follicle, with higher concentrations detected in healthy bovine follicles compared to atretic follicles (Hernandez-

Coronado et al., 2015). Similarly, in humans, depleted levels of S1P in the follicular fluid are associated with ovarian hyperstimulation syndrome, a condition characterised by follicular fluid leakage and rupture of follicles due to the loss of endothelial barrier integrity within the ovary (Scotti et al., 2016).

S1P in the ovary appears to originate from supporting cells of the follicle in response to maternal signalling rather than the maternal blood supply. Bovine granulosa cells can upregulate S1P production by pSphK1 in response to treatment with VEGF and follicle stimulating hormone (FSH) (Hernandez-Coronado et al., 2016). Treatment of isolated bovine granulosa cells with VEGF or FSH increases production of S1P by pSphK1, thus promoting proliferation. Treatment of these cells with 0.1-1 μM S1P increases cellular proliferation, while treatment with 10 μM S1P decreases cell number by increasing apoptosis, suggesting that there is a fine balance of endogenous S1P signalling in the ovary that controls follicular growth and survival.

The addition of 200-400 μM S1P increases survival and decreases apoptosis of *in vitro* cultured primordial and secondary follicles in human ovarian tissue (Guzel et al., 2018; Soleimani et al., 2011). Similarly, S1P prevents apoptosis of ovarian cells induced by stressors such as chemotherapy (Li et al., 2014; Tan et al., 2014), H_2O_2 (Nakahara et al., 2012), and cryopreservation (Tsai et al., 2014) in a range of species. S1P also protects ovarian cells from apoptosis *in vivo*. In rats, S1P protects ovaries from radiation-induced tissue injury when co-administered with x-ray radiotherapy, attenuating follicular atrophy and maintaining serum anti-Mullerian hormone levels (Zhao et al., 2020). Radiation decreases expression of mitochondrial genes necessary for mitochondrial respiration, Ca^{2+} homeostasis, and inhibition of oxidative stress, and S1P protects ovarian function by preventing this decrease in gene expression (Zhao et al., 2020).

1.5.2 Role of S1P in oocyte maturation and fertilisation

S1P signalling is important for bi-directional communication between the oocyte and surrounding granulosa cells that is essential for maturation and developmental competence of the oocyte (Turathum et al., 2021). Treatment of mouse oocytes with 100-500 nM S1P during *in vitro* maturation (IVM) has no effect on oocyte maturation or fertilisation, but increases subsequent blastocyst formation from 40% to 62-66%, and decreases apoptosis from 10.7% to 5.4-6.5% in the blastocyst (Jee et al., 2011). This suggests S1P improves the developmental competence of the oocyte but does not directly impact the maturation

process. However, S1P does have a direct effect on oocyte maturation, both *in vivo* and *in vitro*: treatment of porcine oocytes with 50 nM S1P improves maturation from 64% in control to 84% and fertilisation from 35% in control to 50%, but has no effect on subsequent embryo development (Lee & Koo, 2012). Endogenous S1P production by pSphK1 can be stimulated by luteinising hormone/EGF/EGFR signalling in mouse granulosa cells within cumulus-oocyte complexes (COCs) (Yuan et al., 2022). Increased S1P in the COC decreases the binding affinity of natriuretic peptide receptor 2 for its ligand, thus releasing the cGMP-mediated arrest of the oocyte and allowing oocyte maturation to proceed (Yuan et al., 2022).

There are also interactions between EGF and S1P in porcine oocyte maturation. Both S1P and the S1P analogue phytosphingosine 1-phosphate (P1P) improve meiotic maturation of porcine oocytes from 64% to 77-86%, though P1P requires the presence of EGF (Lee & Koo, 2012; Park et al., 2019). S1P and P1P also improve blastocyst formation in the presence of EGF (Lee & Koo, 2012; Park et al., 2019). Oocytes matured with 1 μM P1P subsequently have higher percentages of cleavage and blastocyst formation, as well as increased blastocyst cell number from approximately 70 to 100 cells (Park et al., 2019). Mechanistically, P1P acts through a number of mechanisms to improve oocyte maturation and developmental competence, including; (i) reduction of reactive oxygen species, (ii) upregulation of genes relating to cumulus expansion, antioxidant regulation, and developmental competence, (iii) activation of pro-developmental ERK1/2 and Akt signalling, and (iv) downregulation of pro-apoptotic JNK signalling (Park et al., 2019). As blastocyst formation is also improved by addition of S1P to IVM medium in the presence of EGF (Lee & Koo, 2012), it is likely that S1P acts via some or all of these mechanisms in the oocyte.

Upon maturation of an oocyte within a preovulatory follicle, ovulation occurs, expelling the oocyte into the oviduct (Figure 1.1). S1P/S1PR1 and S1PR3 signalling are implicated as treatment of human granulosa cells with 300 nM S1P *in vitro* results in a 3-4-fold increase in cellular communication network factor 2 (*Ccn2*) mRNA and protein, leading to 5-6-fold increase in cyclooxygenase 2 (COX2) protein and a 5-fold increase in prostaglandin E2 (PGE2) production, both of which are crucial for ovulation (Cheng et al., 2016; Hu et al., 2019). Following ovulation, the follicle undergoes tissue reorganisation and neovascularisation to form the corpus luteum, a structure essential for hormone synthesis and secretion as the oocyte travels to the uterus. S1P promotes neovascularisation of the

corpus luteum *in vitro* through stimulation of endothelial proliferation and angiogenesis, and stimulates migration of human granulosa lutein cells through S1P/S1PR3/RAC1 signalling (Becker et al., 2011; von Otte et al., 2006).

S1P is involved in fertilisation of the oocyte, particularly in the acrosome reaction in sperm that facilitates penetration of the zona pellucida and fusion of the gametes, though the mechanisms are unclear. Several enzymes involved in sphingolipid metabolism are present in human spermatozoa, including sphingomyelinase within the head, and SphK1 and ceramidase within the acrosome of the capacitated sperm cell (Matsumoto et al., 2005; Suhaiman et al., 2010; Vaquer et al., 2020). Both S1P and ceramide induce a rise in intracellular Ca^{2+} that triggers the exocytosis of the acrosomal vesicle (Suhaiman et al., 2010; Vaquer et al., 2020). However, despite ceramide being a precursor to S1P, ceramide does not induce exocytosis through increasing the synthesis of S1P, as inhibition of SphKs does not prevent the ceramide-induced acrosome reaction (Vaquer et al., 2020). This implies that S1P and ceramide trigger the acrosome reaction through two distinct pathways. However, the conditions upon which each pathway is activated, and their purpose *in vivo* are not fully understood.

Finally, S1P has a protective effect on ovarian tissue and oocytes. Supplementation of bovine IVM medium with 50 nM S1P prevents both thermal stress-mediated decreases in cleavage and blastocyst formation (Roth & Hansen, 2004), and 10 nM S1P prevents apoptosis triggered by ceramide and chemotherapeutic agents in oocytes and ovarian tissue (Jurisicova et al., 2006; Perez et al., 2005). Thermal stress, ceramide, and chemotherapeutic agents are all associated with translocation of Bax to the mitochondria, triggering apoptosis (Gu et al., 2015; Panaretakis et al., 2002; Perez et al., 2005). In Jurkat cells, S1P reduces apoptosis from 60% in control to 40% by blocking this translocation to the mitochondria in a MEK/ERK-dependent manner (Betito & Cuvillier, 2006), and this pathway is likely how S1P protects oocytes from apoptosis.

1.5.3 S1P in preimplantation embryo development

In humans, both SphK1 and S1PR1 are expressed in the epithelial cells of the fallopian tube, and S1P itself is present in fallopian tube tissue at a concentration of 382 ng/L (Gao et al., 2013). S1PR1 is expressed by the oocyte and all stages of mouse preimplantation development (Rayhanna, 2019). If expression of SphK1 and S1PR1 is consistent between species, S1P produced by oviductal cells could act in both an autocrine manner on oviductal

cells, and a paracrine manner on the developing embryo to regulate early stages of preimplantation development.

Exogenous S1P increases the basal tone of both human and rat *ex vivo* oviducts, suggesting S1P signalling may be important in the movement of the embryo to the uterus (Gao et al., 2013). However, the concentration of S1P in the luminal fluid of the oviduct has not been measured in either humans or mice and so it is unclear whether S1P is secreted into the luminal fluid to act on the embryo *in vivo*. *In vitro,* exogenous S1P promotes cleavage of mouse embryos, with the addition of 25 nM S1P to mouse embryo culture increasing the developmental rate of embryos to all cleavage stages (Span, 2015). However, addition of 100 nM S1P to mouse embryo culture reduces blastocyst formation while culture of porcine embryos with 50 nM S1P does not affect embryo cleavage, but does improve blastocyst formation from 20% in control to 30% in S1P, and also reduces the number of apoptotic cells from 5.1 in control to 2.8 (Lee & Koo, 2012; Span, 2015). There are also differences in the protective effect S1P has on embryos between murine and human studies. While 25-100 nM S1P protects mouse preimplantation embryos from ceramide-induced apoptosis (Guo et al., 2013), supplementation of human embryo culture medium with 20 μM S1P reduces fertilisation from 67% in control to 52%, but also reduces embryo fragmentation (Hannoun et al., 2010). This discrepancy is likely due to the differences in the concentrations used or other methodological differences, though could suggest species-specific differences in expression of the S1PRs and therefore activation of different downstream signalling pathways.

Ceramide generation is increased by stressors such as thermal shock in oocytes and embryos (Lee et al., 2021; Taha et al., 2006). Addition of 50 μM ceramide to mouse embryo culture induces apoptosis through activation of caspase-9, subsequently inhibiting proliferation and reducing blastocyst formation from 89% in control to 29% (Guo et al., 2013). *Asah1* encodes acid ceramidase that catalyses the conversion of ceramide to the S1P precursor sphingosine. Knockout of *Asah1* induces a 2-cell block in mice, which can be partially alleviated by treatment with 2 μM S1P (Eliyahu et al., 2007), suggesting that production of S1P by the embryo is necessary for normal progression through the cleavage stages of development. Embryo-derived S1P signalling via S1PR2 and the mammalian target of rapamycin (mTOR) and PI3K/Akt pathways is required for cavitation and the later stages of preimplantation development in mice (Chi et al., 2020). This implies S1P signalling

is present throughout preimplantation development but the expression and localisation of the SphKs and S1PR2-5 required for endogenous S1P signalling in the embryo has not been investigated.

1.5.4 S1P in implantation and placentation

Enzymes involved in S1P metabolism are present in the endometrial tissue of the uterus and are dysregulated in disorders such as endometriosis and uterine cancers in humans and mice (Santulli et al., 2012; Skaznik-Wikiel et al., 2006). Both *Sphk1* and *Sphk2* mRNAs are detectable in human endometrial cells, with *Sphk1* mRNA being much more abundant than *Sphk2* (Bernacchioni et al., 2021; Santulli et al., 2012). The mRNA for enzymes involved in degradation of S1P, including *Sgpl1*, *Sgpp1*, and *Sgpp2* ae also present in human endometrial cells (Santulli et al., 2012). Additionally, mRNA for all five S1PRs are expressed in the human endometrium, with that for *S1pr3* being most abundant, followed by *S1pr1* (Bernacchioni et al., 2021; Brunnert et al., 2014). The presence of both S1P turnover enzymes and S1PRs implies that there is autocrine or paracrine S1P signalling in the uterus, and the S1P produced could act on the embryo to support implantation.

Expression of constituents of the S1P pathway are altered during decidualisation of the uterine endometrium. *S1pr1-3* mRNA and enzymes involved in S1P turnover, including *Sphk1*, *Sgpl1* and *Sgpp1*, are upregulated during decidualisation of endometrial cells, suggesting that higher turnover of S1P and increased S1P signalling are required for normal decidualisation process in both humans and mice (Brunnert et al., 2014; Skaznik-Wikiel et al., 2006). Immunohistochemical staining for S1PR1 and S1PR3 shows that during decidualisation in mice, expression of these receptors is upregulated in the microvascular network of the mesometrium, where it is thought to be involved in coordination of uterine mesometrial angiogenesis during embryo implantation (Skaznik-Wikiel et al., 2006). SphK-deficient female mice ($Sphk1^{-/-}$, $Sphk2^{+/-}$) display impaired uterine decidualisation due to increased decidual cell death, decreased proliferation in stromal cells, and haemorrhage of decidual blood vessels (Mizugishi et al., 2007). This results in abnormal development and lethality by E7.5.

S1PRs are also expressed by the extravillous trophoblast (EVT) cells of the embryo during early pregnancy humans, though there is conflicting evidence for the role of S1P in EVT cell migration: 1-10 nM S1P promotes the migration and invasion of human EVT cells through activation of the S1PR1 and subsequently the MEK-ERK signalling pathway (Yang

et al., 2014). However, activation of the S1P/S1PR2/Rho axis by 0.1-10 μM S1P inhibits EVT cell migration, and this effect is abolished in the presence of vitamin D (Westwood et al., 2017). These conflicting results could be due to the different concentration of S1P used, as well as the use of primary human trophoblasts in the latter study as opposed to the HTR8/SVneo extravillous trophoblast cell line which contains a mixed population of trophoblast and stromal cells (Abou-Kheir et al., 2017).

After implantation of the embryo, S1P is heavily involved in the formation and maintenance of the placenta. In mice, knockout of alkaline ceramidase 2, which prevents the conversion of ceramide to sphingosine, decreases placental sphingolipid levels and compromises vascular integrity due to foetal blood vessel atrophy (Li et al., 2020a). This results in placental haemorrhage and embryonic lethality in 50% of E12.5 mice. Similarly, in humans, dysregulation of S1P signalling results in preeclampsia due to disruption of the integrity of the placental vasculature (Dobierzewska et al., 2016; Gaudio et al., 2020b). There is a reduced *S1pr1*, *S1pr3*, and *Sphk1* mRNA in preeclamptic patients, and a corresponding increase in anti-angiogenic, pro-inflammatory *S1pr2* mRNA in the chorionic villous tissue, highlighting the role of S1P in maintenance of placental vascular integrity (Dobierzewska et al., 2016). The vasoprotective effect of S1P in the placenta is mediated by ERK and phospholipase C (PLC) signalling, stimulating endothelial cell proliferation (Gaudio et al., 2020c) and attenuating TNFα-mediated inflammation (Gaudio et al., 2020a).

1.6 Conclusions

The ability of S1P to activate different signalling pathways according to the localisation of its production and differential receptor binding enables its involvement in many aspects of reproduction. As in other cell types, S1P in the reproductive system primarily acts to promote cell proliferation, survival, and migration both *in vivo* and *in vitro*, though in many cases the signalling pathways underpinning this are complex and poorly understood. Further studies on the function and mechanism of S1P in processes such as embryogenesis could be important in addressing the decreased success rates when replicating these processes *in vitro*.

1.7 Aims

The first aim of this thesis is to confirm the mRNA and protein expression of S1PR1 in mouse preimplantation embryos and determine if exogenous and embryo-derived S1P signals via S1PR1 to promote embryo development *in vitro*.

The second aim is to investigate the mRNA and protein expression of the other four S1PRs, S1PR2-5, as potential mediators of S1P signalling in the embryo, and to determine the function of S1PR2-5 in preimplantation embryo development by using small molecule inhibitors of these receptors individually and in combination.

The final aim is to determine if SphKs are expressed in the embryo as a mechanism for endogenous production of S1P, and to confirm if the S1P produced is required for embryo development *in vitro* using small molecule inhibitors of the SphKs.

CHAPTER 2

Methods and materials

2.1 Animal husbandry and superovulation

2.1.1 Animal husbandry

Experiments involving the use of animals were conducted in accordance with the Australian Code of Practice for Use of Animals in Research and approved by the University of Sydney Animal Care and Ethics Committee (approval numbers 1569, 1876, 1877, 2160). Quackenbush Swiss (QS) mice were used in experiments and housed in a controlled, pathogen-free environment in 12 × 15 × 35 cm cages. Housing was under a 12-h light-dark cycle and maintained at 20-22 ˚C and 55% relative humidity. Female mice were caged in groups of up to 7, while male stud mice were caged individually. All animals were allowed access to standard chow and water *ad libitum*.

2.1.2 Superovulation of female mice

Superovulation was induced in female mice by intraperitoneal injection of 10 IU pregnant mare's serum gonadotrophin (PMS; Folligon, Intervet), followed by 10-20 IU human chorionic gonadotrophin (hCG; Chorulon, Intervet) 48 ± 2 h later. To obtain zygotes, after hCG injection, female mice were placed individually with a male stud aged 2-10 months for mating. The presence of a vaginal plug 24 h later indicated successful mating. Female mice were not mated prior to oocyte isolation.

2.2 Preparation of embryo isolation and culture media

2.2.1 Stock solutions

Stock solutions required for embryo isolation and culture were prepared according to table 2.1. All reagents used were obtained from Sigma Aldrich unless otherwise specified. All stocks were made using MilliQ® water. Glucose, pyruvate, $NaHCO_3$, and penicillin were made fresh for each use, while all other stocks were stored at 4 ˚C. NaCl, KCl, and $NaHCO_3$ were adjusted to 2000 mOsm, 200 mOsm, and 2000 mOsm respectively using a vapour pressure osmometer (Vapro 5600, Wescor, USA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) stock was adjusted to a pH of 7.4 using 2 M NaOH.

Table 2.1. Stock solution for preparation of embryo isolation and culture media

Reagent	Concentration	Amount/volume of H_2O
NaCl	1 M	2920 g/50 mL
KCl	0.1 M	370 mg/50 mL
Na_2EDTA	0.001 M	18.6 mg/50 mL
HEPES	0.154 M	1835 mg/50 mL
KH_2PO_4	0.1 M	136.1 mg/10 mL
$MgSO_4 \cdot 7H_2O$	0.1 M	246.5 mg/10 mL
$CaCl_2 \cdot 2H_2O$	0.17 M	251.5 mg/10 mL
$NaHCO_3$	1 M	420.1 mg/5 mL
Na Pyruvate	0.1 M	55 mg/5 mL
Glucose	0.1 M	90.1 mg/5 mL
Penicillin	6 mg/mL	30 mg/5 mL
NaOH	2 M	800 mg/10 mL
HCl	1 M	1 mL/10 mL

2.2.2 Embryo media preparations

The medium used for embryo and oocyte isolations was HEPES-buffered modified human tubal fluid (HEPES ModHTF) medium. Embryos were then cultured in modified human tubal fluid (ModHTF) medium. Stock reagents were combined according to Table 2.2. HEPES ModHTF and ModHTF medium were buffered to pH 7.4 using 2M NaOH and 1M HCl respectively and adjusted to an osmolality of 270 mOsm using MilliQ® water. Bovine serum albumin (BSA) was added to both media at 0.3 mg/mL before media was filtered with a 0.22 μm sterile filter (Millipore). Media were kept at 4 ˚C for a maximum of two weeks before discarding.

Table 2.2. Composition of ModHTF and HEPES ModHTF media

Reagent	Final concentration	
	ModHTF	**HEPES-ModHTF**
Lactic Acid (60%)	21.4 mM	21.4 mM
NaCl	85 mM	85 mM
KCl	4.6 mM	4.6 mM
KH_2PO_4	0.4 mM	0.4 mM
$MgSO_4 \cdot 7H_2O$	0.2 mM	0.2 mM
$NaHCO_3$	25 mM	4 mM
Na_2EDTA	0.11 mM	-
Na Pyruvate	0.4 mM	0.4 mM
Glucose	2.8 mM	2.8 mM
Penicillin	0.06 mg/mL	0.06 mg/mL
$CaCl_2 \cdot 2H_2O$	2.04 mM	2.04 mM
HEPES	-	21 mM

2.3 Embryo isolation and culture

2.3.1 Isolation of mouse oviducts

Female mice were euthanised by cervical dislocation 24 h post hCG injection to obtain zygotes or 13 h post hCG to obtain oocytes. The abdomen was soaked with ethanol and a midline abdominal incision made to expose the underlying peritoneum. Two transverse incisions were made to open the peritoneum and the visceral organs moved aside to expose the ovaries, oviducts, and uterine horns of the reproductive tract. The mesometrium was separated from the reproductive tract using fine forceps and the ovary was gently separated from the oviduct. Finally, the lateral section of the uterine horn was cut, and the oviduct transferred to a 35 mm dish containing phosphate buffered saline (PBS).

2.3.2 Isolation and culture of oocytes and zygotes

Microdissection of the oviducts was conducted using a dissection microscope (Zeiss, Germany) with a heated stage. Dissected oviducts were transferred to pre-warmed HEPES

ModHTF and treated with hyaluronidase to disperse cumulus cells. After washing, embryos were cultured in ModHTF medium overlaid with mineral oil. Medium was prewarmed and equilibrated to 37 ˚C and 5% CO_2. Embryos were cultured either in groups of 10 per 10 µL droplet for HD culture, or individually in each well of a round bottom 96-well plate (Corning) containing 100 µL of medium for LD culture. Embryos were cultured as per Table 2.3 to the required developmental stage.

Table 2.3. Collection timing for *in vitro* and *in vivo* developed embryos

Embryo stage	*In vitro* (h post hCG)	*In vivo* (h post hCG)
Oocyte	-	13
Zygote	-	24
2-cell	48	46
4-cell	60	52
8-cell	76	64
Morula	92	80
Early blastocyst	106	92
Hatched blastocyst	144	-

2.3.3 Isolation of *in vivo* developed embryos

To obtain *in vivo* developed embryos at the relevant developmental stage, mice were euthanised according to the times in Table 2.3. Oviducts were isolated (section 2.3.1), with the uterine horns additionally dissected to account for embryos that had travelled into the uterus during the later stages of development. Dissected reproductive tissues were placed in cold HEPES-ModHTF and media flushed through using a 30-gauge needle. Embryos were washed in HEPES-ModHTF prior to collection.

2.4 Immunofluorescence staining of oocytes and embryos

2.4.1 Embryo collection and treatment

Zygotes were isolated and cultured in ModHTF medium to each developmental stage. Oocytes and embryos were treated with pronase at 37 ˚C for 2-3 min to dissolve the zona pellucida before fixing in 4% paraformaldehyde for 30 min at room temperature.

Oocytes and embryos were then washed three times in PBS + 1 mg/mL polyvinyl alcohol (PVA). Oocytes and embryos were then permeabilised in PBS + 1mg/mL PVA + 0.3% Triton X100 for 30 min before being washed in PBS + 1 mg/mL PVA. Oocytes and embryos were blocked in PBS + 1 mg/mL PVA + 0.7% BSA + 0.1% Tween-20 for 30 min.

2.4.2 Co-localisation of pSphK2 and S1PR5 with autophagosomes

Zygotes were cultured at HD in ModHTF medium to the 4-cell and 8-cell stages before treatment with pronase to remove the zona pellucida (section 2.4.1). Embryos were then stained with CYTO-ID Autophagy detection kit 2.0 (1:500; Enzo Life Sciences #ENZ-KIT175-0050) before fixing and staining to visualise the pSphK2 or S1PR5 (section 2.4).

2.4.3 Immunostaining with primary and secondary antibodies

Primary and secondary antibodies were diluted to working concentrations in PBS + 1 mg/mL PVA + 0.7% BSA + 0.1% Tween-20. Oocytes and embryos were incubated in primary antibody overnight at 4 ˚C and then washed three times with PBS + 1 mg/mL PVA + 0.7% BSA + 0.1% Tween-20, with the last wash lasting 30 min. Oocytes and embryos were then incubated in secondary antibody for 1-2 h at room temperature in the dark before being washed in PBS + 1 mg/mL PVA + 0.7% BSA + 0.1% Tween-20, with the last wash lasting 30 min. Following staining, oocytes and embryos were mounted onto coverslips in 3-4 µL Vectashield containing DAPI and sealed with Vaseline.

2.4.4 Confocal microscopy

Confocal microscopy was used to visualise secondary antibody fluorescence as well as nuclear fluorescence. Slides were imaged using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany) at 20× objective magnification.

2.5 Quantification of genes relating to S1P signalling by RT-qPCR

2.5.1 Sample extraction and cDNA synthesis

The PCR protocol used in this thesis was adapted from (Joglekar et al. 2010). Samples of 18-30 embryos were run in triplicate, with at least 3 samples per developmental stage. RNA was isolated using 1 µL glycogen and 5 µL chloroform. Samples were manually shaken for 1 min, then centrifuged at 12,000 *g* and 4 ˚C for 15 min. The aqueous phase was transferred to a 0.2 mL PCR tube and 10 µL isopropanol added. Samples were then incubated for 10 min at room temperature, followed by centrifugation at 12,000 *g* and 4 ˚C for 15 min. The supernatant was then removed, and the pellet washed with 100 µL of 75%

ethanol before centrifugation at 12,000 *g* and 4 ˚C for 15 min. The RNA pellet was resuspended in RNAase-free H_2O (Baxter Healthcare). cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Massachusetts, US, #4368814). Positive and negative reverse transcriptase cDNA samples were reverse transcribed using a thermocycler (Eppendorf Mastercycler gradient) under the following cycling conditions:

1. 25 ˚C for 10 min
2. 37 ˚C for 2 h
3. 70 ˚C for 10 min
4. 4 ˚C hold

cDNA was stored at -20 ˚C until use.

2.5.2 RT-qPCR

qPCR was performed using TaqMan™ primers (Table 4.3; Applied Biosciences, Thermo Fisher). Samples were run in duplex with *Rn18s* as a housekeeping gene for normalisation. Master mixes were made for each gene of interest and contained TaqMan™ FAST Universal PCR Master Mix (2x; #4352042), Taqman™ primer for the gene of interest, *Rn18s* primer, and RNAase-free H_2O. 4 µL master mix and 1 µL cDNA were added to each well of a 364-well PCR plate (Applied Biosystems, #4349606). The PCR plate was sealed with an adhesive plate seal, vortexed, and then centrifuged at 12,000 *g* and 4 ˚C for 5 min. qPCR reactions were run using the QuantStudio v7 Flex under the following conditions:

1. 95 ˚C for 20 seconds
2. 45 cycles of:
    i. 95 ˚C for 3 seconds
    ii. 60 ˚C for 30 seconds

2.5.3 RT-qPCR analysis

Thresholds for data analysis were set using Design and Analysis Software v2.6.0 (Applied Biosystems, Thermo Fisher) and CT values exported to Microsoft Excel. ΔΔCT values were calculated from the triplicate mean for each gene of interest and *Rn18s*.

CHAPTER 3

The expression and function of S1PR1 in the mouse preimplantation embryo

3.1 Introduction

S1P is a growth factor in the mammalian maternal reproductive tract and follicular fluid, and regulates of many basic cellular processes, including cell proliferation, migration, and apoptosis (Hernandez-Coronado et al., 2015; Nakahara et al., 2013; Yuan et al., 2021). S1P levels must be tightly regulated for normal cellular function, and this is primarily regulated by pSphKs which phosphorylate sphingosine to produce S1P, and sphingosine lyase which irreversibly degrades S1P (Figure 1.3). While there is constitutive basal activity of pSphKs, their expression and activity can be upregulated by cytokines, hormones, and growth factors (Pitson et al., 2003). S1P produced within the cell can signal intracellularly or can be translocated out of the cell to act at a family of five G-protein coupled receptors, S1PR1-5 (Figure 1.4). While the S1PRs are highly homologous, they have different expression patterns and signal via different G-proteins, facilitating the activation of many different downstream pathways (Mendelson et al., 2014). S1PR1 is the most widely expressed of the S1PRs and is primarily coupled to $G_{i/o}$, thus activating classical signalling pathways that modulate cell proliferation and survival, such as the Ras/MAP kinase cascade, phosphatidylinositol-3-phosphate kinase (PI3K)/Akt, and inhibition of adenylyl cyclase (Badawy et al., 2017; Brinkmann, 2007; Okamoto et al., 2011).

S1P is present at approximately 170 nM S1P in the follicular fluid surrounding the oocyte (von Otte et al., 2006). Follicular S1P concentration is positively correlated with follicle health, with healthy bovine follicles containing threefold higher concentration of S1P than atretic follicles (Hernandez-Coronado et al., 2015). *In vitro,* addition of S1P to culture media improves rodent embryo development by increasing developmental rate to all cleavage stages and reducing fragmentation and apoptosis (Hannoun et al., 2010; Jee et al., 2011; Span, 2015). However, the pathway by which S1P acts to improve embryo development has not been investigated but is likely to occur via activation of the S1PRs. Nor has the concentration of S1P in the oviductal and uterine fluids been measured.

S1PR1 is expressed throughout the female reproductive system, including in the granulosa cells of the ovary (Liu et al., 2022), and the uterine endometrium (Santulli et al., 2012). S1PR1 is necessary for normal reproductive functioning, with dysregulation of S1P/S1PR1 signalling associated with reproductive disorders such as endometriosis and preeclampsia (Gaudio et al., 2020b; Santulli et al., 2012). S1PR1 is expressed in the mouse oocyte as well as all stages of preimplantation development and treatment of cleavage stage

embryos with S1P induces changes in S1PR1 localisation, implying that the receptor is functional in the embryo (Rayhanna, 2019). However, it is unknown whether S1PR1 signalling is required for preimplantation development, or if the S1P-mediated increase in cleavage rate is mediated by S1PR1.

S1PR1 is essential for post-implantation embryo development. In mice, genetic deletion of S1PR1, but not S1PR2 and S1PR3, results in embryonic lethality by E14.5 due to vascular malformation (Kono et al., 2004). Deletion of either S1PR2 or S1PR3 alongside S1PR1 deletion produces more severe defects than S1PR1 null embryos, and deletion of all three S1PRs results in embryonic lethality by E12.5. This suggests that while there are redundancies in function within the S1PR family, S1PR1 signalling is essential for embryo development, including formation of the vascular system. S1PR1 signalling has also been implicated in other processes necessary for embryonic development, such as placentation. S1P-mediated activation of S1PR1 promotes trophectoderm cell invasion via the mitogen-activated protein kinase (MAPK)/ERK pathway (Yang et al., 2014). Knockdown of *Sphk1* or *S1pr1* mRNA or protein are associated with a number of conditions involving poor placentation, including preeclampsia and recurrent spontaneous abortion, while upregulation of S1PR1 occurs in choriocarcinomas, a malignant hyperinvasive trophectoderm tumour (Dobierzewska et al., 2016; Miao et al., 2020). While S1PR1 signalling is involved in blastocyst implantation and placentation, and subsequent vasculogenesis in the embryo, the role of S1PR1 signalling in preimplantation development has not been investigated.

The current study aimed to determine the localisation of S1PR1 in the oocyte and preimplantation embryo, as well as the role of exogenous and embryo-derived S1P signalling via the S1PR1 throughout development. This will facilitate understanding of the molecular mechanisms underpinning preimplantation embryo development, and potentially improve outcomes for *in vitro* embryo culture in clinical, research, and agricultural settings.

3.2 Methods

3.2.1 Animals

ARC Swiss mice (Animal Resource Centre, Perth) were used in parts of this study and housed as per section 2.1. All other experiments were conducted with QS mice.

3.2.2 Immunofluorescence staining of oocytes and embryos for the S1PR1

Zygotes were isolated and cultured at HD to the zygote, 2-cell, 4-cell, 8-cell, morula, and hatched blastocyst stages as per Table 2.3. Oocytes and embryos were collected and immunostained (section 2.4). All preimplantation developmental stages were stained to visualise S1PR1, and blastocysts were additionally stained to visualise GATA4, a marker of hypoblast cells (Table 3.1)

Table 3.1. Primary and secondary antibody dilutions for immunofluorescence staining of S1PR1

Primary antibody	Dilution	Secondary antibody	Dilution
S1PR1 Antibody (R&D Systems #713412)	1:75	Alexa Fluor 488 Goat anti-Rat IgG (Cell Signalling #4416S)	1:100
		Alexa Fluor 546 Goat anti-Rat IgG (#A-11081)	1:100
GATA4 antibody (C-20) (Santa Cruz #sc-1237)	1:100	CruzFluor 488 donkey anti-goat IgG (sc-362255)	1:100
FITC Rat IgG2a, κ Isotype Control antibody (BioLegend #400505)	1:50	-	-

3.2.3 Culture of embryos in the presence of S1PR1 inhibitor

NIBR0213 is a potent and selective S1PR1 antagonist (Quancard et al. 2012). Zygotes were cultured at either HD or LD in ModHTF medium containing 0.1% dimethyl sulfoxide (DMSO; vehicle control), or 1, 2.5, 5 or 10 μM NIBR0213 (Sigma Aldrich) to the hatched blastocyst stage. Additionally, embryos were cultured at LD in the presence of 10 μM NIBR0213 during only the zygote to 8-cell stages (up to 78 h post hCG injection), or only during the 8-cell to blastocyst stages (from 78 h post hCG onwards). Embryo development was scored daily and the percentage of embryos that had reached each developmental stage recorded.

3.2.4 Culture of S1P-treated embryos in the presence of S1PR1 inhibitor

Zygotes were cultured at LD in ModHTF medium or ModHTF medium containing 0.1% DMSO (vehicle control), or 25 nM S1P, or 2.5 µM NIBR0213, or 25 nM S1P + 2.5 µM NIBR0213 to the early blastocyst stage. Wells were not overlaid with mineral oil to prevent S1P partitioning into the oil. Embryo development was scored daily and the percentage of embryos that reached each developmental stage recorded. Additionally, development was scored at 68 h post-hCG and the percentage of 4-cell embryos compared to 5-8-cell embryos was recorded.

3.2.5 Analysis of translocation of S1PR1 following treatment with S1P

Embryos were cultured at LD for 24 h to the 2-cell stage then treated with pronase to remove the zona pellucida (section 2.4.1). Embryos were treated with 25 nM S1P for 5, 10, 15, 30, or 120 min, or treated with 25 nM S1P ± 2.5 µM NIBR0213 for 10 min. Embryos were fixed and stained for the S1PR1 (sections 2.4 and 3.2.2). Fluorescence intensity was quantified using Fiji by ImageJ software and normalised to background fluorescence. Measurements were taken at three locations within each blastomere – at the cell membrane, within the cytoplasm, and within the nucleus. Three measurements were taken for each location within a single blastomere and a ratio of the fluorescence values at the cell membrane and within the cytoplasm was calculated.

3.2.6 Co-culture attachment assays of embryos with Ishikawa cells

3.2.6.1 Ishikawa cell line

Ishikawa cells (passage 14) are an endometrial adenocarcinoma cell line that possesses endometrial receptivity and were used in this study for blastocyst co-culture attachment assays (Nishida et al., 1985; Zhang et al., 2012). Co-culture of mouse blastocysts and Ishikawa cells is an established model of early implantation (Zhang et al., 2012). Cryopreserved Ishikawa cells were kindly provided by Dr Samson Dowland (University of Sydney).

3.2.6.2 Preparation of cell-culture medium

Sterile media were prepared (Table 3.2) and stored at 4 ˚C. Complete cell medium was used for the culture of Ishikawa cells prior to blastocyst transfer. Serum-starved medium was used for co-culture of blastocysts with Ishikawa cells for attachment assays to mitigate the effects of growth factors present in serum.

3.2.6.3 Plating Ishikawa cells for co-culture attachment assays

Cells were seeded onto 24-well plates at a density of ~50,000 cells/well in complete medium. Plates were incubated at 37 ˚C and 5% CO_2 until cells were confluent, with medium replaced every 1-2 days.

Table 3.2. Composition of cell culture media

Reagent	Complete medium (10% FBS)	Serum-starved medium (0.01% FBS)
Dulbecco's Modified Eagle Medium (DMEM) [+] D-glucose, L-glutamine [-] sodium pyruvate	89% (v/v)	99% (v/v)
Foetal Bovine Serum (FBS)	10% (v/v)	0.01% (v/v)
Penicillin	100 IU/mL	100 IU/mL
Streptomycin	100 µg/mL	100 µg/mL

3.2.6.4 Co-culture of mouse blastocysts with Ishikawa cells

Zygotes were cultured at LD in plain ModHTF medium or ModHTF medium containing 0.1% DMSO (vehicle control), or 25 nM S1P, or 2.5 µM NIBR0213, or 25 nM S1P + 2.5 µM NIBR0213 to the early blastocyst stage. 1-2 h prior to transfer of blastocysts onto the cells, the medium on the cells was changed to serum-starved medium. Between 5-8 early blastocysts were transferred into each well of Ishikawa cells and co-cultures were incubated for 72 h. Blastocyst attachment was scored every 24 h. Blastocysts were considered to be attached if they were not displaced when medium was blown at them using a pulled glass pipette.

3.2.7 Statistical analysis

Embryos were randomly allocated into control and treatment groups. All statistical analysis was carried out using GraphPad Prism software. S1PR1 fluorescence images were obtained from at least 4 independent experiments with 2-10 embryos per stage per experiment. Fluorescence data for analysis of translocation of S1PR1 across locations within embryo blastomeres were analysed using a one-way ANOVA with Tukey's multiple comparisons test. Culture experiments investigating the percentage of embryos to reach a particular developmental stage were repeated in at least 3 independent experiments with a minimum of 10 and 12 embryos per condition per experiment for HD and LD experiments,

respectively, and data were pooled and analysed using a chi-squared test. Blastocyst attachment data was pooled from 4 independent experiments with 4-19 blastocysts per condition per experiment and data were analysed using a chi-squared test.

3.3 Results

3.3.1 S1PR1 is present across all stages of preimplantation embryo development

S1PR1 was present across all stages of preimplantation development. The oocyte showed strong staining in the membrane or cortical cytoplasm. The zygote had a complete ring of staining around the plasma membrane, while the 2-cell to 8-cell stages showed an incomplete ring of staining (Figure 3.1, Appendix 1). There was little or no staining at the junction between blastomeres at any of the stages. Membrane and cortical staining decreased in the morula, becoming more cytoplasmic. Weak nuclear staining was present at the morula stage and was stronger in the nuclei of cells at the outer surface of the embryo (Figure 3.1, Appendix 1). IgG isotype and secondary only negative controls demonstrate no staining (Figure 3.2). In blastocysts, staining was localised to the nucleus and cytoplasm of trophectoderm cells and hypoblast cells, but not epiblast cells (Figure 3.3, Appendix 2). IgG isotype and secondary only negative controls have been performed previously and demonstrate no staining (data not shown).

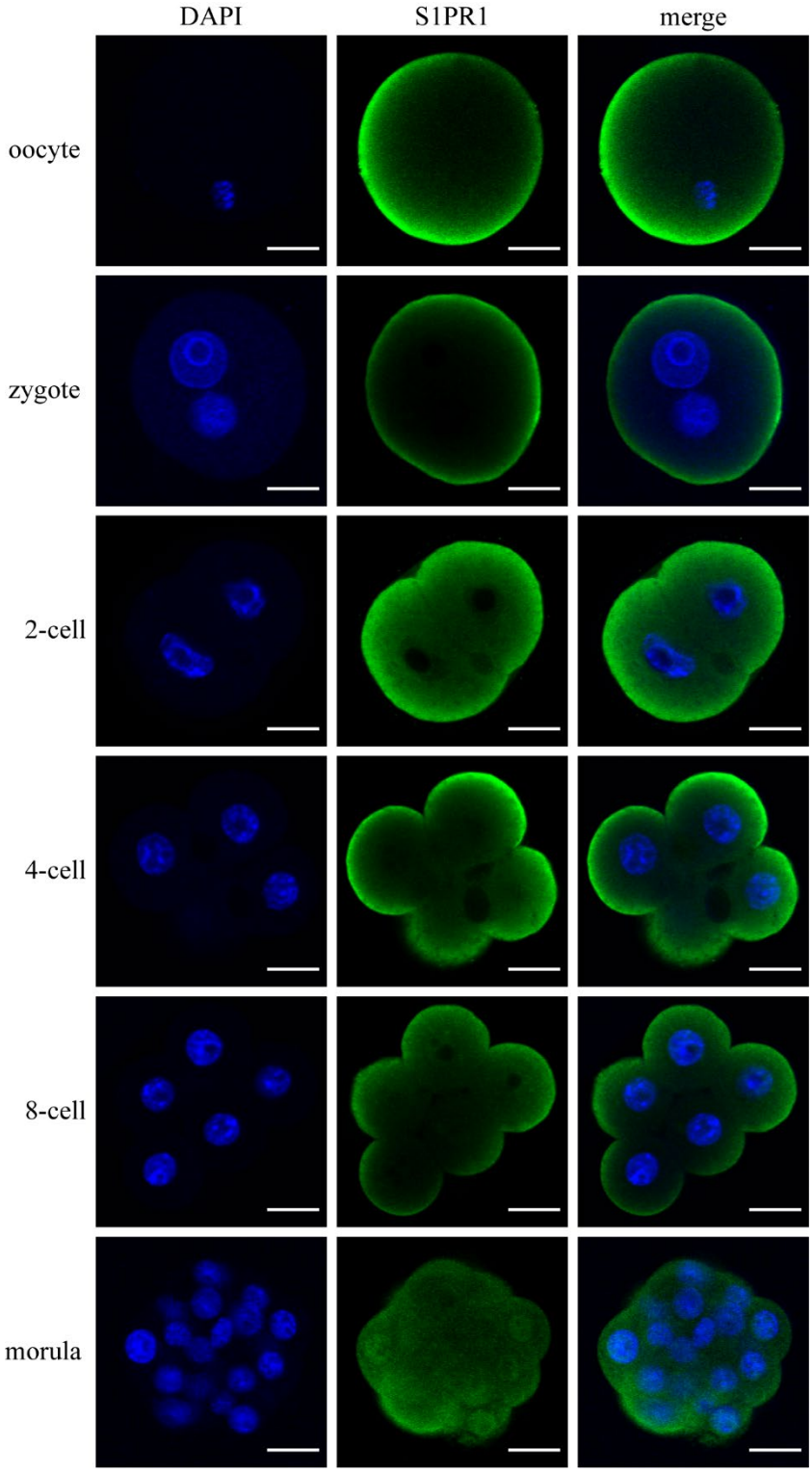


Figure 3.1. Localisation of S1PR1 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured *in vitro* at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote) 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for the S1PR1. Nuclei were stained with DAPI. Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results for zygote to 8-cell embryos were obtained from 3 independent experiments with 5 embryos per stage per experiment, while results for oocytes and morula were obtained from 2 independent experiments with 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.

