# Investigating the in vitro effect of S1P on trophoblast migration and establishing a reliable method for examining the in vivo effect of S1P

Vitamin D and S1P as essential mediators of human placental development

**Word Count: 5931**

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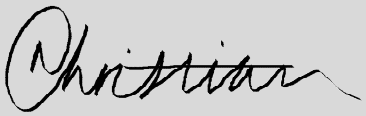
Declaration: 

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**ABBREVIATIONS**

| | |
|---|---|
| CK7 | cytokeratin 7 |
| CT | control |
| CTB | cytotrophoblast |
| DAB | 3,3'diaminobenzidine |
| DMEM F:12 | 1:1 mixture of Dulbecco's Modified Eagle's medium and Ham's F-12 Nutrient Mixture |
| dNK | decidual natural killer cells |
| EVT | extravillous trophoblast |
| FBS | fetal bovine serum |
| FTY720 | S1PR agonist |
| H&E | haematoxylin and eosin |
| HLAG | human leukocyte antigen G |
| HRP | horseradish peroidase |
| IL | interleukin |
| IUGR | intrauterine growth restriction |
| JTE-013 | [selective sphingosine-1-phosphate receptor 2 antagonist] |
| MMP | matrix metalloproteinase |
| NBF | neutral buffered formalin |
| S1P | sphingosine-1-phosphate |
| S1PR | sphingosine-1-phosphate receptor |
| SCID | severe combined immunodeficiency |
| STB | syncytiotrophoblast |
| TBS | tris buffered solution |
| VPC23019 | [specific sphingosine-1-phosphate receptor 1 and 3 antagonist] |

## ABSTRACT

The in vitro effect of the bioactive lipid sphingosine-1-phosphate (S1P) on the development of human placental tissue was studied by examining how the antagonism of its receptors changes the migratory response of trophoblasts to S1P. Explants were selected from first trimester placental tissue (<9 weeks) and incubated in DMEM F:12 culture media with 1% fetal bovine serum (FBS). At 24 hours, explants received treatments of S1P, VPC (S1PR1 and S1PR3 antagonist) and S1P or JTE (selective S1PR2 antagonist) and S1P with controls receiving 1% FBS culture media only. Photos of the explants were taken at 24, 48, 72 and 96 hours. The area covered by migrating trophoblasts under the influence of these treatments was measured and compared at each time point. Results suggested that antagonism of S1PR2 abolished the inhibitory effect of S1P but antagonism of S1PR1/3 was inconclusive due to the small number of samples making the result insignificant.

The second part of this project was focused on finding a reliable method for studying the effect of S1P on trophoblast migration in vivo. Immunohistochemistry was used to assess whether insertion of human placental tissue under the renal capsules of SCID mice led to trophoblast invasion of the renal parenchyma. The results of staining the kidneys with CK7 were inconclusive as there was no significant positive staining despite promising results in preliminary haematoxylin and eosin (H&E) staining. Nevertheless this project has provided a basis for further testing of alternative methods.


## INTRODUCTION

### Structure and early development of the placenta

The placenta is a semi-permeable, highly vascular organ that facilitates communication between mother and fetus.[1] It was once thought to be a passive barrier but studies have demonstrated that the placenta is a complex organ that adapts to meet the needs of the developing fetus by modifying the input of the maternal environment. [2-6] Among its many functions are gas exchange, nutrient and electrolyte exchange, and delivery of maternal antibodies as well as production of hormones to stimulate maturation of the uterine endothelium and development of the mammary glands.[7]

The full-term placenta is a discoid structure that covers 15% to 30% of the internal surface of the uterus termed decidua.[7] Figure 1 shows the gross structure of the placenta. The fetal aspect is formed by the chorionic plate, and the chorionic vessels that branch from the umbilical vessels are visible through this thin membrane. Septa from the uterine endothelium divide the maternal aspect into 10 - 40 slightly bulging units called cotyledons, as shown in Figure 1.[8] The umbilical arteries receive deoxygenated fetal blood and branch until they enter a cotyledon.

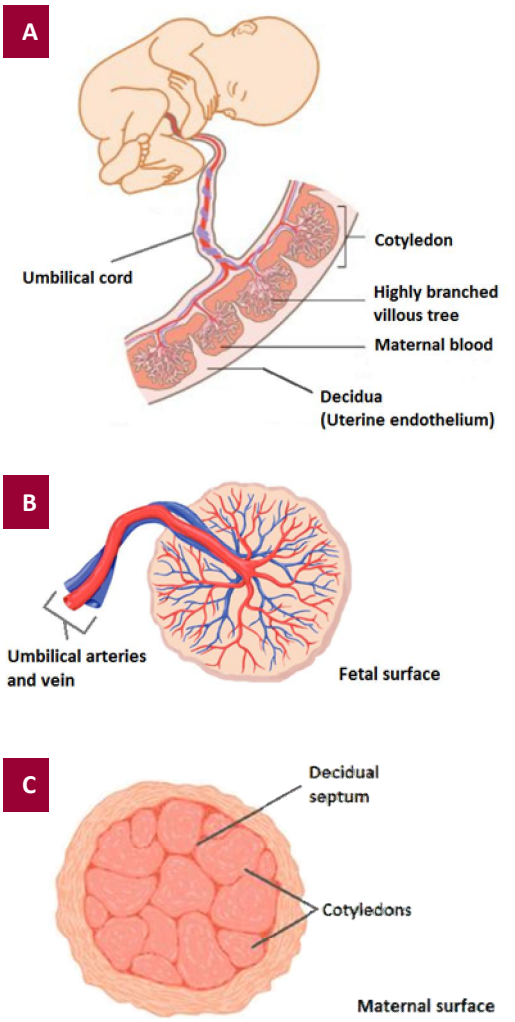


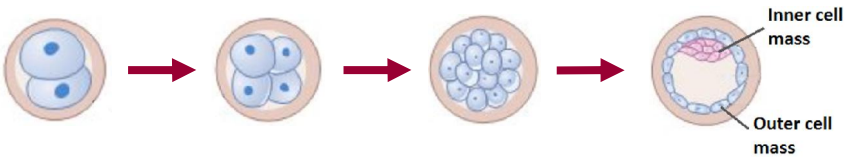

**Figure 1**
**Two umbilical arteries (blue) carrying deoxygenated blood branch into chorionic arteries visible. Chorionic veins converge into the umbilical vein (red) that returns nutrient-rich blood to the fetus. (A) Gross structure of the placenta; (B) Fetal surface of the placenta; (C) Maternal surface of the placenta [Adapted from (1)]**

Within each cotyledon, spiral arteries from the uterine endometrium supply maternal blood that bathes the capillaries of the villous tree. The thin placental membrane allows a high rate of diffusion so that the blood that returns to the fetus is rich in oxygen, glucose, amino acids and other substances. A mature structure is already formed by the end of the first trimester hence the first weeks of development are crucial for appropriate fetal development.[1]

Following fertilisation, the newly formed zygote undergoes a series of cleavage divisions in which these cells, now called blastomeres, halve in size after each mitotic division as shown in Figure 2.[1, 7] The cells form tight junctions with each other in the process of compaction.[7] They are held securely together in this 16-cell morula with cells on the inside surrounded by a layer of cells on the outside.

