NOTTINGHAM TRENT UNIVERSITY

Differential expression of C/T antigens and 5-HT receptors in Normotensive and Pre-eclamptic placentae, and their role in trophoblast invasion.

A Thesis Submitted by

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To

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Copyright statement

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Dedicated to

My beloved Grandpa (Memey) and Grandma (Mam)….

My heroes for lifetime

My Dad (Apa) and Mom (Mommy)….

"No one can finish studying…
One life is not enough to gather all the knowledge on this earth."

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Abbreviations

5-FU	5-fluorouracil
5-HT	5-Hydroxytryptamine
ACTH	Adreno Corticotropic Hormone
AP1	Activator Protein-1
ARNT	Aryl-hydrocarbon Receptor Nuclear Translocator
BUC11	Breast-associated UniGene Cluster 11
C/T	Cancer Testis
CAGE	Cancer-Associated Gene
CAGE1	Cancer Antigen 1
CSF1	Colony Stimulating Factor1
CTAG3/CT3	Cancer/Testis Antigen 95 and Cancer/Testis antigen 3
CTB	Cytotrophoblast
DEAD	Asp-Glu-Ala-Asp Motif
DLBCL	Diffuse Large B-Cell Lymphoma
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine Monophosphate
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ERK	Extra-cellular Signal-regulated Kinases
EVT/ eCTB	Endovascular Cytotrophoblast
FATE1	Fetal and Adult Testis Expressed 1
FdUMP	FluorodeoxyUridine MonoPhosphate
FdUTP	Fluorodeoxyuridine triphosphate
FSH	Follicle-Stimulating Hormone
FT	First Trimester
FUTP	FluoroUridine TriPhosphate
GAGE	G Melanoma Antigen Family
GAGEC1/ PAGE4	Prostate Associated Antigen 4
GAGEE1/ PAGE5	Prostate Associated Antigen 5
HAGE	Helicase Antigen
HIF	Hypoxia-Inducible Factors
HRE	Hypoxia Response Elements
hTERT	human Telomerase Reverse Transcriptase
ICM	Inner Cell Mass
iCTB	interstitial Cytotrophoblast
IGF-1	Insulin-like Growth Factor-1
IHC	Immunohistochemistry
IRS	Immuno-reactivity scoring
IUGR	Intra-Uterine Growth Restriction
IUPHAR	International Union of Basic and Clinical Pharmacology
IVS	Intervillous Space
LDH	Lactate Dehydrogenase
LH	Luteinizing Hormone
MAGE1-4	Melanoma Antigen-1-4
MAGEA	Melanoma Antigen Family

MAPK	Mitogen-Activated Protein Kinase
MCSF	Macrophage-Colony Stimulating Factor
MHD	MAGE Homology Domain
MMP	Matrix Metalloproteinase
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced form of NAD
NT	Normotensive (Normal Placenta)
NY-ESO-1	New York City Esophageal Cancer 1
PASD1	Per ARNT Sim domain containing 1
PDGF	Platelet Derived Growth Factor
PE	Pre-eclampsia
PHD	Proly Hydroxylase Domain protein
pVHL	Von Hippel–Lindau Protein
ROS	Reactive Oxygen Species
SAGE	Sarcoma Antigen
SEREX	serological identification of cDNA expression libraries
SKIP	SKI-interacting Protein
STB	Syncytiotrophoblast
TGF-β	Transforming Growth Factor-beta
TIMP	Tissue Inhibitor of Metallo-Proteinases
TRAIL	TNF-Related Apoptosis-Inducing-Ligand
uPA	Urokinase type Plasminogen activator
VBM	Vascular Basement Membrane
VEGF	Vascular Endothelial Growth Factor
WB	Western blotting

Abstract

Cancer/testis (C/T) antigens are a unique class of tumour associated proteins that are prominently expressed in cancer cells of different histological origin. Placenta is one of the three non-cancerous tissues known to express these antigens. During the first trimester (FT) of pregnancy, placental invasion resembles to that in tumour, and is controlled by several vasoactive substances including 5-hydroxytryptamine (5-HT). Interestingly, in pre-eclampsia (a pregnancy related multi-system complication), the levels of 5-HT are found to be increased, whilst the overall trophoblast invasion is low, when compared to normal (NT) placenta. Previous studies have compared the status of factors that are involved in trophoblast invasion between NT and PE placenta. However, the expression patterns of all known 5-HT receptors in PE placentae have not been fully investigated, even though the 5-HT mediates its various actions via these receptors. Likewise, the status of C/T antigens which are also proposed to be involved in cancer cell proliferation, differentiation, migration and invasion in PE placentae is not known.

On account of the differences in trophoblast invasion between NT and PE placenta, it was hypothesised that the expression of C/T antigens and 5-HT receptors vary. Also, since the circulating 5-HT is increased and the invasive potentials of trophoblast cells is decreased in PE, it was hypothesised the two events may influence one another. Therefore, this case controlled study, compared the C/T antigen and 5-HT receptor expression profiles amongst 11 FT, 13 NT, and 12 PE placentae. The study also focused on the effects of 5-HT on trophoblast invasion using placental cell lines.

Significant differences in the status of the C/T antigens were found between NT and PE placentae using quantitative real-time PCR, western blotting and immunohistochemistry (IHC). Especially MAGEA1 ($p<0.0001$) and MAGEA4 ($p<0.001$) antigens are significantly up-regulated in PE placentae. Significant differences in expression were also observed between term (NT) and FT placentae. This is the first study to report the changes in both mRNA and proteins together with the cellular expression patterns of these C/T antigens in NT, PE and FT. On the 5-HT receptor side, 5-HT_{1B} and 5-HT_{2B} receptor were found to be significantly down-regulated in PE. Since very few studies looked at the effects of 5-HT in placental cells, the effects of 5-HT on migration/invasion using placental cell lines were also studied. 5-HT at 20 to 40μM, induced the migration and invasion of these cells. The expressions of the two 5-HT receptors and six C/T antigens, were also up-regulated at these concentrations. Thus, this study suggests 5-HT may be an important factor for trophoblast invasion. Hence, the shallow invasion in PE may be linked to the differential expression of 5-HT receptors and C/T antigens.