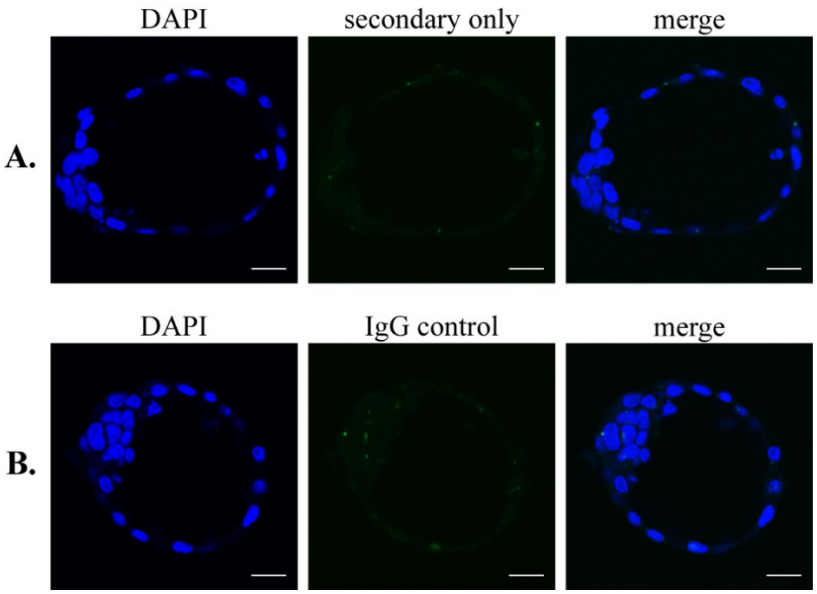


Figure 3.2. Negative controls for immunofluorescence staining in mouse embryos. Zygotes were isolated and cultured at HD (20 embryos/20 μL) in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde. Embryos were stained with (**A**) Alexa Fluor 488 goat anti-rat IgG (secondary only), or (**B**) FITC-labelled rat IgG2a κ isotype control (IgG control) (green). Nuclei were stained with DAPI (blue). Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 1 independent experiment with at least 4 embryos per condition. Scale bar = 20 μm.

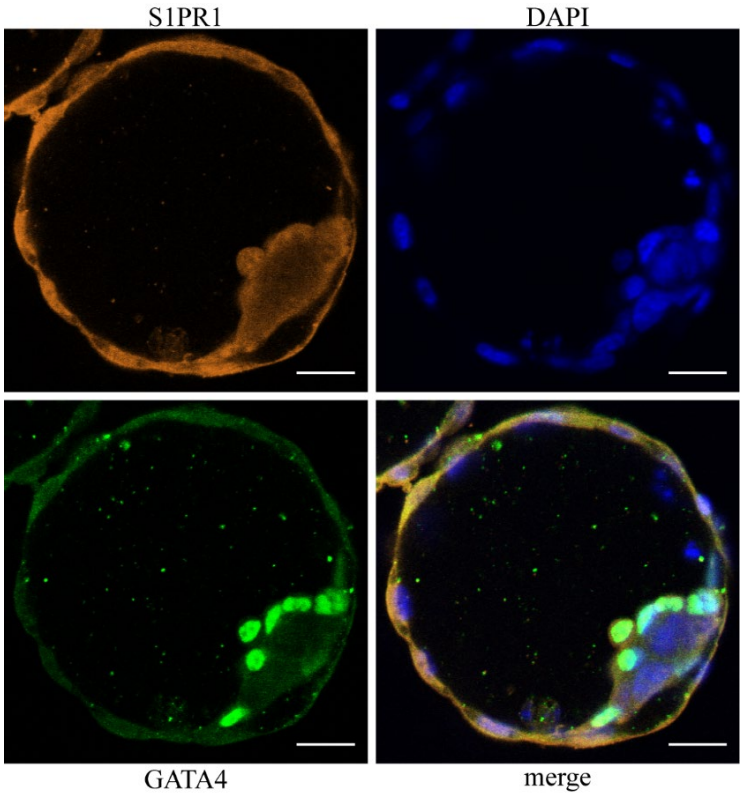


Figure 3.3. Localisation of S1PR1 in mouse blastocysts. Zygotes were isolated and cultured at HD (20 embryos/20 µL) in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde, and immunostained to visualise S1PR1 (orange) and GATA4 (green) as a marker of hypoblast cells. Nuclei were stained with DAPI (blue). Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 4 independent experiments with 6-13 embryos per experiment. Scale bar = 20 µm.

3.3.2 Inhibition of S1PR1 reduces cavitation and blastocyst formation during LD embryo culture

To investigate the role of autocrine S1P/S1PR1 signalling, embryos were cultured at HD or LD in the presence of the S1PR1 inhibitor, NIBR0213, which inhibits binding of S1P to the S1PR1. At LD, 10 μM NIBR0213 decreased the percentage of embryos that cavitated and subsequently reached the blastocyst stage (Figure 3.4A-E). Many embryos compacted early; at the 4-cell stage instead of at 8-16 cells. NIBR0213 had no effect during HD embryo culture on blastocyst formation (Figure 3.4F).

Treatment of embryos with 10 μM NIBR0213 only during the zygote to 8-cell stage also reduced blastocyst formation compared to untreated embryos (Figure 3.5), similar to when embryos were treated from the zygote to blastocyst stage. Treatment during the 8-cell to blastocyst stage had no effect on blastocyst formation (Figure 3.5).

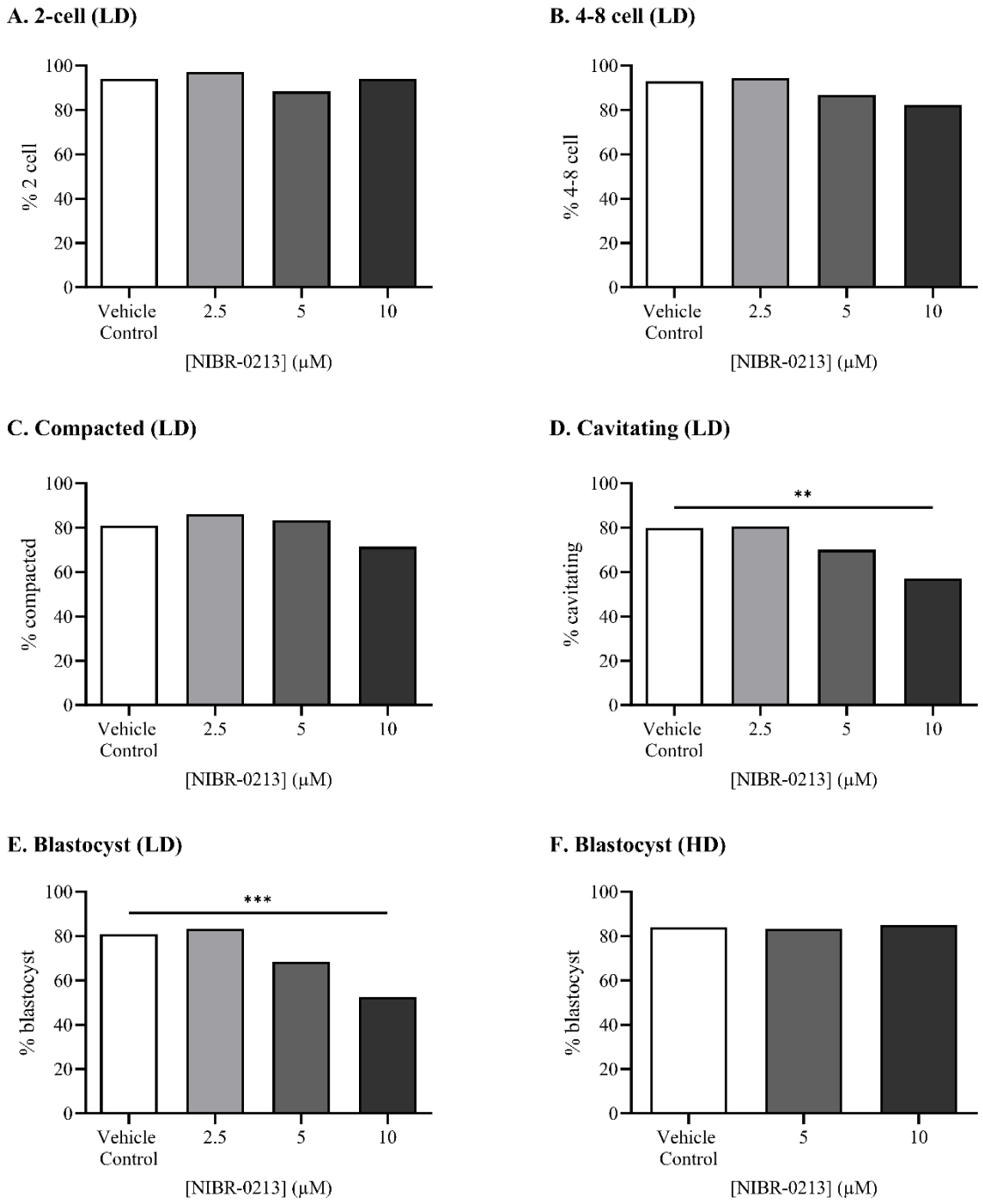


Figure 3.4. The effect of S1PR1 inhibition on mouse preimplantation embryo development. Zygotes were isolated and cultured at either LD (1 embryo/100 μL, panels A-E) or HD (1 embryo/1 μL, panel F). For LD, embryos were cultured in ModHTF medium containing 0.1% DMSO (vehicle control) or 2.5, 5, or 10 μM NIBR0213. Embryos were scored daily and the percentage of embryos that developed normally to the (**A**) 2-cell, (**B**) 4-8-cell, (**C**) compacted, (**D**) cavitating, and (**E**) blastocyst stages were recorded. For HD, embryos were cultured in ModHTF medium containing 0.1% DMSO plus 0, 5, or 10 μM NIBR0213 and the percentage of embryos that developed to the blastocyst stage was recorded (**F**). Data were pooled from at least 3 independent experiments with a minimum of 12 embryos per condition per experiment for LD conditions, or 10 embryos per condition per experiment for HD conditions. Statistical analysis was performed by chi-squared test. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

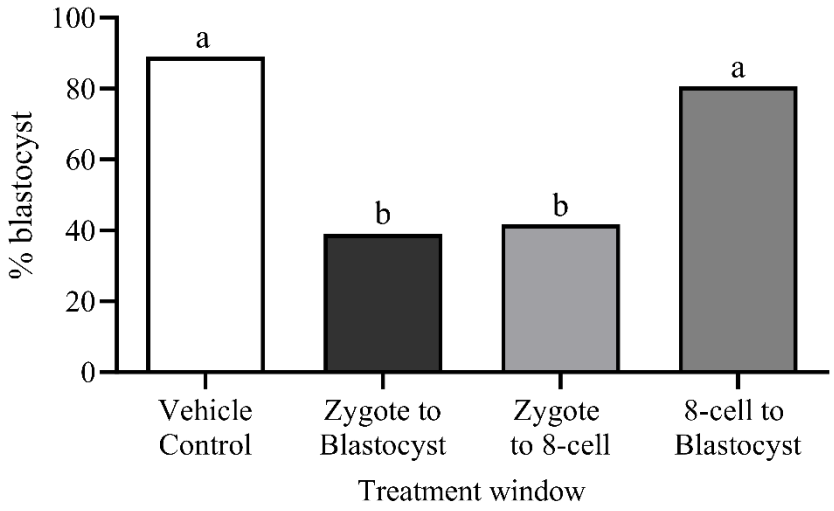


Figure 3.5. The effect of S1PR1 inhibition during early or late preimplantation development on blastocyst formation. Zygotes were isolated and cultured at LD (1 embryo/100 µL) in ModHTF medium. Embryos were treated with 0.1% DMSO (vehicle control) or 10 µM NIBR0213 from the zygote stage until 78 h post-hCG (zygote to 8-cell), from 78 h post-hCG onwards (8-cell to blastocyst) or from the zygote to blastocyst stage. The percentage of embryos that developed to the blastocyst stage was recorded at 144 h. Data were pooled from 3 independent experiments with 12 embryos per condition per experiment. Statistical analysis was performed by chi-squared test. Bars not sharing the same letter are significantly different ($P<0.05$).

3.3.3 Acute S1P treatment alters S1PR1 localisation

In HEK293 cells, S1P treatment induces translocation of the S1PR1 from the plasma membrane to the nucleus within 15 min (Estrada et al. 2009). However, translocation of the S1PR1 in response to S1P treatment has not been examined in the mouse preimplantation embryo. 2-cell embryos were treated with S1P for up to 2 h before fixing and staining for S1PR1. Treatment of ARC Swiss mouse embryos with S1P altered S1PR1 localisation, with a rapid decrease in both nuclear and cytoplasmic staining at 5 and 30 min, as well as a significant decrease in membrane staining at 120 min (Figure 3.6).

Treatment of QS mouse embryos with S1P for 10 min resulted in a small reduction of S1PR1 staining in the membrane, cytoplasm, and nucleus compared to untreated embryos (Figure 3.7), which was not prevented by the presence of NIBR0213. Instead, inhibition of S1PR1 ± S1P resulted in identical reduction in S1PR1 staining across all locations (Figure 3.7).

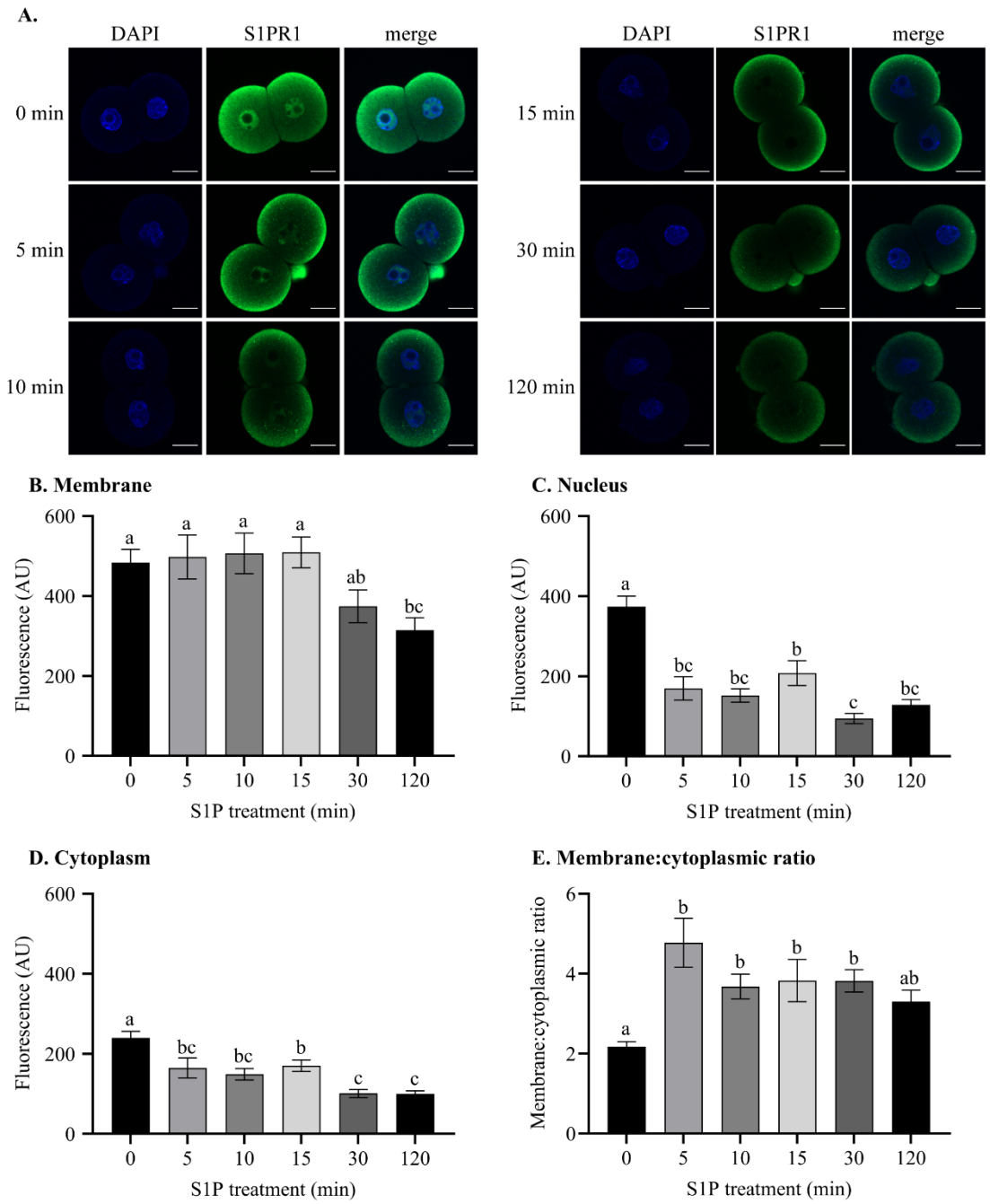


Figure 3.6. The effect of S1P on S1PR1 localisation in 2-cell embryos. Zygotes from ARC Swiss mice were isolated and cultured individually at LD (1 embryo/100 µL) in ModHTF medium for 24 h to obtain 2-cell embryos. Embryos were treated with 25 nM S1P for 0, 5, 10, 15, 30, or 120 min before fixing and immunostaining. (**A**) Representative images of 2-cell embryos at each timepoint, obtained using confocal fluorescence microscopy. Embryos were stained to visualise the S1PR1 (green), and nuclei were stained with DAPI (blue). Scale bar = 20 µm. Image fluorescence was measured using Fiji by ImageJ at the membrane (**B**), within the nucleus (**C**), and within the cytoplasm (**D**) of each blastomere and corrected for background fluorescence. A ratio of fluorescence values at the membrane and within the cytoplasm was calculated (**E**). Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparisons test. Bars not sharing the same letter are significantly different ($P<0.05$). Results were obtained from 3 independent experiments with 3-10 embryos per stage per experiment. Data in this figure was collected in my Honours year and appears in (Rayhanna, 2019).

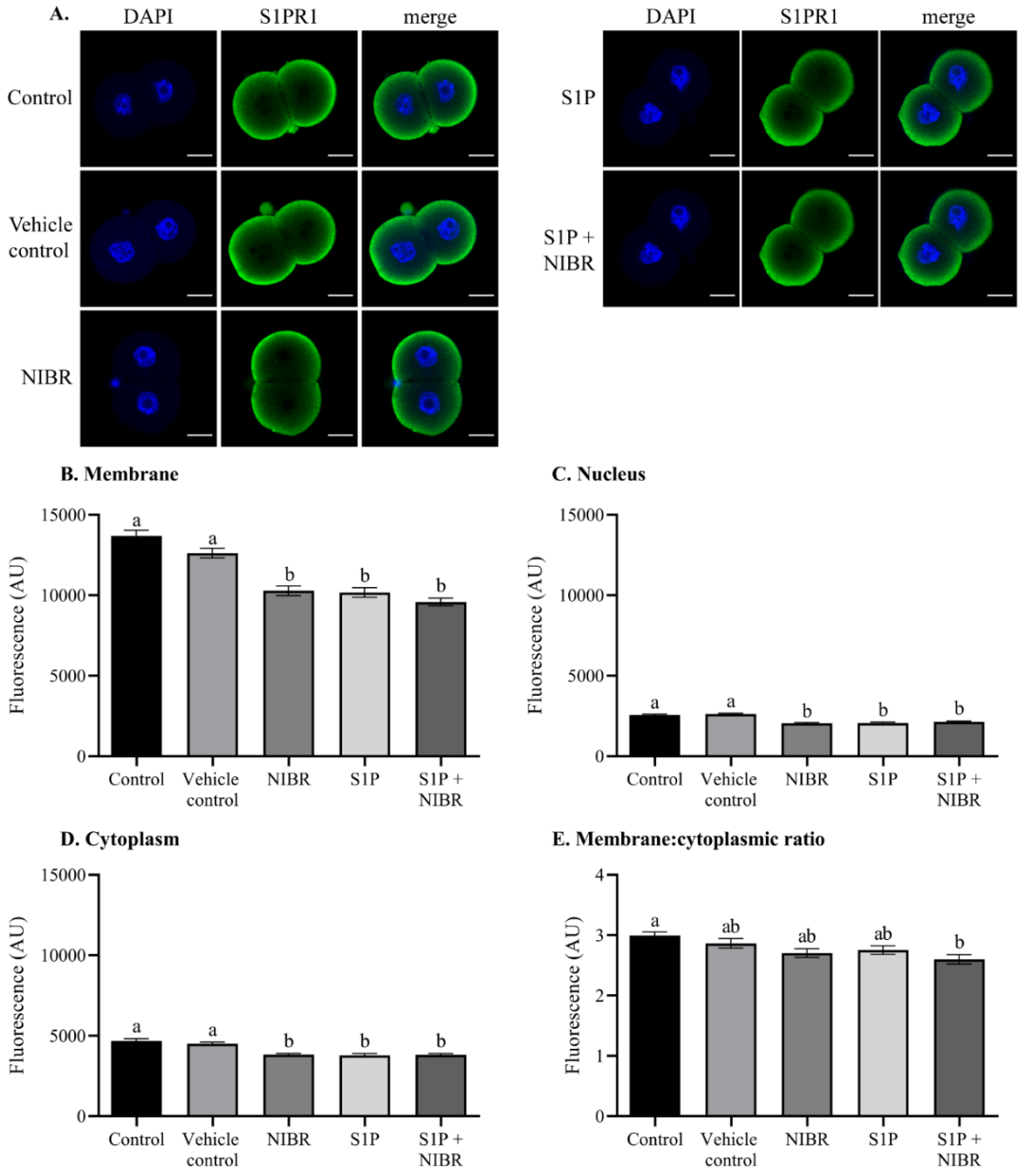


Figure 3.7. The effect of S1PR1 inhibition on S1P-mediated changes in S1PR1 localisation. Zygotes from Quackenbush Swiss mice were isolated and cultured individually at LD (1 embryo/100 µL) in ModHTF medium for 24 h to obtain 2-cell embryos. Embryos were treated for 10 min with ModHTF medium (control) or ModHTF containing 0.1% DMSO (vehicle control), or 2.5 µM NIBR, or 25 nM S1P, or 2.5 µM NIBR + 25 nM S1P before fixing and immunostaining. (**A**) Representative images of 2-cell embryos for each condition, obtained using confocal fluorescence microscopy. Embryos were stained to visualise the S1PR1 (green), and nuclei were stained with DAPI (blue). Scale bar = 20 µm. Image fluorescence was measured using Fiji by ImageJ at the membrane (**B**), within the nucleus (**C**), and within the cytoplasm (**D**) of each blastomere and corrected for background fluorescence. A ratio of fluorescence values at the membrane and within the cytoplasm was calculated (**E**). Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparisons test. Bars not sharing the same letter are significantly different ($P<0.05$). Results were obtained from 3 independent experiments with 3-10 embryos per stage per experiment.

3.3.4 S1P may act via S1PR1 to improve embryo cleavage rate

S1P improves cleavage rate of preimplantation embryos (Span, 2015), but it is not known if this is via S1PR1. To assess this, embryos were treated with 25 nM S1P ± 2.5 μM NIBR0213 during LD culture, and development scored at the 4-8-cell stage (68 h post hCG) as a measure of cleavage rate. Development was also scored at the cavitating stage (96 h post hCG). 2.5 μM NIBR0213 was used as it was the highest concentration that had no effect on development.

S1P or NIBR alone had no effect on cleavage rate, but there were fewer 5-8-cell stage embryos at 68 h when embryos were treated with S1P + NIBR compared to S1P alone (Figure 3.8). Neither S1P nor NIBR0213 alone or in combination affected the percentage of embryos that cavitated (Figure 3.8) There were also no differences in the percentage of embryos that developed to earlier developmental stages (data not shown).

3.3.5 Treatment of embryos with S1P has no effect on subsequent blastocyst implantation

In order to determine whether exposure to S1P during preimplantation development subsequently improves the capacity of the embryo to implant, embryos were cultured with 25 nM S1P ± 2.5 μM NIBR0213 during LD culture to the early blastocyst stage (96 h post hCG) before transfer to Ishikawa cells for 48 h.

Neither S1P nor NIBR0213 alone or in combination affected the percentage of blastocysts that attached after 48 h (Figure 3.9).

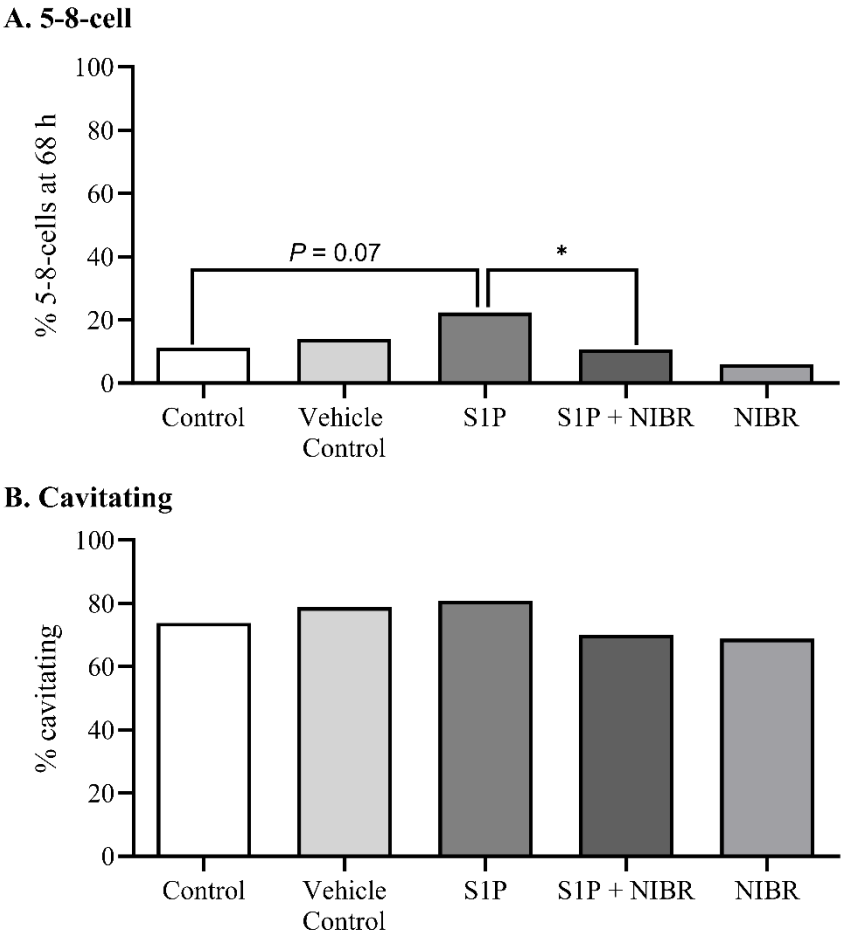


Figure 3.8. The effect of S1P and S1PR1 inhibition on preimplantation embryo development. Zygotes were isolated and cultured at LD (1 embryo/100 µL) in ModHTF medium (control), or ModHTF containing 0.1% DMSO (vehicle control), or 2.5 µM NIBR, or 25 nM S1P, or 2.5 µM NIBR + 25 nM S1P. The percentage of embryos that developed to the 5-8-cell stage at 68 h was scored as a measure of cleavage rate, and the percentage of embryos that cavitated was scored at 96 h. Data were pooled from 4 independent experiments with 20-40 embryos per condition per experiment. Statistical analysis was performed by chi-squared test. * = $P<0.05$

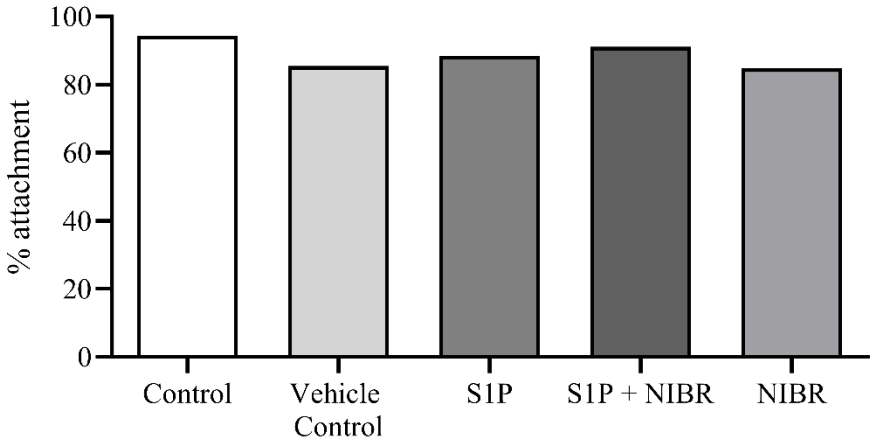


Figure 3.9. The effect of S1P and S1PR1 inhibition during preimplantation embryo development on subsequent blastocyst attachment. Zygotes were isolated and cultured at LD (1 embryo/100 µL) in ModHTF medium (control), or ModHTF containing 0.1% DMSO (vehicle control), or 2.5 µM NIBR, or 25 nM S1P, or 2.5 µM NIBR + 25 nM S1P. Embryos were cultured to the early blastocyst stage (96 h post hCG) then transferred to wells of Ishikawa cells. The percentage of blastocysts that attached to Ishikawa cells was scored after 48 h of co-culture. Data were pooled from 4 independent experiments with 4-19 embryos per condition per experiment. Statistical analysis was performed by chi-squared test.

Discussion

S1P signalling promotes cell proliferation and survival in a number of cell types (Bao et al., 2020; Dai et al., 2022; Liu et al., 2019; Tang et al., 2018; Zhang et al., 2016b). Addition of S1P to embryo culture increases cleavage rate of embryos and decreases apoptosis (Hannoun et al., 2010; Jee et al., 2011; Span, 2015). S1P can act via a family of five GPCRs, of which S1PR1 is the most widely expressed and well-studied (Takuwa et al., 2002). This study investigated the localisation of S1PR1 in mouse embryos and its function in preimplantation development and implantation.

3.4.1 S1PR1 localisation across preimplantation embryo development

S1PR1 was present at all stages of preimplantation development from the oocyte to the blastocyst and was predominately localised subcortically and within the cytoplasm (Figures 3.1 and 3.3). The localisation of S1PR1 has been previously investigated in preimplantation embryos from ARC Swiss mice (Rayhanna, 2019). However, the localisation was different to that seen in QS mice in the current study (Figure 3.1). This is particularly evident in the oocyte, where S1PR1 staining was predominantly cytoplasmic surrounding the meiotic spindle in oocytes from ARC Swiss mice, as opposed to the membranous and subcortical staining seen in oocytes from QS mice. Another key difference is the markedly reduced nuclear staining seen during cleavage stages in QS mice as opposed to strong staining present in ARC Swiss mice. As the same protocol and antibody were used in both studies, this suggests that there are strain-dependent differences in S1PR1 receptor expression and localisation in the oocyte and embryo. While the function of S1PR1 in preimplantation development of ARC Swiss mice has not been elucidated, it is possible that this function could instead be enacted through one of the other S1PRs in QS mice, or even through another growth factor pathway entirely. Therefore, the expression of the other S1PRs in preimplantation embryos from QS mice should be investigated.

3.4.1.1 S1PR1 is expressed and localises to the plasma membrane and subcortical cytoplasm in the oocyte

S1PR1 localised as a complete ring in the membrane and subcortical region of the oocyte (Figure 3.1). This staining pattern could be due to the presence of S1P in the follicular fluid. S1P produced by granulosa cells surrounding the oocyte in humans results in a concentration of ~170 nM S1P in follicular fluid (Hernandez-Coronado et al., 2016; von Otte et al., 2006). Follicular S1P could act via S1PR1 on the surface of the oocyte to promote

maturation prior to ovulation. Additionally, both the oocyte and follicular fluid are expelled into the oviduct upon ovulation, and therefore follicular S1P could be present in the oviductal fluid, where it could act via S1PR1 to promote fertilisation. While S1P promotes oocyte maturation both *in vitro* and *in vivo* (Lee & Koo, 2012; Yuan et al., 2022), it is not known if this occurs via S1PR1 signalling. This could be investigated by maturing and fertilising oocytes *in vitro* in the presence of an inhibitor of S1PR1 to determine whether embryo-derived S1P signalling via S1PR1 has a role in oocyte maturation and/or fertilisation.

3.4.1.2 S1PR1 localisation changes across cleavage stages of development

Immunofluorescence staining revealed expression of S1PR1 in all developmental stages of the embryo as well (Figure 3.1). During the cleavage stages, subcortical staining of S1PR1 was high in the zygote and decreased by the morula, at which point nuclear staining for S1PR1 was present. Localisation of S1PR1 to the junction between cells was absent, as seen with other growth factor receptors in the preimplantation embryo, including growth hormone receptor, EGF receptor, IGF1 receptor, colony stimulating factor-1 receptor, and granulocyte-macrophage colony stimulating factor receptor (Kawamura et al., 2012; Pantaleon et al., 1997). This plasma membrane and subcortical localisation may facilitate binding of maternally-derived ligands.

Membrane localisation of S1PR1 during early cleavage stages suggests that S1P signalling may be present at this stage. *In vivo*, these stages of development occur in the oviduct. In humans, oviductal tissue samples obtained postpartum from normal pregnancies contained 382 nM S1P, though this does not necessarily reflect the concentration in the oviductal fluid (Gao et al., 2013). While the concentration of S1P in oviductal fluid itself has not been measured, human follicular fluid contains 300-400 nM S1P and contributes to the oviductal fluid upon ovulation (Nakahara et al., 2013). However, while inhibition of S1PR1 during only early preimplantation development reduced blastocyst formation (Figure 3.5), S1PR1 inhibition does not affect the percentage of embryos that developed normally during the cleavage stages (Figure 3.4A-C). This suggests that S1PR1 signalling is not required for events that occur during early preimplantation development, but instead is essential for the setup of later stages of development.

3.4.1.3 S1PR1 is localised to the outer cells of morula, and the nuclei of trophectoderm and hypoblast cells in blastocysts

S1PR1 staining was nuclear during the later stages of development, though became restricted to the nucleus of the outer cells of the morula, and the trophectoderm and hypoblast cells of the blastocyst (Figures 3.1 and 3.3). Staining was decreased or absent in cells towards the centre of the morula, which are fated to become the ICM of the blastocyst, and in epiblast cells of the blastocyst. This is similar to the localisation seen in ARC Swiss mouse embryos cultured at LD (Rayhanna, 2019), suggesting that autocrine S1P is sufficient to drive this localisation pattern in the absence of maternal or even paracrine signalling from other embryos.. The localisation to the outer cells of the morula, and in trophectoderm and hypoblast cells suggests a role of S1PR1 in cell differentiation in the embryo. S1P signalling activates yes-associated protein (YAP) and TEA Domain Transcription Factor (TEAD) 4 in late preimplantation development, which is required for the differentiation of totipotent blastomeres into trophectoderm and ICM cells via activation of YAP in cells fated to become trophectoderm (Chi et al., 2020). Differentiation of ICM cells to hypoblast is less understood but requires upregulation of GATA6 and downregulation of Nanog (Carreiro et al., 2021). Both S1P and YAP signalling upregulate GATA6 and supress Nanog in other cells (Donati et al., 2011; Meyer et al., 2023; Price et al., 2015; Zeevaert et al., 2023), suggesting S1P/YAP signalling could also promote differentiation of ICM to hypoblast cells in the embryo. S1PR1 activates YAP in ovarian cancer cells (Tao et al., 2023), and activation of YAP in the outer cells of the morula and in trophectoderm aligns with S1PR1 localisation in the blastocyst, suggesting that S1PR1 could be upstream of YAP in these cells. S1PR1 signalling could also play a role in blastocyst hatching and implantation, though this has not been investigated previously. The effect of S1PR1 inhibition during preimplantation development on subsequent blastocyst attachment is discussed in section 3.4.5.

S1PR1 was nuclear in the morula and blastocyst stage embryo, within the nucleoplasm but not the nucleoli. While the nucleus is composed of several distinct compartments (Weston et al., 2015), the resolution of the images was not high enough to determine the precise localisation of S1PR1 within the nucleus. GPCRs such as S1PR1 typically localise to the plasma membrane to facilitate binding of extracellular ligands and subsequent activation of downstream signalling pathways. However, GPCRs can be internalised upon activation or localise to various intracellular compartments including the nuclear membrane and nuclear bodies within the nucleoplasm (Lee et al., 2004; Morinelli et

al., 2007; Wright et al., 2012). Similar to the staining seen in embryos, S1PR1 localises to the nucleus of other cells, including a variety of malignant and benign human tissue, T-cells, and endothelial cells (Estrada et al., 2009; Liao et al., 2007; Wang et al., 2014). In T-cells and endothelial cells, nuclear S1P1R signalling regulates gene transcription, including promotion of growth factor transcription, though the mechanisms remain obscure (Estrada et al., 2009; Liao et al., 2007). Therefore, S1PR1 could be signalling from the nucleus to promote development in the embryo. In order to determine whether interaction of S1P with nuclear receptors could be responsible for S1PR signalling, inhibitors of Spns2 could be added to culture to prevent S1P secretion, or extracellular S1P could be measured using ELISA or lipidomics to show whether or not S1P is secreted by the embryo.

Nuclear S1PR1 signalling would require the presence of S1P in the nucleus, either transported from the cytoplasm or extracellular space, or synthesised locally. While S1P transporters such as Spns2 have been identified in the plasma membrane (Spiegel et al., 2019), their expression has not been investigated in the embryo, and S1P transporters have not been identified in the nuclear membrane. S1P could also be produced locally by pSphK2, which is expressed in the nucleus of other cells (Diaz Escarcega et al., 2021). In order to determine the source of S1P for nuclear S1PR1 signalling, the expression and localisation of pSphK1 and pSphK2 in the embryo should be determined. This is explored in Chapter 5.

3.4.2 S1PR1 signalling is required for cavitation and blastocyst formation

The importance of S1PR1 signalling in the blastocyst is supported by dose culture experiments, where prevention of binding of S1P to S1PR1 through the use of NIBR0213 during LD culture resulted in a failure of embryos to cavitate and form blastocysts (Figure 3.4). During LD embryo culture, it is assumed that autocrine factors are diluted and therefore their effects on the embryo minimised. However, these results suggest that there is still sufficient embryo-derived S1P acting on the embryo to promote development. This is also seen with other growth factors such as IGF1, with inhibition of the IGF1R during LD culture (1 embryo/100 μL) reducing blastocyst formation, hatching, and blastocyst cell number (Green & Day, 2013).

These results suggest that S1P is not just beneficial when added to culture medium (Span, 2015), but that embryos also require endogenous S1P for normal development. Inhibition of S1PR1 during HD embryo culture had no effect on preimplantation development (Figure 3.4). This suggests that the loss of S1PR1 signalling is able to be

compensated for, either by other S1PRs due to increased S1P concentration during HD culture compared to LD, or by the increased concentration of other autocrine growth factors (Dai et al., 2012). To investigate potential S1PR redundancy, the expression and function of the other S1PRs should be investigated, as well as the effect of inhibiting multiple S1PRs on preimplantation development. This is explored in Chapter 4. Alternatively, the increased concentration of S1P during HD embryo culture could be sufficient to override the competitive inhibition of S1PR1 by NIBR0213. This could be investigated by examining whether the addition of exogenous S1P to embryos treated with 10 μM NIBR0213 during LD culture prevents the detrimental effects of NIBR0213.