The inner cell mass (embryoblast) will develop into the fetus whereas the outer cell mass becomes the trophoblast that will form the placenta.[7]

**Figure 2**
**Formation of the morula by approximately day 4 after fertilisation [Adapted from (1)]**




The trophoblast layer is initially made up of undifferentiated cytotrophoblasts (CTBs) that follow one of two pathways.[9] Some migrate to the embryonic pole of blastocyst, lose their membranes and fuse to form a multinucleated mass of syncytiotrophoblasts (STBs). STBs form a syncytial membrane across which nutrient and gas exchange take place; they are responsible for the endocrine function of the placenta.[7, 9] Other CTBs proliferate and form columns of villous CTBs that penetrate the syncytium as primary villi around day 13 after fertilisation.[7] Some cells of the column detach and migrate into the decidua; these are extravillous trophoblasts (EVTs).

EVTs assume an invasive phenotype. Some become interstitial EVTs that invade the decidua to secure the attachment of the placenta to the uterus.[9] Interstitial EVTs remodel spiral arteries by migrating to them through the decidua whereas endovascular EVTs, a second subset of EVTs, enter the lumina of these arteries where they erode and replace the maternal vascular endothelium. This converts the spiral arteries into high-flow, low-resistance channels. These channels allow increased perfusion of the placenta first with maternal plasma then with maternal blood, consequently tripling the oxygen concentration of the placental bed.[8, 10] There is also evidence for a third group of EVTs called endoglandular EVTs.[11, 12] These are thought to replace the epithelium of the uterine glands in a mechanism comparable to the remodelling of spiral arteries by endovascular EVTs.[11]

Appropriate EVT function is crucial for development of a placenta capable of supporting a healthy growing fetus. Successful invasion of the decidua requires expression of proteinases capable of digesting the extracellular matrix of the endometrium. This includes expression of matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9 that can degrade collagen IV in the basement membrane.[13, 14] There are also a range of adhesion molecules such as cadherins that facilitate anchoring of the cell columns and adhesion between EVTs and decidual endothelial cells.[15, 16] Pro-invasive factors are released from the decidua including IL-1β from maternal macrophages and IL-8 from decidual natural killer cells.[17] These molecules stimulate EVT infiltration and their effect is counteracted by molecules such as IL-10 and IL-12 that suppress MMP expression and cause upregulation of MMP inhibitors.[17]

Regulation of EVT function is complex and abnormalities in any one of the molecular pathways involved leads to a defective placenta. Inadequate invasion of trophoblasts, especially endovascular EVTs, results in failed spiral artery remodelling as up to two-thirds of myometrial vessels remain untransformed. This leads to shallow placentation and a subsequent lack of placental perfusion. These characteristics are observed in pre-eclampsia (PE), a disease affecting 2 – 8% of pregnancies in the UK.[18] In pre-eclampsia, the spiral arteries show greater resistance to blood flow than in normal placentas. This is thought to cause oxidative stress and the release of cytokines and other damaging factors into the maternal circulation.[8] There is an ensuing inflammatory response in the mother characterised by new-onset hypertension, proteinuria and maternal oedema.

Poor placental perfusion also features in intrauterine growth restriction (IUGR). This is a complication in which the fetus' estimated weight is below the 10$^{th}$ percentile for gestational age and there is evidence that this has lifelong consequences for the fetus.[19] It is hoped that deciphering the role of molecules involved in regulation of trophoblast migration could help to improve understanding of the pathogenesis of these diseases.

**Sphingosine-1-phosphate**

Sphingolipids are a diverse class of bioactive lipids that have sphingosine, an eighteen carbon amino alcohol, as their backbone. They were initially discovered in brain extracts but are now known to be found in a range of living tissues where they are an important constituent of plasma membranes.[20] Due to their diversity, they can influence many signalling cascades by regulation of membrane-bound receptors.[21]

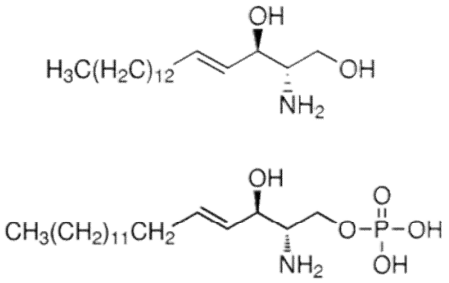



**Figure 3**
**Diagram to show the structures of sphingosine (above) and sphingosine-1-phosphate (below)**

Their catabolism eventually produces sphingosine-1-phosphate (S1P) through phosphorylation of sphingosine by sphingosine kinases 1 and 2 (SK1 and SK2 respectively).[22] S1P binds five G-protein-coupled receptors, termed S1PR1 – 5, each of which regulate intracellular signalling pathways in different ways. The associated G proteins and the distribution of these receptors are displayed in Table 1.[22] The effect of S1P on a cell is dependent on which of these receptors is expressed on the cell surface membrane. Through these receptors, S1P is able to regulate many fundamental cell activities including angiogenesis, proliferation, adhesion and migration.[22]

S1P is known to regulate cell migration in a variety of tissues both in health and in disease.[22-25] As trophoblast migration is central to placental development, it is not surprising that the role of S1P in early pregnancy has become a topic of great interest over the past decade.[26-28]

| Receptor | G protein | Expression |
|---|---|---|
| S1PR1 | Gi | Widespread |
| S1PR2 | Gi,Gq, $G_{12/13}$ | Widespread |
| S1PR3 | Gi, $Gq_q$, $G_{12/13}$ | Widespread |
| S1PR4 | Gi, $G_{12/13}$ | Lungs, lymphoid tissues |
| S1PR5 | Gi, $G_{12/13}$ | Brain, spleen |

**Table 1**
**Functions of S1PR1-5**

There is evidence to suggest that S1P can modulate trophoblast migration indirectly through activation of S1PR5 on decidual natural killer cells (dNK).[27] Treatment of dNK cells with FTY720, a molecule that causes down-regulation of S1PR expression, led to a reduction in S1PR5 expression and an associated impairment in EVT migration.[27] Studies have also shown that S1P modulates EVTs directly.[28] EVTs have been shown to express S1PR1 – 5.[28] S1P is known to inhibit trophoblast migration through S1PR2, which is consistent with models of S1P migration in other tissues.[22, 28] S1P has also been shown to promote EVT migration through S1PR1/3, another finding that correlates to S1P control of migration in other cells.[22, 26] The implications of these conclusions for treatment of diseases due to failure of EVT migration are exciting; however the findings are supported by a small number of studies. Additionally, there is now a new selective S1PR1/3 antagonist, VPC23019, that has not previously been used in the study of S1P in trophoblast migration. This project hopes to confirm the in vitro effect of S1P antagonists, including the new S1PR1/3 antagonist VPC23019, on S1P-mediated control of EVT migration.