Chapter 1
Introduction

1.0 Introduction

The placenta has been a curiosity for many researchers due to its close resemblance to cancer (Ferretti *et al*, 2007; Fest *et al*, 2008; Spellman and Fottrell, 1973). The invasion patterns of tumour cells and placental cells during early pregnancy are remarkably similar. Similar angiogenic agents, growth factors and immune regulators are involved in both processes (Soundararajan and Rao, 2004). However, unlike tumour, placental invasion is tightly regulated. Thus, an understanding of the mechanisms controlling regulated placental invasion has been of interest in order to implicate the same practice in tumour cells.

1.1 Placenta

The placenta is a specialised multifunctional organ that develops during pregnancy. The word 'placenta' originates from two terms, firstly from the Latin term '*placenta*' meaning "a flat cake" and secondly from a Greek term '*plakoenta*' meaning "*flat*" (**www[1]**). Both terms refer to its round-flat shape as seen in humans. The placenta is developed from both foetal and maternal components (as depicted in Figure 1.1) which form a vital connection for the sustenance of the foetus (James *et al*, 2005). It is a villous organ that allows the exchange of nutrients and gases from maternal blood to the placental trophoblast cells, thereby enabling the growth of the foetus and playing an important role in the continuous supply of nutrients, exchange of waste materials and the development of foetal immunity (Red-Horse *et al*, 2004). It also functions as an endocrine organ which produces hormones to protect the foetus against maternal immunity. The development of the placenta and the embryo are closely linked.

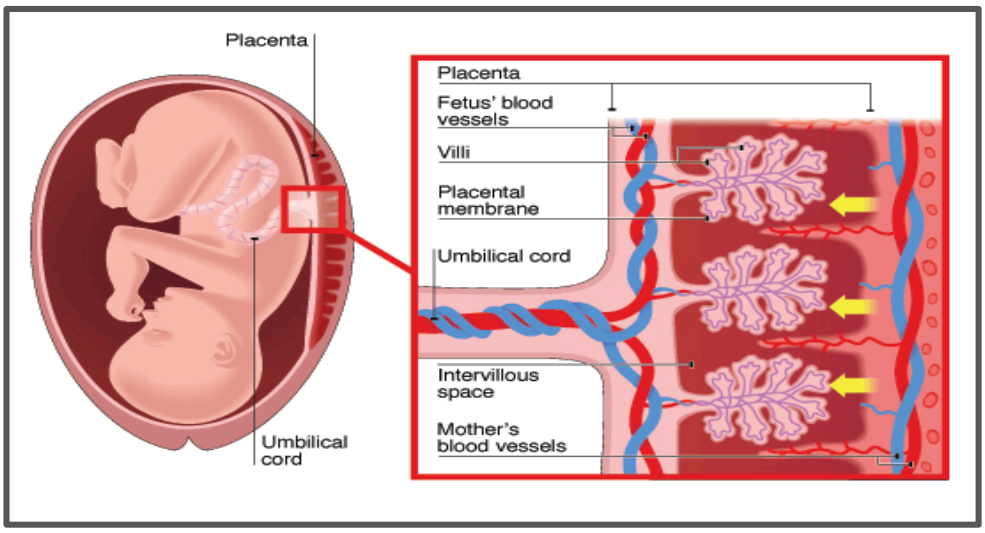


Figure 1.1: Placenta - The interface between mother and foetus.
The figure depicts the localisation of the placenta in the uterus and the cross section of placenta showing the blood ciruclation. *[Figure adapted from* **www[2]***]*

1.1.1 Formation of placenta (placentation)

Placental formation follows an intricate sequence of events initiated by the fertilisation of the oocytes. Figure 1.2 represents the stages of placental development starting from ovulation to implantation of the blastocyst.

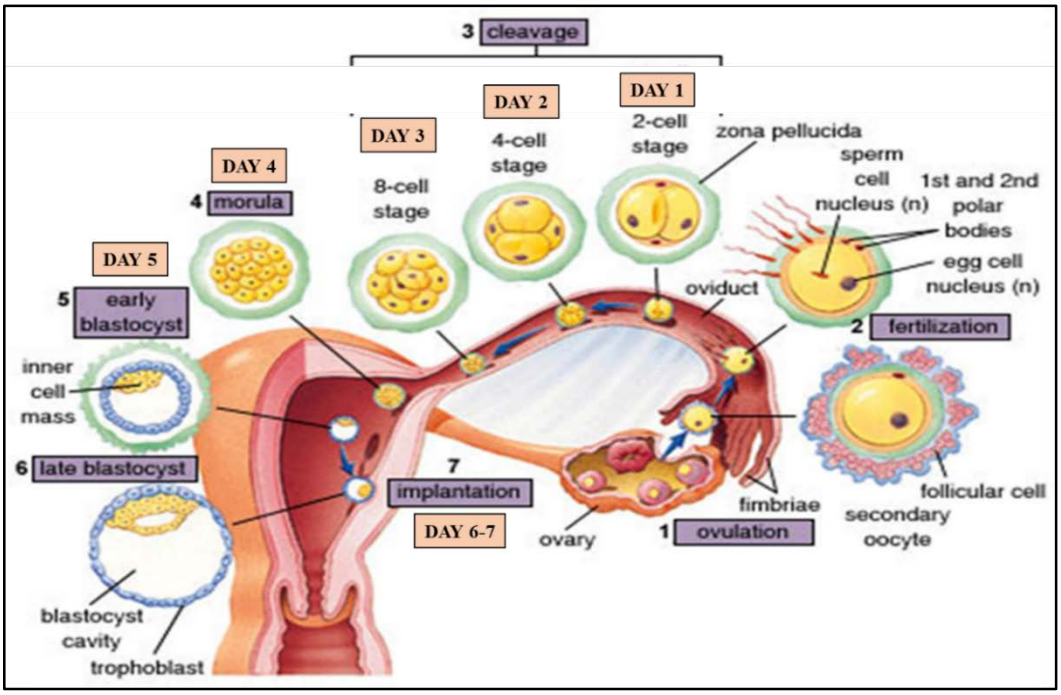


Figure 1.2: Representation of the early stages of placental development starting from ovulation to implantation.
[Figure revised from **www[3]**]