S1PR1 signalling may affect blastocyst formation by affecting compaction of the blastomeres and formation of tight junctions prior to cavitation. In the mouse embryo, cavitation begins at the 32-cell stage and requires the differentiation of totipotent cells into two cell populations – a polar outer layer fated to become trophectoderm cells, and an apolar inner cluster that will form the ICM (Mihajlovic & Bruce, 2017). Maturation of tight junctions between the outer layer of trophectoderm-fated cells allows formation of the fluid-filled blastocoel (Stephenson et al., 2012). At this point, S1PR1 localises to the cytoplasm and nuclei of the outer cells and inhibition of S1PR1 signalling prevents cavitation (Figures 3.1 and 3.4). S1P and the S1PR1 are both involved in the maintenance of tight junctions in blood vessels (Lee et al., 2006). In human umbilical vein endothelial cells, exogenously added S1P acts via the S1PR1 to promote translocation of proteins such as zonula occludens-1 (ZO-1) and formation of tight junctions (Lee et al., 2006). Similarly, knockout of the S1PR1 gene or inhibition of S1PR1 protein in adult mice results in alterations in the subcellular distribution of tight junction proteins in the microvessels of the brain, compromising barrier integrity (Yanagida et al., 2017). ZO-1 is expressed in the mouse preimplantation embryo from the 8-cell stage and accumulates in the membrane at the 16-cell stage, and knockdown of ZO-1 expression with siRNA causes a dose-dependent decrease in blastocyst formation (Wang et al., 2008). It is possible that S1P/S1PR1 signalling is involved in formation and maintenance of tight junctions in the embryo, with inhibition of S1PR1 resulting in abnormal ZO-1 localisation, thus preventing cavitation and blastocyst formation. However, inhibition of S1PR1 up to the 8-cell stage impaired blastocyst formation but its addition at the 8-cell to blastocyst stages did not (Figure 3.5). This implies that the effect of S1PR1 on tight junctions may not just be an acute effect during cavitation (as occurs in vascular cells where S1P-induced ZO-1 translocation within 30 min (Lee et al.,

2006)), but also that S1PR1 signalling is required for the induction of ZO-1 expression and its localisation within the 8-cell embryo or earlier. To investigate this, the effect of S1PR1 inhibition at specific stages during early preimplantation development on subsequent localisation of ZO-1 should be examined.

3.4.3 Ligand-induced activation of S1PR1 alters receptor localisation

Treatment of 2-cell embryos from ARC Swiss mice with 25 nM S1P resulted in a decrease in S1PR1 staining in the nucleus and cytoplasm within 5 min, while membrane staining only decreased after 2 h (Figure 3.6). However, future experiments should assess changes in S1PR1 staining in untreated embryos over the 2 h time course to rule out normal fluctuations that are not due to exogenous S1P. Further, 2-cell embryos from QS mice were treated for 10 min with S1P in the presence or absence of the S1PR1 inhibitor NIBR0213 (Figure 3.7). Similar to ARC Swiss, treatment of QS embryos with S1P caused a rapid decrease in S1PR1 staining in the nucleus and cytoplasm, but membrane staining also decreased within 10 min. This was not prevented by inhibiting the S1PR1, which instead caused a similar decrease in staining across all cellular compartments regardless of whether exogenous S1P was present (Figure 3.7). This decrease is likely due to increased receptor degradation, which requires internalisation of S1PR1 (Gatfield et al., 2014). Activation of S1PR1 induces receptor internalisation and translocation in other cells (Estrada et al., 2009; Gatfield et al., 2014). For example, treatment of human umbilical vein endothelial cells (HUVECs) with 1 μM S1P for 1 h induced β-arrestin-dependent internalisation of S1PR1 to the Golgi compartment, where the receptor can undergo ubiquitin-mediated degradation (Gatfield et al., 2014). Lower concentrations of S1P also induced internalisation of S1PR1. However, this was transient, and no receptor degradation occurred. Similarly, in HEK293 cells and HUVECs, S1P treatment resulted in rapid internalisation and nuclear translocation of S1PR1, followed by recycling to the plasma membrane after 2 h (Estrada et al., 2009). In the nucleus, S1PR1 localised to the nuclear membrane and regulated growth-factor transcription, though the mechanism by which it translocated to the nucleus is unknown. S1PR1 does not contain a nuclear localising sequence, but other GPCRs shuttle to the nucleus via vesicular-transport pathways involving importins, small GTPases, and sorting nexin proteins (Bhosle et al., 2019).

The decrease in staining in all cellular compartments seen in this study when embryos were treated for 10 min with S1P in the presence or absence of S1PR1 inhibitor

suggests that there is increased degradation of the receptor (Figure 3.7). S1P induces rapid internalisation and degradation of S1PR1 in HUVECs (Gatfield et al., 2014). GPCR antagonists can also cause receptor internalisation, potentially by inducing or stabilising a conformation change that mediates internalisation independent of G-protein activation (Pheng et al., 2003; Roettger et al., 1997), which could lead to receptor degradation. However, this has not been reported with NIBR0213 (Quancard et al., 2012). While immunostaining of fixed tissue can determine localisation of the S1PR1, it is not well-suited for determining rates of degradation or for tracking the dynamic process of translocation. In order to track translocation, fluorescence imaging of live embryos could be performed by tagging the extracellular domain of S1PR1 or using a fluorescently labelled ligand (Sridharan et al., 2014). Receptor degradation could also be more accurately measured and quantified using western blotting in order to determine if activation of S1PR1 by S1P causes receptor degradation in the preimplantation embryo. Future experiments could also repeat these experiments on embryos cultured at HD or developed *in vivo* in order to determine whether culture environment affects S1PR1 localisation in response to exogenous S1P.

3.4.4 S1PR1 may be required for the S1P-mediated improvement in embryo cleavage rate

The addition of 25 nM S1P or 2.5 μM NIBR0213 to embryo culture medium had no effect on embryo cleavage rate to the 5-8 cell stage (Figure 3.8). S1P not affecting cleavage rate conflicts with data showing S1P increased the developmental rate of mouse embryos to all cleavage stages (Span, 2015). However, as this result was close to significance ($P = 0.07$), further repetitions to increase the sample size of this experiment could reveal a significant increase in developmental rate in the presence of S1P. This discrepancy could also be due to embryos in this study only being scored at a single time point, rather than the hourly live-cell imaging used previously. Embryos did develop slower when treated with S1P + NIBR0213 compared to S1P alone, with fewer embryos reaching the 5-8-cell stage by 68 h (Figure 3.7), suggesting S1P increases cleavage rate via S1PR1 signalling. S1P/S1PR1 signalling promotes proliferation in endothelial cells via PI3K/Akt signalling (Wang et al., 2018), and this pathway could also be operating in the embryo to increase cleavage rate. This experiment should be repeated using the live-cell imaging technology such as the IncuCyte to confirm that inhibition of S1PR1 prevents the S1P-mediated increase in developmental rate.

The addition of 25 nM S1P or 2.5 μM NIBR0213 alone or in combination to embryo culture medium did not impact cavitation (Figure 3.8). Addition of S1P to porcine embryo culture medium improves the percentage of embryos that form blastocysts from 20% in control to 30% (Lee & Koo, 2012). This was not seen in this study, though embryos were scored during the process of cavitation prior to transfer to Ishikawa cells. It is possible that scoring embryos at the late blastocyst stage would yield a difference in blastocyst formation between control and S1P-treated embryos.

3.4.5 Treatment of embryos S1P and S1PR1 inhibitors during preimplantation development has no effect on blastocyst attachment

Treatment of embryos with S1P during preimplantation development did not impact their subsequent ability to attach to Ishikawa cells, suggesting S1P does not prime embryos for attachment (Figure 3.9). Instead, S1P treatment during preimplantation development could promote other aspects of implantation such as trophoblast invasion. This could be investigated using blastocyst outgrowth assays, in which embryos treated with S1P are transferred to glass coverslips and allowed to outgrow before measurement of outgrowth area (Binder et al., 2015; Kim et al., 2020).

Treatment of embryos with NIBR0213 alone also had no effect on blastocyst attachment (Figure 3.9). This may be due to the concentration used, as 2.5 μM NIBR0213 also had no obvious effect on preimplantation embryo development while higher concentrations impaired development (Figure 3.4). Therefore, these experiments should be repeated with a higher concentration of NIBR0213 to determine if S1PR1 inhibition during preimplantation development affects the ability of the blastocyst to attach. S1PR1 signalling may not prime the embryo for attachment, but instead have an acute effect on blastocyst attachment. This could be investigated by treating co-cultures of blastocysts and Ishikawa cells with NIBR0213 during attachment itself.

3.4.6 Conclusions

In summary, S1PR1 is present across all stages of preimplantation development, though there appears to be strain-dependent differences in mice. Embryo-derived S1P signalling via the S1PR1 is essential for cavitation and blastocyst formation, though the effect of S1PR1 activation occurs prior to the 8-cell stage. S1PR1 is perhaps required in setting up tight junctions required for blastocoel formation. Additionally, ligand-stimulated activation of S1PR1 resulted in a decrease in S1PR1 staining across all cellular

compartments, though the mechanism behind this is unclear. Together, these results provide evidence that S1P/S1PR1 signalling is active in the mouse preimplantation embryo and required for normal development *in vitro*.

CHAPTER 4

The expression and function of S1PR2-5 in the mouse preimplantation embryo

4.1 Introduction

S1P is a growth factor present in the female reproductive tract that increases preimplantation cleavage rate when added to culture media, though the mechanism by which it does so is not clearly understood (Span, 2015). In other cell types, S1P is produced by the phosphorylation of sphingosine by pSphK1 and 2. S1P produced within the cell can be secreted via membrane transporters such as Spns2, ABC transporters, and MFSD2B (Ihlefeld et al., 2015; Kobayashi et al., 2018; Spiegel et al., 2019). Once outside the cell, S1P acts as a ligand for the S1PR family in an autocrine or paracrine manner. While S1PR1 is the most widely expressed and well-studied of the S1PRs, there are four other GPCRs in the S1PR family. In other cells, each of the S1PRs demonstrate different expression patterns and G-protein coupling properties that allow activation of many different downstream signalling pathways (Mendelson et al., 2014).

S1PR2 and S1PR3 are ubiquitously expressed in adult tissue and couple to G_i, $G_{12/13}$, and G_q (Figure 1.4) (Takuwa et al., 2002). Expression of S1PR4 and S1PR5 is much more restricted than S1PR1-3, with S1PR4 expressed in lymphoid tissue and S1PR5 in the brain and immune cells (Takuwa et al., 2002). Both S1PR4 and S1PR5 couple to G_i and $G_{12/13}$, but not G_q (Figure 1.4). While S1P/S1PR signalling is predominantly associated with increased proliferation and survival, this varies based on S1PR isoform and cell type. S1PR3 is almost exclusively reported to promote proliferation in a range of cell types (He et al., 2021; Shen et al., 2019; Tang et al., 2018; Wang et al., 2018). However, S1PR2 has a more nuanced effect on cell proliferation dependent on cell type, in that it promotes proliferation in some cells (Cheng et al., 2018; Liu et al., 2020; Wang et al., 2023a) but inhibits proliferation in others (Kitada et al., 2016; Price et al., 2015; Zhou et al., 2022). Similarly, while much less is known about S1PR4 and S1PR5, there are also differences in the effect of S1P on proliferation. S1PR4 signalling inhibits T-cell proliferation in breast and colorectal cancer cells via modulation of PI3K signalling (Olesch et al., 2020) but promotes proliferation of rheumatoid arthritis fibroblast-like synoviocytes via IL-17/STAT3 signalling (Zhang et al., 2023). S1PR5 regulates mitotic progression via G_i/PI3K/Akt/Polo-like kinase 1 (PLK1) (Andrieu et al., 2017) and inhibits proliferation in mouse embryonic fibroblasts (Talmont et al., 2022) and oesophageal cancer cells (Hu et al., 2010). These differences highlight the complexity of S1P/SIPR signalling in modulation of cell growth and proliferation, though little is known about the role of S1P in the preimplantation embryo.

S1PR1 is present in the oocyte and across all stages of preimplantation development, and is indispensable for normal embryo development (Chapter 3, Rayhanna, 2019). From Chapter 3, embryos require embryo-derived S1P signalling via the S1PR1 for development, with inhibition of S1PR1 during LD but not HD culture reducing blastocyst formation. This could be due to a higher concentration of autocrine growth factors present during HD embryo culture, or there could be redundancy between the S1PRs in that S1P could instead act at the other S1PRs to exert its beneficial effect in the absence of S1PR1 signalling. Despite differences in localisation and signalling between S1PR1-3, redundancy between these receptors occurs in later stages of development in mice (Kono et al., 2004). Genetic deletion of S1PR2 or S1PR3 does not result in embryonic lethality whereas S1PR1-null mice die at E13.5-14.5 due to vascular malformation (Kono et al., 2004). However, S1PR1 knockout along with either S1PR2 or S1PR3 demonstrated more severe phenotypic defects than S1PR1 single null embryos, and generation of S1PR2/S1PR3 double null embryos resulted in low numbers of viable offspring and perinatal lethality. Additionally, S1PR1/S1PR2/S1PR3 triple null embryos exhibited more severe defects than single or double null embryos, with vascular defects at E10.5 and embryonic lethality by E12.5 (Kono et al., 2004). These studies did not look at the role of S1PR4 and S1PR5, but it is possible that redundancy exists with these receptors and the rest of the S1PR family, which could potentially have mitigated the effects of S1PR1-3 knockout during earlier developmental stages. There is little information on redundancy in S1PR4 and S1PR5 in other cell types, likely due to the restricted expression of these receptors in the adult.

As only the presence of S1PR1 has been confirmed in the preimplantation embryo, this study aims to identify the expression of other S1PRs in the embryo, as well as the role of S1PR signalling in embryo development *in vitro*. This will provide a better understanding of growth factor signalling required for development, which could inform addition of growth factors such as S1P to culture media to improve the viability of embryos *in vitro.*

4.2 Methods

4.2.1 Immunofluorescence staining of oocytes and embryos for S1PR2-5

Zygotes were isolated and cultured at HD to the zygote, 2-cell, 4-cell, 8-cell, morula, and hatched blastocyst stages as per Table 2.3. Immunofluorescence staining was carried out as in section 2.4. All developmental stages were stained to visualise S1PR2-5 and blastocysts were additionally stained to visualise GATA4, a marker of hypoblast cells (Table 4.1).

Table 4.1. Primary and secondary antibody dilutions for immunofluorescence staining of S1PRs

Primary antibody	Dilution	Secondary antibody	Dilution
S1PR2 antibody (Proteintech #21180-1-AP)	1:50	Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies #A11034)	1:100
		Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies #A11037)	1:100
S1PR3 antibody (Novus Bio #NBP2-24762)	1:50	Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies #A11034)	1:200
		Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies #A11037)	1:200
S1P4/EDG-6 antibody (Affinity Biosciences #DF4872)	1:200	Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies #A11034)	1:200
		Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies #A11037)	1:200
S1PR5 antibody (Proteintech #13874-1-AP)	1:100	Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies #A11034)	1:200
		Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies #A11037)	1:200
GATA-4 antibody (C-20) (Santa Cruz #sc-1237)	1:100	CruzFluor 488 donkey anti-goat IgG (sc-362255)	1:100

4.2.2 Co-localisation of S1PR5 with autophagosomes

Zygotes were cultured at HD to the 4-cell and 8-cell stages before treatment with pronase to remove the zona pellucida (section 2.4.1). Embryos were then stained for autophagosomes using the CYTO-ID Autophagy detection kit 2.0 (1:500; Enzo Life Sciences #ENZ-KIT175-0050) before fixing and staining (section 2.4) to visualise S1PR5.

4.2.3 Culture of embryos in the presence of inhibitors of S1PR2-4

Zygotes were cultured at either HD or LD in ModHTF medium containing inhibitors of S1PR2-4 to the hatched blastocyst stage (Table 4.2). There are no suitable inhibitors for S1PR5, and so these experiments could not be performed.

Table 4.2. S1PR inhibitor concentrations used for dose culture experiments.

Receptor	Inhibitor	Solvent	Concentrations used	Source
S1PR2	JTE013	DMSO	0-5 µM	Sigma Aldrich
S1PR3	TY52156	DMSO	0-10 µM	Sigma Aldrich
S1PR4	CYM50358	DMSO	0-1000 nM	Sigma Aldrich

Embryos were cultured at LD in the presence of inhibitors of S1PR1-3 (2.5 µM NIBR0213 + 1 µM JTE013 + 1 µM TY52156) or S1PR1-4 (2.5 µM NIBR0213 + 1 µM JTE013 + 1 µM TY52156 + 500 nM CYM50358). These concentrations were selected as the highest concentration of the inhibitors that individually had no discernible effect on embryo development in dose-culture experiments.

Embryos were also cultured at LD in the presence of 2.5 µM JTE013, 10 µM TY52156, or 1 µM CYM50358 during only the zygote to 8-cell stages (up to 78 h post hCG injection), or only during the 8-cell to blastocyst stages (from 78 h post hCG onwards). Embryo development was scored daily and the percentage of embryos that had reached each developmental stage recorded.

4.2.4 Quantification of S1PR gene expression in the embryo

Oocytes and embryos were isolated (section 2.3.2), and embryos were cultured *in vitro* at HD to the zygote, 2-cell, 4-cell, 8-cell, morula, or early blastocyst stages per the timings in Table 2.3. *In vivo* developed embryos were flushed from the reproductive tract (section 2.3.3) as per the timings in Table 2.3. Oocytes and embryos were collected in groups of 18-30 in 15 µL Trizol, then snap frozen in liquid nitrogen and stored at -80 ˚C. Gene expression of S1PR1-5 was quantified by RT-qPCR (section 2.5) to using *Rn18s* as the reference gene (Table 4.3).

Table 4.3. Genes analysed using RT-qPCR for S1PRs

Gene name	Protein	Assay ID	Catalogue #	Reporter	Amplicon length
S1pr1	S1PR1	Mm02619656_s1	#4453320	FAM	154
*S1pr2**	S1PR2	-	-	FAM	-
S1pr3	S1PR3	Mm02620181_s1	#4448892	FAM	105
S1pr4	S1PR4	Mm00468695_s1	#4448892	FAM	60
S1pr5	S1PR5	Mm02620565_s1	#4448892	FAM	146
Rn18s	18S ribosomal RNA	Hs03003631_g1	#4453320	VIC	69

* primers were custom made using TaqMan® Assay Design Tool. Amplicon length unknown.

4.2.5 Statistical analysis

All statistical analysis was carried out using GraphPad Prism software. Embryos were randomly allocated into treatment groups. Fluorescence images were obtained from at least 3 independent experiments with 5 embryos per developmental stage per experiment. Data for inhibitor dose culture experiments were obtained in at least 3 independent experiments with 10 or 12 embryos per treatment per experiment for HD and LD experiments, respectively. Data from these experiments were pooled and analysed using a chi-squared test.

4.3 Results

4.3.1 S1PR2 is present across all stages of preimplantation embryo development

S1PR2 was present at all stages of preimplantation development, with punctate cytoplasmic staining at all stages, and strong nuclear staining present at the 2-cell stage (Figures 4.1 and 4.2, Appendix 1). In the oocyte, staining of S1PR2 in the microvilli-free region overlying the meiotic spindle was stronger than that of the microvilli-rich region of the membrane (Figure 4.1).

Cytoplasmic staining was uniform in zygote to 8-cell stage embryos, though staining at the zygote and 8-cell stages was low. Note that multiple stages were stained in the same well containing antibody to control for variability in staining between stages. Weak nuclear staining was present in the nucleoplasm but not the nucleoli at all stages except the morula, and nuclear staining was strong at the 2-cell stage. In the morula, S1PR2 staining was stronger on the outer surface of the embryo (Figure 4.1, Appendix 1). At the blastocyst stage, staining was uniform across the cytoplasm and nucleus, and was present in both the trophectoderm and ICM (Figure 4.2, Appendix 2). IgG isotype and secondary only negative controls have been performed previously and demonstrate no staining (data not shown).

4.3.2 Inhibition of S1PR2 reduces cavitation and blastocyst formation during LD embryo culture

To investigate the effect of autocrine S1P/S1PR2 signalling, embryos were cultured at HD or LD in the presence of JTE013, an S1PR2 inhibitor. At LD, treatment with 5 μM JTE013 significantly reduced development to all developmental stages, while 2.5 μM JTE013 reduced cavitation and blastocyst formation (Figure 4.3A-E). At HD, 5 μM JTE013 reduced the percentage of embryos that developed to the blastocyst stage, but 2.5 μM JTE013 had no effect (Figure 4.3F).

Treatment of embryos with 2.5 μM JTE013 during only the zygote to 8-cell stage or only the 8-cell to blastocyst stage also reduced blastocyst formation compared to untreated embryos (Figure 4.4). However, blastocyst formation when embryos were treated during the 8-cell to blastocyst stage was significantly higher than embryos treated from the zygote to the blastocyst stage (Figure 4.4).

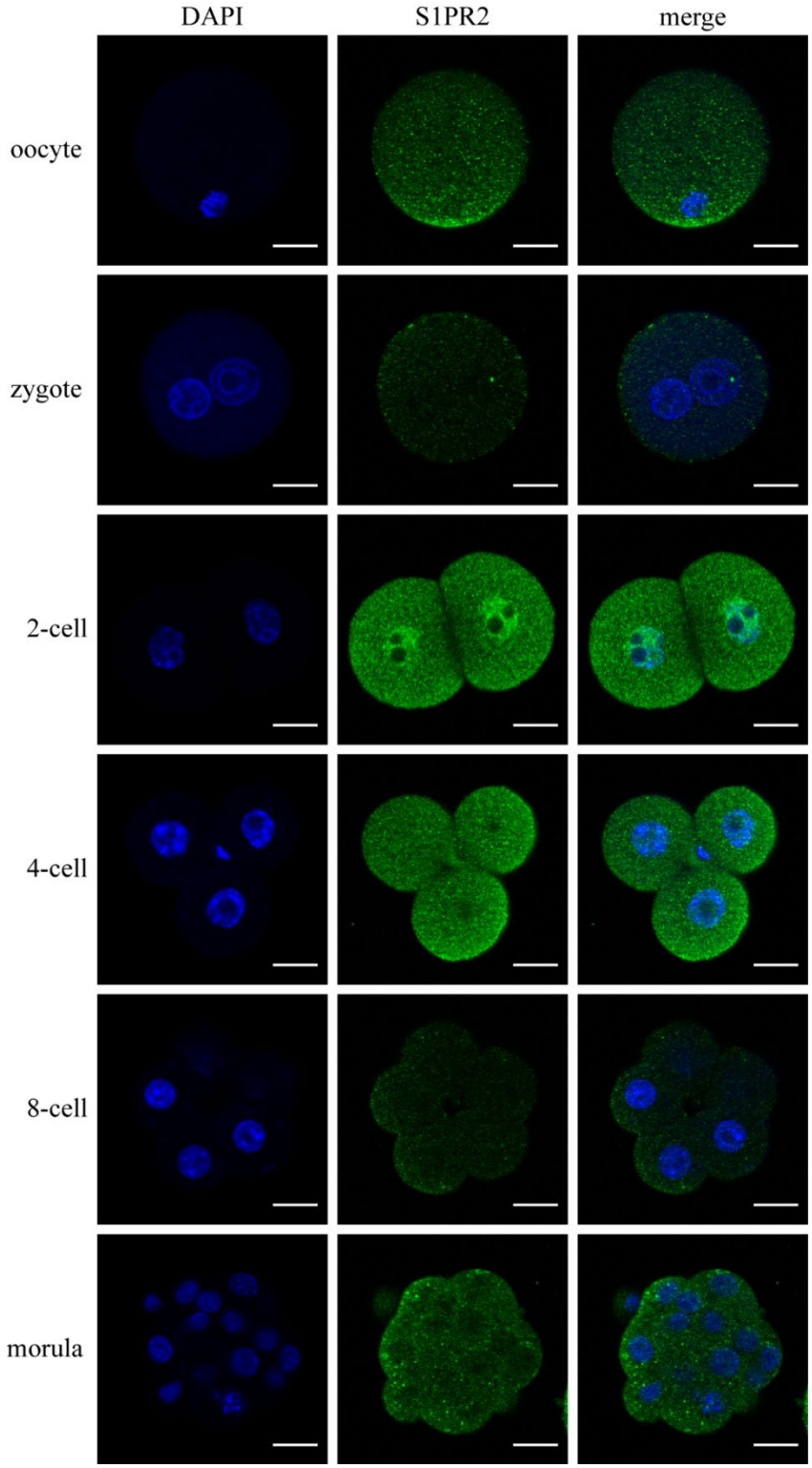


Figure 4.1. Localisation of S1PR2 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured *in vitro* at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote), 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for the S1PR2. Nuclei were stained with DAPI. Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.

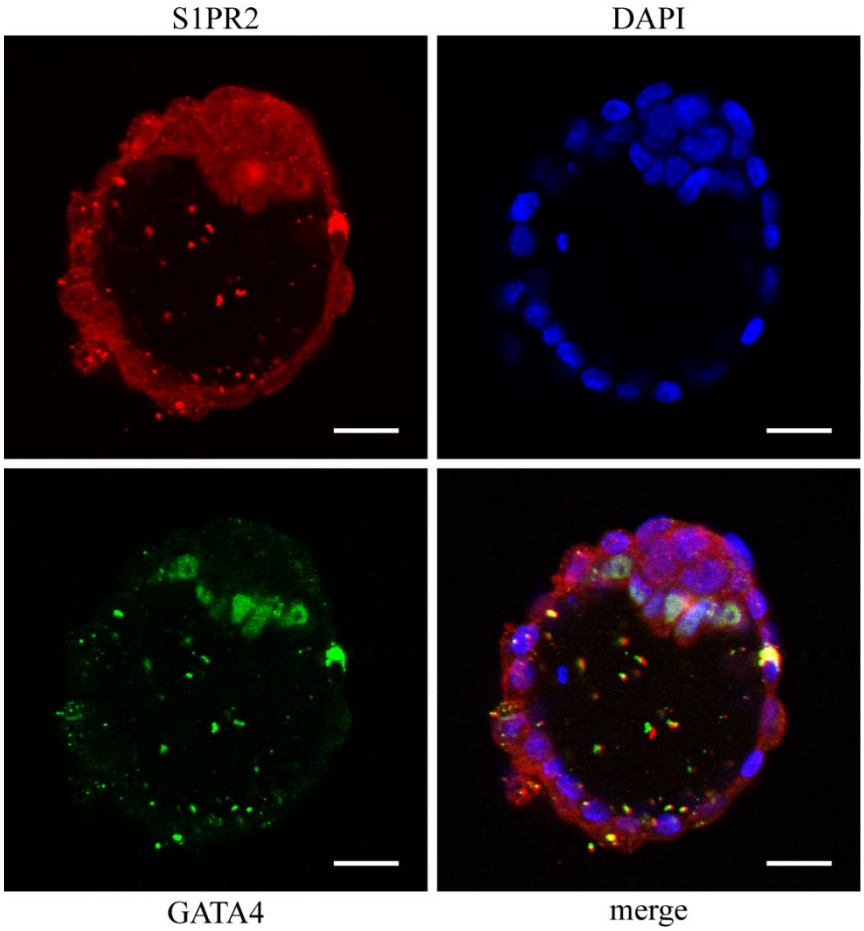


Figure 4.2. Localisation of S1PR2 in mouse blastocysts. Zygotes were isolated and cultured individually at a density of 20 embryos/20 µL in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde and immunostained to visualise S1PR2 (red) and GATA4 (green) as a marker of hypoblast cells. Nuclei were stained with DAPI (blue). Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.

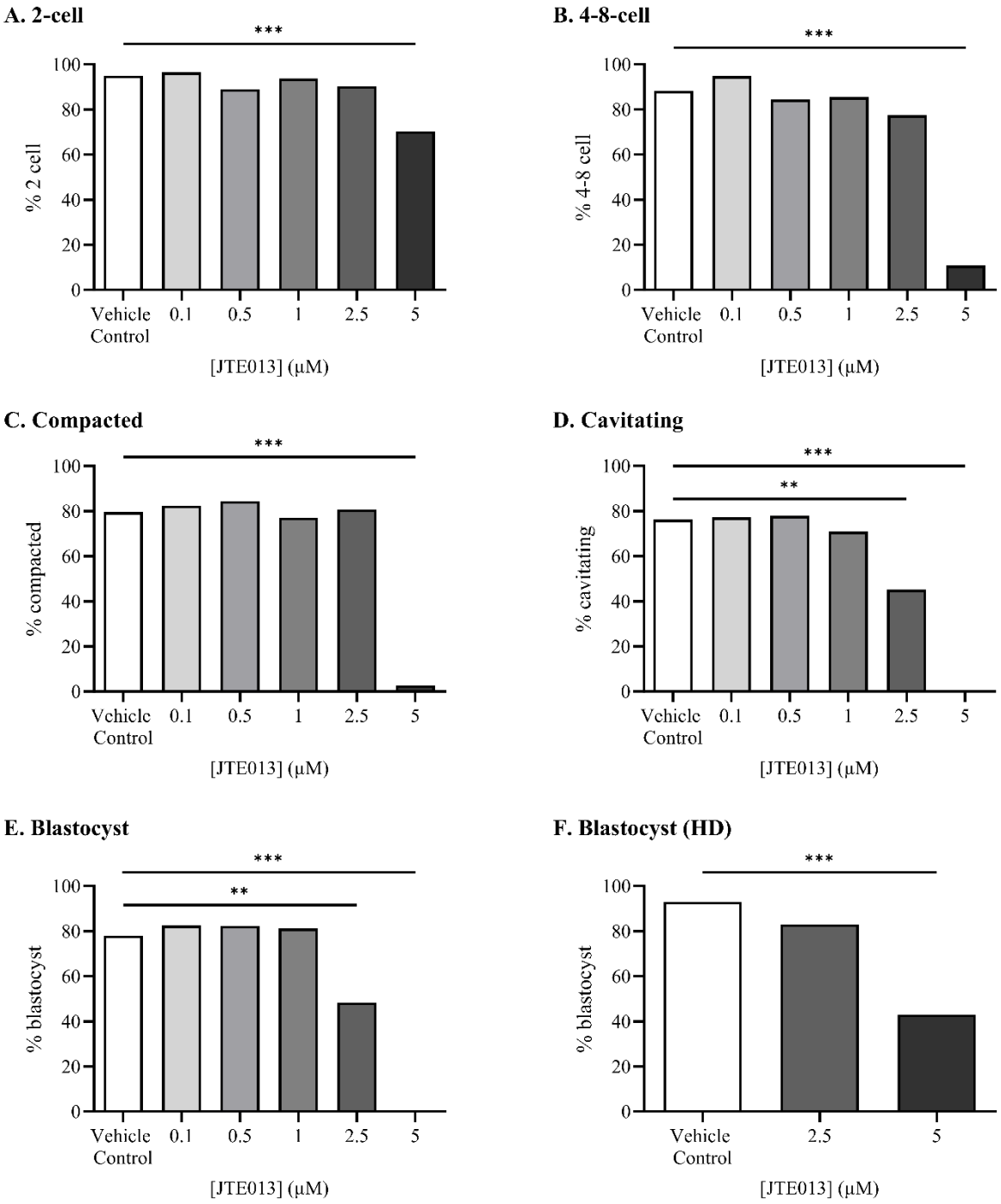


Figure 4.3. The effect of S1PR2 inhibition on mouse preimplantation embryo development. Zygotes were isolated and cultured at either LD (1 embryo/100 μL, panels **A-E**) or HD (1 embryo/1 μL, panel **F**). For LD, embryos were cultured in ModHTF medium containing 0.1% DMSO (vehicle control) or 0.1, 0.5, 1, 2.5, or 5 μM JTE013. Embryos were scored daily and the percentage of embryos that developed normally to the (**A**) 2-cell, (**B**) 4-8-cell, (**C**) compacted, (**D**) cavitating, and (**E**) blastocyst stages were recorded. For HD, embryos were cultured in ModHTF medium containing 0.1% DMSO plus 0, 2.5 or 5 μM JTE013 and the percentage of embryos that developed to the blastocyst stage was recorded (**F**). Data were pooled from at least 3 independent experiments with a minimum of 12 embryos per condition per experiment for LD conditions, or 10 embryos per condition per experiment for HD conditions. Statistical analysis was performed by chi-squared test. ** = $P<0.01$, *** = $P<0.001$.

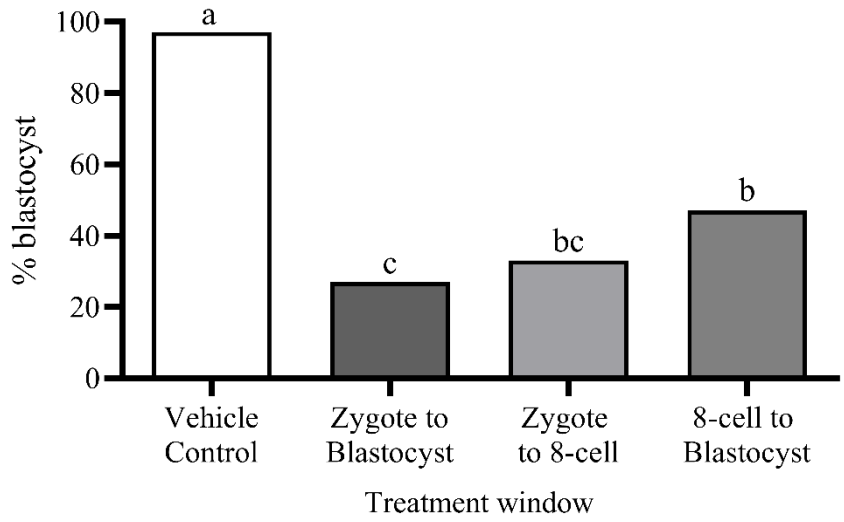


Figure 4.4. The effect of S1PR2 inhibition during early or late preimplantation development on blastocyst formation. Zygotes were isolated and cultured at LD (1 embryo/100 µL) in ModHTF medium. Embryos were treated with 0.1% DMSO (vehicle control) or 2.5 µM JTE013 from the zygote stage until 78 h post-hCG (zygote to 8-cell stage), from 78 h post-hCG onwards (8-cell to blastocyst) or from the zygote to blastocyst stage. The percentage of embryos that developed to the blastocyst stage was recorded at 144 h. Data were pooled from at least 3 independent experiments with a minimum of 12 embryos per condition per experiment. Statistical analysis was performed by chi-squared test. Bars not sharing the same letter are significantly different ($P<0.05$).

4.3.3 S1PR3 is present across all stages of preimplantation embryo development

S1PR3 was present at all stages of preimplantation development, predominantly within the cytoplasm and nucleus (Figures 4.5 and 4.6, Appendix 1 and 2). In the oocyte, S1PR3 staining was punctate throughout the cytoplasm, with stronger staining in the microvilli-free region overlying the meiotic spindle (Figure 4.5, Appendix 1).

At the zygote stage, S1PR3 staining was present subcortically and was relatively weaker in the cytoplasm and nucleus. Subcortical staining was also present in 2-cell to 8-cell stage embryos and these stages had strong staining in the nucleus, localised to the nucleoplasm but not the nucleoli (Figure 4.5, Appendix 1). In the morula, staining was restricted to the cytoplasm of cells along the outer surface of the embryo (Figure 4.5, Appendix 1). In blastocysts, S1PR3 staining was predominantly nuclear in all cells of the trophectoderm and ICM, though weak cytoplasmic staining was present (Figure 4.6, Appendix 2). IgG isotype and secondary only negative controls have been performed previously and demonstrate no staining (data not shown).

4.3.4 Inhibition of S1PR3 reduces embryo cleavage and blastocyst formation during LD embryo culture

To investigate the effect of autocrine S1P/S1PR3 signalling, embryos were cultured at HD or LD in the presence of TY52156, an S1PR3 inhibitor. During LD embryo culture, 10 μM TY52156 reduced the percentage of embryos that developed to all stages compared to untreated embryos, while 5 μM TY52156 reduced development from the 4-8-cell stage onwards (Figure 4.7A-E). Embryos in 10 μM TY52156 appeared blackened and lysed at the zygote to 2-cell stage. This effect was not seen during HD embryo culture, with no reduction in blastocyst formation in the presence of 5 μM or 10 μM TY52156 (Figure 4.7F).

Treatment of embryos with 10 μM TY52156 only during the zygote to 8-cell stage also reduced blastocyst formation compared to untreated embryos, similar to embryos treated from the zygote to blastocyst stage (Figure 4.8). Treatment of embryos with 10 μM TY52156 only during the 8-cell to blastocyst stage had no effect on blastocyst formation (Figure 4.8).

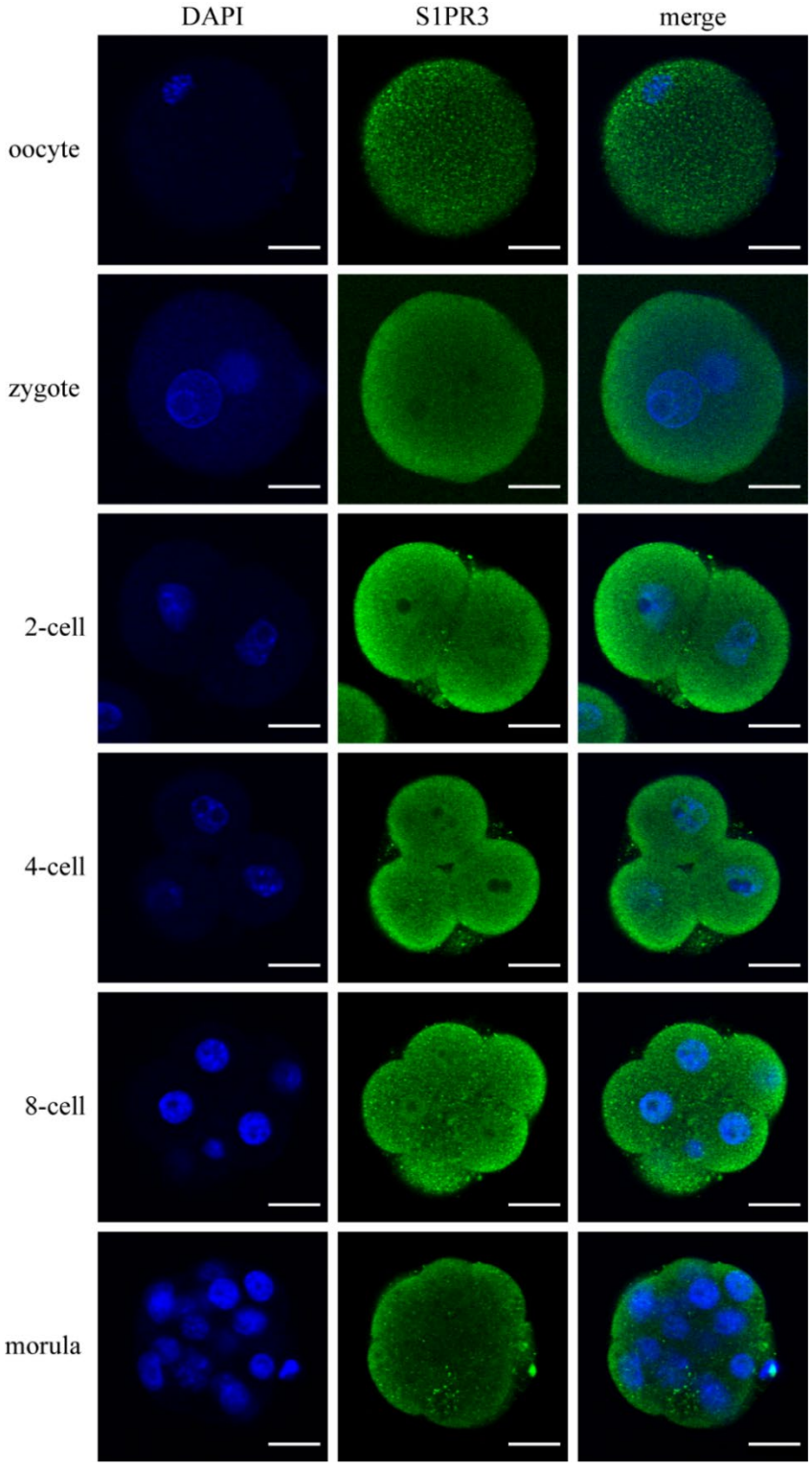


Figure 4.5. Localisation of S1PR3 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured *in vitro* at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote), 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for the S1PR3. Nuclei were stained with DAPI. Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.

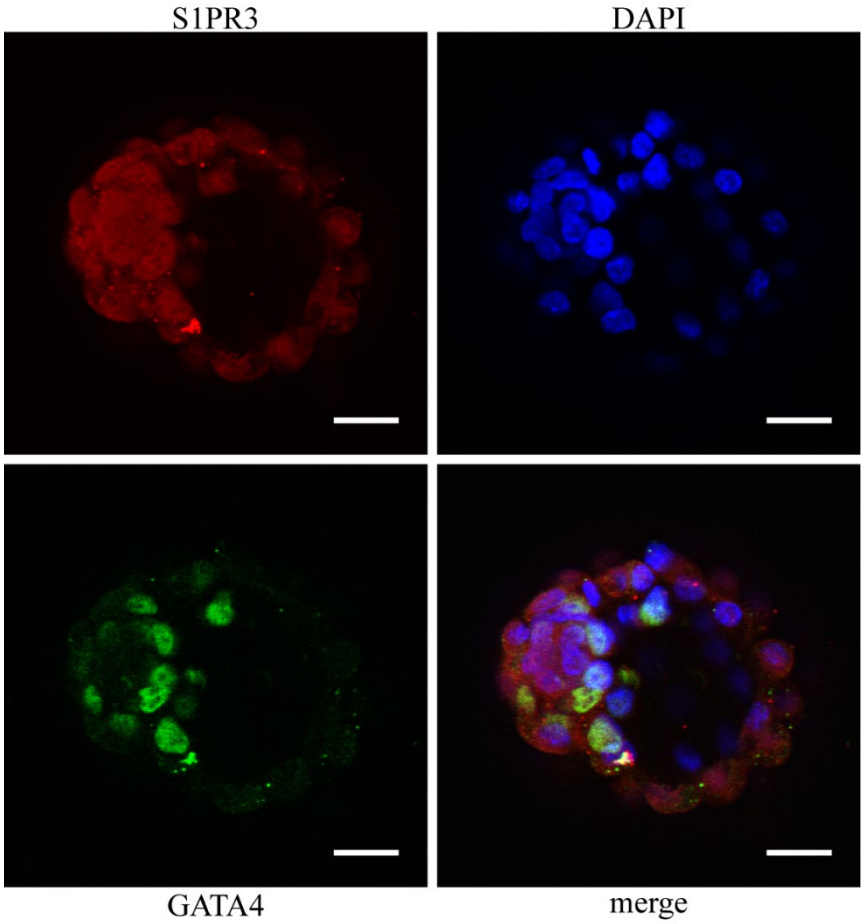


Figure 4.6. Localisation of S1PR3 in mouse blastocysts. Zygotes were isolated and cultured individually at HD (20 embryos/20 µL) in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde and immunostained to visualise S1PR3 (red) and GATA4 (green) as a marker of hypoblast cells. Nuclei were stained with DAPI (blue). Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.