It has been found that vitamin D attenuates the in vitro inhibition of EVT migration by S1P action on S1PR2, and in vitro EVT invasion is increased by treatment with vitamin D. These findings strengthen the prospects of modulating S1P-mediated regulation of EVT invasion in PE and IUGR. In order to understand the clinical possibilities, these findings must be studied in vivo. This project proposes to test a new model for investigation of trophoblast invasion in vivo with the aim of using this model to examine the validity of the conclusions discussed in vivo.

**Hypotheses**

(a) **Antagonism of S1PR1/3 has no effect on the inhibitory effect of S1P on trophoblast migation**

(b) **Antagonism of S1PR2 abolishes the inhibitory effect of S1P on trophoblast migration**

(c) **Insertion of trophoblast under the renal capsule of SCID mice leads to trophoblast invasion of the kidney parenchyma**

1. Investigate the in vitro effect of S1P receptor antagonists on the ability of S1P to inhibit trophoblast migration
2. Use histological methods to examine SCID mouse kidneys for trophoblast invasion of the renal parenchyma

Objectives

1. Continue to investigate the in vitro effect of S1P on trophoblast migration
   (i) Dissect explants from the first trimester placentas of informed and consented patients undergoing elective medical or surgical terminations of pregnancy at St. Mary's Hospital
   (ii) Culture the explants on plates of collagen gel for 24 hours. At 24 hours, photograph the explants under a microscope and assign to one of the following treatment groups:
   (iii) Culture the explants for a further 72 hours. At 48, 72 and 96 hours, photograph the explants
   (iv) Quantify the outgrowth of the explants at each time point and compare the percentage area of outgrowth for each treatment group

2. Use histological methods to examine SCID mouse kidneys for trophoblast invasion of renal parenchyma
   (i) Section harvested SCID mouse kidneys embedded in paraffin wax
   (ii) Stain sections of each kidney with haematoxylin and eosin (H&E) to identify which part of the kidney may have trophoblast
   (iii) Compare the reliability of cytokeratin 7 (CK7) and human leukocyte antigen G (HLAG) in identifying trophoblast infiltration
   (iv) Once the trophoblast has been located, stain sections close by with either CK7 or HLAG to confirm human origin

## METHODS

Placental was collected from women undergoing elective medical or surgical termination at St. Mary's Hospital, Manchester. These patients were recruited with written informed consent by research nurses. Samples were from 5 – 9 weeks gestation. Placentas were washed in sterile PBS and explants were dissected either for culture or transplantation as discussed below.

### Explant Culture

Materials:

- First trimester placental tissue
- Serum free culture medium – 1:1 mixture of Dulbecco's Modified Eagle's medium and Ham's F-12 Nutrient Mixture (DMEM F-12) with glutamine, a mixture of antibiotics (penicillin and streptomycin) and an antifungal agent (amphotericin B)
- Control (CT) culture medium (DMEM F:12 as above containing 1% fetal bovine serum) – the concentration of FBS was reduced from the standard cell culture medium concentration of 10% FBS so the S1P present in the serum would not interfere with the experiment
- Rat tail collagen type I – to provide extracellular matrix for outgrowth
- 7.5% $NaHCO_3$ solution – allows gel to set and neutralises the collagen
- 24-well flat-bottomed sterile cell culture plate

Collagen gels were made up using serum free DME F:12, type I rat tail collagen and $NaHCO_3$. For one 24-well plate, 1ml of collagen was mixed with 50µl of serum free DMEM F:12. 100µl of $NaHCO_3$ was mixed in well and around 60µl of gel was deposited into each well of the plate. The plate was transferred to a humidified 5% $CO_2$ incubator at 37°C to set for at least half an hour.

Before being dissected, placental tissue was washed in sterile PBS to remove the blood. Then in a sterile Petri dish, the tissue was examined under a microscope and explants were selected using

forceps and a small pair of scissors. Favourable explants had two to four terminal villi branches for optimal attachment to the collagen, and extravillous trophoblast cell columns which are visible as opaque areas at the villous tips (Figure 5). The explants were transferred to the collagen gels, given 300µl of CT culture medium and left for 3 to 4 hours to adhere to the collagen. Then a further 700µl of 1% serum medium was added and the explants were allowed to grow overnight.

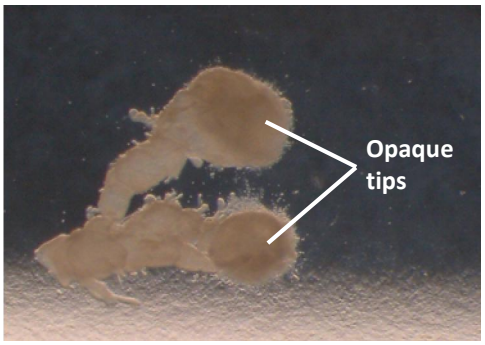



**Figure 4**
**Opaque tips were found to be more likely to contain extravillous trophoblasts.**

24 hours later, images were taken of each explant under a microscope at x20.0 magnification. Each explant was judged qualitatively on how likely it was to form measurable outgrowth. Some explants were excluded at this point, usually because they have detached from the collagen gel or in one case because a fungal infection developed. Then explants were randomly allocated to each treatment group such that each group had an equal number of promising and questionable explants. Not every explant formed outgrowth so this qualitative assessment was done to reduce the possibility that differences between treatment groups were due to some groups having explants more likely to grow. Treatments were added and the explants were incubated at 37°C. The S1P treatment had a concentration of 100nM. The concentrations of VPC and JTE used were also 100nM and explants in these treatment groups also received S1P at a concentration of 100nM.

Photos were taken at 48, 72 and 96 hours. After 96 hours, the media from each well was collected separately to be frozen at -20°C for future analysis. The explants were washed in PBS, fixed in 4% neutral buffered formalin for 15 minutes at room temperature and washed twice again in PBS. Finally, 1ml of PBS was added to each well, and the plates are covered with film and stored in a cold room according to the Human Tissue Act.

The outgrowth area of the explants was measured using Image J processing software. An outline of the outgrowth was drawn using the polygon selection tool and the area was measured in pixels as shown in Figure 5. The percentage change in the outgrowth areas at 48, 72 and 96 hours from 24 hours was calculated for each explant. Then for each treatment group, the percentage change at the different time points was averaged to give a mean.

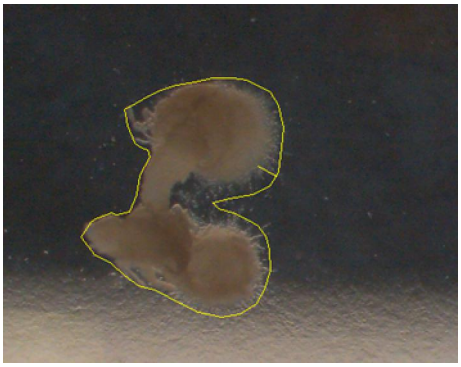


**Figure 5**
**Image shows the same explant in Figure 4 now at 48 hours with yellow outline of outgrowth area drawn with Image J.**

**Transplantation of villous tissue in SCID mice**

All animal experiments were performed under the UK Animals (Scientific Procedures) Act 1986 by two qualified, Home Office-approved scientists. Explants dissected as above were stored in CT medium overnight before the surgery the next morning. Female SCID mice at 8 – 16 weeks of age were anaesthetised with isoflurane and their left flanks were shaved and cleaned.