The stages seen in Figure 1.2 are described in detail below:

1.1.1.1 Ovulation to formation of blastocyst:

During ovulation, the mature ovum (haploid oocyte) is released from Graafian follicles in the ovary into the oviducts and then into the fallopian tube. This process is initiated and controlled by the secretion of endocrine hormones such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Fertilisation takes place in the fallopian tube where the mature oocytes are fertilised by a sperm cell to form a diploid zygote. This is counted as Day 0 of pregnancy or placental development where the nucleus of the ova and the sperm unite (Hamilton and Boyd, 1960). After fertilization, the zygote moves along the fallopian tube into the uterus and undergoes several stages of cleavage and differentiation. The mitotic division starts with 2-cell stage (Day 1), 4-cell stage (Day 2), 8-cell stage (Day 3), and continues until the zygote reaches the uterus. After multiple mitotic divisions, a round mass of cells (blastomere) is formed with 12-16 cells (Day 4) which is called morula (**www[4]**). The outer lining zona pellucida is rigid and does not allow dividing cells to expand. This results in a collection of cells, which look like a mulberry from where the name morula is derived.

At Day 4-5 the morula rolls from the fallopian tube to the uterine cavity, where it transforms into a blastocyst. The cells on the outer side of the morula thicken and flatten out while the cells inside become more compact and adhere to one another forming a cavity (Hamilton and Boyd, 1960). This eventually gives rise to an outer layer of trophoblast cells and an inner cell mass. The blastocyst hatches from the zona pellucida by constant contraction and expansion (Day 5-6). The free blastocyst then aligns along the uterine wall. The inner cell mass forms the foetus while the outer trophoblast layer leads to the implantation of the blastocyst into the uterus to form the placenta.

1.1.1.2 Implantation

At day 6-7, the blastocyst implants into the endometrium of the uterus. The implantation is completed by day 10. The implantation stage takes place in three steps:

- **Apposition:** the blastocyst positions itself with the inner cell mass (ICM) against the uterine wall, but the interaction is not stable. This stage is called apposition. At this stage, initial adherence is sustained by the presence of microvilli on the syncytiotrophoblast layer (a multinucleated layer formed by the fusion of cytotrophoblast cells), which comes in contact with the micro-protrusions called pinopodes on the uterine epithelium (Figure 1.3, Panel I).
- **Adhesion:** physical interaction occurs between the blastocyst and the epithelium to develop a stable adhesion.
- **Invasion:** immediately after adhesion, the syncytiotrophoblast cells release lytic enzymes and factors that erode the epithelium as well as basal lamina in order to start invading the stroma of the uterus. By day 10, the blastocyst is fully embedded in the uterus and the epithelial layer grows over the blastocyst concealing it. The trophoblast cells undergo multiple changes to facilitate invasion and placental development.

1.1.1.3 Trophoblast differentiation

As mentioned in Section 1.1.1.2, during invasion, trophoblast cells lying directly under the inner mass undergo differentiation to form a multinucleated syncytiotrophoblast which displays invasive properties (Huppertz, 2008). This syncytiotrophoblast penetrates the uterine epithelium crossing the basal lamina and embeds the embryo into the stroma (Figure 1.3 Panel II). This usually takes place within a certain time period called the “window of receptivity” (Lunghi *et al*, 2007), which is controlled by the levels of oestrogen.

The remaining mononucleated trophoblast cells are now referred to as cytotrophoblasts. They form the second layer and do not come into contact with the maternal interface.

Cytotrophoblasts have, however, a major function for placental development as they act as stem cells for syncytiotrophoblast cells, which upon rapid division and fusion ultimately lead to the expansion of the placenta. Eight days after implantation, lacunae (large fluid filled spaces) are formed within the syncytiotrophoblast (Figure 1.3 Panel III). Some masses of syncytiotrophoblast are found in between the lacunae and are termed trabeculae, which develop further into the villous tree of the placenta. Once the embryo and the surrounding tissues are completely embedded in the endometrium and the syncytiotrophoblast are fully developed, the extra-embryonic mesodermal cells migrate over the top of the inner surface of the cytotrophoblast to form the chorion (Norwitz *et al*, 2001). The cytotrophoblasts then penetrate the trabeculae and migrate towards the maternal side of the placenta. These cells differentiate into extravillous and villous pathways. The pathway for trophoblast differentiation is represented in Figure 1.4.

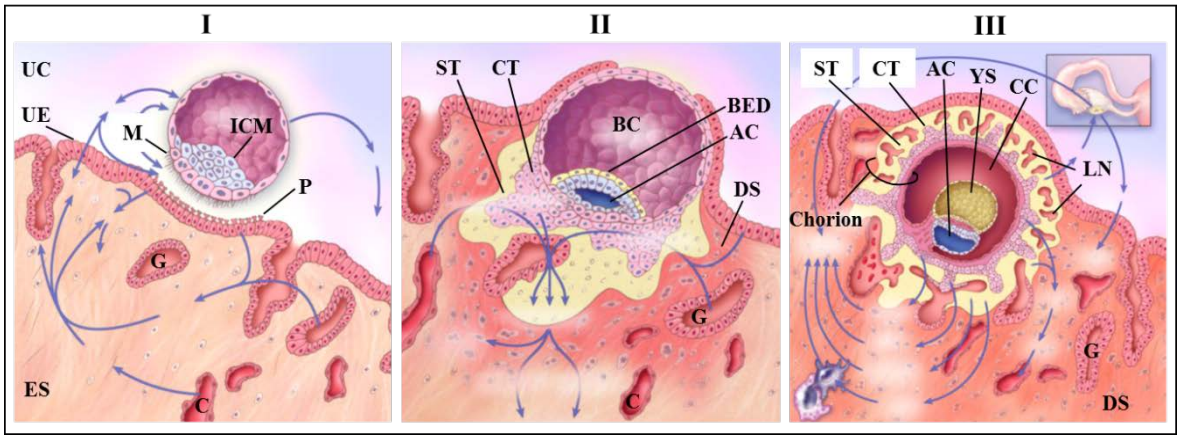


Figure 1.3: Implantation of blastocysts into the endometrium.