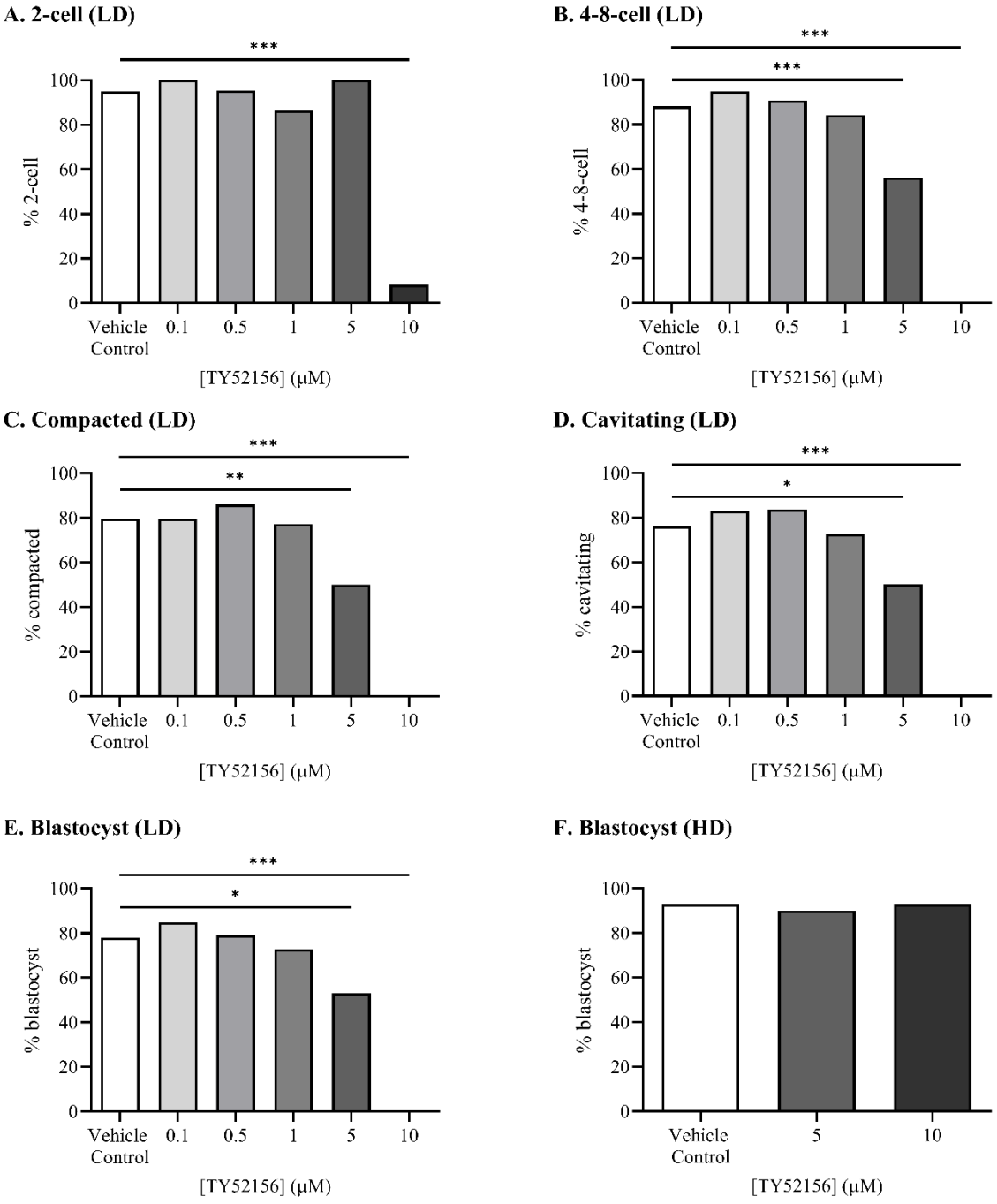


Figure 4.7. The effect of inhibition of S1PR3 on mouse preimplantation embryo development. Zygotes were isolated and cultured at either LD (1 embryo/100 μL, panels **A-E**) or HD (1 embryo/1 μL, panel **F**). For LD, embryos were cultured in ModHTF medium containing 0.1% DMSO (vehicle control) or 0.1, 0.5, 1, 5, or 10 μM TY52156. Embryos were scored daily and the percentage of embryos that developed normally to the (**A**) 2-cell, (**B**) 4-8-cell, (**C**) compacted, (**D**) cavitating, and (**E**) blastocyst stages were recorded. For HD, embryos were cultured in ModHTF medium containing 0.1% DMSO plus 0, 5 or 10 μM TY52156 and the percentage of embryos that developed to the blastocyst stage was recorded (**F**). Data were pooled from at least 3 independent experiments with a minimum of 12 embryos per condition per experiment for LD conditions, or 10 embryos per condition per experiment for HD conditions. Statistical analysis was performed by chi-squared test. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

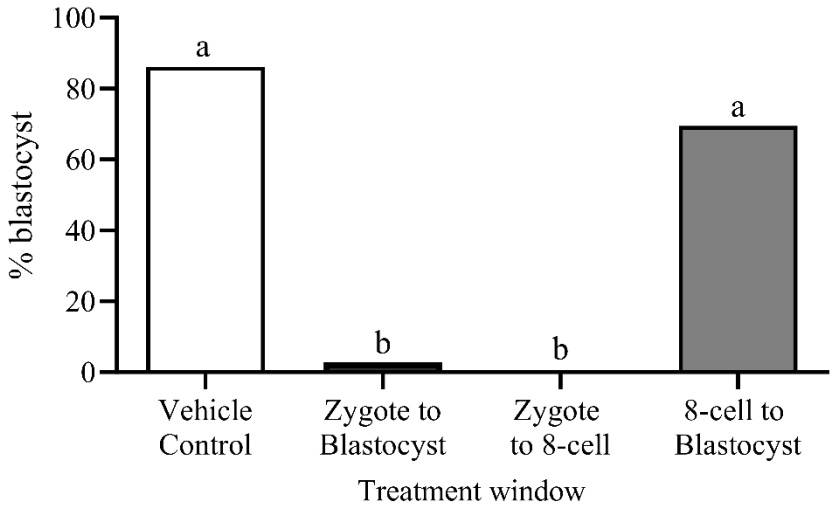


Figure 4.8. The effect of S1PR3 inhibition during early or late preimplantation development on blastocyst formation. Zygotes were isolated and cultured at LD (1 embryo/100 µL) in ModHTF medium. Embryos were treated with 0.1% DMSO (vehicle control) or 10 µM TY52156 from the zygote stage until 78 h post-hCG (zygote to 8-cell), from 78 h post-hCG onwards (8-cell to blastocyst) or from the zygote to blastocyst stage. The percentage of embryos that developed to the blastocyst stage was recorded at 144 h. Data were pooled from 3 independent experiments with 12 embryos per condition per experiment. Statistical analysis was performed by chi-squared test. Bars not sharing the same letter are significantly different ($P<0.05$).

4.3.5 S1PR4 is present across all stages of preimplantation development

S1PR4 was present at all stages of preimplantation development and was predominantly punctate and cytoplasmic before the blastocyst stage (Figure 4.9, Appendix 1). Nuclear staining was weak in the 2-cell, and 4-cell stage embryo, stronger at the zygote and 8-cell stage, and absent in the morula. Subcortical staining was present particularly at the zygote and 2-cell stage, as well as in the outer cells of the morula.

In the blastocyst, S1PR4 localised to the trophectoderm, and the hypoblast cells of the ICM (Figure 4.10, Appendix 2). In trophectoderm cells, staining was predominantly membranous, while in hypoblast cells, S1PR4 staining was nuclear. IgG isotype and secondary only negative controls have been performed previously and demonstrate no staining (data not shown).

4.3.6 Inhibition of S1PR4 reduces cavitation and blastocyst formation during LD embryo culture

To investigate the effect of autocrine S1P/S1PR4 signalling, embryos were cultured at HD or LD in the presence of CYM50358, an S1PR4 inhibitor. During LD embryo culture, 1000 nM CYM50358 reduced the percentage of embryos that cavitated and formed a blastocyst compared to untreated embryos (Figure 4.11A-E). Embryos in 1000 nM CYM50358 appeared healthy up to the morula stage but were blackened and lysed 24 h later. This effect was not seen at HD, where 1000 nM CYM50358 had no effect on blastocyst formation (Figure 4.11F).

Treatment of embryos with 1000 nM CYM50358 only during the 8-cell to blastocyst stage also reduced blastocyst formation compared to untreated embryos (Figure 4.12), similar to embryos treated from the zygote to blastocyst stage. Treatment of embryos with 1000 nM CYM50358 only during the zygote to 8-cell stage had no effect on blastocyst formation (Figure 4.12).

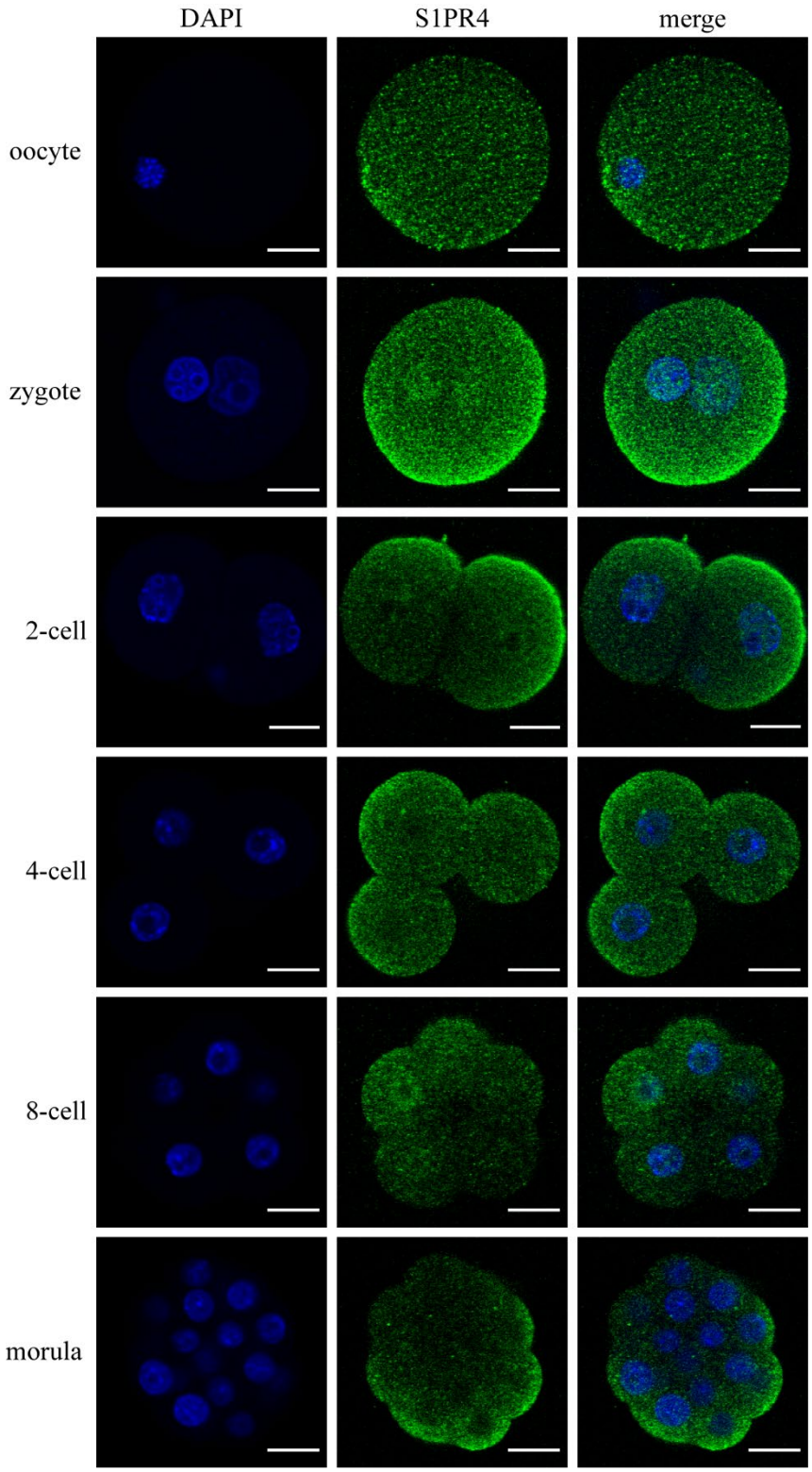


Figure 4.9. Localisation of S1PR4 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured *in vitro* at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote), 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for the S1PR5. Nuclei were stained with DAPI. Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.

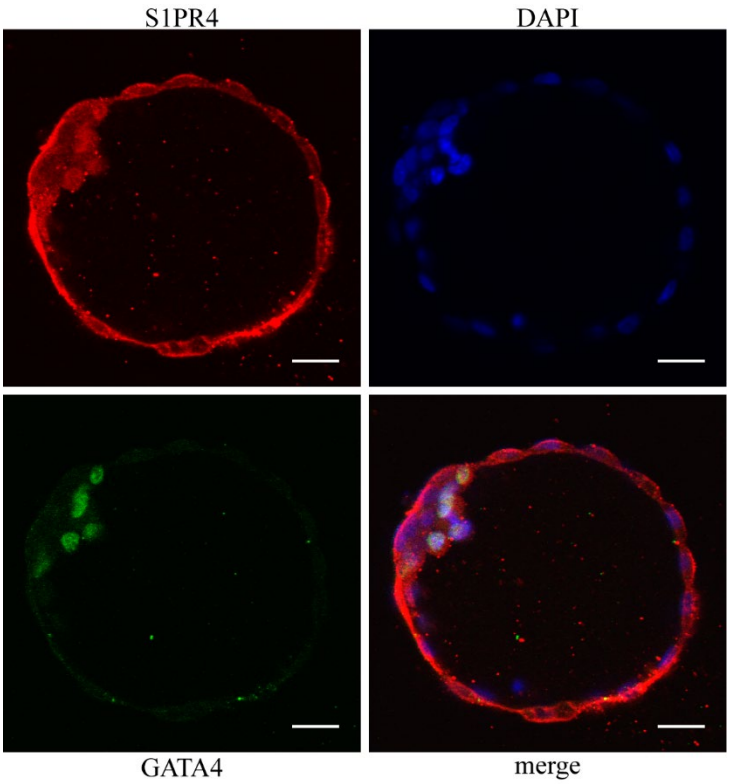


Figure 4.10. Localisation of S1PR4 in the mouse blastocyst. Zygotes were isolated and cultured at HD (20 embryos/20 µL) in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde and immunostained to visualise S1PR4 (red) and GATA4 (green) as a marker of hypoblast cells. Nuclei were stained with DAPI (blue). Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.

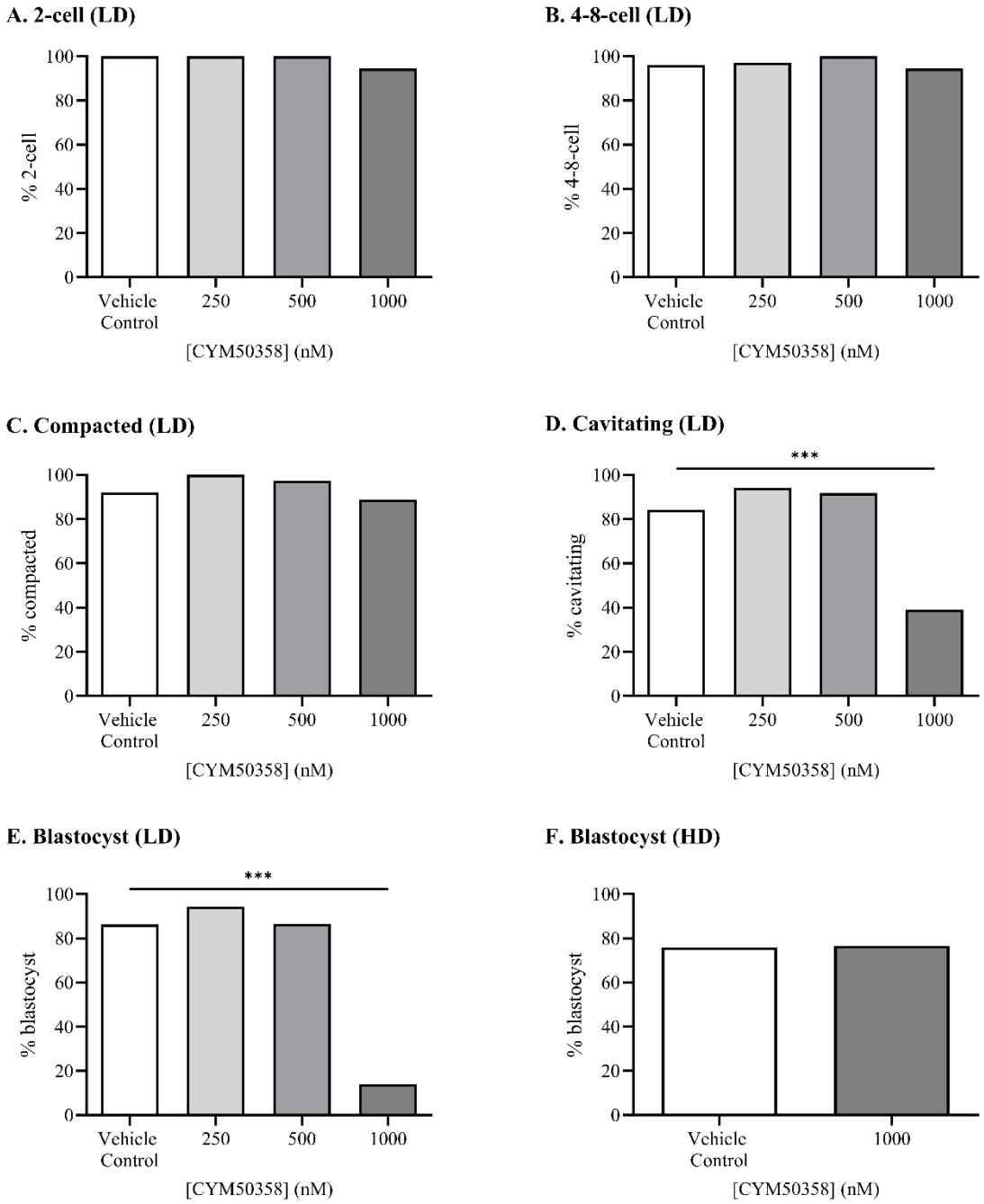


Figure 4.11. The effect of inhibition of S1PR4 on mouse preimplantation embryo development. Zygotes were isolated and cultured at either LD (1 embryo/100 µL, panels **A-E**) or HD (1 embryo/1 µL, panel **F**). For LD, embryos were cultured in ModHTF medium containing 0.1% DMSO (vehicle control) or 250, 500, or 1000 nM CYM50358. Embryos were scored daily and the percentage of embryos that developed normally to the (**A**) 2-cell, (**B**) 4-8-cell, (**C**) compacted, (**D**) cavitating, and (**E**) blastocyst stages were recorded. For HD, embryos were cultured in ModHTF medium containing 0.1% DMSO plus 0 or 1000 nM CYM50358 and the percentage of embryos that developed to the blastocyst stage was recorded (**F**). Data were pooled from at least 3 independent experiments with a minimum of 12 embryos per condition per experiment for LD conditions, or 10 embryos per condition per experiment for HD conditions. Statistical analysis was performed by chi-squared test. *** = $P<0.001$.

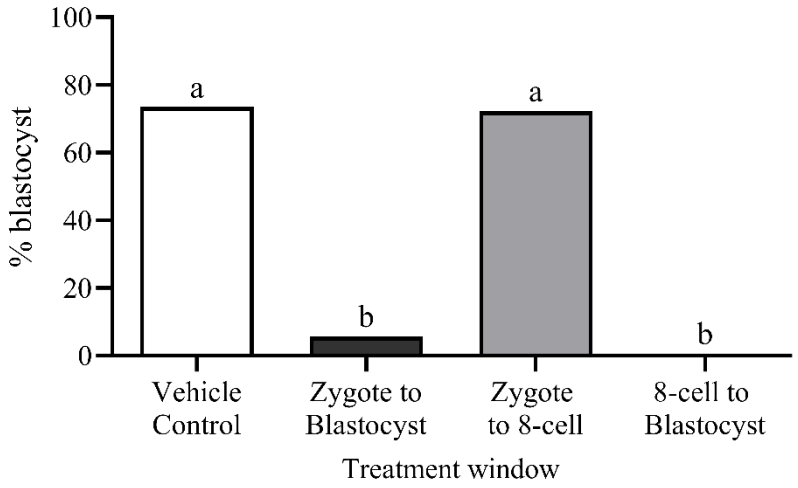


Figure 4.12. The effect of S1PR4 inhibition during early or late preimplantation development on blastocyst formation. Zygotes were isolated and cultured at LD (1 embryo/100 µL) in ModHTF medium. Embryos were treated with 0.1% DMSO (vehicle control) or 1000 nM CYM50358 from the zygote stage until 78 h post-hCG (zygote to 8-cell), from 78 h post-hCG onwards (8-cell to blastocyst) or from the zygote to blastocyst stage. The percentage of embryos that developed to the blastocyst stage was recorded at 144 h. Data were pooled from 3 independent experiments with 12 embryos per condition per experiment. Statistical analysis was performed by chi-squared test. Bars not sharing the same letter are significantly different ($P<0.05$).

4.3.7 S1PR5 is present across all stages of preimplantation embryo development

S1PR5 was present at all stages of preimplantation development, with staining appearing membranous or subcortical during early stages of development, and punctate within the cytoplasm during later stages (Figures 4.13 and 4.14, Appendix 1 and 2). From the oocyte to the 4-cell stage, S1PR3 staining was cytoplasmic, with stronger staining within the membrane and subcortically (Figure 4.13, Appendix 1). Punctate cytoplasmic staining was present from the 4-cell stage onwards (Figures 4.13 and 4.14, Appendix 1 and 2). Note that multiple stages were stained in the same well containing antibody to control for differences in staining between stages. In the morula, staining was stronger on the outer edge of the embryo. In blastocysts, staining was restricted to the trophectoderm, with no staining present in the hypoblast or epiblast cells of the ICM (Figure 4.14, Appendix 2). No staining was present in the nucleus at any stage of development. IgG isotype and secondary only negative controls have been performed previously and demonstrate no staining (data not shown).

4.3.8 S1PR5 is co-localised to autophagosomes in 4-cell and 8-cell embryos

To investigate whether the S1PR5 was present within autophagosomes, embryos were co-stained using the CYTO-ID Autophagy Detection Kit and the S1PR5 antibody. The punctate cytoplasmic staining observed with S1PR5 staining co-localised to puncta dyed using the CYTO-ID kit (Figure 4.15).

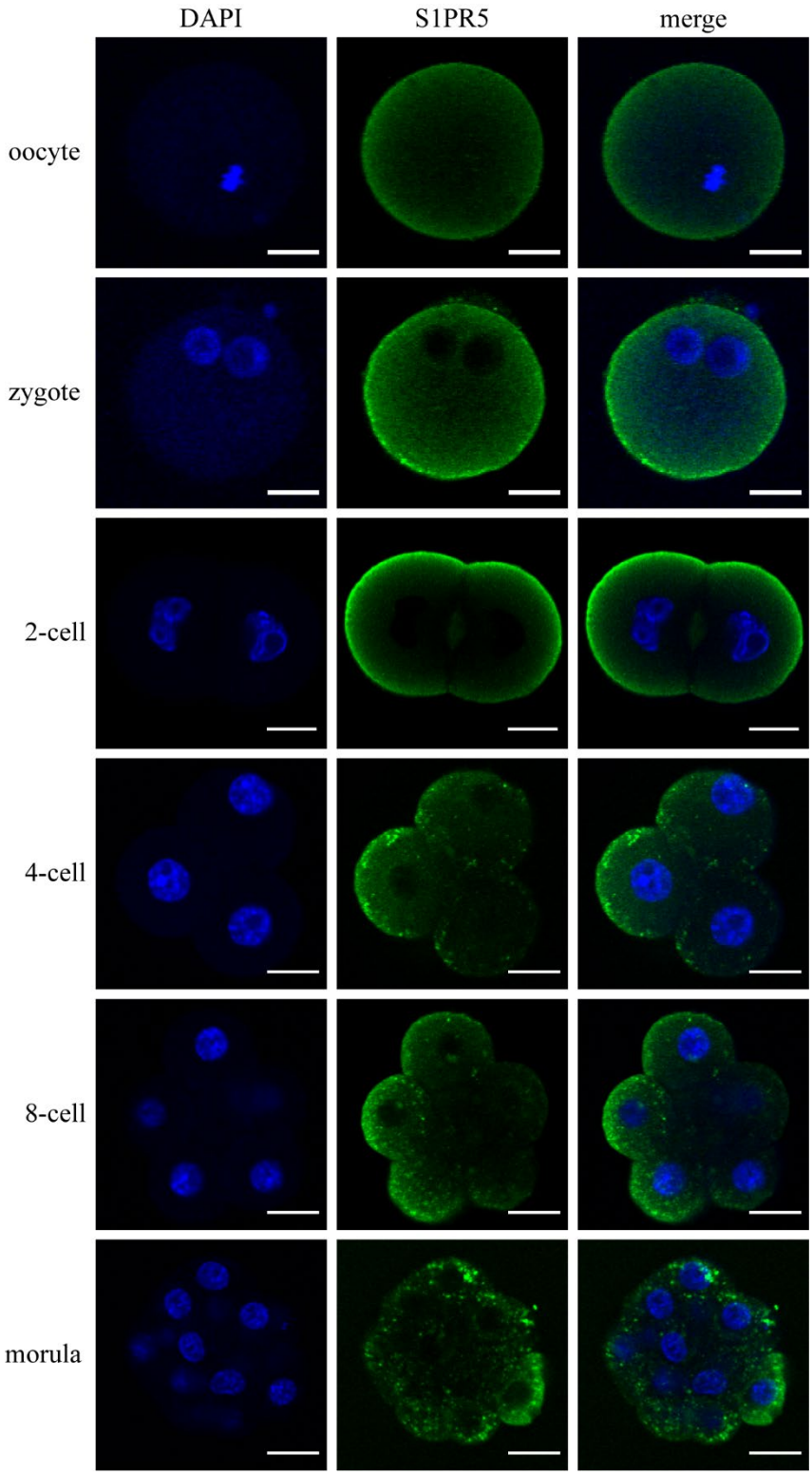


Figure 4.13. Localisation of S1PR5 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured *in vitro* at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote), 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for the S1PR5. Nuclei were stained with DAPI. Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.

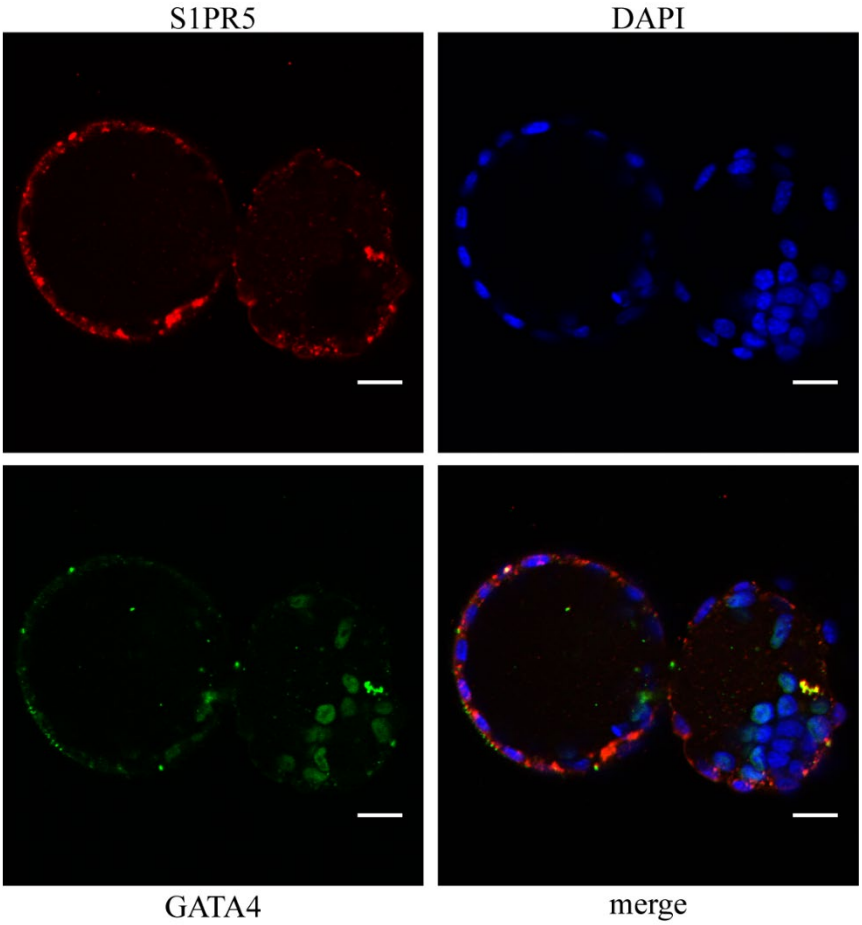


Figure 4.14. Localisation of S1PR5 in the mouse blastocyst. Zygotes were isolated and cultured at HD (20 embryos/20 µL) in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde and immunostained to visualise S1PR5 (red) and GATA4 (green) as a marker of hypoblast cells. Nuclei were stained with DAPI (blue). Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.

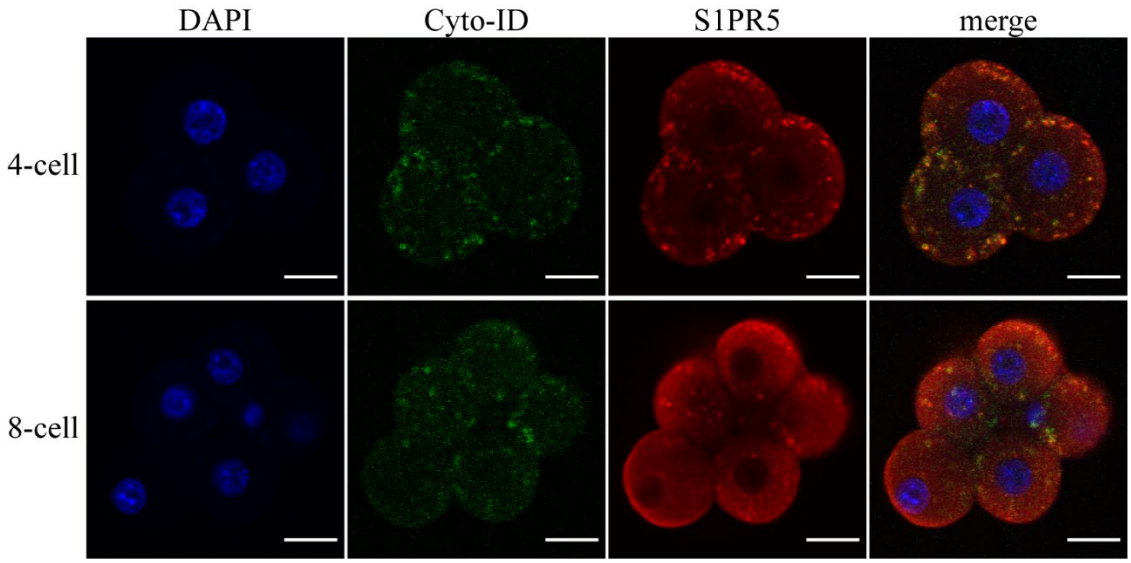


Figure 4.15. Co-localisation of S1PR5 with autophagosomes in 4-cell and 8-cell embryos. Zygotes were isolated and cultured at HD (20 embryos/20 µL) in ModHTF medium to the 4-cell and 8-cell stage. The zona pellucida was removed and embryos were cultured in CYTO-ID autophagy dye to visualise autophagosomes (green). Embryos were then fixed and immunostained to visualise the S1PR5 (red) and nuclei (blue). Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.

4.3.9 Inhibition of S1PR1-3 reduces cavitation and blastocyst formation during LD embryo culture

Redundancy occurs between S1PR1-3 during later stages of mouse foetal development, with knockout of all three receptors resulting in embryonic lethality despite knockout of individual receptors having no effect (Kono et al., 2004). To investigate redundancy between S1PR1-3 during preimplantation development, embryos were cultured in the presence of inhibitors of S1PR1-3 (2.5 μM NIBR0213 + 1 μM JTE013 + 1 μM TY25156). Inhibition of S1PR1-3 during LD culture reduced the percentage of embryos that cavitated and formed blastocysts compared to untreated embryos (Figure 4.16A-E). This effect was not seen at HD, where inhibition of S1PR1-3 had no effect on blastocyst formation (Figure 4.16F).

4.3.10 Inhibition of S1PR1-4 reduces embryo development past the 2-cell stage during LD embryo culture

To investigate redundancy between S1PR1-4 during preimplantation development, embryos were cultured in the presence of inhibitors of S1PR1-4 (2.5 μM NIBR0213 + 1 μM JTE013 + 1 μM TY25156 + 500 nM CYM50358). Inhibition of S1PR1-4 during LD culture reduced the percentage of embryos that developed past the 2-cell stage compared to untreated embryos (Figure 4.17A-E). This effect was not seen at HD, where inhibition of S1PR1-4 had no effect on blastocyst formation (Figure 4.17F).

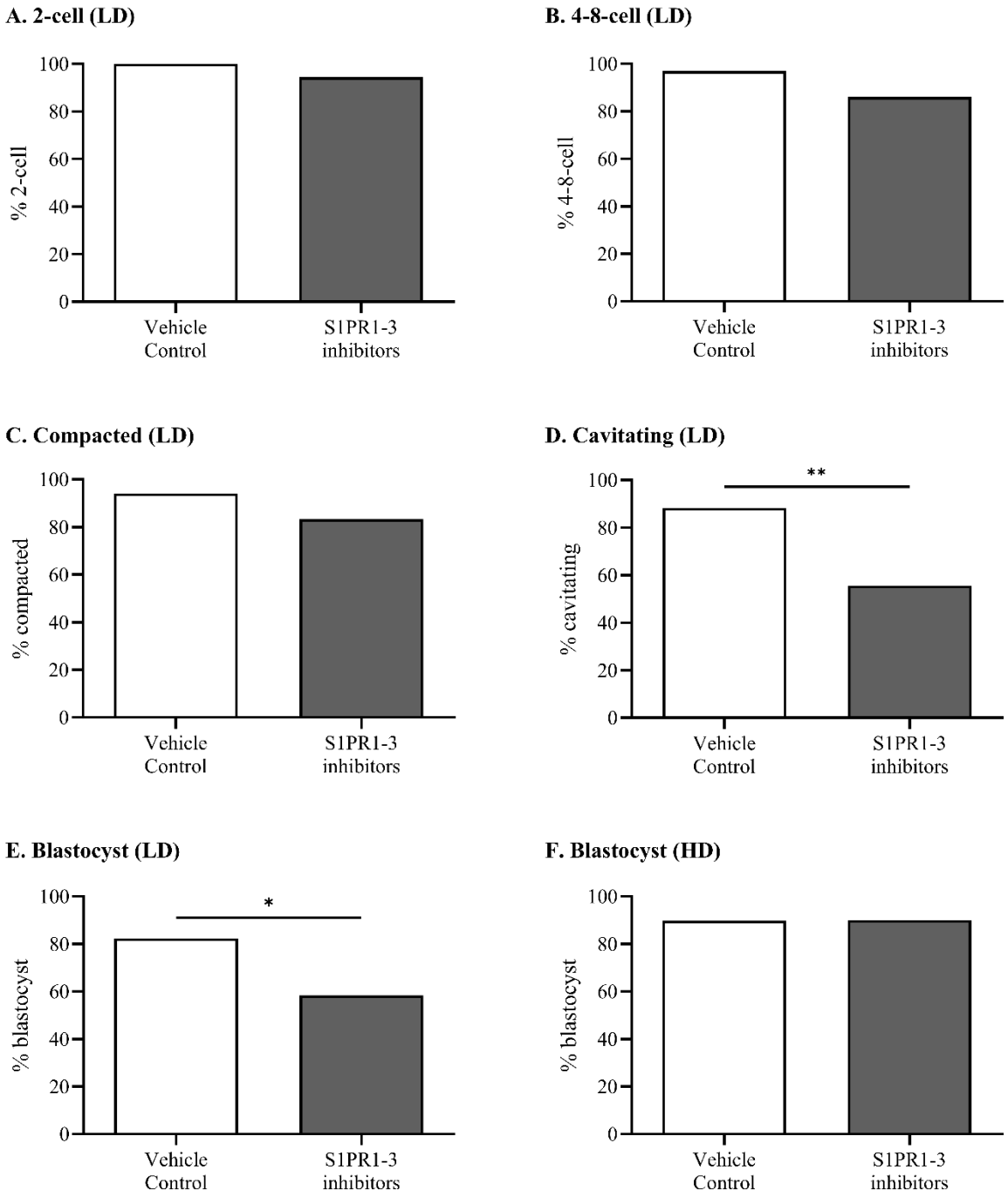


Figure 4.16. The effect of inhibition of S1PR1-3 on mouse preimplantation embryo development. Zygotes were isolated and cultured at either LD (1 embryo/100 µL, panels **A-E**) or HD (1 embryo/1 µL, panel **F**). Embryos were cultured in ModHTF medium containing 0.1% DMSO (vehicle control), or ModHTF medium containing inhibitors of S1PR1-3 (2.5 µM NIBR0213 + 1 µM JTE013 + 1 µM TY25156, respectively). For LD, embryos were scored daily and the percentage of embryos that developed normally to the (**A**) 2-cell, (**B**) 4-8-cell, (**C**) compacted, (**D**) cavitating, and (**E**) blastocyst stages were recorded. For HD, the percentage of embryos that developed to the blastocyst stage was recorded (**F**). Data were pooled from at least 3 independent experiments with a minimum of 12 embryos per condition per experiment for LD conditions, or 10 embryos per condition per experiment for HD conditions. Statistical analysis was performed by chi-squared test. * = $P<0.05$, ** = $P<0.01$.