The left kidney of was exposed outside of the body. A small incision was made into the renal capsule and the explant material was carefully placed under the membrane. The kidney was returned to the abdominal cavity and the opening was sealed with absorbable sutures. In the initial experiments, the mice were then immediately culled and the kidneys were harvested. These mice acted as time point zero controls. In following experiments, the transplant was left to invade the kidneys for 7 days. The kidneys were bisected and fixed in 4% NBF for 24 hours before being washed in PBS and processed. Then they were embedded in paraffin wax and stored for histological analysis. The kidneys were serially sectioned into 5µM sections. H&E staining was used first at a number of sections throughout the kidney to locate the transplanted tissue. Then CK7 or HLAG staining was used to confirm the human origin of the transplant in hopes of quantifying the invasion.

**Haematoxylin and Eosin Staining**

Reagents:

- Histological clearing solution
- Alcohol solutions at 70%, 95% and 100% concentrations
- Acid / alcohol solution (1ml hydrochloric acid in 50ml of 70% ethanol solution)
- Harris' haematoxylin solution
- Water-soluble eosin solution

First the paraffin sections were warmed for 10 minutes in a 60°C oven to melt the wax. Then the sections were cleared of wax in a series of Histoclear solutions, and rehydrated in a series of alcohol solutions followed by a rinse in tap water. After being left in tap water for 5 minutes, the slides were soaked in haematoxylin for 15 minutes. Then the sections were dipped quickly in the acid/alcohol solution to differentiate the nuclei and rinsed under running tap water for 5 minutes. At this point, the staining was checked to ensure the nuclei were well stained blue. Then the slides were soaked in eosin for 10 minutes, washed in cold tap water and the staining was checked again to ensure the cytoplasm of the cells were well stained pink. Then the sections were dehydrated and mounted with a glass cover slip and clear mounting medium. Figure 6 shows different stages of this process.

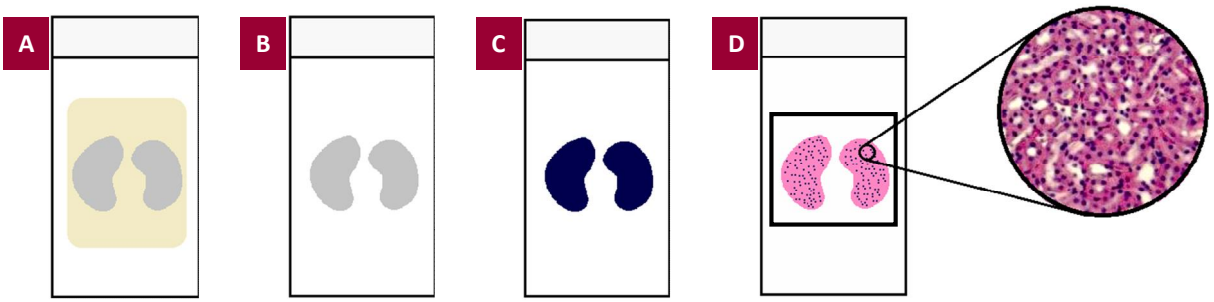



**Figure 6**
**Different stages of H&E staining process. (A) Section in paraffin wax; (B) Section with wax removed after rehydration; (C) Section stained with haematoxylin; (D) Section stained with eosin – expected final result**

**CK7 and HLAG staining**

- Histological clearing solution
- Alcohol solutions at 70%, 95% and 100% concentrations
- Acid / alcohol solution (1ml hydrochloric acid in 50ml of 70% ethanol solution)
- Sodium citrate buffer solution pH 6.0
- 0.6% Tween / TBS solution; 0.1% Tween / TBS solution; TBS solution
- 3% hydrogen peroxide solution
- Endogenous peroxidase block (Abcam ab127055 kit)
- Rodent block (Abcam ab127055 kit)
- Mouse on mouse horseradish peroxidase polymer (Abcam ab127055 kit)
- Harris' haematoxylin solution
- 3'3, diaminobenzidine (DAB) chromogen

Antibodies:

- Monoclonal mouse anti-human CK7 solution (1 in 100 dilution)
- Monoclonal mouse anti-human HLAG solution (1 in 50 dilution)

First the sections were cleared and rehydrated as explained for H&E staining. Then the slides were heated in a microwave with 400ml sodium citrate buffer pH 6.0 to expose the epitopes for binding. The sections were washed with a series of TBS/Tween solutions, then the endogenous peroxidase was blocked first using a 3% hydrogen peroxide solution then using the peroxidase block from the kit. After another series of washes, a rodent block from the kit was applied for 30 minutes, followed by another sequence of washes and the primary antibody solutions containing either CK7 (1 in 100), HLAG (1 in 50) or neither as a control were applied and left for 2 hours in the dark. After further washing, the mouse on mouse horseradish peroxidase (HRP) polymer was applied for 30 minutes. Then the sections were washed and the DAB stain was applied. Finally the sections were

counterstained with haematoxylin, dehydrated and mounted with a glass cover slip. Figure 7 shows the expected results.

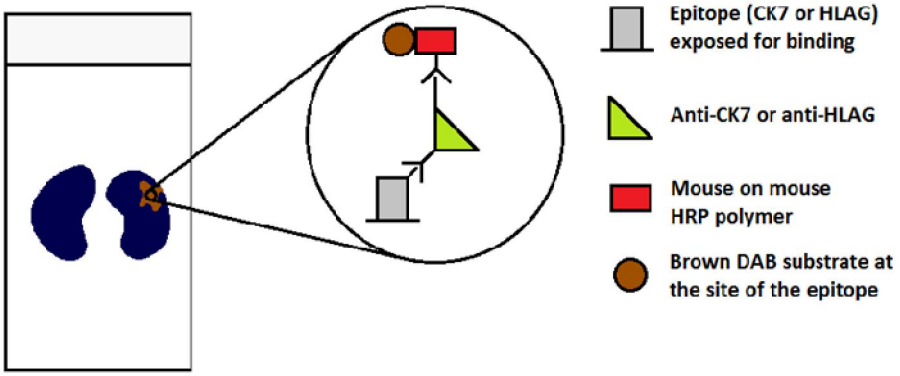



**Figure 7**
**The expected final result of the CK7 and HLAG staining was a blue kidney section with a well-defined brown area of positive antibody staining to indicate trophoblast invasion.**

## RESULTS

### The effect of JTE and VPC on S1P mediated inhibition of migration

Figure 8 shows the images of an explant that formed outgrowth over 96 hours. The yellow outlines quantify the outgrowth area. Explants that did not form outgrowth were excluded from the results. Some explants digested the collagen gels to form cavities. The outgrowth areas have been calculated to exclude cavities where they occurred.