Panel I. Apposition and adhesion of the blastocyst on the uterine epithelium with the help of microvilli on the surface of syncytiotrophoblast cells and pinopodes on the surface of epithelial cell (Day 6-7). **Panel II**. The invasion of the syncytiotrophoblast into the endometrial stroma (Day 8-9). **Panel III**. Blastocyst fully embedded and concealed within the endothelium (Day 10). There is formation of the lacunar network in the chorion and the syncytiotrophoblasts called the trabeculae. The cytotrophoblasts invade the trabeculae and migrate towards the maternal side. The blue arrows indicate the circulation of endocrine hormones, growth factors and cytokines which help with the maintainance of the pregnancy at its early stages. [*Figure slightly revised from Norwitz et al, 2001*].

AC- Amniotic Cavity; **BC**- Blastocyst Cavity; **BED**- Bilaminar Embryonic Disk; **C**- Capillary; **CC**- Chorionic Cavity; **CT**- Cytotrophoblast; **DS**- Decidualised Stroma; **ES**- Endometrial Stroma; **G**- Endometrial Glands; **ICM**- Inner Cell Mass; **LN**- Lacunar Network; **M**- Microvilli; **P**- Pinopodes; **ST**- Syncytiotrophoblast; **UC**- Uterine Cavity; **UE**- Uterine Epithelium; **YS**- Yolk Sac

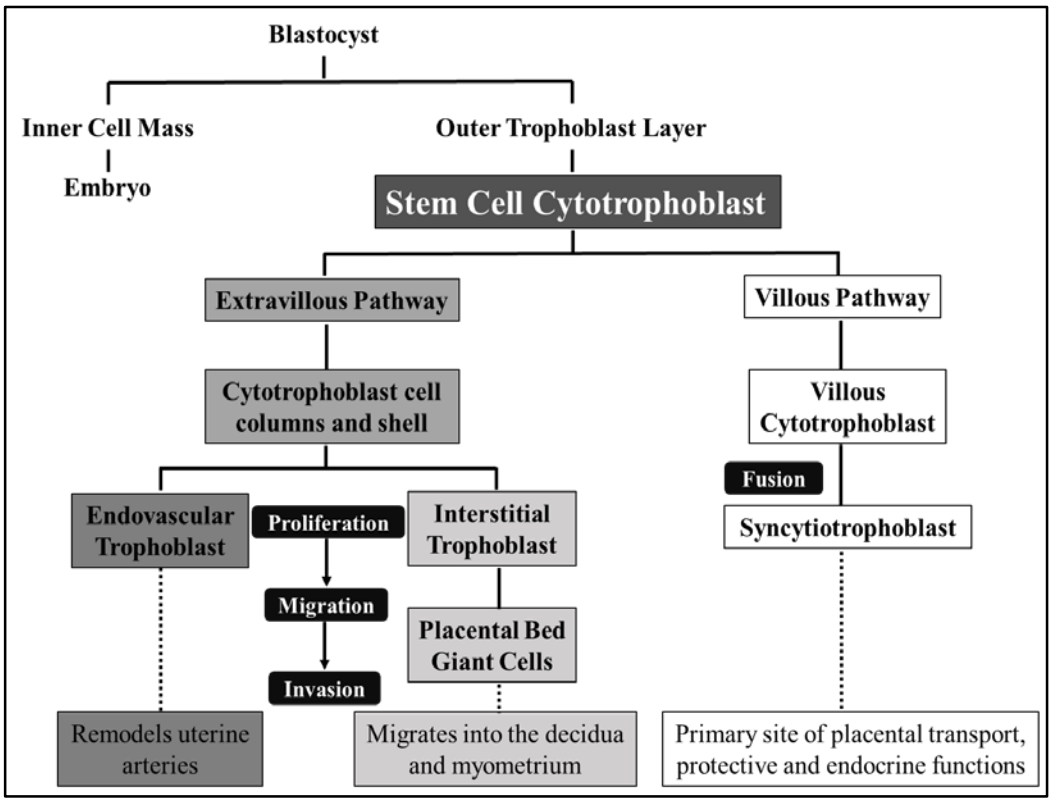


Figure 1.4: Pathway representing trophoblast differentiation.

The outer trophoblast layer of the blastocyst gives rise to stem cell cytotrophoblast which differentiates into two major pathways with autonomous functions. The differentiation processes that the cells undergo are highlighted in black boxes. *[Figure adapted from Gude et al, 2004 and Tarrade et al, 2001]*

The interstitial trophoblast (Interstitial Cytotrophoblast, iCTB) further invades the stroma whereas the endovascular trophoblast (EVT; Endovascular Cytotrophoblast, eCTB) invades the spiral arteries of the uterus. The 'physiological changes of pregnancy' begin when a sub-population of EVT moves as a column from the anchoring villi spreading laterally to form the trophoblastic shell and longitudinally invading the spiral arteries of the decidua (James *et al*, 2005) (Figure 1.5). Around 100 to 150 spiral arteries are transformed extending to the inner third of the myometrium. The invading EVT first plugs the maternal spiral arteries and then subsequently replaces the maternal endothelial lining and the myometrial section of the vessels (processes called endovascular and interstitial invasion, respectively) (Soundararajan and Rao, 2004; Ramsey *et al*, 1976). These processes transform the vessels into large bore conduits by the loss of their musculo-elastic structure and into high capacitance vessels by their increase in diameter converting them from "high resistance, low capacitance" to "low resistance, high capacitance" vessels (Johns *et al*, 2006). The fibrinoid layer replaces the muscular and elastic components of maternal blood vessels.

This transformation leads to adequate supply of maternal blood flow, required for the growth of the placenta/foetus; thereby leading to placentation. At the 10th week of pregnancy, the plug dissipates, thus establishing direct contact between the spiral arteries and the placental villi. The net result is the formation of the placenta with low resistance materno-foetal interface and the establishment of a blood supply between the foetus and the mother.