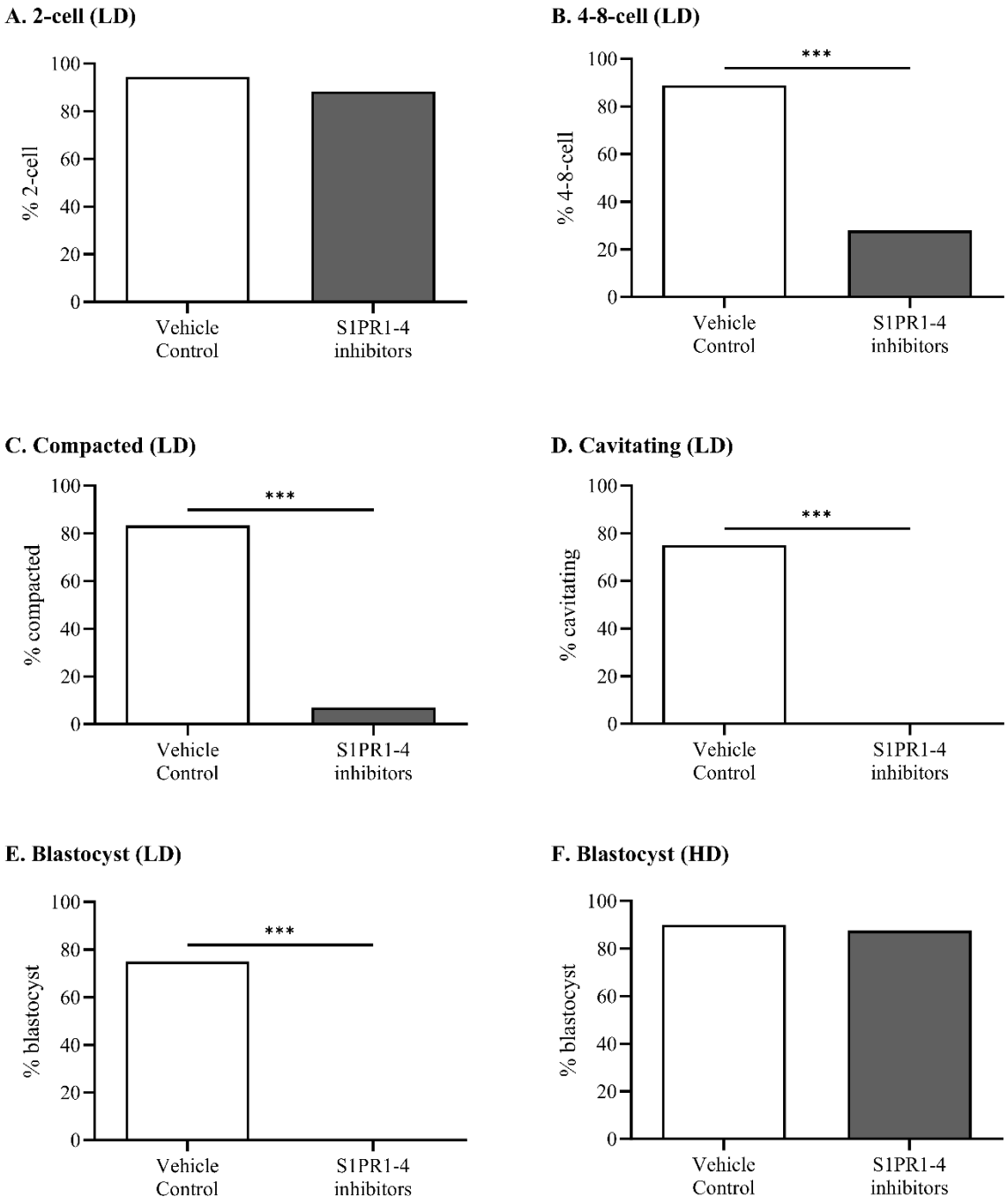


Figure 4.17. The effect of inhibition of S1PR1-4 on mouse preimplantation embryo development. Zygotes were isolated and cultured at either LD (1 embryo/100 µL, panels **A-E**) or HD (1 embryo/1 µL, panel **F**). Embryos were cultured in ModHTF medium containing 0.1% DMSO (vehicle control), or ModHTF medium containing inhibitors of S1PR1-4 (2.5 µM NIBR0213 + 1 µM JTE013 + 1 µM TY25156 + 500 nM CYM50358, respectively). Embryos were scored daily and the percentage of embryos that developed normally to the (**A**) 2-cell, (**B**) 4-8-cell, (**C**) compacted, (**D**) cavitating, and (**E**) blastocyst stages were recorded. For HD, the percentage of embryos that developed to the blastocyst stage was recorded (**F**). Data were pooled from at least 3 independent experiments with a minimum of 12 embryos per condition per experiment for LD conditions, or 10 embryos per condition per experiment for HD conditions. Statistical analysis was performed by chi-squared test. *** = $P<0.001$.

4.3.11 Expression of S1PR genes in *in vivo* and *in vitro* developed embryos

To investigate the expression of *S1pr* genes in preimplantation embryos, as well as the effect of *in vitro* culture on *S1pr* expression, qPCR was performed on embryos developed both *in vivo* and *in vitro*. Experiments were run in triplicate with 3 independent experiments for each gene. Transcripts of most *S1pr* genes were not detectable (C_t value ≥ 38) in the samples using qPCR, with signal detected in 45 out of 315 wells from *in vivo* samples, and 12 out of 315 from *in vitro* samples (Table 4.4). *S1pr1* mRNA was not detected at any stage regardless of condition. *S1pr2* was detected at the 4-cell and blastocyst stages *in vivo* but not *in vitro*. *S1pr3* was detected at the 8-cell stage both *in vivo* and *in vitro*. *S1pr4* was detected at the 8-cell and blastocyst stages for *in vivo* developed embryos. *S1pr5* was detected at all developmental stages except for the 8-cell and blastocyst *in vivo*, but only the 4-cell and morula stages *in vitro*.

Table 4.4. Expression of *S1pr* genes in embryos analysed using RT-qPCR

Stage	*In vivo*	*In vitro*
Oocyte	S1PR5 (3/9)	
Zygote	S1PR5 (7/9)	
2-cell	S1PR5 (6/9)	
4-cell	S1PR2 (3/9), S1PR5 (4/9)	S1PR5 (6/9)
8-cell	S1PR3 (2/9), S1PR4 (5/9)	S1PR3 (2/9)
Morula	S1PR5 (6/9)	S1PR5 (4/9)
Blastocyst	S1PR2 (5/9), S1PR4 (4/9)	

N.B. Numbers in parentheses represent the number of wells the gene was detected in out of the total number of wells analysed for that gene. Genes detected in 0/9 wells not shown (C_t value ≥ 38).

4.4 Discussion

Embryo-derived S1P signalling via the S1PR1 is essential for mouse preimplantation embryo development *in vitro* (Chapter 3). However, there are also potential redundancies between S1PR1 and the other four S1PRs. This study investigated the expression and localisation of S1PR2-5 as well as the function of S1PR2-4 in the mouse embryo and found that all four S1PRs were present at all stages of the embryo and were required for normal development.

4.4.1 S1PR2 signalling is required for cavitation and blastocyst formation

Punctate cytoplasmic staining for S1PR2 was present throughout preimplantation development, though staining was very weak at the zygote and 8-cell stages (Figure 4.1). In the oocyte, staining was stronger over the meiotic spindle (Figure 4.1) in the microvilli-free region (Eager et al., 1976; Runge et al., 2007), suggesting the involvement of S1PR2 in chromosomal division, cytokinesis, or the location of the spindle in the oocyte. S1P/S1PR2 signalling promotes proliferation in other cell types, including intestinal epithelial cells, satellite cells, and cancer cells (Chen et al., 2017; Cheng et al., 2018; Loh et al., 2012; Pitman et al., 2022), and this may also occur in the mouse oocyte. However, the microvilli-free region of the oocyte membrane is extruded upon formation of the second polar body at fertilisation, and so it is possible that S1PR2 is involved in oocyte development prior this stage and localises to this region of the mature oocyte to facilitate disposal. Neither human mature oocytes nor human or mouse immature oocytes have a microvilli-free region, and instead have surfaces completely covered in microvilli, or rarely containing microvilli, respectively (Kryzak et al., 2013; Santella et al., 1992; Yang et al., 2009). Investigation of the localisation of S1PR2 in other species or other stages of oogenesis could provide insight into the role of S1PR2 in the oocyte.

There was strong staining for S1PR2 in the nucleus and cytoplasm of 2-cell embryos, as well as in the cytoplasm of 4-cell embryos (Figure 4.1). Staining was decreased at the 8-cell stage before increasing in the cytoplasm in the morula and blastocyst. This cyclic expression suggests that S1PR2 signalling may be necessary during both early and late stages of preimplantation development. Inhibition of S1PR2 with 2.5 μM JTE013 during LD embryo culture resulted in failure of embryos to cavitate (Figure 4.3), suggesting a role for S1PR2 signalling in blastocyst formation. This is consistent with inhibition of S1PR2 with 1 μM JTE013 causing a morula block in C57BL/6J X C3He mouse embryos (Chi et

al., 2020). The difference in concentration that resulted in arrest of the embryos could be due to subtle differences in S1PR2 expression between strains of mice or differences in culture medium or embryo culture density, the latter of which was not reported. Inhibition of S1PR2 during HD embryo culture did not affect development, which could be due to increased concentration of S1P or other autocrine growth factors present, as discussed in section 3.4.2 (Dai et al., 2012).

Inhibition of S1PR2 could impair blastocyst formation by disrupting differentiation. In the mouse early blastocyst, S1P/S1PR2 signalling is involved in the differentiation of blastomeres into trophectoderm and ICM cells (Chi et al., 2020). This study demonstrated that activation of S1PR2 leads to downstream activation of mTOR, thus facilitating the transcription of *Tfap2c*. Within pre-trophectoderm cells of the early blastocyst, TFAP2C interacts with YAP and TEAD4 to form a nuclear complex which upregulates the expression of trophectoderm-specific genes, such as *Cdx2* (Chi et al., 2020). This is accompanied by Hippo signalling in the ICM which prevents nuclear accumulation of YAP and subsequent differentiation into trophectoderm cells. Collectively, this permits formation of these two cell populations.

Similar to S1PR1, as discussed in Chapter 3, S1PR2 inhibition could impair blastocyst formation by disrupting the formation and maintenance of tight junctions. S1PR2 signalling upregulates junctional proteins, including occludin (OCLN) and ZO-1, in epidermal and intestinal epithelial cells (Chen et al., 2018; Igawa et al., 2021; Paszti-Gere et al., 2016). Both ZO-1 and OCLN are essential for embryo compaction and subsequent blastocyst formation (Kim et al., 2004; Wang et al., 2008), and so abnormalities in expression of these proteins might be expected to result in failure to cavitate as seen with inhibition of S1PR2 (Figure 4.3). To investigate this, the effect of S1PR2 inhibition on localisation of tight junction proteins should be determined.

Inhibition of S1PR2 impaired blastocyst formation regardless of whether inhibition occurred only in early or late stages of development (Figure 4.3). This could be due to S1P/S1PR2 signalling being necessary during early preimplantation development to prime the embryo for tight junction assembly through upregulation of junctional proteins, and necessary during late preimplantation development for the maintenance of tight junctions as the embryo undergoes cavitation.

The selectivity of JTE013 for S1PR2 is not high and it can inhibit S1PR4 at a concentration of 10 μM, only 4 times that used here (Long et al., 2010). Thus, if JTE013 also significantly inhibits S1PR4 at 2.5 μM, then some or all of the effects of JTE013 during later stages of preimplantation development may be mediated by S1PR4 rather than S1PR2, as inhibition of S1PR4 reduces embryo cavitation and blastocyst formation (Figure 4.11).

JTE013 also inhibits enzymes involved in sphingolipid metabolism including SphKs and is particularly potent against pSphK2 (Pitman et al., 2022). However, this was tested using 5 μM JTE013 and so it is unclear if the 2.5 μM JTE013 used here would also have a significant effect on pSphK2 activity. 5 μM JTE013 did impair blastocyst formation during both HD and LD embryo culture (Figure 4.3), which could be due to inhibition of S1PR4 and pSphK2 in addition to S1PR2. Inhibition of pSphK2 would reduce the amount of S1P available to act on other S1PRs, thus having broader effects on S1P signalling than intended, and so other methods of S1PR2 inhibition such as small molecule inhibitor GLPG2938 (Mammoliti et al., 2021), genetic knockout, or siRNA knockdown should be considered. However,

4.4.2 S1PR3 signalling is required for embryo cleavage

S1PR3 staining in the oocyte was very similar to S1PR2, suggesting a coordinating function in meiotic division in the mature oocyte (Figure 4.5). S1PR3 is also present at all stages of preimplantation development, and staining was punctate and cytoplasmic, with subcortical and nuclear staining in the cleavage stages (Figures 4.5 and 4.6). Inhibition of S1PR3 during LD embryo culture from the zygote to blastocyst stages reduced the percentage of zygotes that cleaved to form a 2-cell embryo, while inhibition of S1PR3 during HD embryo culture did not affect development (Figure 4.7), potentially be due to increased concentration of S1P or other autocrine growth factors present during HD culture, as discussed in section 3.4.2 (Dai et al., 2012).

This suggests that S1PR3 signalling is already established in the zygote stage and is required for cell survival and cleavage. S1PR3 is important for proliferation in other cells, with inhibition of S1PR3, using 10 μM TY52156, resulting in reduced proliferation of osteosarcoma cell lines and induction of apoptosis (Shen et al., 2019). S1P/S1PR3 signalling promotes nuclear localisation of YAP in these cells, leading to downstream regulation of gene expression and thus promoting cell survival and proliferation. However, it is unlikely that this is the signalling pathway that occurs in the preimplantation embryo. While

YAP/TEAD-mediated transcription is essential for degradation of maternal mRNA at the 2-cell stage in mice (Sha et al., 2020), inhibition of YAP does not prevent cleavage to the 2-cell stage as seen with inhibition of S1PR3 in this study (Figure 4.7). Instead, embryos lacking maternal YAP progress more slowly through the 2-cell stage and arrest at the 4-cell stage (Yu et al., 2016). This suggests that embryonic death following S1PR3 inhibition is not solely due to disruption of YAP, and that S1PR3 is involved in other pathways that regulate cell survival and cleavage.

S1PR3 signalling regulates cell cycle in other cell types, including muscle satellite cells and lung cancer cell lines (Fortier et al., 2013; Lin et al., 2022). In satellite cells, S1PR3 signalling supresses cell-cycle progression and is associated with cell quiescence (Fortier et al., 2013). However, in lung cancer cells, S1PR3 signalling has the opposite effect in that it promotes proliferation by binding to PBX1, which in turn binds to the S1PR3 promoter region to increase transcription of S1PR3 (Lin et al., 2022). Inhibition of S1PR3 or PI3K/Akt signalling in these cells results in G1/S phase arrest, supporting a role for S1PR3/PBX1/PI3K/Akt signalling in progression of cell cycle. Similar to cancer cells, embryos are highly proliferative, and it is possible that S1P/S1PR3 signalling in the embryo acts via the same pathway it does in cancer cells to promote proliferation and cleavage.

Alternatively, embryo culture exacerbates production of reactive oxygen species (ROS) which results in oxidative stress (Hardy et al., 2021), and S1PR3 signalling protects human granulosa cells from oxidative stress-induced apoptosis through activation of PI3K/Akt and ERK and inhibition of caspase-3 (Nakahara et al., 2012). It is possible that loss of protective S1PR3 signalling sensitises the embryo to these stressors, resulting in rapid cell death. Inhibition of S1PR3 only affects embryo development during early stages, whereas inhibition during later stages has no effect (Figure 4.8). This could be due to upregulation of antioxidant genes during later stages of embryo development which could protect embryos from oxidative stress-induced cell death in the absence of S1PR3 signalling (Amin et al., 2014).

By the blastocyst stage, S1PR3 staining was predominantly restricted to the nuclei of cells, though some weak cytoplasmic staining was present (Figure 4.6). Inhibition of S1PR3 during later stages of development has no effect on blastocyst formation, suggesting S1P/S1PR3 signalling is only present during early stages despite S1PR3 also being expressed during later stages (Figure 4.8). Alternatively, the loss of S1PR3 signalling could

be compensated for by other S1PRs or other growth factor pathways during later stages, but not during early stages. S1PR3 signalling could also affect processes beyond blastocyst formation such as implantation. It is unknown if S1PR3 signalling can occur from the nucleus, or if S1PR3 signalling during earlier stages, when it is localised subcortically, sets the embryo up for implantation. To confirm this, the effect of S1PR3 attachment on blastocyst attachment and invasion should be investigated.

4.4.3 S1PR4 signalling is required for cavitation and blastocyst formation

Similar to S1PR2 and S1PR3, S1PR4 was also present over the second meiotic spindle of the mature oocyte (Figure 4.9). This again suggests that there is a role for S1PR signalling in division of the oocyte. S1P/S1PR4 signalling activates Cdc42 in S1PR4-transfected cells (Kohno et al., 2003). Cdc42 signalling is important in the oocyte for normal spindle positioning during fertilisation, and so it is possible that S1PR4 activates Cdc42 in the oocyte. This should be investigated by using small molecule inhibitors or siRNA to inhibit S1PR4 during oocyte maturation and/or fertilisation to determine the role of S1PR4 signalling in the oocyte.

In this study, inhibition of S1PR4 during LD embryo culture prevented cavitation and subsequent blastocyst formation (Figure 4.11). This was not seen during HD embryo culture, likely due to increased concentrations of S1P and other growth factor present during HD culture, as discussed in section 3.4.2. This effect was not seen when S1PR4 was inhibited during only early preimplantation development, suggesting that S1PR4 signalling has an acute role in the morula (Figure 4.12). This suggests that S1PR4 signalling has an acute role in the morula despite S1PR4 also being expressed by earlier embryo stages, or that loss of S1PR4 signalling early in development is able to be compensated for by other S1PRs or other growth factor signalling pathways. However, little is known about S1PR4 signalling outside immune cells, where it is involved in activation and migration of these cells. S1PR4 is highly expressed by immune cells, and in CHO cells transfected with S1PR4, S1PR4 signalling regulates cell shape and motility through cytoskeletal remodelling mediated by activation of RhoA/Rock (Graler et al., 2003). The contractile forces generated by the cytoskeleton is important for cell differentiation in the compacting embryo (Maitre et al., 2016). Disruption of the cytoskeleton by deletion of maternal myosin impairs embryo compaction and alters YAP localisation, making the blastomeres take on an ICM phenotype regardless of their positioning in the embryo. Despite this, these embryos are still viable and

form blastocysts and offspring. In addition, acute inhibition of actin-filament formation at the zygote stage causes a dose-dependent reduction in blastocyst formation (Tan et al., 2015). However, reduction of blastocyst formation to 10% coincided with a decrease in the percentage of embryos that cleaved, which was not seen with inhibition of S1PR4. As neither of these phenotypes align with S1PR4 inhibition, this suggests that S1PR4 signalling does not have a large role in cytoskeletal remodelling in the embryo, though this could be confirmed by performing immunostaining for cytoskeletal components such as myosin and actin on embryos treated with an S1PR4 inhibitor.

Instead, the loss of S1PR4 signalling could be preventing blastocyst formation by more niche interactions with the cytoskeleton, such as interfering with tight junctions. S1PR4 is expressed in brain microvascular endothelial cells that comprise the blood brain barrier, where it plays a role in endothelial barrier integrity (Hansen et al., 2022). Even partial knockdown of S1PR4 induced transcriptional downregulation of junctional proteins including vascular endothelial cadherin, Claudin 5, OCLN and ZO-1. As previously identified, tight junctions are important for cavitation, and so inhibition of S1PR4 could prevent cavitation by impairing tight junction formation and maintenance. The effect of S1PR4 inhibition on the expression and localisation of junctional proteins such as ZO-1 could be investigated through immunostaining experiments.

4.4.4 S1PR5 is present in the embryo and colocalises to autophagosomes

S1PR5 is present throughout preimplantation development, and was predominantly membranous in the oocyte, zygote, and 2-cell embryo (Figures 4.13 and 4.14), suggesting classical ligand-mediated GPCR signalling. S1P/S1PR5 signalling promotes mitotic progression via PI3K signalling and the mitotic kinase PLK1 in HeLa cells (Andrieu et al., 2017). PLK1 is essential for the first cell division in the embryo, and inhibition of PLK1 results in mitotic arrest at the metaphase/anaphase transition prior to the 2-cell stage (Baran et al., 2013). Therefore, S1PR5 signalling via PI3K/PLK1 could promote cleavage in the early embryo. This could be investigated through inhibition of S1PR5, though there are no small-molecule inhibitors currently available. Instead, other techniques could be used, such as siRNA knockdown.

S1PR5 was present in the trophectoderm cells but not the hypoblast or epiblast cells of the blastocyst (Figure 4.14), suggesting a role for S1PR5 in blastocyst hatching or implantation. While no prior studies have investigated S1PR5 expression or localisation in

the blastocyst, S1PR5 is expressed in in D3 and R1 embryonic stem cells, cell lines derived from the ICM of E4 blastocysts (Kleger et al., 2007; Rodgers et al., 2009), suggesting S1PR5 would be expressed in the ICM of the early blastocyst. Blastocysts in this study were imaged at a later stage, at which point the ICM has differentiated into hypoblast and epiblast cells. Future studies should look at the presence of S1PR5 in the ICM of the early blastocyst by S1PR5 immunostaining.

S1PR5 was co-localised to autophagosomes in 4- and 8-cell embryos (Figure 4.15), and presumably in morula and blastocyst stages as well due to similar staining patterns observed from the 4-cell to blastocyst stages (Figures 4.13 and 4.14). S1PR5 signalling is linked to autophagosome formation in prostate cancer cells (Chang et al., 2009), but it is unclear if this occurs through insertion of S1PR5 into the autophagosome membrane. In prostate cancer cells, S1PR5-induced autophagosome formation is triggered in response to endoplasmic reticulum (ER) stress and occurs via activation of G_i signalling – specifically, activation of PI3K and PLC (Huang et al., 2014), and plays an important role in cell survival (Ogata et al., 2006; Yorimitsu & Klionsky, 2007). *In vitro* culture conditions induce ER stress in the embryo (Latham, 2016), which could in turn induce S1PR5-mediated protective autophagy. Alternatively, localisation of S1PR5 to these autophagosomes could be normal and not part of a stress response. Autophagosome formation is essential for preimplantation development and is induced following fertilisation and then suppressed until the 2-cell stage, after which it is gradually upregulated throughout preimplantation development to the blastocyst stage (Leung et al., 2022; Tsukamoto et al., 2008). Knockout of ATG5, a key autophagosome protein, results in developmental arrest at the 4-8-cell stage (Tsukamoto et al., 2008), which coincides with the appearance of S1PR5 in autophagosomes, suggesting that S1PR5 may have a role in autophagosome formation in the embryo. S1PR5 may also be contained in autophagosomes for degradation, though persistence of S1PR5 over several days from the 4-cell to blastocyst stages makes this unlikely. To determine whether the presence of S1PR5 in autophagosomes is a result of stress induced by *in vitro* culture, colocalization experiments should be repeated on *in vivo* developed embryos.

4.4.5 S1PR redundancy in the embryo

Inhibition of S1PR1-3 at LD reduced the percentage of embryos that cavitated and formed a blastocyst, despite each drug being present at a concentration that had no effect on embryo development individually (Figure 4.16). This suggests that there is redundancy

between S1PR1-3 in the preimplantation embryo as seen in other models, including later stages of foetal development (Kono et al., 2004). When S1PR4 was inhibited alongside S1PR1-3, embryos arrested earlier at the 2-4 cell stage (Figure 4.17), suggesting that S1PR4 is able to partially compensate for the loss of S1PR1-3 signalling as well. Due to the limited expression of S1PR4 in the adult, there is very little data on redundancy between S1PR4 and S1PR1-3 in other cell types, and so it is unclear if S1PR4 redundancy is a phenomenon exclusive to the embryo due to its unique expression pattern. The phenotype produced by S1PR1-4 inhibition does not align with inhibition of any single receptor, suggesting there may be a cumulative effect of inhibiting two or more S1PRs. The most likely S1PR involved is S1PR3 as inhibition of S1PR3 reduces the percentage of embryos that successfully cleave (Figure 4.7). Inhibition of S1PR4 could sensitise embryos to loss of S1PR3 signalling due to cumulative activation of shared signalling pathways, suggesting redundancy between these two receptors. Interactions and redundancies between S1PRs should be further investigated by inhibiting different pairs and trios of S1PRs, including S1PR3 and S1PR4.

4.4.6 Investigation of genes associated with S1P signalling in the embryo

In vitro culture affects genes relating to other signalling pathways, such as *Igf1* (Stojanov et al., 1999; Stojanov & O'Neill, 2001). However, expression of genes associated with S1P signalling or the effect of culture on these genes is not known. To investigate this, RT qPCR was performed on embryos that were cultured *in vitro* or developed *in vivo*. However, very little information could be obtained from the data due to the low signal, which could be due to the low number of embryos used per sample. From the data that was obtained, genes encoding the S1PRs were more likely to be detected in embryos that developed *in vivo* rather than *in vitro*, suggesting that embryo culture disrupts expression of *S1pr* genes as was seen with genes relating to IGF1 signalling (Stojanov et al., 1999; Stojanov & O'Neill, 2001). While gene expression does not always correlate to protein expression, lower expression of S1PR genes *in vitro* could contribute to poorer development due to loss of signalling pathways activated by S1PRs that promote cell proliferation and survival.

PCR and RT-PCR have previously been used to investigate *S1pr* expression in porcine COCs (Park et al., 2019): immature oocytes express *S1pr2* and *S1pr3* and cumulus cells express *S1pr1-3*. *S1pr4* expression was not detected in either oocytes or cumulus cells, and *S1pr5* expression was not investigated. However, detection of these genes required 150

COCs per sample, suggesting that expression was low. This is also confirmed by previous RNAseq and microarray studies using preimplantation embryos that produced similar results, in that expression of *S1pr* genes was very low, if not undetectable (Deng et al., 2014; Fan et al., 2020; Hamatani et al., 2004; Jeong et al., 2006). Increasing the signal obtained by performing RNAseq on groups on embryos rather than single cells could allow investigation of *S1pr* gene expression in the embryo.

4.4.7 Conclusions

In conclusion, S1PR2-5 were identified in mouse preimplantation embryos, and the localisation differed between receptors. Inhibition of S1PR2-4 also revealed differences in function between these receptors. Inhibition of S1PR2 and S1PR4 impaired embryo cavitation, possibly due to alteration of tight junction formation in the morula, while inhibition of S1PR3 prevented embryo cleavage. These differences in localisation and function suggest that receptors within the S1PR family have different roles in preimplantation embryo development but are all individually required for normal development.

CHAPTER 5

The expression and function of SphK1 and SphK2 in the mouse preimplantation embryo

5.1 Introduction

S1PR signalling is indispensable for preimplantation development *in vitro* (Chapter 3 and 4), suggesting that embryos can produce S1P which acts in an autocrine manner to improve development. In other cells, most if not all S1P is produced from the phosphorylation of sphingosine, which is in turn derived from plasma-membrane sphingomyelins (Spiegel & Milstien, 2007). The phosphorylation of sphingosine to form S1P is catalysed by the two isoenzymes of SphK, SphK1 and SphK2, that are transcribed from genes on different chromosomes (Spiegel & Milstien, 2007).

Mammalian cells have low basal levels of S1P due to intrinsic pSphK activity. In addition, cytokines, growth factors, hormones, and angiogenic factors all upregulate pSphK activity, including IGF1 (Martin et al., 2009; Wu et al., 2019), EGF (Hait et al., 2005; Johnstone et al., 2005), VEGF (Lee et al., 2014), and oestradiol (Sukocheva et al., 2015).

SphK1 is primarily cytosolic and is activated upon phosphorylation at Ser225 (Pitson et al., 2003). Multiple kinases have been implicated in the activation of SphK1, including ERK1/2, PKC, protein kinase A (PKA), and, most recently, PKR (Johnson et al., 2002; Johnstone et al., 2005; Qiao et al., 2021; Rius et al., 1997). Upon phosphorylation, pSphK1 undergoes calcium-dependent translocation to the plasma membrane, leading to an increase in S1P. pSphK1/S1P signalling is associated with the promotion of cell proliferation and survival in a range of cell types (Hernandez-Coronado et al., 2016; Wang et al., 2019).

In contrast, SphK2 is present in various intracellular compartments, including the nucleus, mitochondria, and endoplasmic reticulum. In human cell lines, SphK2 is phosphorylated at Ser351 and Thr578 by ERK1 (Hait et al., 2007) and at Ser419 and Ser421 by protein kinase D (Ding et al., 2007). Phosphorylation by PKC also occurs in mice and humans (Ebenezer et al., 2019; Hait et al., 2007). Many of the targets of S1P generated by pSphK2 are intracellular. However, S1P can also be released from the cell to bind to S1PRs (Wang et al., 2016).

Early studies reported that pSphK2 has an opposing role to that of pSphK1 in that it promoted cell-cycle arrest and apoptosis, but many of these studies relied on forced overexpression of SphK2 (Chipuk et al., 2012; Igarashi et al., 2003; Liu et al., 2003; Maceyka et al., 2005). More recently, SphK2 promotes survival and proliferation in a range of cell types, including hepatocytes, fibroblasts, cardiomyocytes, and cancer cells (Antoon et al., 2011; Dai et al., 2022; Lee et al., 2015; Schnitzer et al., 2009; Selvam et al., 2015;

Selvam et al., 2018; Zhang et al., 2016c). Abrogation of pSphK2/S1P signalling results in increased apoptosis, cell cycle arrest, and attenuation of tumour growth both *in vivo* and *in vitro* (Dai et al., 2018; Dai et al., 2022).

SphK1 and SphK2 are present in mouse COCs and are activated in response to luteinising hormone signalling (Yuan et al., 2022). Inhibition or depletion of both SphK1 and SphK2 in the granulosa cells of COCs resulted in infertility due to impaired oocyte maturation and ovulation, while depletion of either SphK1 or SphK2 had no effect on fertility (Yuan et al., 2022). In the embryo, *Sphk1* and *Sphk2* mRNA are present from E7 onwards (Liu et al., 2000). Knockout of one or the other gene has no effect on embryo development or health of the resulting pups, but knockout of both is lethal between E11.5 and E13.5 due to cranial haemorrhage (Mizugishi et al., 2005). These studies suggest that SphKs are required for oocyte maturation and later stages of development, and that there is redundancy between the SphK isoenzymes, though the expression of these isoenzymes in preimplantation embryos has not been investigated.

Due to the importance of embryo-derived S1P signalling via S1PRs in early embryo development, this study aimed to identify the presence of pSphK1 and pSphK2 in the embryo. Redundancy between SphK1 and SphK2 was also investigated. It was hypothesised that activity of both pSphK1 and pSphK2 would be required in the embryo, and that functional redundancy would exist between the isoenzymes similar to that seen in the oocyte and later stages of development.

5.2 Methods

5.2.1 Immunofluorescence staining of oocytes and embryos

Zygotes were isolated and cultured at HD to the zygote, 2-cell, 4-cell, 8-cell, morula, and hatched blastocyst stages as per Table 2.3. Immunofluorescence staining was carried out as in section 2.4. All developmental stages were stained to visualise SphK1, pSphK1, SphK2, and pSphK2 (Table 5.1).

Table 5.1. Primary and secondary antibody dilutions for immunofluorescence staining of SphKs

Primary antibody	Dilution	Secondary antibody	Dilution
SphK1 antibody (Bioss Antibodies #BS-2652R)	1:100	Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies #A11034)	1:200
pSphK1(Ser 225) antibody (ECM Biosciences #SP1641)	1:100	Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies #A11034)	1:100
SphK2 antibody (Abcam #ab37977)	1:50	Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies #A11034)	1:200
pSphK2 (Thr578) antibody (ECM Biosciences #SP4631)	1:100	Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies #A11034)	1:200

5.2.2 Co-localisation of pSphK2 with autophagosomes

Zygotes were cultured at HD to the 4-cell and 8-cell stages before treatment with pronase to remove the zona pellucida (section 2.4.1). Embryos were then stained with CYTO-ID Autophagy detection kit 2.0 (1:500; Enzo Life Sciences #ENZ-KIT175-0050) before fixing and staining (section 2.4) to visualise the pSphK2.

5.2.3 Culture of embryos in the presence of inhibitors of SphK1 and SphK2

Zygotes were cultured at either HD or LD in ModHTF medium containing 0-20 µM PF543 (Sigma Aldrich) or 0-100 µM ABC294640 (Sigma Aldrich) to the hatched blastocyst stage. Embryos were also cultured in ModHTF medium containing 1 µM PF543 + 60 µM ABC296460 in order to investigate redundancy between SphKs in the embryo. Embryo development was scored daily and the percentage of embryos that had reached each developmental stage recorded.

5.2.4 Quantification of SphK gene expression in the embryo

Oocytes and embryos were isolated (section 2.3.2), and embryos were cultured *in vitro* at HD to the zygote, 2-cell, 4-cell, 8-cell, morula, or early blastocyst stages per the timings in Table 2.3. *In vivo* developed embryos were flushed from the reproductive tract (section 2.3.3) as per the timings in Table 2.3. Oocytes and embryos were collected in groups of 18-30 in 15 µL Trizol, then snap frozen in liquid nitrogen and stored at -80 ˚C. Gene expression of SphK1 and SphK2 was quantified by RT-qPCR (section 2.5) using *Rn18s* as the reference gene (Table 5.2).

Table 5.2. Genes analysed using RT-qPCR for SphKs

Gene name	Protein	Assay ID	Catalogue #	Reporter	Amplicon length
Sphk1	SphK1	Mm00448841_g1	#4453320	FAM	65
Sphk2	SphK2	Mm00445021_m1	#4453320	FAM	113
Rn18s	18S ribosomal RNA	Hs03003631_g1	#4453320	VIC	69

5.2.5 Statistical analysis

All statistical analysis was carried out using GraphPad Prism software. Embryos were randomly allocated into treatment groups. Fluorescence images were obtained from at least 3 independent experiments with 5 embryos per developmental stage per experiment. Data for inhibitor dose culture experiments were pooled from at least 3 independent experiments with 10 or 12 embryos per treatment per experiment for HD and LD experiments, respectively. Data from these experiments were analysed using a chi-squared test.

5.3 Results

5.3.1 SphK1 and pSphK1 are present across all stages of preimplantation embryo development

SphK1 was present at all stages of preimplantation embryo, predominantly in the cytoplasm and nucleus (Figures 5.1 and 5.2, Appendix 3 and 4). In the oocyte, SphK1 staining was punctate throughout the cytoplasm, with stronger staining in the microvilli-free region overlying the meiotic spindle. Following fertilisation, SphK1 staining was strongly nuclear and cytoplasmic throughout development (Figure 5.1, Appendix 3). Cytoplasmic puncta were present subcortically at the 8-cell stage, and to a lesser extent in the cytoplasm of the 4-cell and morula stages. In the blastocyst, staining was predominantly nuclear with weaker cytoplasmic staining, and staining appeared stronger in the ICM than the trophectoderm (Figure 5.2, Appendix 4).

The phosphorylated form of SphK1 was also present at all developmental stages, almost exclusively in the cytoplasm (Figures 5.3 and 5.4, Appendix 3 and 4). In the oocyte, pSphK1 staining was localised to the meiotic spindle (Figure 5.3, Appendix 3). Some nuclear staining was evident in the 4-cell and 8-cell embryo. Weak pSphK1 staining was present in all cells of the blastocyst and was restricted to the cytoplasm (Figure 5.4, Appendix 4).

IgG isotype and secondary only negative controls have been performed previously and demonstrate no staining (data not shown).

5.3.2 Inhibition of SphK1 reduces embryo compaction and cleavage during LD embryo culture

To investigate production of S1P by SphK1 in the embryo, embryos were cultured at HD or LD in the presence of PF543, a selective SphK1 inhibitor. At LD, 10 and 15 μM reduced the percentage of embryos that compacted, and there was a dose-dependent reduction in the percentage of embryos that cavitated in the presence of 5 μM PF543 or above (Figure 5.5A-E). 10 μM PF543 additionally reduced the percentage of embryos that developed to the 4-8-cell stage. No effect on blastocyst formation was seen at HD when embryos were cultured in the presence of 20 μM PF543 (Figure 5.5F).

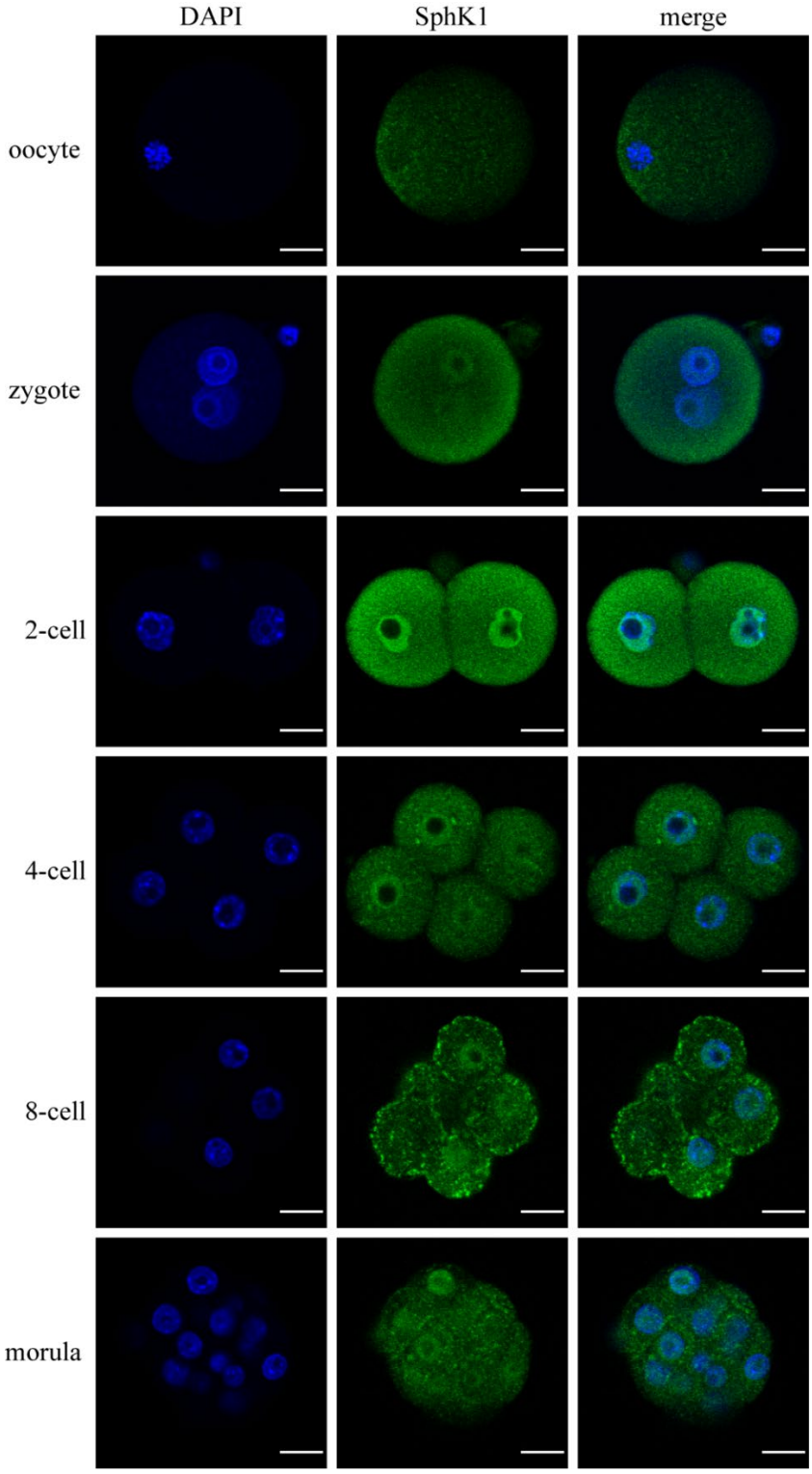


Figure 5.1. Localisation of SphK1 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured *in vitro* at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote), 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for SphK1. Nuclei were stained with DAPI. Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.

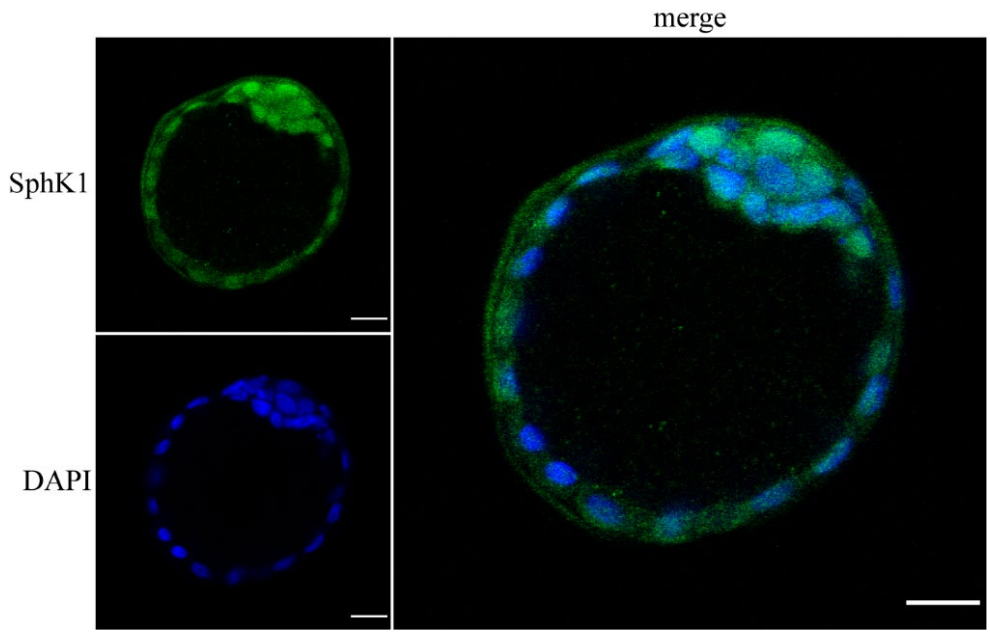


Figure 5.2. Localisation of SphK1 in mouse blastocysts. Zygotes were isolated and cultured at HD (20 embryos/20 µL) in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde and immunostained for SphK1. Nuclei were stained with DAPI. Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.