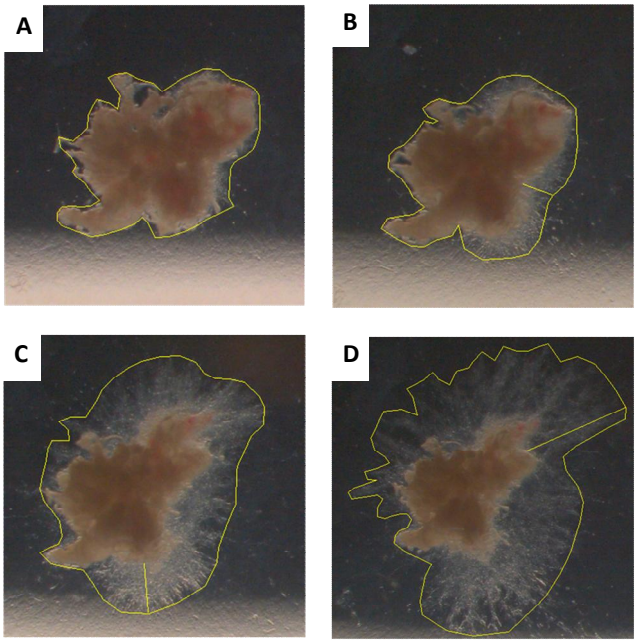



**Figure 8**
**The outgrowth areas and migration distances of each explant were measured at (A) 24 hours, (B) 48 hours, (C) 72 hours and (D) 96 hours**

Figure 9 displays the results of the experiments performed as part of this project. There was a total of N = 3 in each treatment group where N is the number of placentas from which explants were taken therefore 1 plot on the graph is the average percentage change in outgrowth area from the explants of one placenta. The graph shows that across all time points, S1P treated explants had a lower median level of migration compared to control explants. The percentage change in outgrowth of the VPC group was also lower than controls and JTE treated explants had greater increases in outgrowth compared to S1P treated explants. These results are not significant but they do support the hypotheses that antagonism of S1PR2 abolishes the inhibitory effect of S1P whereas antagonism of S1PR1/3 does not.

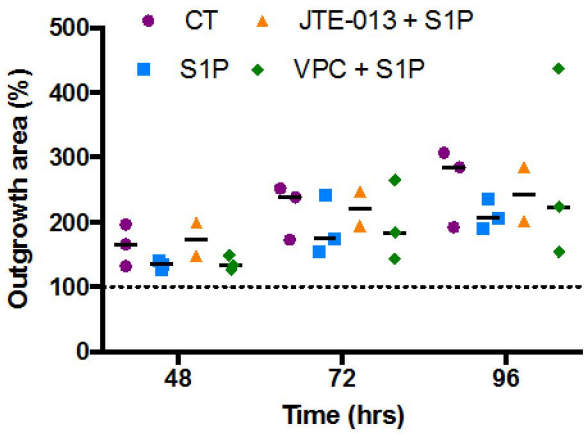



**Figure 9**
**Comparison of the mean percentage change in outgrowth area for the control (CT), S1P, JTE and VPC treatment groups. N = 3 in each group.**

Figure 10 combines the data from this project with the results of previous experiments carried out in an identical way, giving a total of N = 15 in the control and S1P treatment groups, N = 9 in the JTE treatment group and N = 3 in the VPC treatment group. The data was analysed using a two-way ANOVA statistical test with the time point and the treatment group as the independent variables, and the mean percentage change in outgrowth as the dependent variable. The only significant finding (**) is that S1P treated explants had lower levels of EVT migration than control explants, which is in accordance with findings in the literature. These results are mostly in agreement with the results of this project apart from the VPC columns. At all time points, the VPC treatment group has greater areas of outgrowth than the S1P treatment group, opposite to what was hypothesised. This result is unexpected but the VPC data from Figure 9 shows great variability with the data from one placenta being much higher than the other two. It is likely that the results from this one sample have skewed the overall distribution of the VPC group.

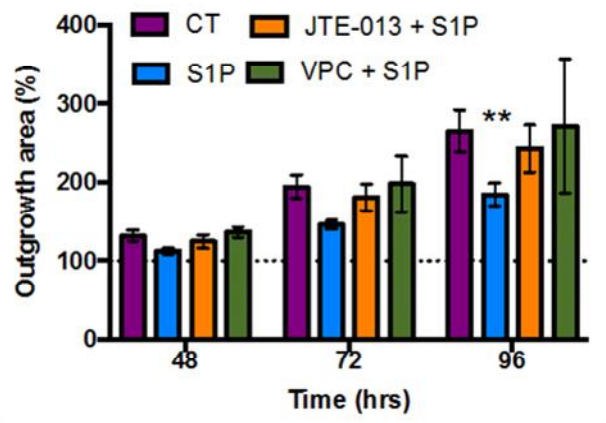



**Figure 10**
**Comparison of the mean percentage change in outgrowth area for the control (CT), S1P, JTE and VPC treatment groups. N = 3 in each group. **P=0.01**

**New trophoblast invasion model**

For the first experiment, the kidneys were harvested directly from mouse 1 after transplanting the human placental villi. Figure 11 shows the results of H&E staining to locate the transplanted tissue. The staining showed the suspected explant tissue, which was structurally different to the kidney and clearly visible underneath the renal capsule.

Sections from the same kidney were stained with CK7 and HLAG to confirm the human origin of the transplant. First trimester placenta was also stained to provide a positive control.

The CK7 and HLAG staining on the sections of placenta were positive with trophoblasts clearly stained brown. On the kidney sections, the CK7 stained positive in what appeared to be the transplanted tissue. However the transplanted tissue was not present in the HLAG stained and negative kidney sections, emphasising the fragility of the tissue.

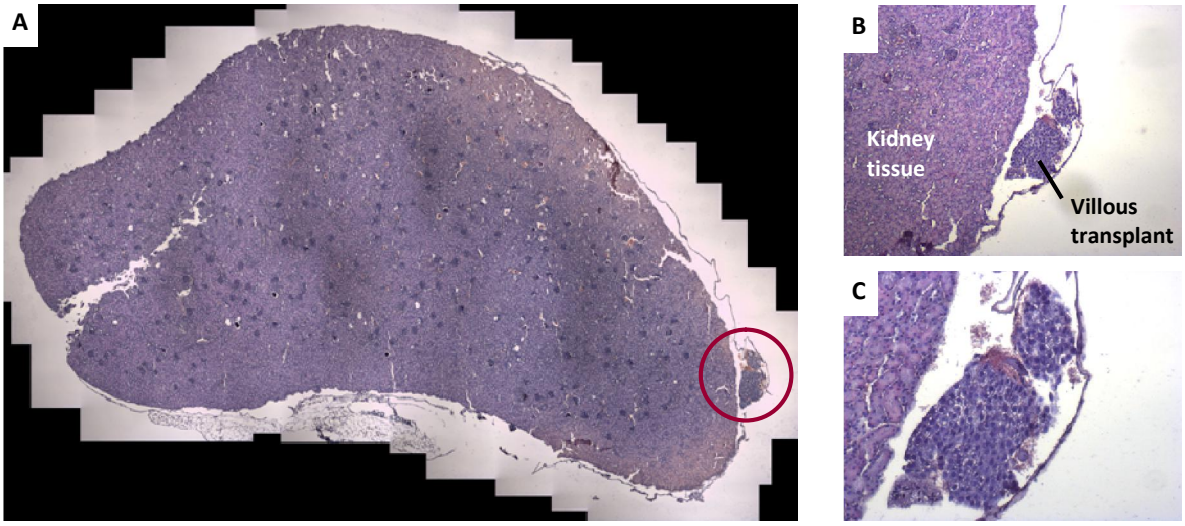



**Figure 11**
**Location of transplant under the renal capsule of mouse 1 using H&E staining. (A) Tiled image of H&E stain (B) Transplanted human villous tissue at x4 magnification; (C) Same area at x10 magnification**