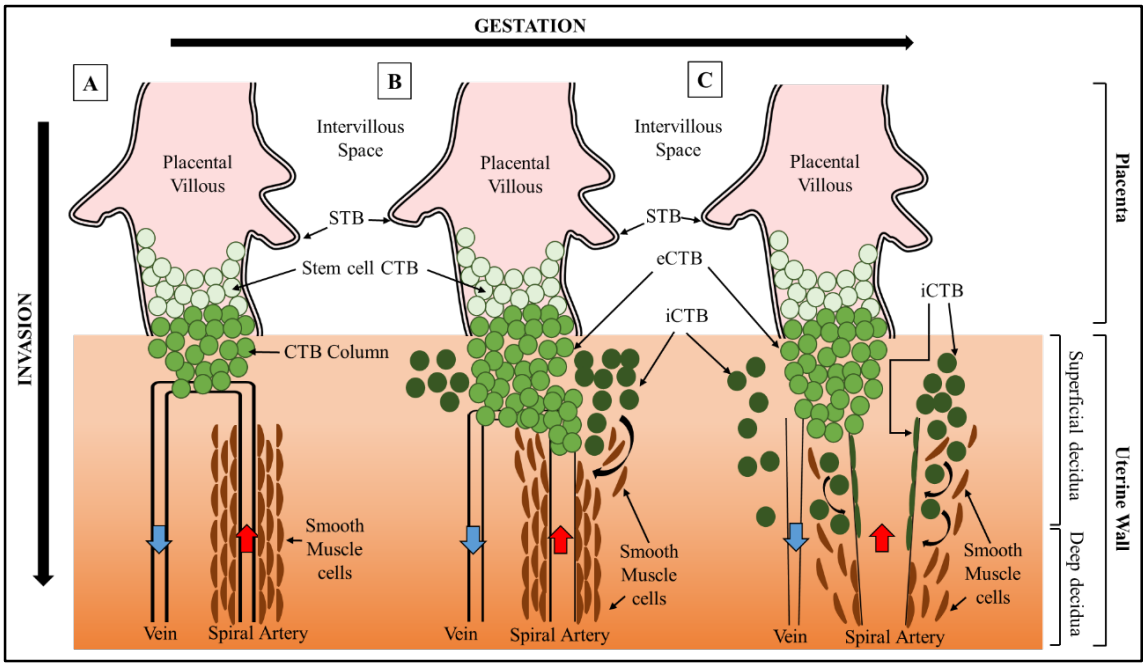


Figure 1.5: Invasion of spiral arteries by endovascular trophoblast.

The stem cell CTB (light green) in tertiary villous form CTB columns invading the uterine wall [A]. Syncytiotrophoblast (STB) forms the outer layer of the villi, which is in connection with the intervillous spaces (IVS). As the pregnancy progresses, CTB cells differentiate into endovascular CTB (eCTB; green) and interstitial CTB (iCTB; dark green) [B]. The eCTB forms a plug on the spiral arteries and builds the pressure. This helps the iCTB and eCTB to replace the endothelial lining and displaces the smooth muscle cells of the artery [C]. The plug disappears once the spiral arteries are remodelled and there is sufficient blood supply to the placenta. *[Figure revised from Red-Horse et al*, 2004*]*

1.1.2 Pregnancy stages and placental anatomy

Pregnancy is divided into three stages known as First, Second and Third Trimesters, and the placental development is often termed according to these stages.

1.1.2.1 First trimester (Week 1-13)

The first trimester of pregnancy begins with the implantation of the blastocyst into the uterine wall and marks the beginning of angiogenesis. The first trimester is the most critical one, as it determines the development of the foetus. Exchange of materials in the early embryo is done through diffusion and osmosis, but as the demand of the foetus increases with development, a more defined materno-foetal circulation needs to develop for sufficient

exchange of nutrients and gases. Consequently, placental villi systems start developing, which bring the maternal and foetal blood circulations closer. However, there is no direct mixing of the two blood circulations. As explained earlier in Figure 1.3, Panel III, large spaces are formed in the syncytiotrophoblast layer called lacunae. This mechanism occurs around day 9-10 post conception (p.c.). Figure 1.6[I] also represents the lacunar stage while the cytotrophoblast penetrates the syncytiotrophoblast around the day 11-13 p.c. forming the primary villi of the placenta (Figure 1.6[II]). The mesoblast from the foetal side grows into the cytotrophoblast forming the outer-most layer of syncytiotrophoblast followed by layers of cytotrophoblast and inner mesoblast. This forms the secondary villi around the day 16 p.c. (Figure 1.6[III]). By the end of the first trimester, the secondary villi gives rise to the tertiary villi with the foetal capillary network penetrating the mesoblast as seen in figure 1.6[IV]. The tertiary villi continues to develop and grow until the end of the pregnancy. The cytotrophoblast also starts differentiating at the end of the first trimester, as explained earlier in Section 1.1.1.3.

1.1.2.2 Second trimester (Week 14-26)

The second trimester is marked by vascularisation of the chorionic villi system (Chaddha *et al*, 2004). The tertiary villi form numerous branches. Some of them are anchored to the decidua and are called anchoring villi, from where the eCTB plugs the spiral arteries followed by iCTB lining the arteries (Figure 1.5, Section 1.1.1.3). Some branches of the villi do not anchor to the decidua and are thus called floating villi. The tertiary villi change to intermediate immature villi, which mature into intermediate villi and which further give rise to either stem villi or terminal villi (mesenchymatous). The stem villi give the structural support to the villous tree, whereas the terminal villi elongate the tree. At the end of the 4^{th} month, the cytotrophoblast in the tertiary villi starts to disappear and the space between the capillaries and the intervillous space (IVS) is lined only by the syncytiotrophoblast layer. These villi are called free villi (Figure 1.6[V]). Very few studies have been conducted on the importance of chorionic villi development during pregnancy.

1.1.2.3 Third trimester (Week 27 to 40)

In the third trimester, the eCTB are almost absent. The iCTB cells invade two thirds of the myometrium. The tertiary villus system is maintained until the end of the pregnancy in order to maintain the surface for exchange. The capillaries move closer to the syncytiotrophoblast layers as seen in free villi, ensuring closer connection between the maternal and foetal blood circulations.