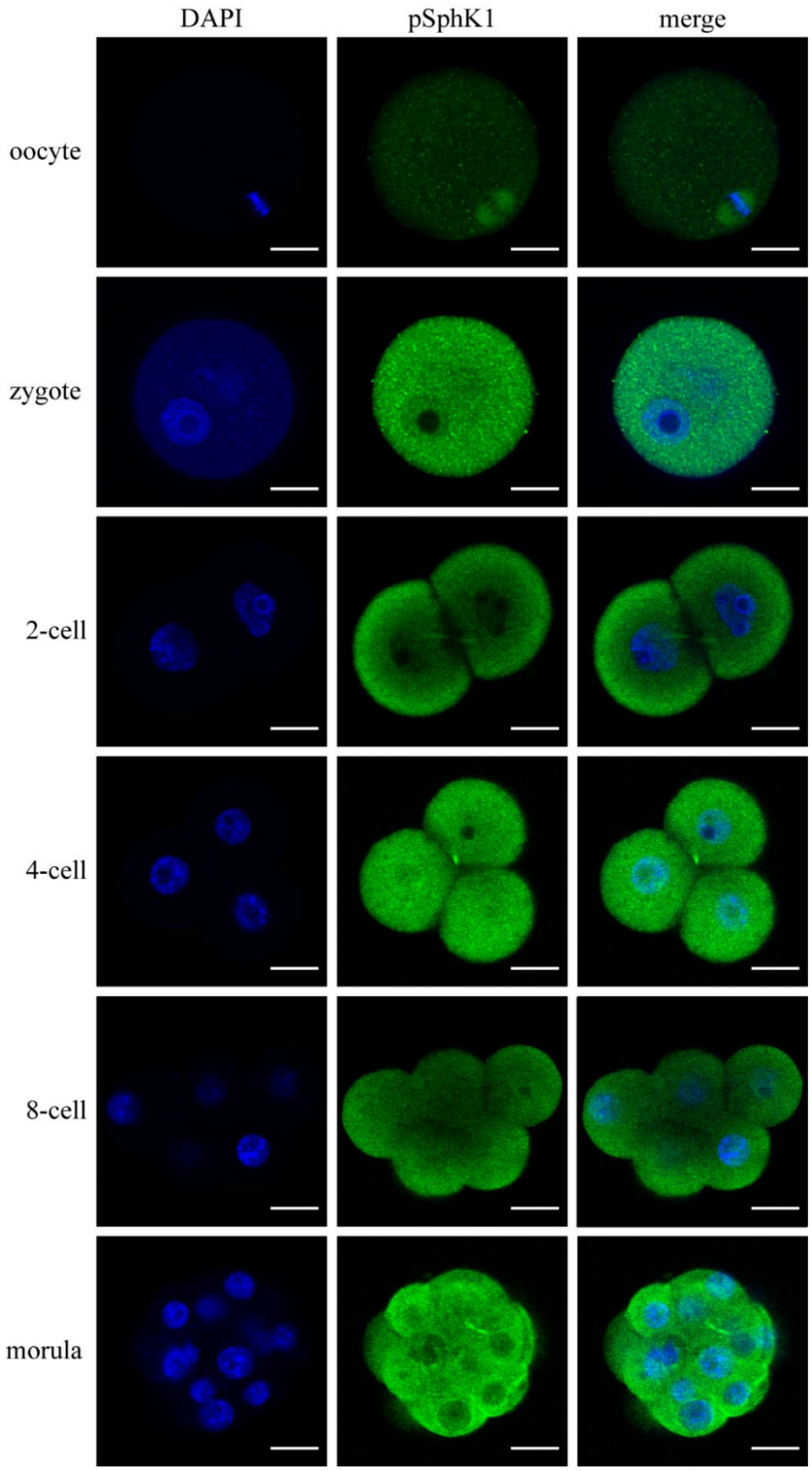


Figure 5.3. Localisation of pSphK1 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured *in vitro* at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote), 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for phosphorylated SphK1 (pSphK1). Nuclei were stained with DAPI. Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.

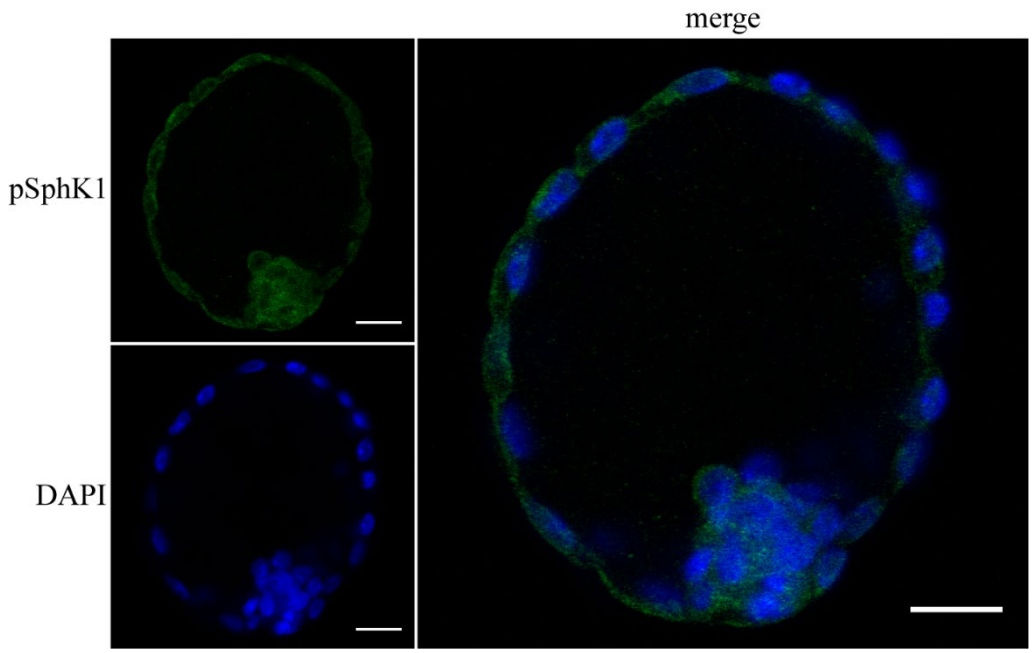


Figure 5.4. Localisation of pSphK1 in mouse blastocysts. Zygotes were isolated and cultured at HD (20 embryos/20 μL) in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde and immunostained to visualise phosphorylated SphK1 (pSphK1). Nuclei were stained with DAPI. Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 μm.

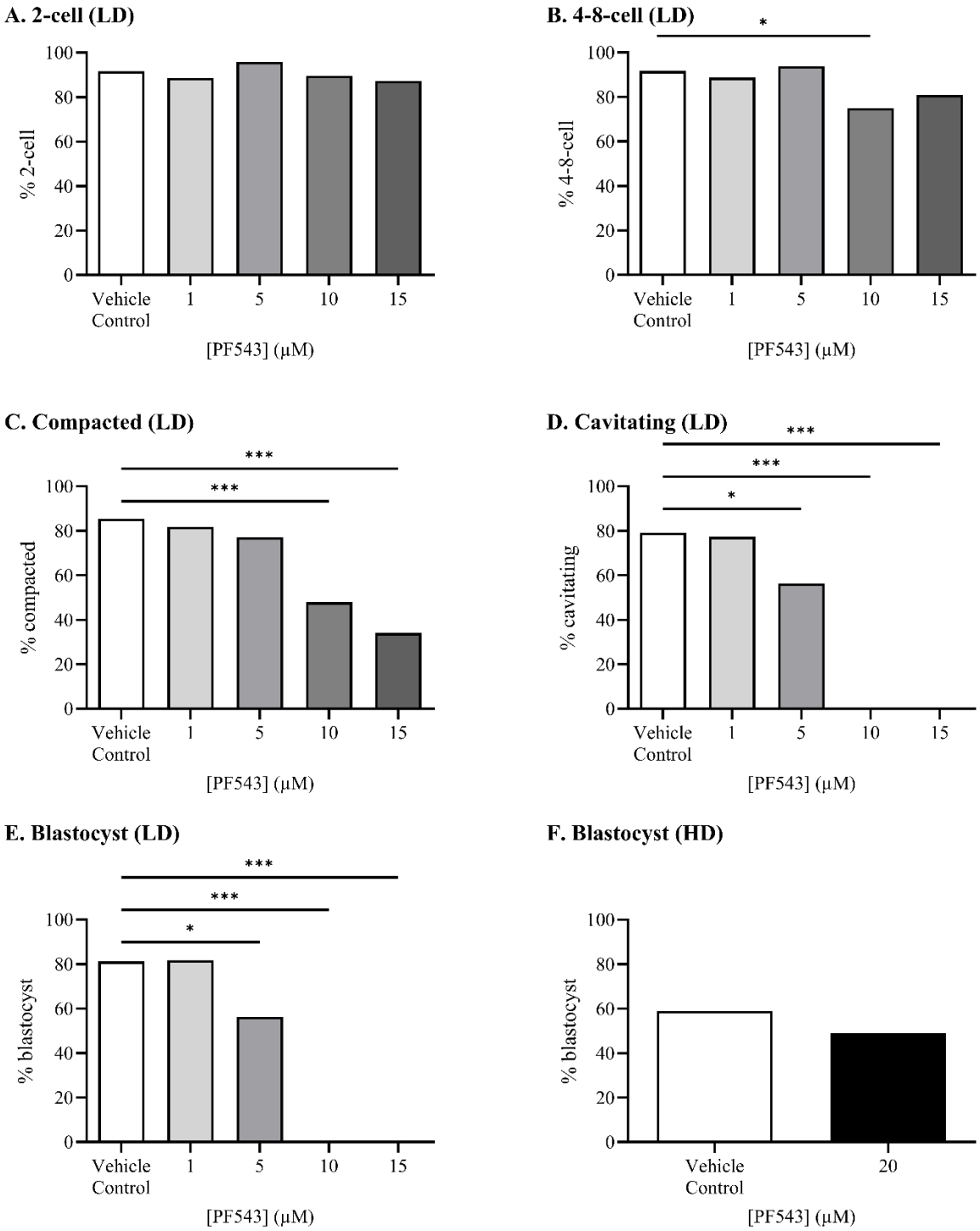


Figure 5.5. The effect of SphK1 inhibition on mouse preimplantation embryo development. Zygotes were isolated and cultured at either LD (1 embryo/100 μL, panels **A-E**) or HD (1 embryo/1 μL, panel **F**). For LD, embryos were cultured in ModHTF medium containing 0.1% DMSO plus 0, 1, 5, 10, or 15 μM PF543. Embryos were scored daily and the percentage of embryos that developed normally to the (**A**) 2-cell, (**B**) 4-8-cell, (**C**) compacted, (**D**) cavitating, and (**E**) blastocyst stages were recorded. For HD, embryos were cultured in ModHTF medium containing 0.1% DMSO plus 0 or 20 μM PF543 and the percentage of embryos that developed to the blastocyst stage was recorded (**F**). Data were pooled from at least 3 independent experiments with a minimum of 12 embryos per condition per experiment for LD conditions, or 10 embryos per condition per experiment for HD conditions. Statistical analysis was performed by chi-squared test. * = $P<0.05$, *** = $P<0.001$.

5.3.3 SphK2 and pSphK2 are present across all stages of preimplantation embryo development

SphK2 was present at all stages of preimplantation development and staining was predominantly punctate in the cytoplasm and nucleus (Figures 5.6 and 5.7, Appendix 3 and 4). In the oocyte, there was increased staining in the cytoplasm overlying the second meiotic spindle (Figure 5.6, Appendix 3). From the 2-cell stage onwards, nuclear staining was stronger than cytoplasmic staining, and cytoplasmic puncta were present subcortically at the 4-cell stage. In the blastocyst, there was weak staining in the cytoplasm and nucleus (Figure 5.7, Appendix 4).

The phosphorylated form of SphK2 was also present at all developmental stages, and was restricted to the plasma membrane and nucleus (Figures 5.8 and 5.9, Appendix 3 and 4). Membrane staining was reduced after the 2-cell stage, which coincided with cytoplasmic puncta present subcortically in 4-cell, 8-cell, and morula stage embryos (Figure 5.8, Appendix 3). pSphK2 staining was reduced in the blastocyst and was predominantly nuclear and cytoplasmic in the ICM cells (Figure 5.9, Appendix 4).

IgG isotype and secondary only negative controls have been performed previously and demonstrate no staining (data not shown).

5.3.4 pSphK2 does not co-localise to autophagosomes in 4-cell and 8-cell stage embryos

Based on the punctate cytoplasmic staining observed with pSphK2, presence of pSphK2 in autophagosomes was investigated. Embryos were co-stained with the CYTO-ID Autophagy Detection Kit and pSphK2 antibody. pSphK2 staining did not co-localise to puncta dyed by the CYTO-ID kit (Figure 5.10).

5.3.5 Inhibition of SphK2 reduces embryo cleavage and cavitation during LD embryo culture

To investigate production of S1P by SphK2 in the embryo, embryos were cultured at HD or LD in the presence of ABC294640, a selective SphK2 inhibitor. Treatment with 100 μM ABC294640 at LD reduced the percentage of embryos that developed to all developmental stages, and 80 μM ABC294640 at LD reduced the percentage of embryos that cavitated and subsequently formed a blastocyst (Figure 5.11A-E). Treatment of embryos with 80 or 100 μM ABC294640 at HD had no effect on blastocyst formation (Figure 5.11F).

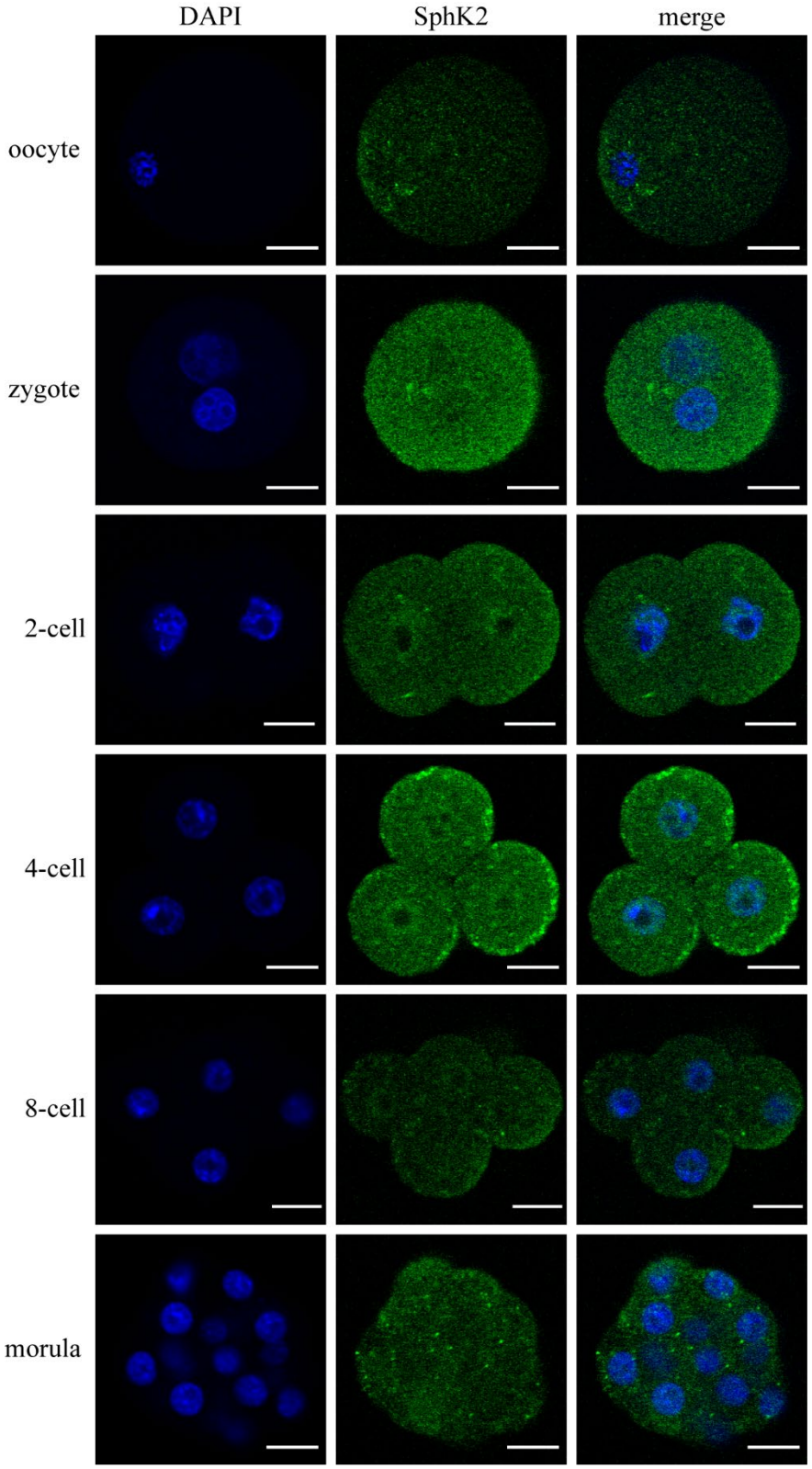


Figure 5.6. Localisation of SphK2 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured *in vitro* at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote), 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for SphK2. Nuclei were stained with DAPI. Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.

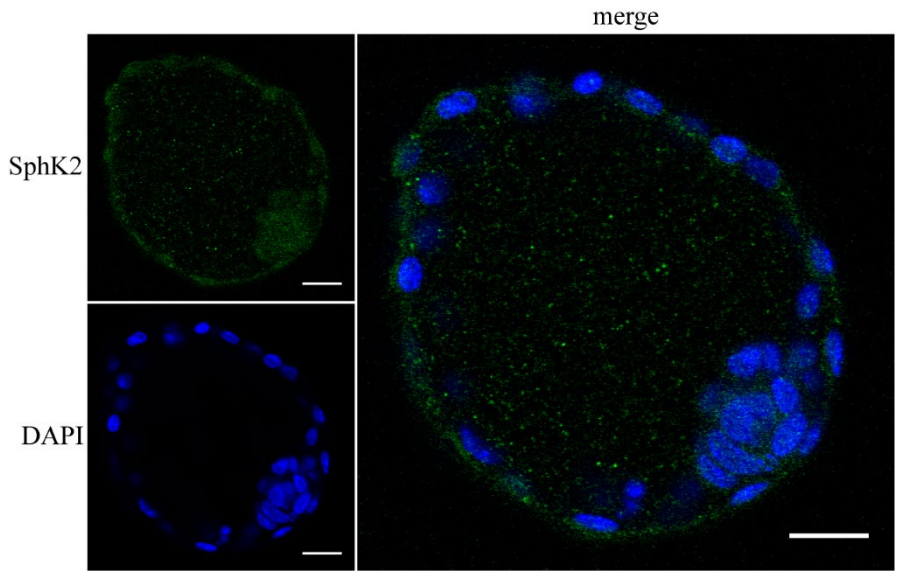


Figure 5.7. Localisation of SphK2 in mouse blastocysts. Zygotes were isolated and cultured at HD (20 embryos/20 µL) in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde and immunostained to visualise SphK2. Nuclei were stained with DAPI. Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.

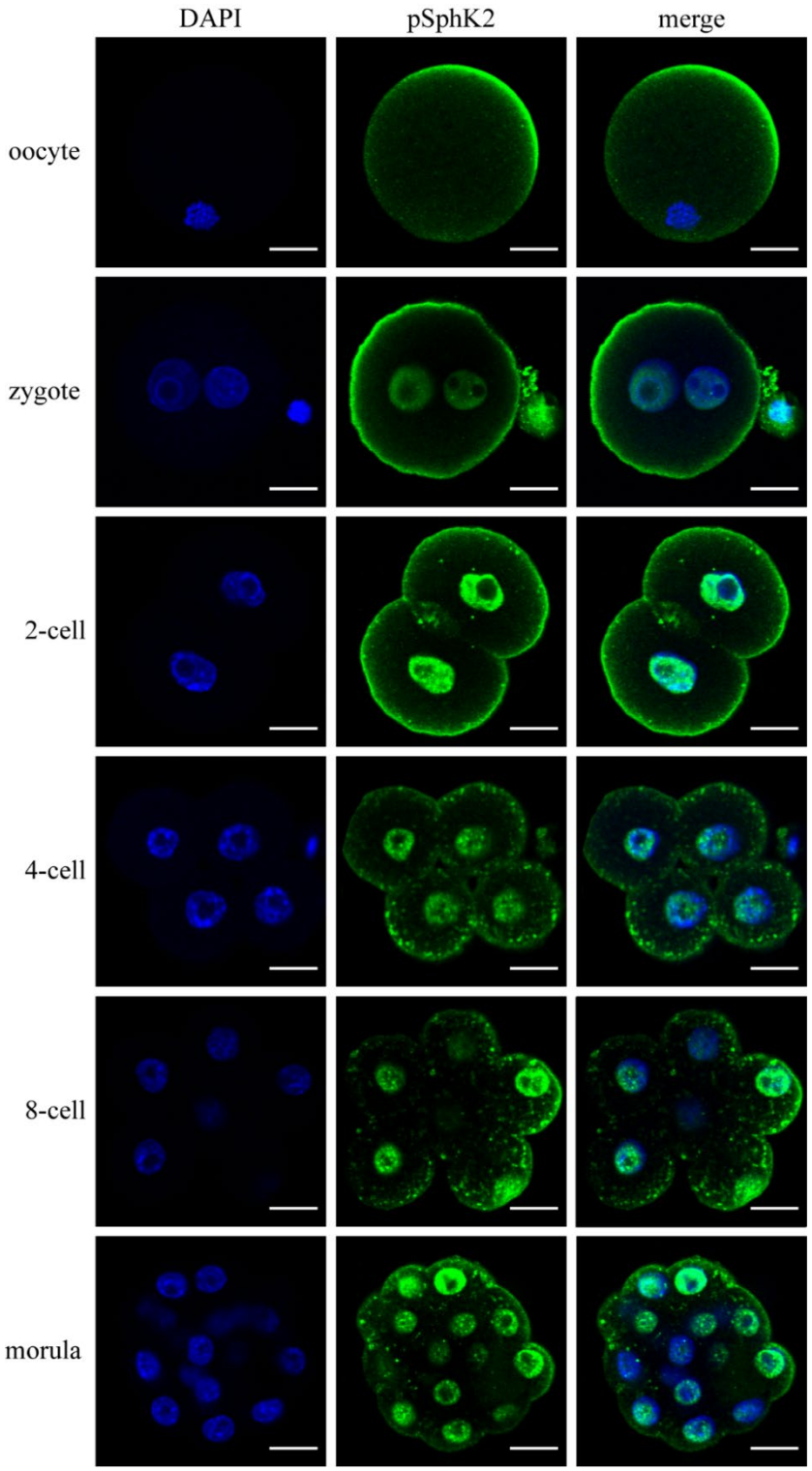


Figure 5.8. Localisation of pSphK2 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured *in vitro* at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote), 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for phosphorylated SphK2 (pSphK2). Nuclei were stained with DAPI. Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.

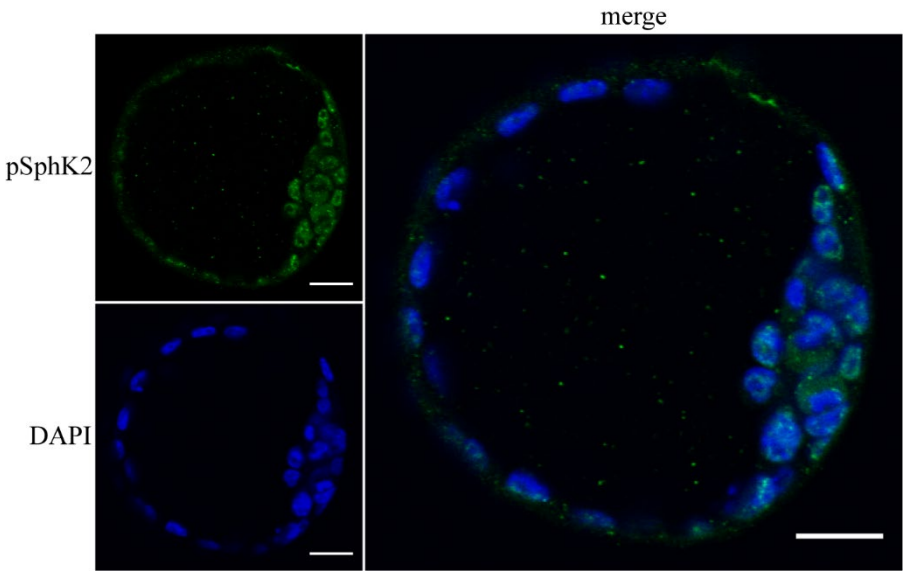


Figure 5.9. Localisation of pSphK2 in mouse blastocysts. Zygotes were isolated and cultured at HD (20 embryos/20 µL) in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde and immunostained to visualise phosphorylated SphK2 (pSphK2). Nuclei were stained with DAPI. Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.

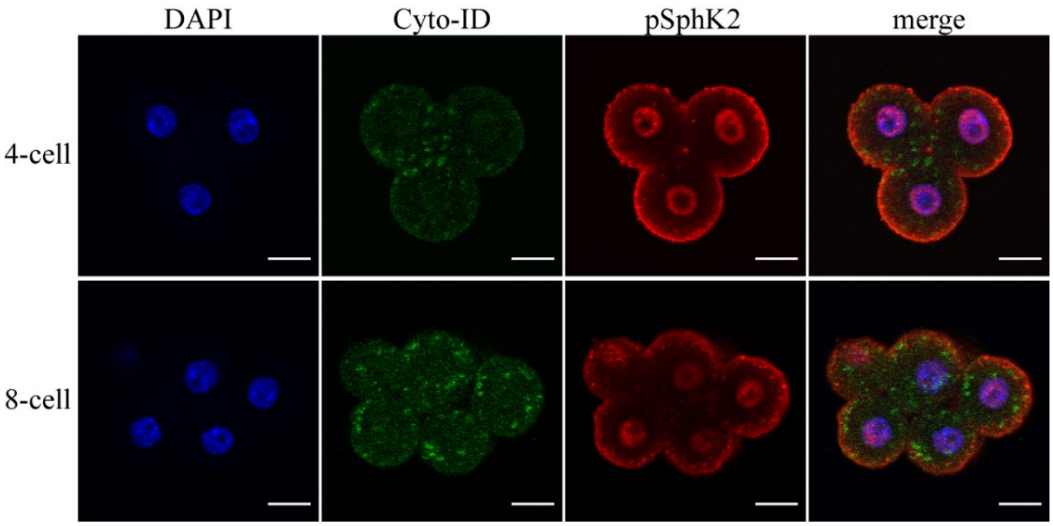


Figure 5.10. Localisation of pSphK2 and autophagosomes in 4-cell and 8-cell embryos. Zygotes were isolated and cultured at HD (20 embryos/20 µL) in ModHTF medium to the 4-cell and 8-cell stage. The zona pellucida was removed and embryos were cultured in Cyto-ID Autophagy dye to visualise autophagosomes (green). Embryos were then fixed and stained to visualise the pSphK2 (red) and nuclei (blue). Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.

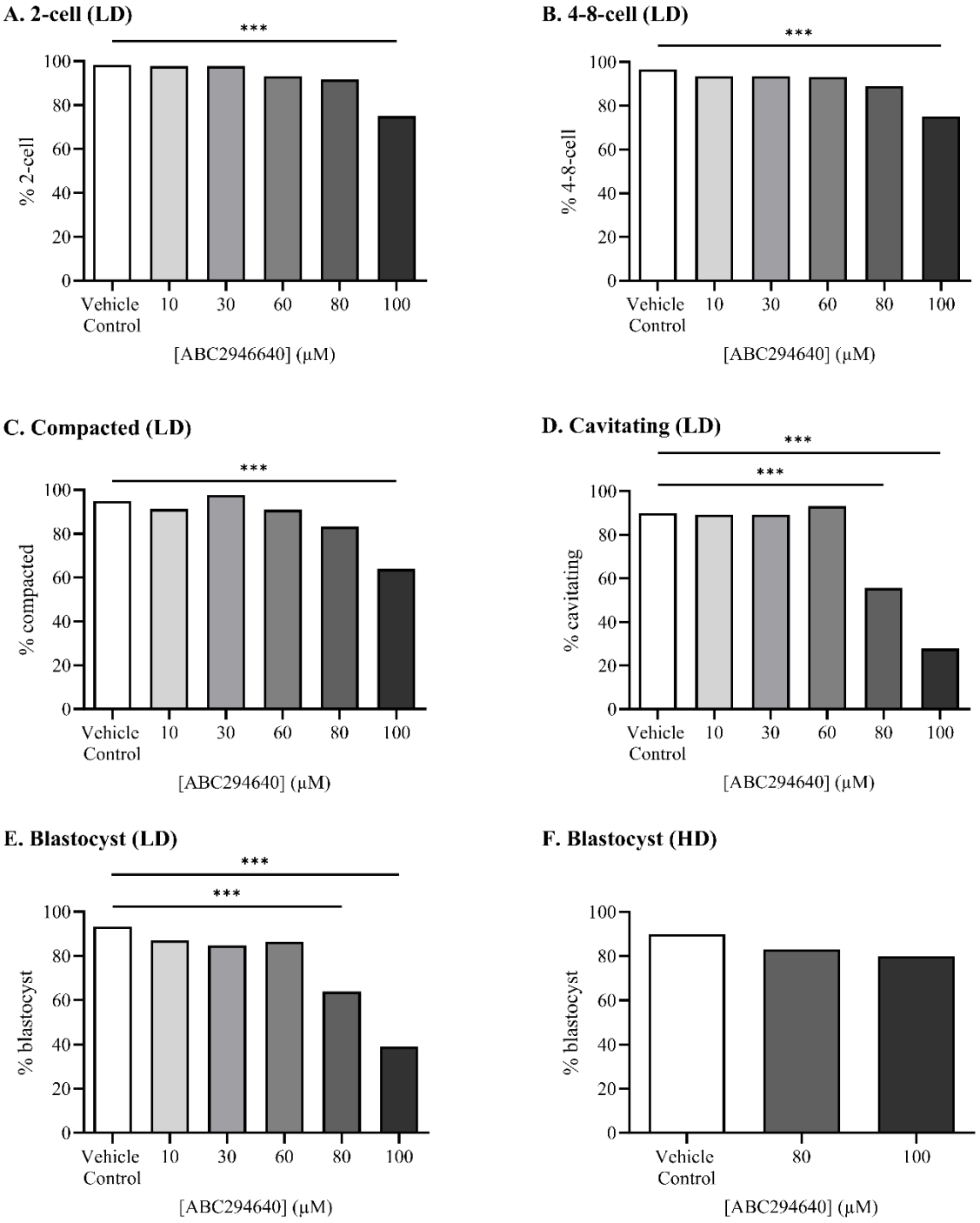


Figure 5.11. The effect of SphK2 inhibition on mouse preimplantation embryo development. Zygotes were isolated and cultured at either LD (1 embryo/100 μL, panels **A-E**) or HD (1 embryo/1 μL, panel **F**). For LD, embryos were cultured in ModHTF medium containing 0.1% DMSO (vehicle control) or 10, 30, 60, 80, or 100 μM ABC294640. Embryos were scored daily and the percentage of embryos that developed normally to the (**A**) 2-cell, (**B**) 4-8-cell, (**C**) compacted, (**D**) cavitating, and (**E**) blastocyst stages were recorded. For HD, embryos were cultured in ModHTF medium containing 0.1% DMSO plus 0, 80 or 100 μM ABC294640 and the percentage of embryos that developed to the blastocyst stage was recorded (**F**). Data were pooled from at least 3 independent experiments with a minimum of 12 embryos per condition per experiment for LD conditions, or 10 embryos per condition per experiment for HD conditions. Statistical analysis was performed by chi-squared test. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

5.3.6 Combined inhibition of SphK1 and SphK2 reduces embryo cavitation and blastocyst formation

To investigate redundancy between SphK1 and SphK2, embryos were cultured in the presence of 1 μM PF543 + 60 μM ABC294640. These concentrations were selected as the highest concentration of the inhibitors that individually had no discernible effect on embryo development in dose-culture experiments. Treatment of embryos with 1 μM PF543 + 60 μM ABC294640 during LD embryo culture reduced the percentage of embryos that cavitated and formed a blastocyst compared to untreated embryos (Figure 5.12A-E). This effect was also seen at HD, where treatment of embryos with 1 μM PF543 + 60 μM ABC294640 reduced blastocyst formation (Figure 5.12F).

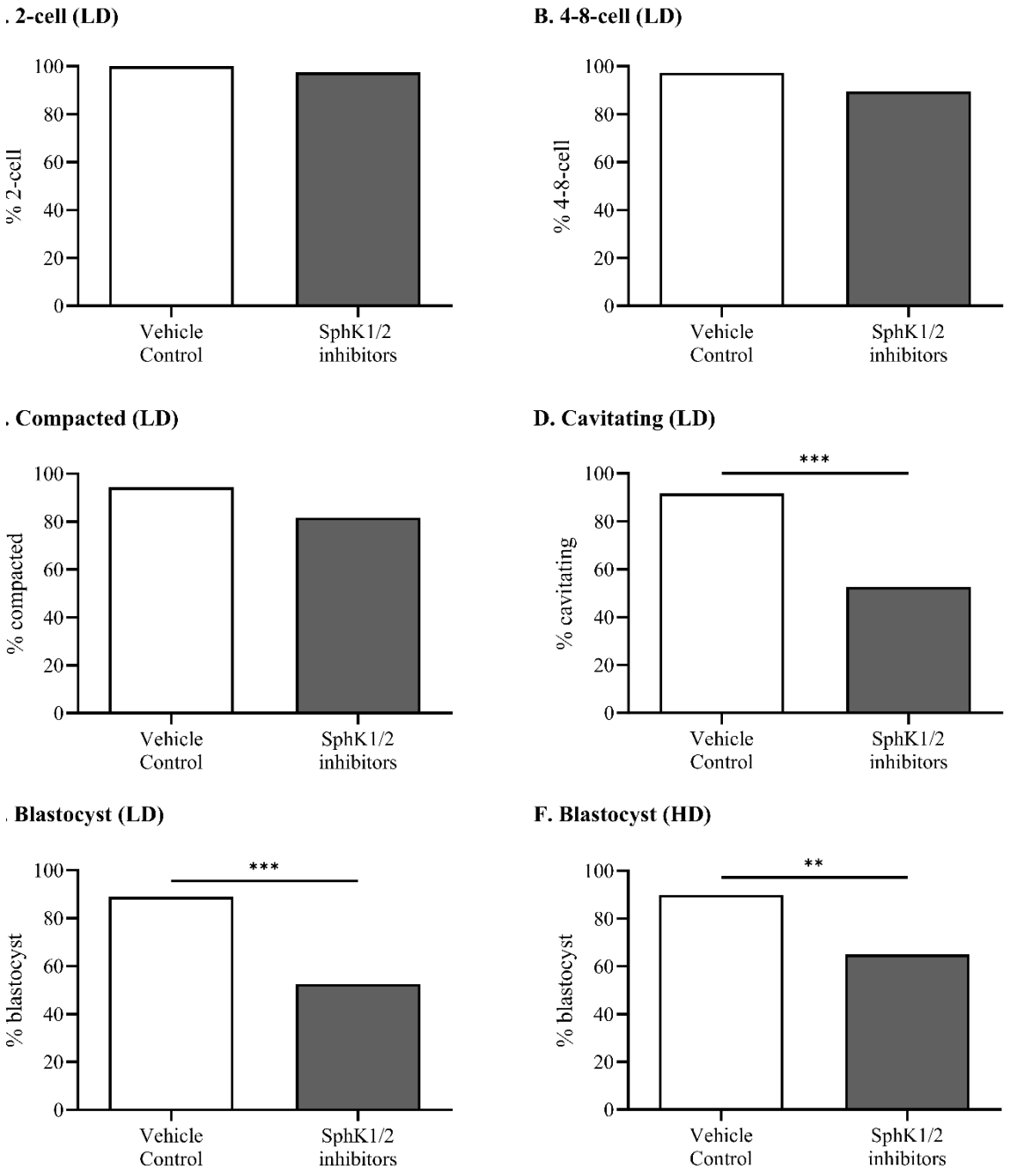


Figure 5.12. The effect of combined SphK1 and SphK2 inhibition on mouse preimplantation embryo development. Zygotes were isolated and cultured at either LD (1 embryo/100 µL, panels **A-E**) or HD (1 embryo/1 µL, panel **F**). Embryos were cultured in ModHTF medium containing 0.1% DMSO (vehicle control) or 1 µM PF543 + 60 µM ABC294640. For LD, embryos were scored daily and the percentage of embryos that developed normally to the (**A**) 2-cell, (**B**) 4-8-cell, (**C**) compacted, (**D**) cavitating, and (**E**) blastocyst stages were recorded. For HD, the percentage of embryos that developed to the blastocyst stage was recorded (**F**). Data were pooled from at least 3 independent experiments with a minimum of 12 embryos per condition per experiment for LD conditions, or 10 embryos per condition per experiment for HD conditions. Statistical analysis was performed by chi-squared test. *** = $P<0.001$.

5.3.7 Expression of SphK genes in *in vivo* and *in vitro* developed embryos

To investigate the expression of *Sphk* genes in preimplantation embryos, as well as the effect of *in vitro* culture on *Sphk* expression, qPCR was performed on embryos developed both *in vivo* and *in vitro*. Experiments were run in triplicate with 3 independent experiments for each gene. *Sphk1* mRNA was not detected (C_t value ≥ 38) at any stage regardless of whether embryos were developed *in vivo* or *in vitro*. *Sphk2* mRNA was only detected in some of the samples at the oocyte, 4-cell, and blastocyst stages *in vivo*, and some of the samples at the 4-cell and blastocyst stages *in vitro* (Table 5.3).

Table 5.3. Expression of *Sphk2* in embryos analysed using RT-qPCR

Stage	*In vivo*	*In vitro*
Oocyte	SphK2 (6/9)	– *
Zygote	–	–
2-cell	–	–
4-cell	SphK2 (4/9)	SphK2 (4/9)
8-cell	–	–
Morula	–	–
Blastocyst	SphK2 (7/9)	SphK2 (2/9)

N.B. Numbers in parentheses represent the number of wells *Sphk2* was detected in out of the total number of wells analysed.

* Not detected (C_t value ≥ 38) in any of the 9 wells.

5.4 Discussion

Embryo-derived S1P signalling via S1PRs is required for preimplantation development *in vitro* (Chapters 3 and 4). For this to occur, embryos must produce S1P that can act back on S1PRs expressed by the embryo. SphKs are likely the dominant, if not sole, source of S1P in the embryo, as E11.5 embryos deficient in both SphK1 and SphK2 have no measurable S1P (Mizugishi et al., 2005). Here we show that SphK1 and SphK2, as well as their phosphorylated forms, are present across all preimplantation stages, and confirmed that both kinases are required for normal development.

5.4.1 SphK1 and pSphK1 are present at all stages of preimplantation embryo development

SphK1 is located in both the cytoplasm and nucleus across all stages of development (Figures 5.1 and 5.2). In the oocyte, SphK1 staining was stronger over the second meiotic spindle (Figure 5.1), as also seen with S1PR2-4 in Chapter 4. pSphK1 specifically localised to the meiotic spindle (Figure 5.3). Similar staining occurs with other kinases in the mature oocyte, including MAPK (Goto et al., 2002), Fyn kinase and other Src family kinases (Levi et al., 2011; McGinnis et al., 2007), phospholipase D2 (Liu et al., 2017), mitofusin-2 (Zhang et al., 2016a), and casein kinase 2 (ShiYang et al., 2020). Many of these enzymes are involved in proliferation or spindle assembly and chromosome segregation, suggesting that SphK1 may be involved in normal meiotic division of the oocyte.

Following fertilisation, SphK1 was predominantly localised to the nucleus and pSphK1 predominantly in the cytoplasm (Figures 5.1 and 5.3). An identical staining pattern for SphK1 and pSphK1 occurs in HEK293T cells, in which SphK1 translocates from the nucleus of cells to the cytoplasm upon phosphorylation by PKR (Qiao et al., 2021). In the cytoplasm, pSphK1 produces S1P and therefore activates pro-survival signalling pathways via S1PR1 but can also directly interact with PKR to downregulate the cellular stress response that leads to cell death and apoptosis (Qiao et al., 2021). These pathways could be present in the embryo and regulate cellular homeostasis to promote normal cell development. However, embryos developed *in vitro* do so in the absence of maternal support which is stressful to the embryo. This stress could upregulate the PKR-mediated stress response and therefore alter SphK1 expression, activity (via phosphorylation), and localisation. Expression, activity, and localisation of pSphK1 in *in vivo* developed embryos may differ from *in vitro* and so should be investigated.

Staining for pSphK1 appeared to increase across the cleavage stages, suggesting that the embryo requires S1P production for early stages of preimplantation development (Figure 5.3), though this should be confirmed with enzyme activity assays. While follicular fluid contains S1P derived from supporting cells of the follicle (von Otte et al., 2006; Yuan et al., 2022), it is unclear if there is maternal S1P production in the oviduct and uterus that could act on the embryo during early development. Instead, increased pSphK1 staining in the early embryo suggests that the embryo itself produces S1P that can act in an autocrine manner to promote development. However, inhibition of pSphK1 had no effect on embryo cleavage, and instead impaired development from the compaction stage onwards during LD embryo culture (Figure 5.5), indicating S1P signalling is required for late preimplantation development instead. It is possible that the concentration of PF543 used to inhibit pSphK1-mediated S1P production was insufficient, resulting in S1P still being produced and acting in an autocrine manner to support development at these earlier stages. Therefore, testing the effect of concentrations above 15 μM PF543, or using more precise methods of SphK1 inhibition such as siRNA silencing or genetic knockout, could reveal a role for S1P in early development. These experiments could be performed on *Sphk2*$^{-/-}$ embryos to further elucidate the role of pSphK1 without interference from pSphK2-derived S1P.