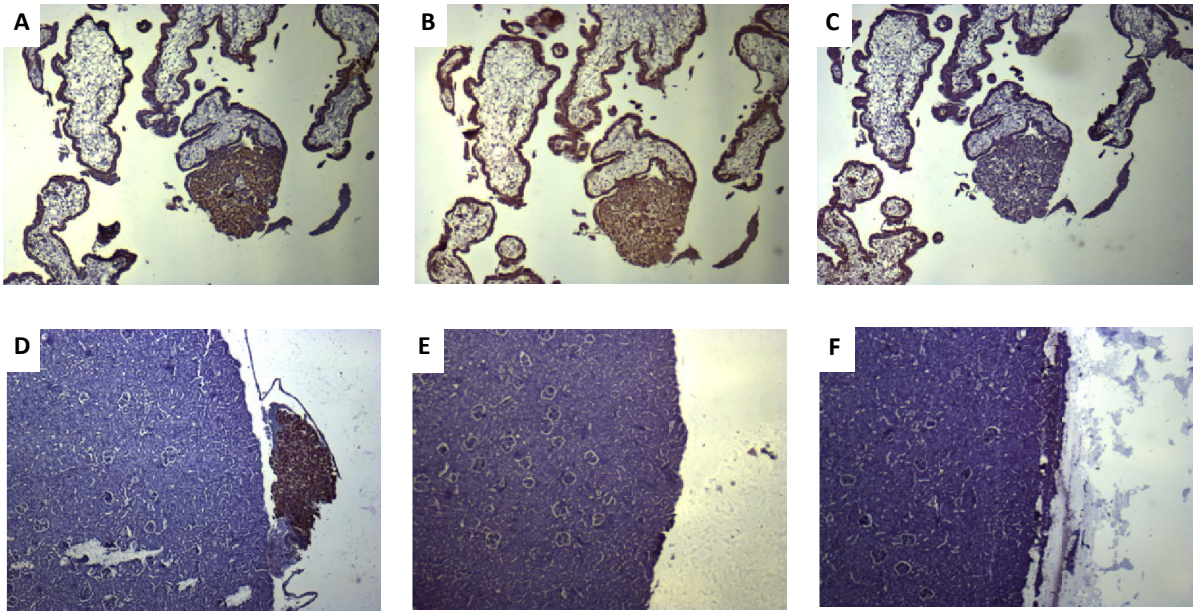



**Figure 12**
**CK7 and HLAG staining for the presence of trophoblast was positive (A) Early placenta CK7 staining x10; (B) Early placenta HLAG staining x10; (C) Early placenta negative control x10; (D) Positive CK7 staining on kidney section; (E) Missing transplant on HLAG stained kidney section; (F) Missing transplant on negative control kidney section**

This experiment was repeated and again, the kidneys were harvested straight after insertion of the first trimester tissue. Figure 8 shows the results of the H&E staining and figure 9 displays the results of the CK7 and HLAG staining on the kidney sections from these mice. In these H&E sections the transplanted tissue was clearly visible next to the kidney tissue with some renal capsule still intact. The transplanted tissue stained positive for CK7 and for HLAG. The CK7 staining was much stronger than the HLAG staining so it was decided that CK7 staining only would be used going forward.

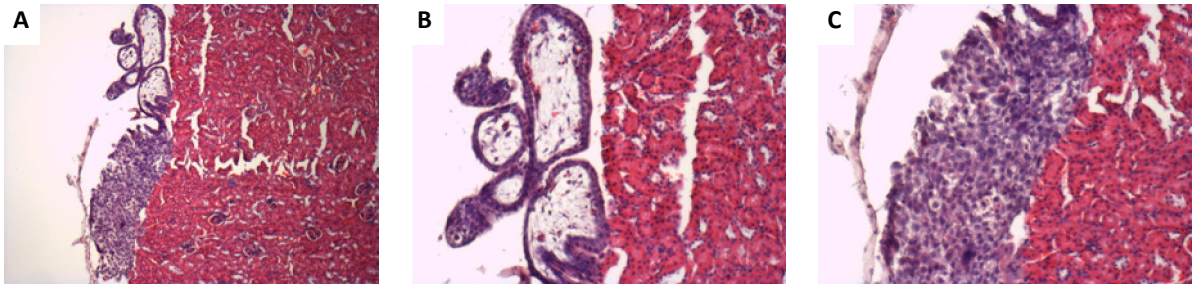



**Figure 12**
**H&E staining showing location of transplanted villous tissue. (A)Transplant at x4 magnification; (B) Top half of transplant at x10 magnification; (C) Bottom half of transplant at x10 magnification**

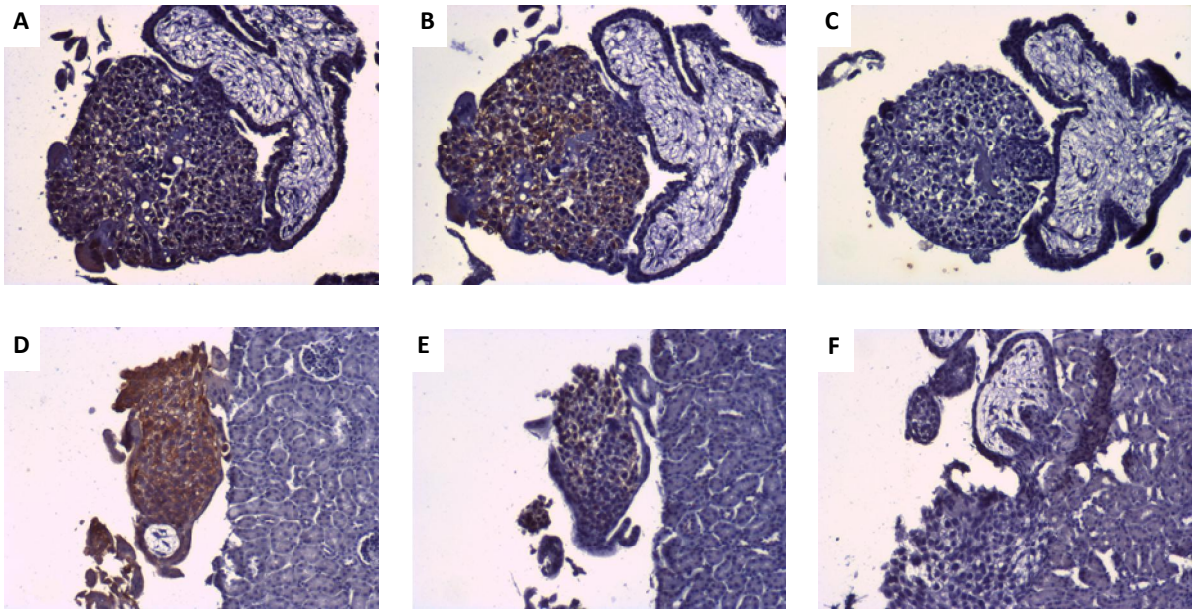



**Figure 13**
**CK7 and HLAG staining for the presence of trophoblast was positive (A) Early placenta CK7 staining x10; (B) Early placenta HLAG staining x10; (C) Early placenta negative control x10; (D) Positive CK7 staining on kidney section; (E) Positive HLAG staining on kidney sections; (F) Negative control kidney section**

In the next round of experiments, the transplanted tissue was allowed to invade in SCID mice for 7 days prior to collection for histological analysis. Figure 10 shows the results of the H&E staining.