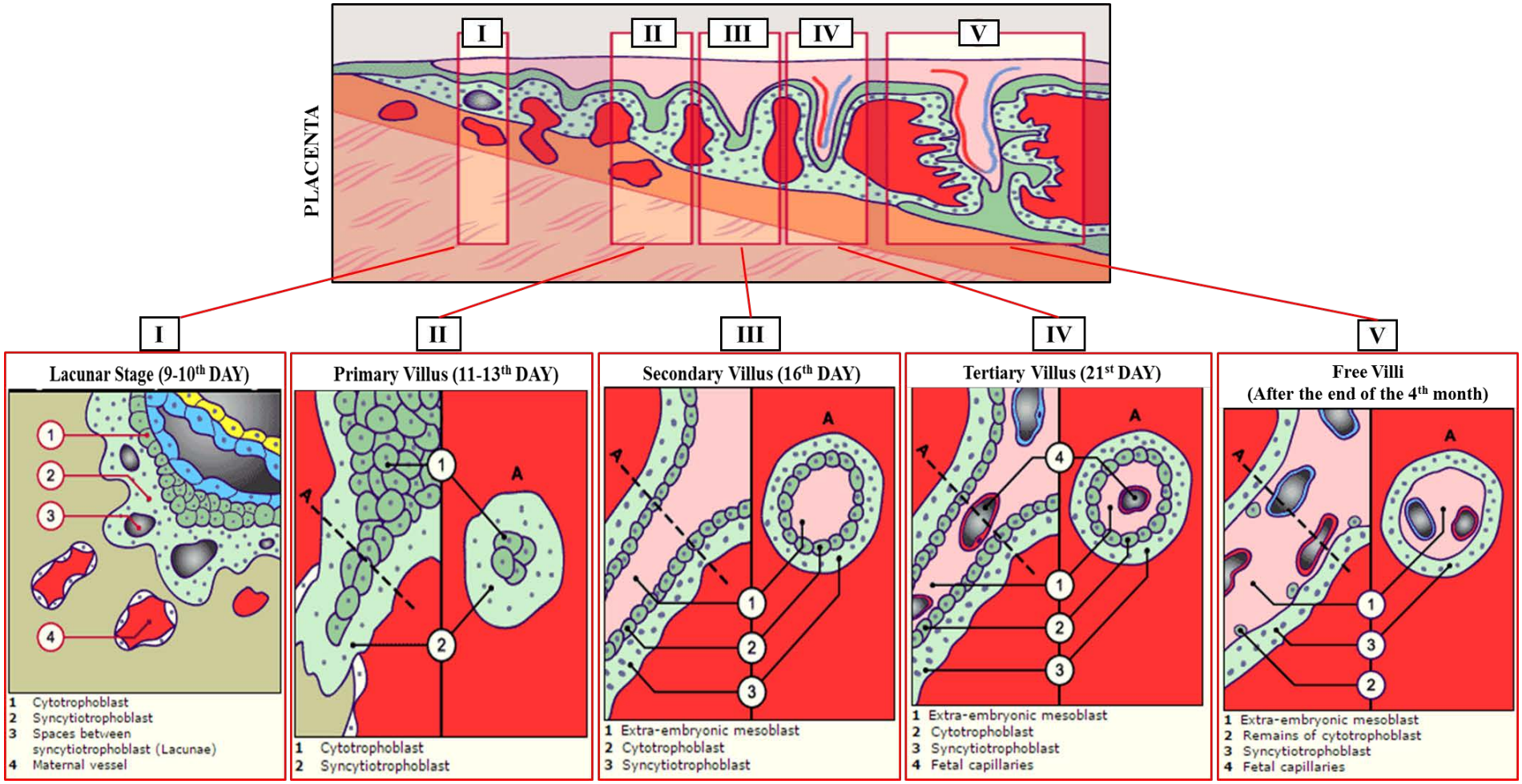


Figure 1.6: Different types of villus formation during placental development.

[I] Lacunar stage shows formation of large spaces called lacunae in the syncytiotrophoblast layer. [**II**] Primary villus shows cytotrophoblast penetrating the syncytiotrophoblast. [**III**] The mesoblast from the foetal side fills up the cytotrophoblast forming the innermost core of the secondary villus. [**IV**] Foetal capillaries form a mesh in the mesoblast core of the tertiary villi. [**V**] The cytotrophoblast layer starts disappearing in the free villi thus reducing the distance between the foetal capillaries and the maternal circulation. *[Figure adapted from* **www**[4]*]*

1.1.3 Structure of full term placenta

A mature full-term placenta is around 22cm in diameter with a central thickness of 2.5cm and an average weight of 470g (Huppertz, 2008). These parameters vary from one placenta to another, but the weight of it is directly related to the foetus. The placenta has two surfaces; the maternal and the foetal surface.

- **Maternal surface:**

The surface, attached to the uterine wall forms the maternal surface. It has an irregular surface lined with different trophoblast cells and maternal cells and covered with grooves and clefts as seen in Figure 1.7.A.

- **Foetal surface:**

The surface, which has an umbilical cord connected to its center, is the foetal surface (Figure 1.7.B). It contains the chorionic plate which is covered by the amnion. The umbilical cord is a continuation of the chorionic vessels which arise from the chorionic mesenchyme. The chorionic arteries branch from the two umbilical arteries and supply the villous trees. The villous trees continue to the chorionic veins and finally to a single umbilical vein.