Staining for pSphK1 appeared to decrease in the late blastocyst compared to earlier stages (Figure 5.4). Since immunofluorescence is primarily qualitative a method, these results should be confirmed with a protein quantification method such as western blotting before conclusions can be drawn. A decrease in pSphK1 at the blastocyst stage could be due to the embryo preparing for implantation and thus relying on paracrine S1P from the maternal endometrium, rather than autocrine S1P. *Sphk1* and *Sphk2* are expressed in endometrial cells, and are upregulated during endometrial decidualisation (Bernacchioni et al., 2021; Brunnert et al., 2014; Santulli et al., 2012; Skaznik-Wikiel et al., 2006). This suggests that S1P production by the maternal system is important for blastocyst implantation and could act on the blastocyst to replace autocrine signalling present during preimplantation development.

5.4.2 SphK2 and pSphK2 are present at all stages of preimplantation embryo development

SphK2 and pSphK2 were also present at all developmental stages and localised to the cytoplasm and nucleus, and plasma membrane and nucleus, respectively (Figures 5.6

and 5.8). Given that the total and phosphorylated SphK2 staining was quite different, it would be worth confirming total SphK2 staining with another SphK2 antibody.

Nuclear SphK2 has been reported in a range of cell types (Hait et al., 2020; Igarashi et al., 2003). In cancer cells, S1P produced in the nucleus by pSphK2 promotes hypoxia-induced histone acetylation by inhibition of HDAC1/2, resulting in increased transcription of hypoxia inducible factors HIF-1α and HIF-2α (Hait et al., 2010; Hait et al., 2020). S1P in the nucleus can also bind directly to HIF-1α to promote transcription of HIF-2α and HIF target genes such as VEGF and OCT4 in breast cancer cells to promote cell proliferation and survival (Hait et al., 2020). This pathway could be active in the embryo and promote normal preimplantation development. Alternatively, or perhaps additionally, S1P produced by nuclear pSphK2 could bind to telomerase reverse transcriptase (TERT), as seen in lung cancer cells (Selvam et al., 2015), which prevents telomere shortening and delays senescence. However, unlike pSphK2 which had stronger staining prior to the blastocyst stage (Figure 5.8), telomerase activity in the oocyte and early cleavage stages is low, and increases by the blastocyst stage, suggesting its role in early preimplantation development is limited (Liu et al., 2007). Additionally, knockout of telomerase in mice does not affect embryo development or reproductive parameters such as litter size directly (Liu et al., 2002). Instead, knockout of telomerase impairs telomere function, which manifests in later generations as impaired fertilisation and embryo development due to shortened telomeres (Liu et al., 2002). As acute inhibition of SphK2 impairs embryo development (Figure 5.11) and knockout of telomerase does not, it is unlikely that pSphK2 promotes embryo development solely through binding to TERT and stabilising telomerase within the nucleus.

While SphK2 is commonly found in the cytoplasm and nucleus, association of pSphK2 with the plasma membrane, as observed here in the embryo, has been reported in other cells, where it has been linked to oncogenic transformation and cell proliferation (Maceyka et al., 2005; Neubauer et al., 2016). Presence of SphK2 in the plasma membrane of other cells requires extracellular growth factors (Maceyka et al., 2005), suggesting autocrine growth factor signalling in the embryo may be causing its localisation to the membrane. Since pSphK1 was not localised to the plasma membrane in embryos, pSphK2 may be responsible for the production of S1P that can be translocated out of the cell to act at the S1PRs and thereby promote embryo development.

The punctate staining of pSphK2 observed at the 4-cell and 8-cell stages did not colocalise to autophagosomes (Figure 5.10). It is possible that the pSphK2 is instead contained within transport vesicles. Transport vesicles such as clathrin-coated pits and caveolae are endocytic structures that facilitate that transport of cargo between the plasma membrane and other parts of the cell. In mammalian cells, SphK1 associates with early and late endocytic membranes, and pSphK1 activity facilitates membrane trafficking (Lima et al., 2017; Shen et al., 2014; Young et al., 2016). It is possible that pSphK2 also associates with endocytic membranes in the preimplantation embryo. Similar staining to that of pSphK2 in the embryo has been observed for the amino-acid transporter, B0AT1 (Treleaven et al., 2022). B0AT1 appears to localise to subcortical vesicles prior to insertion into the plasma membrane and similar staining occurs for pSphK2 (Figure 5.8). It is possible that these vesicles are transport vesicles, and both of these proteins are ready to be inserted into the plasma membrane at the appropriate time of development or in response to the correct signal. Given the pSphK2 requires growth factor signalling to associate with the plasma membrane (Maceyka et al., 2005), localisation of pSphK2 to transport vesicles could be due to lack of maternal growth factors in the *in vitro* culture environment. pSphK2 was present in the plasma membrane only at the oocyte, zygote, and 2-cell embryo. These stages were either fixed immediately after isolation from the maternal reproductive tract or cultured for 24 h. The loss of maternal growth factor support associated with longer culture times could result in internalisation of pSphK2 into transport vesicles, as the *in vitro* culture environment lacks the growth factor signalling required for insertion into the membrane.

5.4.3 SphKs are required for embryo cleavage and cavitation

Inhibition of SphK2 reduced the percentage of embryos that developed to the 2-cell stage onwards, suggesting S1P production by pSphK2 is required for cleavage stages (Figure 5.11). This could be via YAP signalling, which promotes proliferation in many progenitor cell populations in the later embryo, including cardiomyocytes (Heallen et al., 2011) and lung epithelial cells (Lin et al., 2017). YAP also has a more specific role in embryonic genome activation (EGA). In mice, the EGA occurs at the 2-cell stage and YAP derived from maternal mRNA is a key activator of this process (Yu et al., 2016). YAP signalling is controlled by many factors, which could be why only partial arrest is seen with inhibition of SphK2. One such factor that mediates YAP function in the embryo is lysophosphatidic acid (LPA), a mitogenic phospholipid derivative that shares many similarities with S1P. Exogenous LPA promotes nuclear localisation of YAP and expression of genes downstream

of YAP signalling in the 4-cell embryo (Yu et al., 2016). LPA treatment increases development to the blastocyst stage and increases litter size, suggesting these embryos have increased developmental competence (Yu et al., 2016). S1P promotes YAP signalling during formation of the blastocyst (Chi et al., 2020), and so it is possible that S1P also regulates YAP signalling earlier in development similar to LPA. Therefore, inhibition of SphK2 could impair embryo cleavage by dysregulating YAP signalling, leading to abnormal EGA activation and decreased proliferation. This could be investigated by examining the effect of SphK2 inhibition on the localisation of YAP in the early embryo.

Inhibition of either SphK1 by 5-15 μM PF543 or SphK2 by 80 μM ABC294640 during LD culture resulted in abnormal compaction and cavitation (Figures 5.5 and 5.11). This occurred when embryos were treated with the inhibitors individually or with a combination of the inhibitors used at a concentration that had no effect on compaction alone (Figure 5.12). Combined inhibition of SphK1 and SphK2 also reduced blastocyst formation during HD embryo culture, suggesting that the increased concentration of autocrine growth factors present was unable to compensate for the loss of SphK activity. These results suggest there is an additive effect of the combined inhibitors, and no effect was seen when inhibitors were used individually possibly due redundancy between SphK1 and SphK2 in the early embryo, as seen in later development (Mizugishi et al., 2005). While deletion of either *Sphk1* or *Sphk2* in mice results in offspring that are viable and demonstrate no phenotypic defects, deletion of both *Sphk1* and *Sphk2* is lethal by E11.5, indicating functional redundancy between the isoenzymes (Mizugishi et al., 2005). However, it is unclear how this redundancy occurs given the different subcellular localisation of the isoenzymes in the embryo. Therefore, the effect of increasing concentrations of SphK1 or SphK2 inhibitors on the expression, localisation, and activity of both SphKs should be determined.

While S1PR signalling has been linked to processes required for compaction (Chapter 4), S1P also influences cell survival by reducing levels of pro-apoptotic ceramide and sphingosine within the cell (Pandey et al., 2020). Inhibition of SphKs would prevent conversion of sphingosine to S1P, thus allowing accumulation of ceramide and sphingosine. In mouse embryonic stem cells, knockout of SphK1 and SphK2 results in accumulation of ceramide and sphingosine and G2/M cell-cycle arrest, as well as expression of telomere lengthening proteins (Pandey et al., 2020). This was independent of S1PR signalling as exogenous S1P did not overcome the cell-cycle arrest. Expression of ceramide synthase 2,

which converts sphingosine to ceramide, did partially alleviate the arrest, suggesting that it is the accumulation of sphingosine that induces cell-cycle arrest. Accumulation of sphingosine upon SphK inhibition could explain the abnormal compaction and cavitation in this study as previous work has shown that addition of 2.5 μM sphingosine to preimplantation embryo culture prevents compaction (Zhu et al., 2017). This is due to inhibition of PLC/PKC-mediated phosphorylation of myosin regulatory light chain and its accumulation at the apical domain that is essential for polarisation of cells at the 8-cell stage. Lipidomics could be used to investigate levels of sphingosine and ceramide in embryos treated with SphK inhibitors compared to the concentration of sphingosine required to affect compaction. This would provide insight as to whether inhibition of SphKs is detrimental to embryo development due to loss of S1P signalling or accumulation of sphingosine as seen in embryonic stem cells.

5.4.4 Investigation of genes associated with SphK1 and SphK2 in the embryo

As previously discussed (Chapter 4), use of qPCR to analyse gene expression of *Sphk1* and *Sphk2* in preimplantation embryos had limited success, with most samples showing no expression. This is likely due to the small number of embryos within each sample analysed. qPCR has been used to determine expression of genes relating to S1P signalling in COCs (Park et al., 2019), but with 150 COCs per sample as opposed to the 20 embryos per sample used in the current study. Due to the ethical restrictions and difficulty of collecting large numbers of embryos, replicating the number of embryos per sample used in the initial study was not feasible. Unfortunately, this means that no conclusions can be drawn from the current data. While *Sphk2* expression was only detected in a limited number of samples, *Sphk1* expression was not detected in any sample. This could mean that there is higher expression of *Sphk2* mRNA in the embryo or could simply be due to differences in the efficiencies of the primers. However, RNAseq and microarray analysis of *Sphk* expression throughout preimplantation embryo development have demonstrated the same result, in which only *Sphk2* was detectable in embryos (Deng et al., 2014; Hamatani et al., 2004; Jeong et al., 2006). This supports the theory that pSphK2 is the dominant source of S1P in the embryo, rather than pSphK1 as in other cells.

5.4.5 Conclusions

This study identified the localisation of SphK1 and SphK2 in mouse preimplantation embryos. Both pSphK1 and pSphK2 were individually indispensable for preimplantation

development *in vitro,* though there was also functional redundancy between the enzymes which is also seen in later development and in other cell types. As SphKs may promote development by preventing accumulation of pro-apoptotic sphingosine or by downstream intracellular or extracellular signalling, further investigation into the role of pSphKs in embryo development is required. This would provide greater insight into growth-factor signalling in the embryo and therefore a better understanding of the molecular mechanisms underpinning preimplantation embryo development.

CHAPTER 6

General discussion

In vitro culture is detrimental to embryo viability, resulting in slower development (Harlow & Quinn, 1982), chromosomal abnormalities (Maurer et al., 2015), and altered gene expression (Giritharan et al., 2007). Improving the culture environment to better reflect the environment in which embryos develop *in vivo* would assist in alleviating these issues and allow production of more embryos of higher quality for use in assisted reproductive technologies. Identification of key molecules and signalling pathways present *in vivo* facilitates a greater understanding of the molecular mechanisms underpinning embryo development, and this information can be used to inform decisions regarding the improvement of *in vitro* embryo culture. Exogenous S1P increases the developmental rate of embryos when added to culture medium (Span, 2015), but the mechanism behind this remained unclear. This thesis examined the localisation and function of S1P pathway constituents in the embryo and found that not only is exogenous S1P beneficial to development, but that the preimplantation embryo produces S1P that is required for key developmental processes.

6.1 S1P signalling in the oocyte

In the oocyte, S1PR2-4 all had increased staining in the cytoplasm surrounding the second meiotic spindle, suggesting that these receptors may play a role in meiotic division (Appendix 1). While pSphK2 is localised to the plasma membrane, pSphK1 localises to the metaphase spindle in the oocyte, meaning pSphK1 could provide S1P for any autocrine S1P/S1PR signalling at or near this site (Appendix 3). Maternally-derived S1P could originate from the follicular fluid ovulated with the oocyte, as S1P is produced by the surrounding mural granulosa and cumulus cells within the follicle (Yuan et al., 2022). These cumulus cells are ovulated with the oocyte, and so could also produce S1P that acts on the oocyte during fertilisation.

S1P present in the oviductal fluid would need to enter the cell to act on S1PR2-4 in the cytoplasm as they are not localised to the plasma membrane. The two main transporters of S1P in the plasma membrane of cells are Spns2 and Mfsd2b (Chen et al., 2023; Vu et al., 2017). Spns2 is a facilitated-diffusion uniporter, while Mfsd2b is only associated with S1P export and not S1P import into the cell (Chen et al., 2023; Vu et al., 2017). Expression of *Spns2* mRNA is upregulated in the embryo from the late 2-cell stage onwards, while *Mfsd2b* is not expressed at any stage (Deng et al., 2014). Therefore, Spns2 is more likely to be involved in uptake of S1P from the follicular fluid to act on cytoplasmic S1PRs.

Alternatively, S1P in oviductal fluid could act via S1PR1 and S1PR5, both of which are present in the oocyte membrane. Further studies are needed to measure the concentration of S1P in oviductal fluid, as well as the expression and localisation of S1P transporters in the mature oocyte.

S1P signalling could promote fertilisation through activation of $G_{12/13}$ and downstream RhoA signalling (Figure 6.1). Fertilisation involves a meiotic division that results in extrusion of a polar body. For this asymmetric division to occur, the oocyte must establish cortical polarity prior to anaphase and subsequent cell division (Dehapiot et al., 2021). Cortical polarity is characterised by migration of the meiotic spindle to the cortex, spindle rotation, and cortical reorganisation to form an ectopic actin cap. RhoA signalling is heavily involved in establishing cortical polarity, with knockdown or inhibition of RhoA signalling in the oocyte resulting in defects in spindle rotation and actin cap formation, thus preventing polar body extrusion (Dehapiot et al., 2021; Zhang et al., 2014). S1PR2-5 all couple to $G_{12/13}$ which can activate downstream Rho signalling, and this could promote fertilisation of the oocyte.

While S1P promotes oocytes maturation both *in vivo* and *in vitro* (Lee & Koo, 2012; Yuan et al., 2022), data on the role of S1P in fertilisation is limited to treatment of oocytes during IVM and its effect on subsequent fertilisation, rather than treatment during fertilisation itself (Lee & Koo, 2012). Therefore, the effect of both exogenous and embryo-derived S1P on oocyte fertilisation should be investigated, as well as the involvement of downstream S1PR signalling. This could be conducted using immunofluorescence staining to visualise actin, tubulin, and nucleic material in oocytes fertilised in the presence of S1PR inhibitors to detect abnormalities in oocyte morphology, actin cap formation, polar body extrusion, and the processes of meiosis and cytokinesis. Polarised light microscopy could also be used to visualise the meiotic spindle of live oocytes to determine the effect of S1PR inhibition on spindle migration, rotation, and polar body extrusion (Inoue, 1953; Molinari et al., 2012).

Alternatively, since S1PRs are GPCRs, they may activate canonical PLC signalling pathways and thereby regulate intracellular Ca^{2+} (Figure 6.1). During fertilisation, Ca^{2+} oscillations are required for oocyte activation and are triggered by sperm-derived PLCζ hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to form inositol 1,4,5-trisphosphate (IP3) (Hachem et al., 2017; Yang et al., 2013) which acts on the IP3 receptor

on the endoplasmic reticulum to cause Ca^{2+} release (Yang et al., 2013). Oocyte PLC isoforms are also involved in fertilisation, including PLCβ1, β3, and γ (Dupont et al., 1996). Knockdown or overexpression of PLCβ1 in the oocyte alters fertilisation-induced Ca^{2+} oscillations, and delays pronuclei formation (Igarashi et al., 2007), suggesting PLCβ1 activity is required for normal oocyte activation. Similarly, activation of oocyte PLCγ results in Ca^{2+} oscillations and formation of pronuclei consistent with oocyte activation (Mehlmann et al., 1998). S1P activates PLC and subsequent Ca^{2+} release in many different cell types (Fujii et al., 2014; Lucki et al., 2012; Suhaiman et al., 2010; Yan et al., 2019). All five S1PRs couple to G_i, and S1PR2 and S1PR3 couple to G_q, both of which can activate downstream PLC signalling and Ca^{2+} release (Figure 1.4). Therefore, S1P signalling via S1PRs could support fertilisation of the oocyte through activation of PLC. This could be investigated by performing calcium imaging experiments on activating oocytes in the presence of S1PR inhibitors to determine their effect on oocyte activation and Ca^{2+} dynamics in the oocyte.

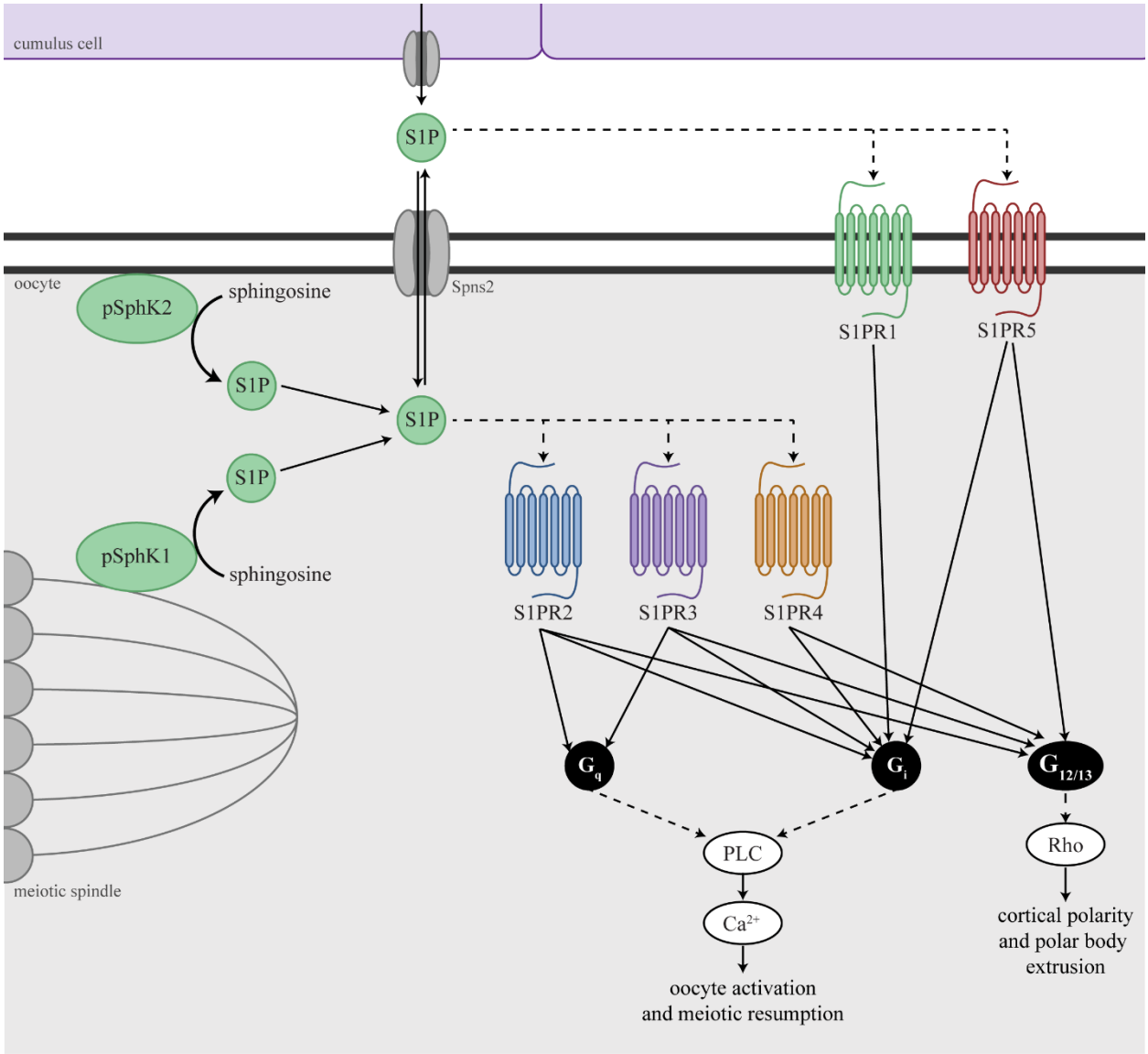


Figure 6.1. Proposed signalling activity mediated by S1P in the oocyte. S1P may be synthesised by pSphK1 and pSphK2 in the oocyte or originate from granulosa cells surrounding the oocyte. S1P could either act intracellularly at S1PR2-4 present in the cytoplasm of the oocyte, or extracellularly at S1PR1 and S1PR5 present in the plasma membrane. These receptors couple to G-proteins that could activate downstream PLC and Rho signalling to promote oocyte activation, meiotic resumption, establishment of cortical polarity, and polar body extrusion. Localisation of SphKs and S1PRs are based on data from the current study. Dashed arrows represent hypothesised signalling mechanisms. Solid arrows represent published data (references in-text).

6.2 S1P signalling in cleavage stage embryos

Inhibition of SphK2 or S1PR3 both reduced the percentage of embryos that cleaved while inhibition of SphK1 or other S1PRs had no effect, suggesting that pSphK2/S1PR3 signalling is present in cleavage-stage embryos (Figures 4.7 and 5.11). During cleavage stages, S1PR3 is predominantly localised to the cytoplasm and subcortically, though there is some localisation to the nucleus, while pSphK2 is present in the plasma membrane and nucleus (Appendix 1 and 3). This means that pSphK2/S1PR3 signalling could either occur through the canonical signalling at the plasma membrane, or within the nucleus (Figure 6.2). A nuclear pSphK2/S1PR3 axis occurs in lung adenocarcinoma cells and promotes cell proliferation, though the downstream signalling pathways associated with this are unknown (Terlizzi et al., 2021). However, inhibition of S1PR3 almost completely prevented development to the 2-cell stage (Figure 4.7), while most embryos successfully developed to the 2-cell stage with inhibition of SphK2 (Figure 5.11). It is possible that the concentration of ABC294640 is not high enough to fully inhibit S1P production, thus allowing embryos to progress to the 2-cell stage. Using purified recombinant SphK2 and a derivatised substrate (NBD-sphingosine), liquid chromatography/mass spectrometry results show that 100 μM ABC294640 only reduced SphK2 activity to approximately 40% (French et al., 2010). Thus, even in the presence of 100 μM ABC294640, production of S1P by pSphK2 is likely still sufficient for embryos to progress to the 2-cell stage. Inhibition of SphK2 using more rigorous techniques, such as genetic knockout or siRNA silencing, could determine the effect of complete loss of pSphK2 activity in the preimplantation embryo. This could result in a phenotype closer to that of S1PR3 inhibition, where embryos fail to develop past the zygote stage rather than the 2-4-cell stage as with SphK2 inhibition in this study.

Collective inhibition of S1PR1-4 resulted in most embryos arresting at the 2-cell stage (Figure 4.17), which coincides with the EGA. S1P is associated with transcription and gene expression in a number of cells, including the mouse blastocyst and human granulosa cells (Cheng et al., 2018; Chi et al., 2020; Estrada et al., 2009; Hu et al., 2019; Li et al., 2020b). In both blastocysts and granulosa cells, S1P signalling results in downstream activation of YAP to facilitate expression of genes required for development of the embryo or follicle. As previously discussed (Chapter 5), YAP is a key activator of the EGA (Yu et al., 2016), and therefore could be the link between disrupted S1P signalling and 2-cell arrest. YAP is expressed at all stages of preimplantation development in mice, and gradually translocates from the cytoplasm to the nucleus during cleavage stages (Yu et al., 2016).

S1PR2 activates YAP signalling in the blastocyst (Chi et al., 2020), and in other cell types S1PR1 and S1PR3 are also linked to YAP activation (Shen et al., 2019; Tao et al., 2023; Wang et al., 2023b).

In ovarian cancer cells, a positive feedback loop occurs between S1PR1 and YAP, in which S1PR1 activates YAP via PDK1, inducing its translocation to the nucleus (Tao et al., 2023). In the nucleus, YAP binds to the *S1pr1* gene promoter and increases S1PR1 expression. This feedback loop regulates senescence of these cells and promotes their proliferation and migration. S1PR3 also activates YAP in osteosarcoma cells (Shen et al., 2019) and lymphoma cells (Wang et al., 2023b).

Whether S1PR4 and S1PR5 can activate YAP has not been investigated, but both of these receptors can couple to $G_{12/13}$ and activate RhoA. YAP can be activated by RhoA in other cells (Liu et al., 2021; Sakabe et al., 2022; Xia et al., 2020), providing a mechanism by which S1PR4 could activate YAP in the embryo. The potential for all five S1PRs to activate YAP could allow for redundancy in S1PR-mediated YAP activation and thus activation of the EGA (Figure 6.2). This would explain why 2-cell arrest was seen with inhibition of S1PR1-4 but not S1PR1-3 (Figures 4.16 and 4.17), as S1PR4 was able to compensate for the loss of S1PR1-3 signalling during early development. Therefore, the effect of inhibition of S1PRs individually or in combination on the localisation of YAP during early development should be investigated. Transcriptomics could also be used to determine the effect of S1PR inhibitors on EGA.

The phenotype produced by inhibition of S1PR1-4 does not align with inhibition of SphK1/2 which inhibited development at cavitation (Figure 5.12). This is surprising, as E11.5 mouse embryos in which both SphK1 and SphK2 are genetically deleted contain no measurable S1P, suggesting SphKs are the dominant, if not sole, source of S1P in the embryo (Mizugishi et al., 2005). While it is possible that S1PR4 could be activated independently of S1P by another ligand or by transactivation of the receptor, this has not been reported for any of the S1PRs. It is more likely that the concentration of SphK inhibitors used in this study is too low, allowing for some production of S1P that can act at the receptors. This could be confirmed performing enzyme activity assays on embryos treated with SphK inhibitors. It is possible that use of more rigorous techniques to inhibit SphK1/2, such as siRNA knockdown, would produce a similar arrest pattern to that seen with inhibition of S1PR1-4.

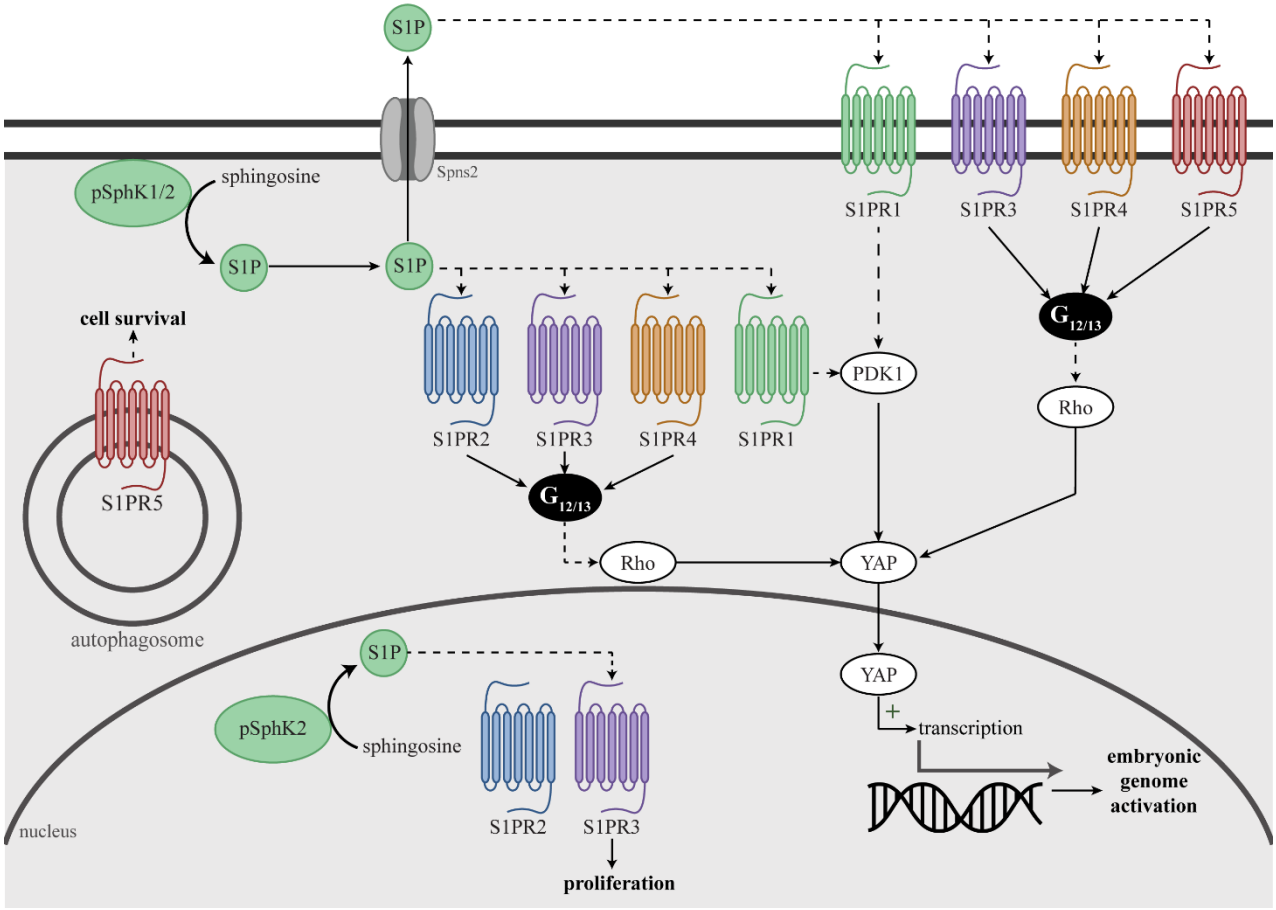


Figure 6.2. Proposed signalling activity mediated by S1P during embryo cleavage stages. S1P may be synthesised by pSphK1 within the cytoplasm or pSphK2 in the nucleus in the cleavage-stage embryo. Cytoplasmic S1P could act on S1PR1-4 in the cytoplasm or be secreted extracellularly to act at S1PR1, 3, 4, and 5 in the plasma membrane. These receptors could act via PDK1 for S1PR1, or $G_{12/13}$/Rho signalling for S1PR3-5, to activate YAP, resulting in nuclear translocation of YAP where it could promote transcription required for EGA. Nuclear S1P could act at S1PR3 to promote proliferation. Localisation of SphKs and S1PRs are based on data from the current study. Dashed arrows represent hypothesised signalling mechanisms. Solid arrows represent published data (references in-text). Note that oviductal cells were not included as they have not been shown to secrete S1P.

6.3 S1P signalling in the morula

Inhibition of S1PR1, S1PR2, and S1PR4 resulted in a morula block, suggesting that these S1PRs are involved in cavitation and blastocyst formation (Figures 3.4, 4.3, and 4.11). All three S1PRs regulate proteins associated with tight junctions and adherens junctions in other cell types (Hansen et al., 2022; Reinhard et al., 2017). In vascular cells, the endothelial barrier is formed by tight junctions and adherens junctions and is important for endothelial barrier integrity, cell-cell contact, and selective permeability to fluids, solutes, and proteins (Komarova et al., 2017). In these cells, S1P activates multiple S1PRs with opposing signalling outcomes - S1PR1 and S1PR4 act via G_i/Rac1 to promote endothelial barrier formation, while S1PR2 acts via $G_{12/13}$/RhoA to disrupt barrier integrity (Hansen et al., 2022; Reinhard et al., 2017). G_i/Rac1 and $G_{12/13}$ /RhoA reciprocally inhibit each other's activity, and it is the balance between these two pathways that determines endothelial barrier integrity. Inhibition of S1PR1/S1PR4/G_i signalling decreases transcription of VE-cadherin (*Cdh5*), OCLN (*Ocln*), claudin 5 (*Cldn5*), and ZO-1 (*Tjp1/ZO-1*) (Hansen et al., 2022), whereas inhibition of S1PR2/RhoA signalling upregulates these proteins (Cao et al., 2019). These junctional proteins are also present in the compacting embryo, and formation of a paracellular seal by tight junctions is crucial for formation of the blastocoel (Canse et al., 2023). Given that inhibition of S1PR1, S1PR2, and S1PR4 all prevented formation of the blastocoel, it seems likely that the same balance of signalling pathways seen in vascular endothelial cells also exists in the embryo (Figure 6.3).

However, inhibition of these receptors during only early or late preimplantation development suggest there is a biphasic role for S1P signalling in tight junction formation (Figures 3.5, 4.4, and 4.12). Inhibition of S1PR1 prevented cavitation when embryos were treated during early development but not when treated during late development (Figure 3.5), while the opposite was true of S1PR4 inhibition (Figure 4.12). On the other hand, inhibition of S1PR2 reduced cavitation when embryos were treated during either early or late development (Figure 4.4). Collectively, these results could be due to a balance of S1PR1/Rac1 and S1PR2/RhoA signalling mediating early set up of tight junctions during the cleavage stages of development, while a balance of S1PR4/Rac1 and S1PR2/RhoA signalling has an acute effect on tight junction assembly during later stages. This should be investigated by confirming that inhibition of S1PR1/S1PR4 and S1PR2 signalling in the embryo disrupts G_i and $G_{12/13,}$ respectively, and then determining the effect of inhibition of

these receptors during early or late development on the expression and localisation of junctional proteins such as cadherins, OCLN, and ZO-1.

Both SphK1 and SphK2 are present and functional at the morula stage, and thus could be providing S1P that acts on the S1PRs. pSphK1 and pSphK2 are both present at the morula stage with opposing localisation – pSphK1 is strongly cytoplasmic, while pSphK2 is present in the nucleus and plasma membrane (Appendix 3). This facilitates production of S1P in all cellular compartments. Inhibition of SphK2 prevented embryo cavitation (Figure 5.11), which aligns with the phenotype produced by inhibition of S1PR1, S1PR2, and S1PR4, and suggests that pSphK2-mediated S1P production is necessary for S1PR signalling in the morula. Inhibition of SphK1 affects embryo development slightly earlier, during the process of compaction (Figure 5.5). Inhibition of the S1PR2-4 did not inhibit compaction, and so S1P produced by pSphK1 at this stage could be acting via S1PR5, or through intracellular signalling. While a small molecule inhibitor selective for S1PR5 is not currently available, inhibition of S1PR5 signalling using genetic knockout or siRNA could be used to investigate whether S1PR5 has a role in embryo compaction.

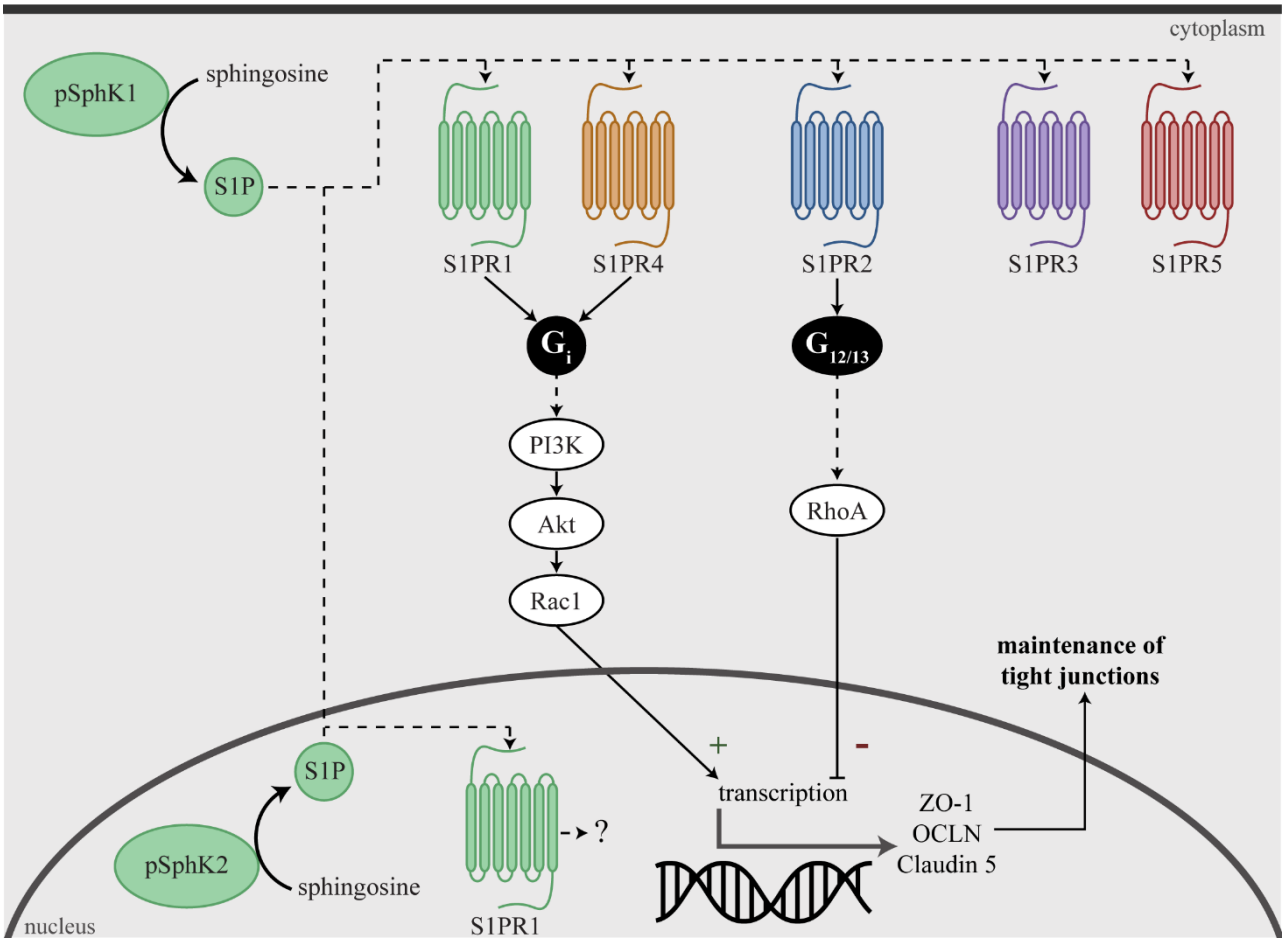


Figure 6.3. Proposed signalling activity mediated by S1P in the morula. S1P may be synthesised by pSphK1 within the cytoplasm or pSphK2 in the nucleus in the morula. S1P could act on S1PR1-5 in the cytoplasm. Activation of G_i via S1PR1 and S1PR4 could activate downstream P13K/Akt signalling to promote transcription of tight junction proteins such as ZO-1, OCLN, and claudin 5, while activation of S1PR1/$G_{12/13}$/RhoA signalling could inhibit this. The balance between these two opposing activities could be required for maintenance of tight junctions in the morula. Localisation of SphKs and S1PRs are based on data from the current study. Dashed arrows represent hypothesised signalling mechanisms. Solid arrows represent data from previous literature (references in-text).

6.4 S1P signalling in the blastocyst

Each of the S1PRs had distinct localisation patterns in the blastocyst, suggesting they play different roles at this stage (Appendix 3). However, pSphK1 and pSphK2 staining was weak in the blastocyst (Appendix 4), suggesting that there is limited production of S1P by the embryo itself. Instead, S1P could be produced by the maternal cells to act on the blastocyst as it comes into close proximity with the uterine wall. Genes encoding enzymes responsible for S1P synthesis (*Sphk1 and Sphk2*) and degradation (*Sgpl1*, *Sgpp1*, and *Sgpp2*), are expressed in endometrial cells and expression is upregulated during the time of implantation (Bernacchioni et al., 2021; Brunnert et al., 2014; Santulli et al., 2012). This upregulation suggests increased S1P turnover during the time of implantation, which could act on the blastocyst to promote attachment and invasion of trophectoderm cells. The concentration of S1P in uterine fluid could be measured using lipidomics or ELISA to determine if maternally derived S1P is secreted into the uterine fluid during implantation.