There was tissue of a different structure and colour on the outside of the kidney that appeared to invade the kidney through the kidney's surface. In one section (Figure 10A), the suspected area of invasion appeared to almost cleave the kidney into two parts.

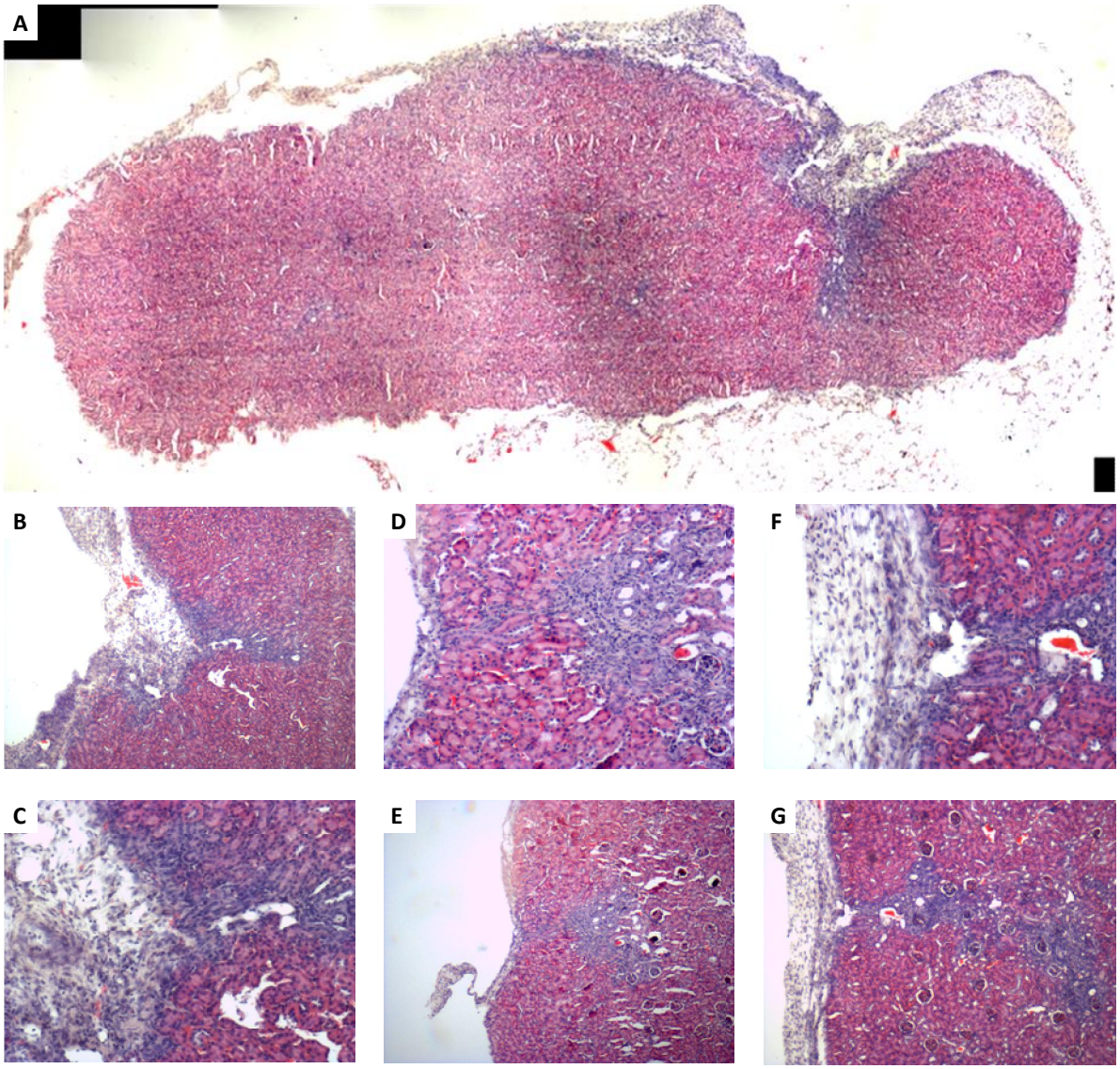



**Figure 14**
**Suspected invading tissue on was visible using H&E staining. (A) Tiled image of H&E section; (B) Invading tissue on section from at x4; (C) Same area at x10 magnification; (D) Invading tissue on a different section at x4; (E) Same area at x10; (F); (G)**

Despite the encouraging results of the H&E staining, the invading tissue could not be differentiated from the kidney tissue in the CK7 staining on these kidneys (Figure 11). Only one section in a second round of CK7 staining performed on these kidneys showed potential (Figure 12). Some cells on the

periphery of the kidney appeared to have positive CK7 staining, however there was no positive staining in the invasion.

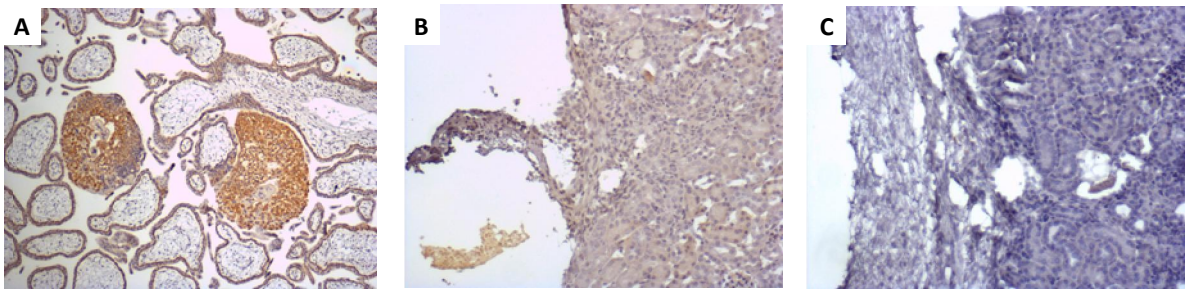



**Figure 15**
**CK7 antibody staining was negative on kidneys with regions of suspected invasion. (A) Positive CK7 staining on early placenta control x4; (B) Region of suspected invading tissue at x4; (C) Same area at x10 magnification**