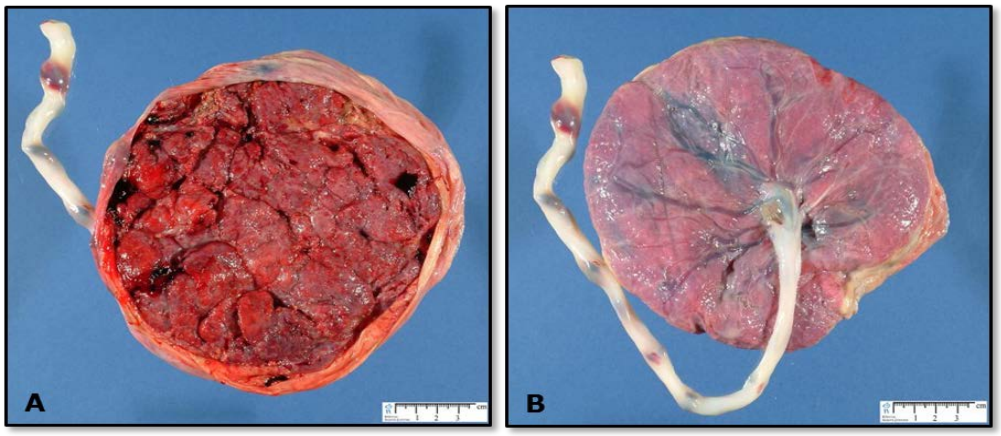


Figure 1.7: Macroscopic anatomy of a normal placenta (28 weeks).
[A] Represents the maternal surface; [B] Represents the foetal surface. *[Figure adapted from* **www**[5]*]*

1.1.4 Function of placenta

The fundamental function of the placenta is to nourish the foetus. The dense network of chorionic villi, lacunae, veins and arteries within the placenta allows it to temporarily perform the following functions for the embryo until its organs develop, or become functional after birth (Donnelly and Campling, 2010; Gude *et al*, 2004) (Figure 1.8):

- Gaseous exchange (foetal lungs)
- Uptake of nutrients (gastrointestinal tract)
    - Glucose
    - Amino acids
    - Fatty acids
    - Calcium
    - Iron
- Fluid regulation and elimination of waste metabolites (kidney function)
- Release of steroid and peptide hormones (endocrine function)
- Protection from xenobiotics.

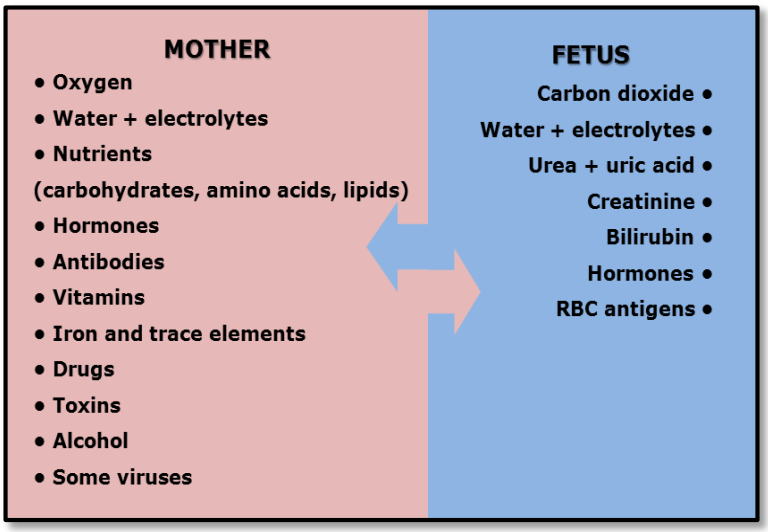


Figure 1.8: Exchange of materials between the mother and the foetus.
[Donnelly and Campling, 2010]

The maternal blood supplies the foetus with nutrients and gas and removes waste materials. However, there is no mixing of the foetal and maternal blood.

1.1.5 Complications during placental development

When compared to other mammalian species, human reproduction is not fully competent in establishing a fully functional placenta (Jauniaux and Burton, 2005). Failure in one of the key aspects of placentation can lead to abnormal placental development. Complications due to abnormal placentation in embryonic or second-trimester lead to foetal death and early pregnancy loss. Complications at the third trimester can lead to three severe diseases: pre-eclampsia (PE), intra-uterine growth restriction (IUGR) and abruption (Chaddha *et al*, 2004). PE is the leading cause of perinatal death and is studied in detail in this thesis.

- **Pre-eclampsia**

Pre-eclampsia (PE) is derived from the Greek word '*eklampsis'*, meaning sudden flash or development (Red-Horse *et al*, 2004). PE is a pregnancy disorder that is diagnosed in 2-3% of pregnancies in the third trimester (Lunghi *et al*, 2007).

- *Clinical features*:

Increased blood pressure and proteinuria are the distinctive clinical features of this disease. Systolic blood pressure (BP) ≥ 140 mmHg and /or diastolic BP≥90 mmHg range is seen in hypertensive patients. Proteinuria is confirmed when the protein in the urine is >300 mg in 24 hours. PE may lead to IUGR in some cases (Fisher *et al*, 2009). A placenta in PE is smaller than in normal pregnancy (NT) as seen below in Figure 1.9.

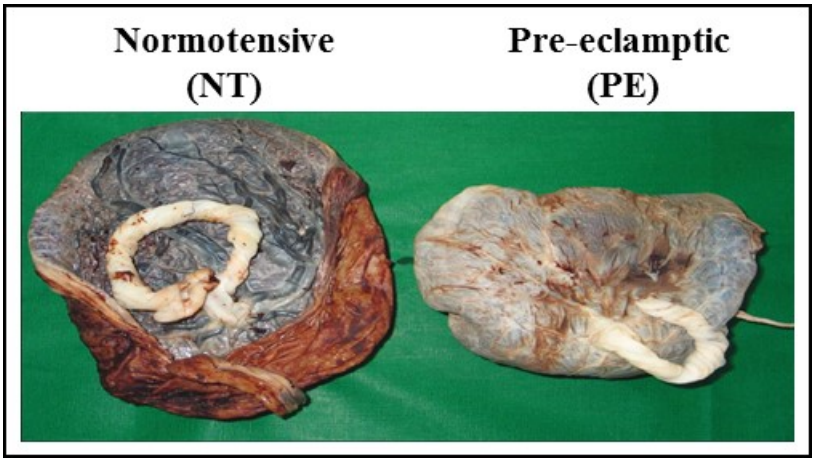


Figure 1.9: Structural differences in NT and PE placentae.
The PE placenta is smaller in size when compared to NT placenta. [*Figure adapted from Akhlaq et al, 2012*]

- *Placentation in PE*:

Current evidence shows that the major factor for the cause of pre-eclampsia is poor placentation (Mohaupt, 2007) and the only cure for PE is removal of the placenta thus confirming this as the root cause (Centlow *et al*, 2010). Unlike normal placentation, poor placentation in PE is shown by inadequate intravascular invasion by the cytotrophoblast, and the resultant defective transformation of the maternal spiral arteries. The trophoblast invasion into the spiral arteries is shallow. Thus, the PE placenta remains in a state of high resistance. PE is also associated with defective plug formation and a thinner trophoblast shell. In a normal placenta, the blood flow takes place from the periphery where the plug formation is incomplete but in a PE placenta, the blood flow mostly takes place from the centre.