S1PR1 and S1PR4 were localised to the outer cells of the morula, as well as trophectoderm cells and hypoblast cells, but not epiblast cells (Appendix 1 and 2). This suggests that these receptors could be involved in cell differentiation in the blastocyst (Figures 6.3-6.6). As previously discussed (Chapter 4), S1P/SIPR2 signalling promotes differentiation of morula-stage blastomeres into trophectoderm and ICM cells via activation of YAP in trophectoderm cells and suppression of YAP in ICM cells (Chi et al., 2020). Activation of YAP in outer cells of the morula and trophectoderm coincides with the localisation of S1PR1 and S1PR4 found in this study, suggesting that these receptors are involved in these differentiation processes upstream of YAP (Figure 6.4). Chi et al. (2020) did not investigate the effect of other S1PRs on YAP in the embryo but, as discussed in section 6.2, S1PR1 and S1PR3 activate YAP in other cell types (Shen et al., 2019; Tao et al., 2023; Wang et al., 2023b) and S1PR4 and S1PR5 could activate YAP via RhoA signalling (Liu et al., 2021; Sakabe et al., 2022; Xia et al., 2020). Therefore, both S1PR1 and S1PR4, and potentially other S1PRs, could regulate YAP signalling and thus differentiation to, and maintenance of, trophectoderm cells. Immunofluorescence staining to visualise YAP in embryos treated with S1PR inhibitors could be used to determine if S1PR signalling regulates YAP activation and thus the localisation of YAP in the nucleus of trophectoderm cells of the blastocyst.

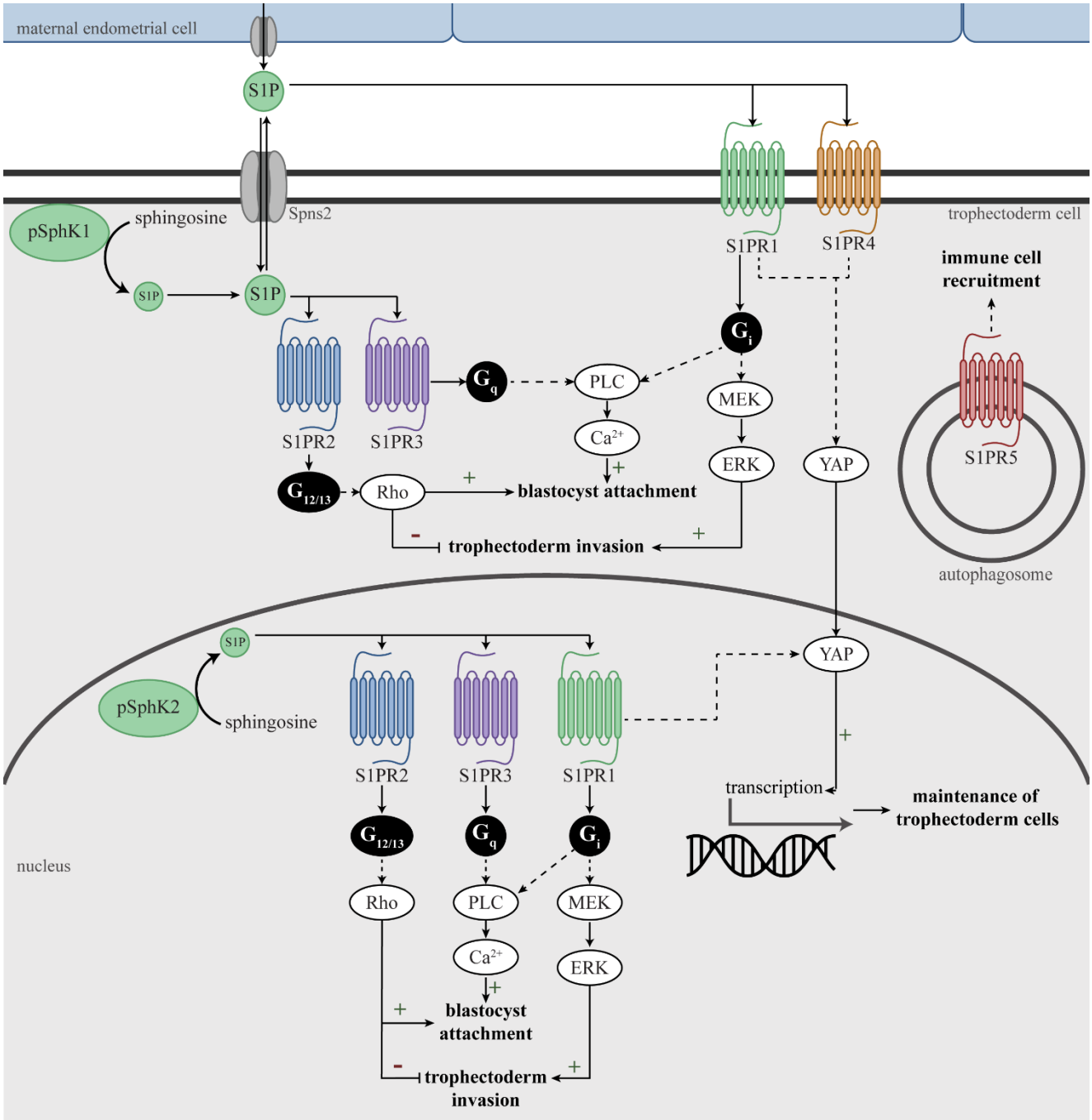


Figure 6.4. Proposed signalling activity mediated by S1P in trophectoderm cells. S1P may be synthesised by pSphK1 within the cytoplasm or pSphK2 in the nucleus of trophectoderm cells or originate from the maternal endometrium as the blastocyst prepares for implantation. Extracellular or cytoplasmic S1P could act at S1PR1 and S1PR4 in the plasma membrane to activate YAP signalling, which could promote transcription required for differentiation to, and maintenance of, trophectoderm cells. Activation of S1PR1 could also promote blastocyst attachment and invasion by downstream G_i/PLC/Ca^{2+} and G_i/MEK/ERK signalling, respectively. Extracellular or cytoplasmic S1P could also activate S1PR2/Rho signalling in the cytoplasm to promote blastocyst attachment and inhibit trophectoderm invasion, or S1PR3/G_q/PLC/Ca^{2+} to promote attachment. Alternatively, nuclear S1P produced by pSphK2 could act at S1PR1-3 in the nucleus to activate these signalling pathways. Localisation of SphKs and S1PRs are based on data from the current study. Dashed arrows represent hypothesised signalling mechanisms. Solid arrows represent data from previous literature (references in-text). Decreased size of S1P circle indicates lower production of S1P by SphKs.

S1PR1 and S1PR4 also localised to hypoblast cells, but not epiblast cells, and could be involved in differentiation of ICM cells to hypoblast (Figure 6.5 and 6.6). Commitment to the hypoblast cell lineage requires expression of GATA6 and suppression of Nanog. In mesenchymal stem cells, S1PR2 signalling via ERK transcriptionally decreases expression of pluripotency factors, including Nanog and Sox2 (Price et al., 2015). YAP also has transcriptional control over Nanog expression, with strong activation of YAP supressing Nanog expression in mouse embryonic stem cells (Meyer et al., 2023). Additionally, both YAP and S1P upregulate expression of GATA6 in human iPSCs and mesoangioblasts, respectively (Donati et al., 2011; Zeevaert et al., 2023). It is possible that S1P acts via S1PR1 and S1PR4, and downstream YAP, to supress Nanog and upregulate GATA6 to promote differentiation to, and maintenance of, hypoblast cells in the mouse blastocyst. This could be confirmed by investigating expression and localisation of S1PR1, S1PR4, and YAP in the early blastocyst as differentiation to hypoblast occurs, as well as investigating the effect of S1PR1 and S1PR4 inhibitors on hypoblast formation by analysis of hypoblast cell markers such as GATA6.

All five S1PRs were localised to trophectoderm cells of the blastocyst, suggesting a role for S1P signalling in blastocyst implantation (Appendix 2). While there is little data on the role of S1P in embryos during the peri-implantation period, the effect of S1P on migration of human extravillous trophoblast cells has been investigated but results are conflicting: 1-10 nM S1P promotes migration and invasion of HTR8/SVneo cells via S1PR1/MEK/ERK signalling (Yang et al., 2014), while 0.1-10 μM S1P inhibits migration of primary trophoblast cells via S1PR2/Rho signalling (Westwood et al., 2017). However, these cells are differentiated trophoblast cells of the placenta derived from much later in development than the blastocyst, and so do not necessarily reflect trophectoderm cells during implantation. If trophectoderm cells do exhibit a similar response to S1P, it is possible that a balance between the pro-invasive S1PR1 signalling and anti-invasive S1PR2 is required *in vivo* to spatially restrict embryo implantation within the uterus (Figure 6.4). Both S1PR1 and S1PR2 are present in trophectoderm cells of the late blastocyst, where they could respond to autocrine or paracrine S1P to regulate blastocyst invasion. S1PR3-5 are also localised to the trophectoderm cells of the blastocyst and promote migration in other cells, particularly immune cells (Donovan et al., 2010; Drouillard et al., 2018; Xiong et al., 2019). If these receptors also promote migration in trophectoderm cells, this suggests a complex network of S1P signalling for regulation of blastocyst invasion.

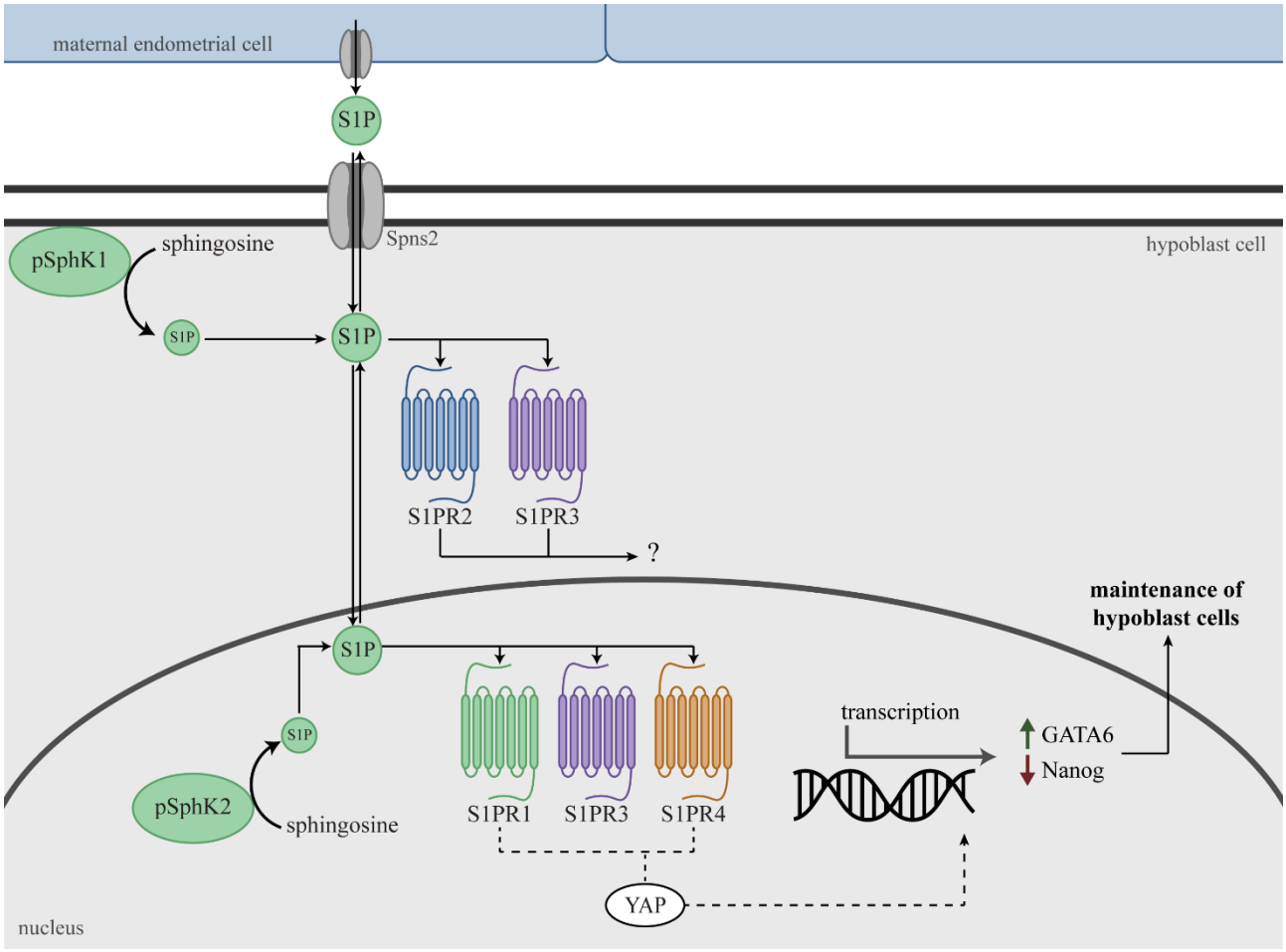


Figure 6.5. Proposed signalling activity mediated by S1P in hypoblast cells. S1P may be synthesised by pSphK1 within the cytoplasm or pSphK2 in the nucleus of hypoblast cells or originate from the maternal endometrium as the blastocyst prepares for implantation. This S1P could act at nuclear S1PR1 and S1PR4 to activate YAP signalling, which could promote transcription required for maintenance of hypoblast cells. Localisation of SphKs and S1PRs are based on data from the current study. Dashed arrows represent hypothesised signalling mechanisms. Solid arrows represent data from previous literature (references in-text). Decreased size of S1P circle indicates lower production of S1P by SphKs.

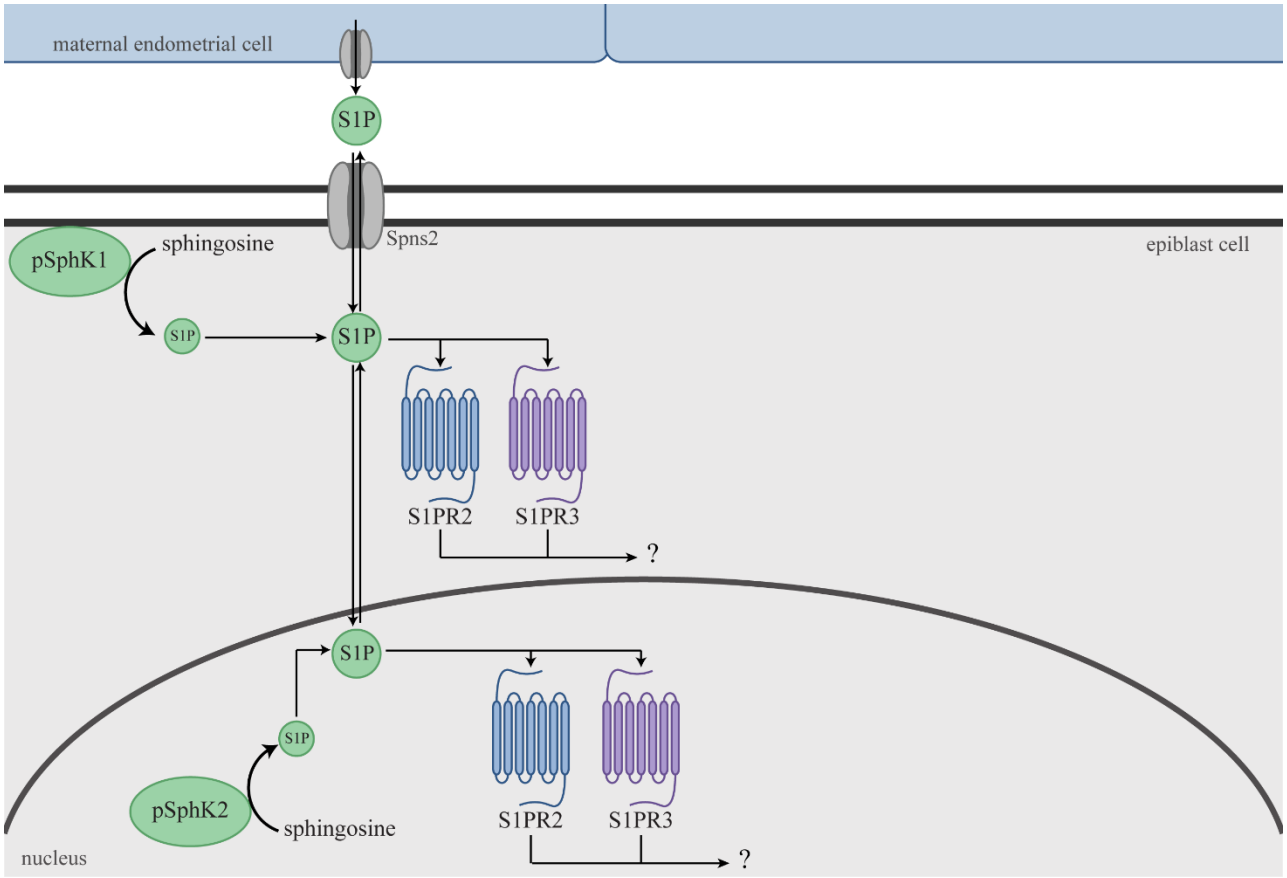


Figure 6.6. S1P signalling in epiblast cells. S1P may be synthesised by pSphK1 within the cytoplasm or pSphK2 in the nucleus of epiblast cells or originate from the maternal endometrium as the blastocyst prepares for implantation. This S1P could act at S1PR2 and S1PR3 receptors within the cytoplasm or nucleus to activate downstream signalling. Localisation of SphKs and S1PRs are based on data from the current study. Decreased size of S1P circle indicates lower production of S1P by SphKs.

Blastocyst invasion could also be promoted by S1PR-mediated upregulation of leukemia inhibitory factor (LIF) (Figure 6.4). In astrocytes, S1P acts via simultaneous S1PR1 and S1PR2/$G_{12/13}$/RhoA signalling to upregulate expression of transcription factors FOS and JUN and, subsequently, LIF (Tran et al., 2020). LIF promotes blastocyst formation, hatching and trophectoderm outgrowth *in vitro*, with treatment of embryos from the 8-cell stage onward with 1000 U/mL LIF increasing hatching from 62% in control to 85% and increasing the percentage of blastocysts that attached and outgrew in culture (Lavranos et al., 1995). Treatment of embryos with 1000 U/mL LIF from the 2-cell stage did not affect early preimplantation development, but did increase blastocyst formation and hatching (Tsai et al., 1999). As S1PR1 and S1PR2 are both present in trophectoderm cells in the blastocyst (Appendix 2), they could upregulate LIF expression in order to promote blastocyst hatching and implantation. This could be investigated by performing blastocyst outgrowth assays, which involve the culture of hatched blastocysts on glass coverslips to facilitate outgrowth

of trophectoderm cells (Binder et al., 2015; Kim et al., 2020). These experiments could be conducted with the addition of S1PR inhibitors to determine their effect on trophectoderm cell migration and invasion.

S1PR signalling could also promote blastocyst attachment (Figure 6.4). S1PR1/3 signalling activates PLC in lung carcinoma epithelial cells and primary arterial placental endothelial cells (Chen et al., 2008; Del Gaudio et al., 2020) and PLCγ activation is essential for trophectoderm attachment during implantation, as intracellular Ca^{2+} signalling downstream of PLC leads to strengthening of trophoblast adhesion to components of the extracellular matrix underlying uterine endometrial cells (Wang et al., 2007). This study concluded that GPCRs were not directly involved in PLCγ activation, but instead requires receptor or non-receptor tyrosine kinases (Gresset et al., 2012). However, S1P can directly activate PLCγ via S1PRs coupled to G-proteins (Matsushita et al., 2004), or by transactivation of RTKs. For example, in breast cancer epithelial cells, S1P signalling via S1PR1/3 results in transactivation of IGF1R and EGFR, though the mechanism behind this is yet to be elucidated (Martin et al., 2009). Therefore, while S1P may not directly activate PLCγ, it could indirectly activate it as part of a larger signalling network that ultimately promotes blastocyst attachment during implantation. The role of S1P signalling in blastocyst attachment could be investigated through *in vitro* attachment assays in the presence of S1PR inhibitors to determine their effect on blastocyst attachment. Additionally, to determine if S1PR signalling leads to downstream activation of PLC, enzyme activity assays could be used to determine the effect of S1PR inhibitors on PLCγ activation in trophectoderm cells.

Localisation of S1PR5 in the blastocyst is unique compared to the other S1PRs, in that it was present only in trophectoderm cells (Appendix 2). Unlike other S1PRs, S1PR5 can signal via JNK in addition to G-proteins, which could provide a mechanism for S1PR5 to promote blastocyst implantation (Figure 6.4). S1PR5 is involved in immune regulation and angiogenesis in other cell types, both of which are crucial for successful implantation. In keratinocytes, JNK signalling results in upregulation of CCL20, a chemokine that attracts dendritic cells and T-cells (Li et al., 2017). Inflammation and the presence of natural killer cells, dendritic cells, and regulatory T-cells are essential for normal blastocyst implantation and placentation (Collins et al., 2009; Hanna et al., 2006; Plaks et al., 2008). Therefore, S1PR5 signalling in trophectoderm cells could be involved in implantation through the recruitment of immune cells to the site of implantation. Knockout or knockdown of S1PR5

could be used in conjunction with blastocyst attachment and outgrowth assays to determine if S1PR5 promotes implantation *in vitro*, though this would not provide information on immune-cell recruitment. Involvement of CCL20 could be determined by quantifying *Ccl20* mRNA and protein using PCR and Western blotting, respectively. Ultimately, *in vivo* implantation studies in which S1PR5 knockout embryos are transferred into the uterus of a mouse would be best for examining the impact of S1PR5 signalling on immune-cell recruitment to the uterus, and whether this promotes blastocyst implantation.

6.5 Conclusions and future directions

Use of ART is rapidly increasing, with the number of initiated ART cycles in Australia increasing from 74,357 in 2016 to 102,157 in 2021, an increase of 37% (Fitzgerald et al., 2018; Newman et al., 2023). However, live births per initiated cycle have remained stagnant around 18% for the past 20 years (Waters et al., 2006). This may be due, at least in part, to the suboptimal environment embryos are cultured in *in vitro*. In order to improve success rates of ART, a better understanding of the molecular mechanisms underpinning embryo development is required in order to improve the *in vitro* culture environment.

This thesis examined S1P signalling in preimplantation embryos. S1P is a growth factor that was previously shown to increase developmental rate when added to culture medium, but the mechanisms behind this were unclear (Span, 2015). In the present study, kinases and receptors in the S1P signalling network were identified throughout preimplantation embryo development. While S1P likely increases embryo cleavage rate via S1PR1, the presence of embryo-derived S1P signalling via other S1PRs in the embryo was also established.

S1PR1-5 and pSphK1 and 2 were present in the oocyte, though their function there was not directly investigated in this study. Exogenous S1P has beneficial effects on oocyte maturation and fertilisation *in vitro* (Jee et al., 2011; Lee & Koo, 2012; Yuan et al., 2022), likely by activation of S1PRs. Small molecule inhibitors of S1P signalling could be used during *in vitro* fertilisation to investigate the downstream signalling pathways involved in exogenous S1P signalling, as well as potentially identify the presence of oocyte-derived S1P signalling via S1PRs, as demonstrated in the embryo.

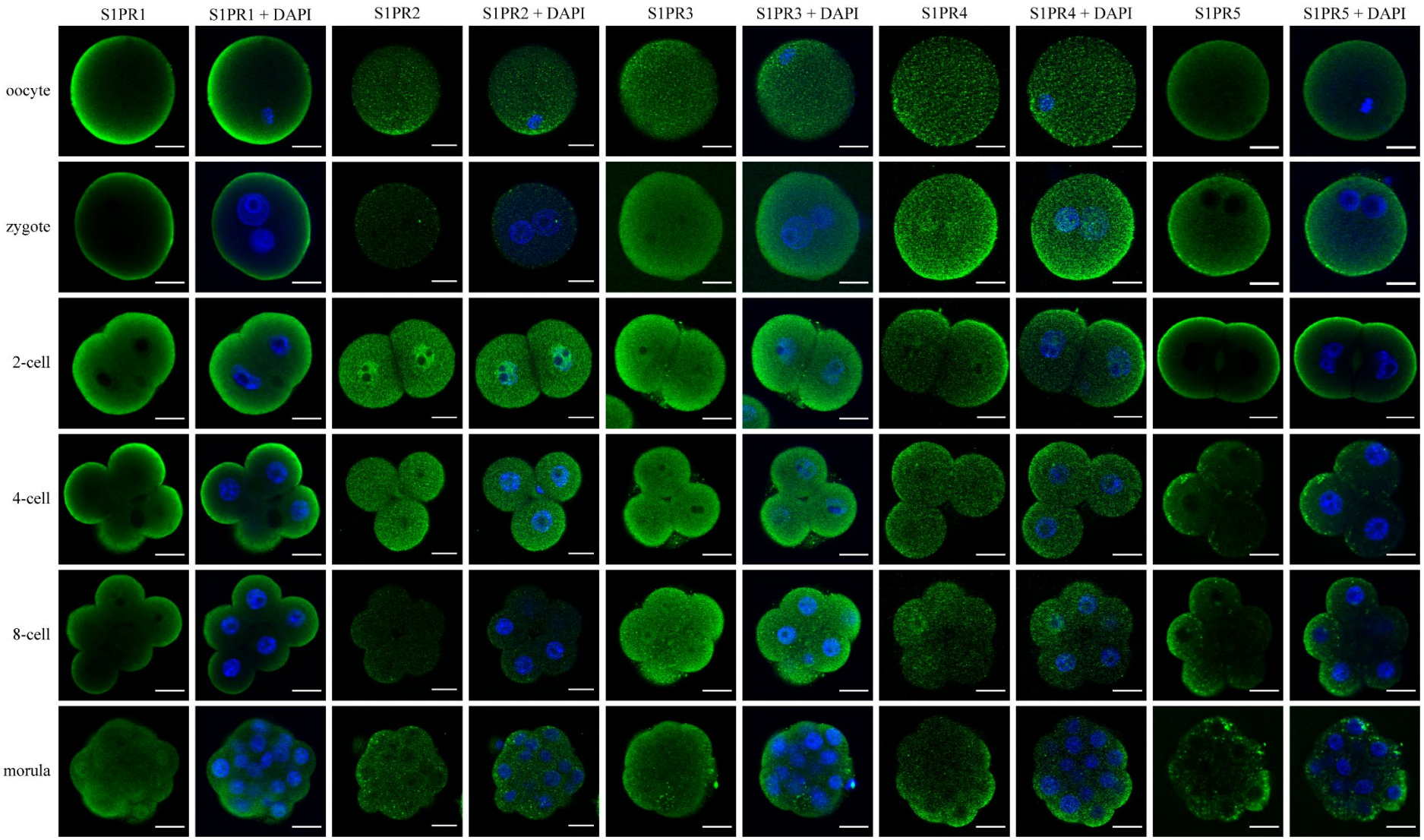
Constituents of S1P signalling pathways were also identified in the embryo, with S1P signalling acting at two main stages of preimplantation development. Firstly, both pSphK2 and S1PR3 are required for the early cleavage stages, and potentially play a role in initiating the EGA. This could occur by activation of YAP signalling, which has been linked to initiation of the EGA in mouse embryos (Yu et al., 2016), or pSphK2/S1PR3 signalling could activate another unidentified signalling pathway in the early embryo. Secondly, S1PR1, S1PR2, S1PR4, and both SphKs play a role in cavitation and blastocyst formation, likely through the establishment and maintenance of tight junctions, as seen in endothelial

cells of the blood brain barrier (Hansen et al., 2022; Reinhard et al., 2017). Data suggest that pSphK2 is the dominant SphK in the embryo, rather than pSphK1 as in other cells.

In the blastocyst, all five S1PRs were present and most had distinctive localisation patterns, suggesting the involvement of S1P/S1PR signalling in multiple processes in the blastocyst. Therefore, the role of S1P signalling in blastocyst hatching and implantation, and in subsequent foetal development should be investigated. Implantation studies should be performed where embryos are cultured in the presence of S1P pathway inhibitors before transfer to receptive uterine epithelial cells or transfer into surrogate mice.

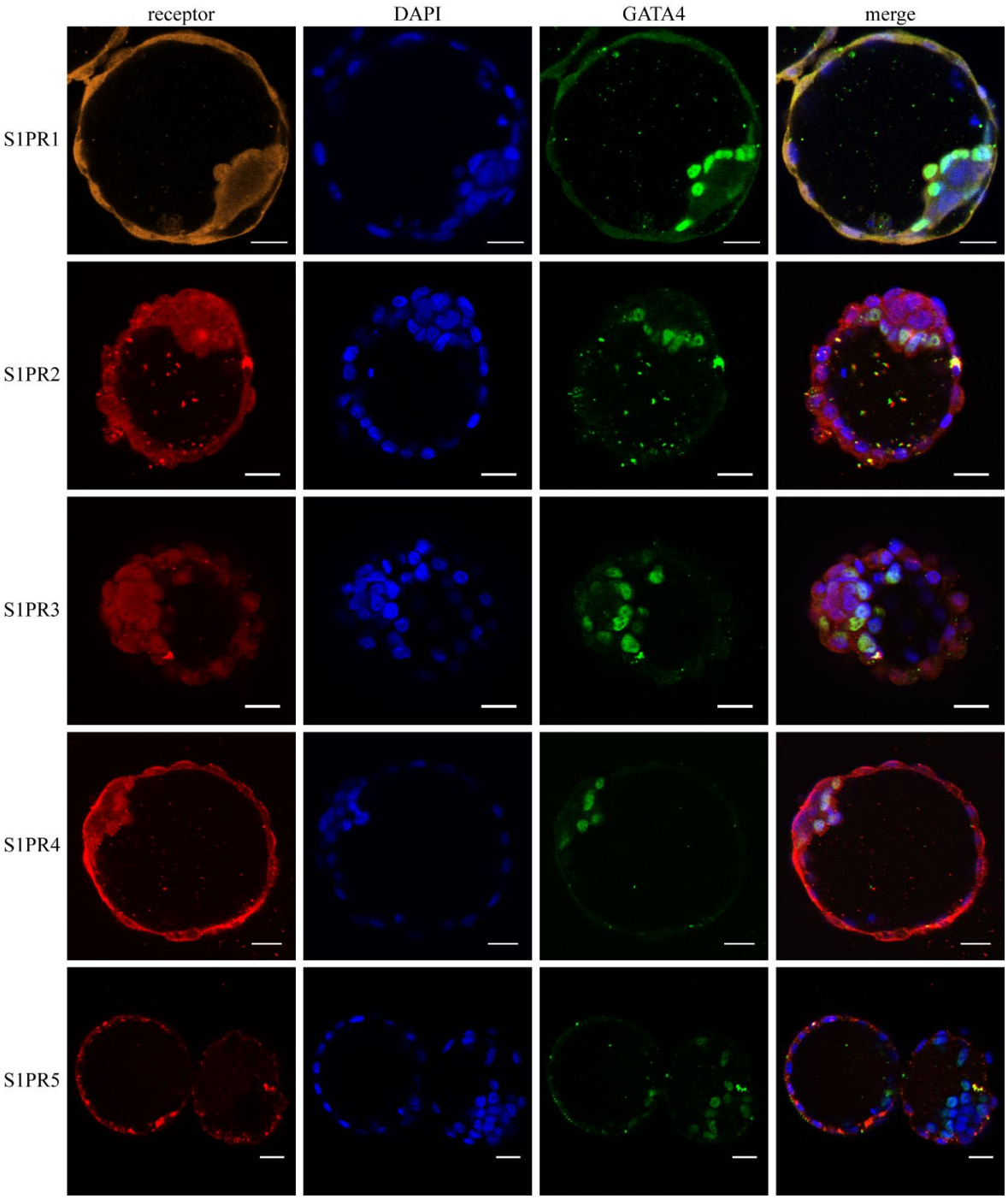
Overall, this thesis provides the foundations for investigating S1P signalling in the preimplantation embryo by identifying SphK1 and 2, and S1PR1-5 in the embryo and establishing the requirement of embryo-derived S1P signalling via S1PRs for preimplantation development. Understanding S1P signalling in the embryo provides a better understanding of the molecular mechanisms that underpin normal embryo development. This information could be used to improve the *in vitro* culture environment and allow for the production of more viable embryos for clinical, research, and agricultural purposes.

Appendix

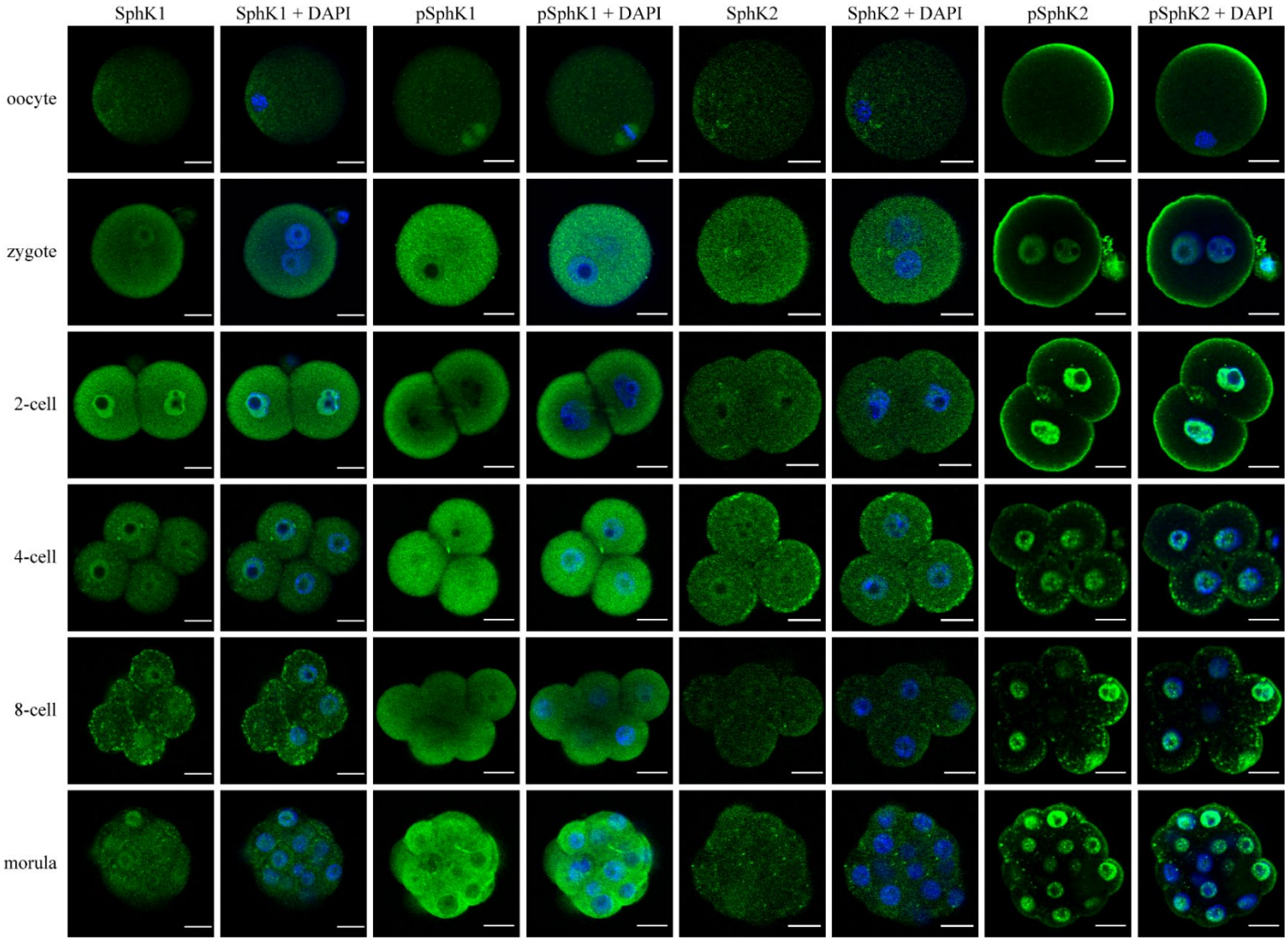


Appendix 1. Localisation of S1PR1-5 in mouse oocytes and preimplantation embryos.

Appendix 1. Localisation of S1PR1-5 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured in vitro at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote), 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for the S1PR1-5 (green). Nuclei were stained with DAPI (blue). Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.

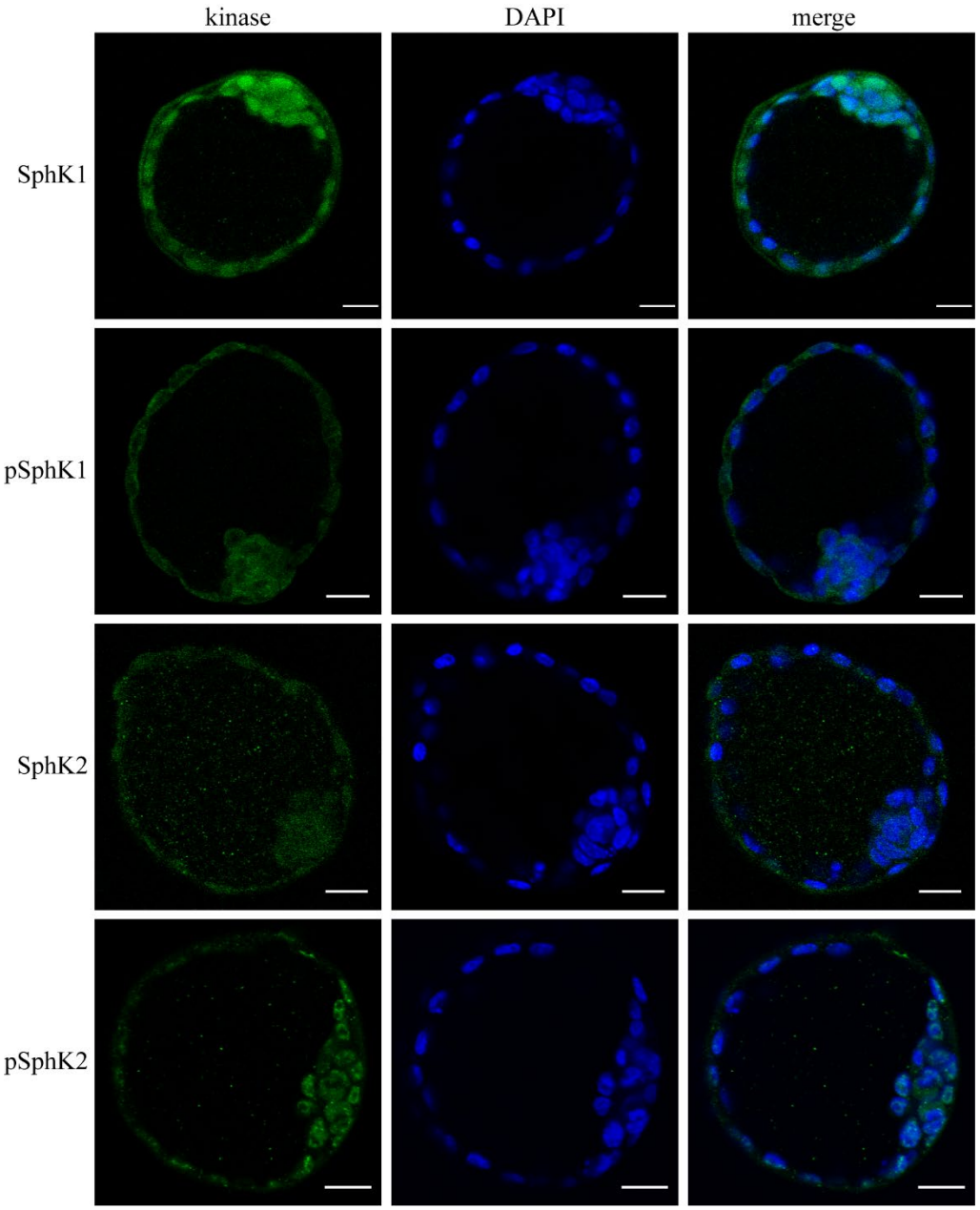


Appendix 2. **Localisation of S1PR1-5 in mouse blastocysts.** Zygotes were isolated and cultured individually at a density of 20 embryos/20 µL in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde and immunostained to visualise S1PR1-5 (orange/red) and GATA4 (green) as a marker of hypoblast cells. Nuclei were stained with DAPI (blue). Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.



Appendix 3. Localisation of SphK1 and SphK2 in mouse oocytes and preimplantation embryos.

Appendix 3. Localisation of SphK1 and SphK2 in mouse oocytes and preimplantation embryos. Oocytes were freshly isolated and collected 13 h post-hCG, while zygotes were isolated 24 h post-hCG and cultured in vitro at HD (20 embryos/20 µL) in ModHTF medium for 1 h (zygote), 24 h (2-cell), 48 h (4-cell), 60 h (8-cell), or 72 h (morula). Oocytes and embryos were fixed and immunostained for SphK1, pSphK1, SphK2, or pSphK2 (green). Nuclei were stained with DAPI (blue). Oocytes and embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 oocytes or embryos per stage per experiment. Scale bar = 20 µm.



Appendix 4. **Localisation of S1PR1-5 in mouse blastocysts.** Zygotes were isolated and cultured individually at a density of 20 embryos/20 µL in ModHTF medium to the hatched blastocyst stage (144 h post hCG) then fixed in paraformaldehyde and immunostained to visualise SphK1, pSphK1, SphK2, or pSphK2 (green). Nuclei were stained with DAPI (blue). Embryos were imaged by confocal microscopy using a 20x objective. Results were obtained from 3 independent experiments with at least 5 embryos per experiment. Scale bar = 20 µm.

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