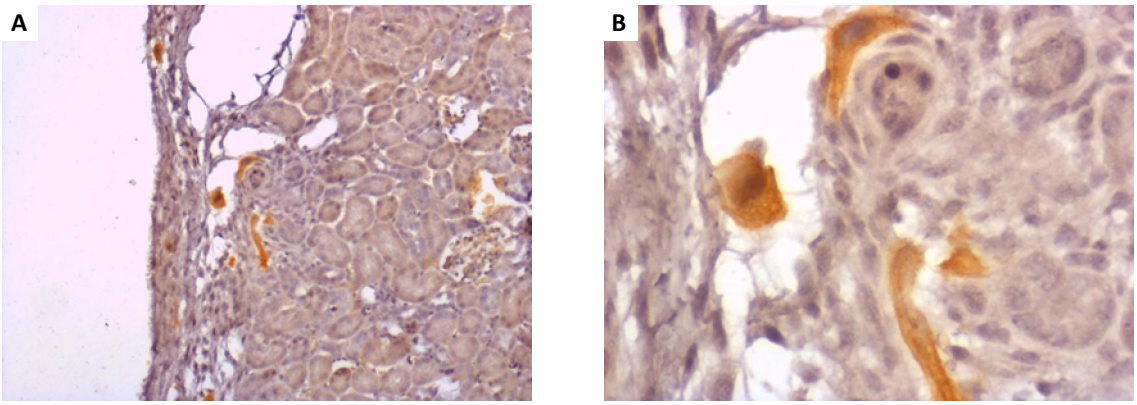



**Figure 16**
**Suspected positive CK7 staining at proposed site of invasion. (A) At x10 magnification. (B) At x40 magnification.**

## DISCUSSION

This project investigates the effect of S1P antagonists JTE and VPC on the ability of S1P to inhibit EVT migration. It reports the use of VPC, a S1PR1/3 antagonist that has never been used to investigate S1P-mediated trophoblast migration, and also reports the trial of a new in vivo model of human trophoblast invasion based on a method used by a previous study.[29]

The main overall limitation for both experiments was the low availability of first trimester tissue. It was expected that there would be a minimum of 3 first trimester placentas available each week. Over the course of 11 weeks, 13 placentas were available within the gestational range required. Additionally it was found that explants placentas from medical terminations of pregnancy were less likely to form outgrowth. Although placentas from a later gestational age did form outgrowth, it was to a lesser extent than the outgrowth from earlier placentas.

### Explant Culture

In the results from the experiments in this project, there was no significant difference in the mean percentage change in outgrowth area between the treatment groups due to the small number of samples used. S1P treated explants showed a lower level of migration compared to CT explants as expected. JTE treated explants showed greater levels of migration than S1P treated explants; however again, this result was not found to be significant. This does suggest that antagonism of S1PR2 eliminates the inhibitory effect of S1P but more samples are needed to confirm this. The VPC treated explants showed lower levels of migration on average, however there is one placenta that produces much higher values at each time point than the others. With a sample of 3 it is not possible to tell whether this is an outlying value.

In the combined results, the S1P treated explants had a significantly reduced mean percentage outgrowth area than control explants owing to inhibition of migration via S1PR1. The VPC treated

explants showed a higher level of migration on average, which was opposite to what was hypothesised. However the figure 9 shows that there was a wide variation in the VPC data with one result much higher than the others. As the result is not significant, the hypothesis that antagonism of S1PR1 and 3 is still inconclusive. However it is thought that the higher data value will be shown to be an outlier when combined with more samples.

The method of explant culture used is well-recognised and reliable with widespread use in the literature.[30] VPC has not been studied in this way before but the use of JTE to study the effects of S1P migration has been conducted where it was used to show that S1P inhibited migration through S1PR2 as suggested by the results of this project.[28]

The main limitation in this experiment was that some explants did not adhere well to the collagen gel so when media was added they partially or fully detached from the gel and were unable to form measurable outgrowth. The number of explants from a sample that would adhere to the collagen could be increased by allowing the explants a full 4 hours to adhere after adding the initial 300µl of 1% serum media. Additionally data from several explants could not be collected because their outgrowth extended beyond the edge of the collagen gel making measurements of percentage area unreliable.

**Staining**

The H&E staining from all SCID mice showed clearly the location of the trophoblast and the proposed site of invasion. There is evidence from the results of the staining to suggest that insertion of the transplanted villous tissue led to invasion of the kidney parenchyma. However the human origin of the invading transplant could not be verified as the CK7 staining was negative.

This method was based on the work of another study looking at how cytotrophoblasts stimulate arterial apoptosis and lymphangiogenesis.[29] In that work, 2mm$^2$ pieces of human placental villi were transplant beneath the kidney capsules of SCID mice in the same way as described in the methods of this project.[29] However the results of immunohistochemistry on the kidneys in this study showed invasion of the renal parenchyma.[29] There are no known reasons at present for the lack of positive CK7 staining at the regions of suspected invasion in this project but there are no grounds to doubt the methods used. The next steps will involve repeat CK7 staining and the use of other antibodies if CK7 is still negative.

One major limitation in the staining was the use of antibodies grown in mice. This led to widespread background staining which contributed to the difficulty in identifying invasion. Another limitation was the fragility of the tissue. In many of the sections, the renal capsules of the kidneys are not visible and it was likely that the capsule was damaged as part of the sectioning process. To manage this, the embedded kidneys were well-cooled before sectioning and handled carefully throughout the staining process. Even then, some sections lacked a visible renal capsule making it harder to confirm the origin of transplanted tissue.

Another limitation was the presence of some patchy staining in the results of both the H&E staining and the CK7 staining. It is likely this was because some sections dried out during the staining process. This was rectified in the rounds of staining that followed.

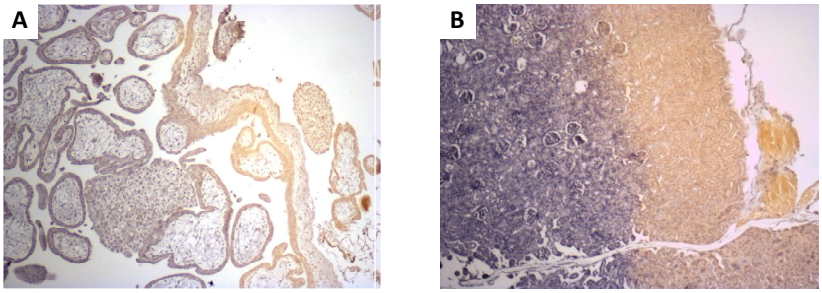



**Figure 16**
**Patchy results of CK7 staining on (A) first trimester placenta and (B) kidney tissue**

## CONCLUSION

The current understanding of the effect of S1P on early placental development continues to be updated. It is clear that S1P is a key determinant of EVT migration and it remains to be seen whether the findings of in vitro studies are replicated in vivo. If so, it could be possible to manipulate the role of S1P by use of vitamin D or by pharmacological agents to improve outcomes in diseases of pregnancy.

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