The spiral arteries of non-pregnant, normal pregnancy and PE vessels are compared in Figure 1.10. The diameter of spiral arteries in normal pregnancy is four times greater than the diameter of the non-pregnant vessels and that of PE are half of the diameter of normal pregnancy.

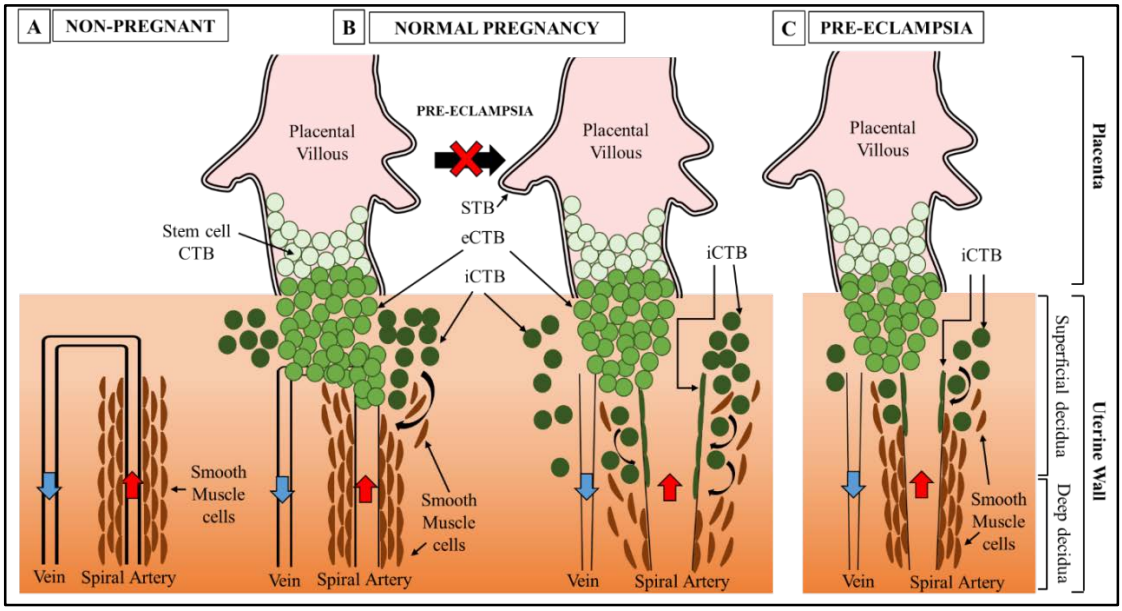


Figure 1.10: Difference in the diameters of spiral arteries in non-pregnant, normal pregnancy and pre-eclampsia.

[Figure revised from Moffett-King, 2002 and Red-Horse *et al*, 2004*]*

The defective remodeling of spiral arteries may be due to the combination of defect in EVT differentiation to the invasive type, rise in apoptosis rate, uncontrolled migratory and invasive function of EVT, and failure of cells to adopt endovascular properties. The net result is poor formation of the placenta and thus miscarriages. The trophoblasts in PE were found to be immature intermediates that failed to aggregate together to form sheets on the spiral arteries (Redline and Patterson, 1995; Fisher *et al*, 2009). These immature trophoblasts remain in round clusters thus leading to irregular oxygen tensions, which eventually cause oxidative stress and formation of reactive oxygen species (ROS). These events cause the maternal body to respond providing clinical features like hypertension and proteinuria (Fisher *et al*, 2009).

- **Immune mechanism in pre-eclampsia**

Placentation involves specific maternal immune responses to fetal alloantigens. Human trophoblast has limited expression of strong Human Leukocyte Antigens (HLA) and express only HLA-E, F, and G (Redman and Sargent, 2010). HLA-G regulates the cells of the maternal immune system to accommodate the foetus, by suppression of CD4+ and CD8+ proliferation, regulating the regulatory T-cells [T(reg)], B cells, NK cells and inflammatory

cells (macrophages and dendritic cells). Any impairment in this immune system at its early stages of development may cause PE. T(reg) cells of the decidua have short term memory of the paternal HLA-C which down-regulates the anti-paternal responses (Redman and Sargent, 2010). Thus, PE is predominantly a disease of the first partum.

In summary, the normal placentation is a highly invasive process, which is tightly controlled in such a way to be a transient event. On the other hand, in PE placentation, the invasion is not fully complete.

As mentioned at the beginning of this chapter (Section 1.1), the process of invasion during tumorigenesis has similar characteristics with trophoblast invasion during placental development, particularly in the first trimester of pregnancy (Ferretti *et al*, 2007; Soundararajan and Rao 2004). The following section will briefly discuss the invasive process during tumorigenesis and compare the subtle differences between the two.

1.2 Tumour

A tumour is defined as an abnormal mass of tissue formed by uncontrolled division of cells. This uncontrolled division takes place due to mutations in the cells leading to defective repair mechanisms. They can be of two types: benign (cause no threat to the body) or malignant (leading to serious damage to body and ultimately death of the patient). Cancer is one of the leading cause of worldwide death in recent years (**www**[6]). Therefore, there is a critical need for exploring new avenues of therapeutic modalities.

1.2.1 Hallmarks of cancer

Six vital alterations in the cell physiology were proposed by Hanahan and Weinberg, in the year 2000, for the transformation of normal cells to malignant cells (Figure 1.11). These 'hallmarks' are shared by most of the tumour cells and are essential for their growth and metastasis (Hanahan and Weinberg, 2011). These six hallmarks are described below.

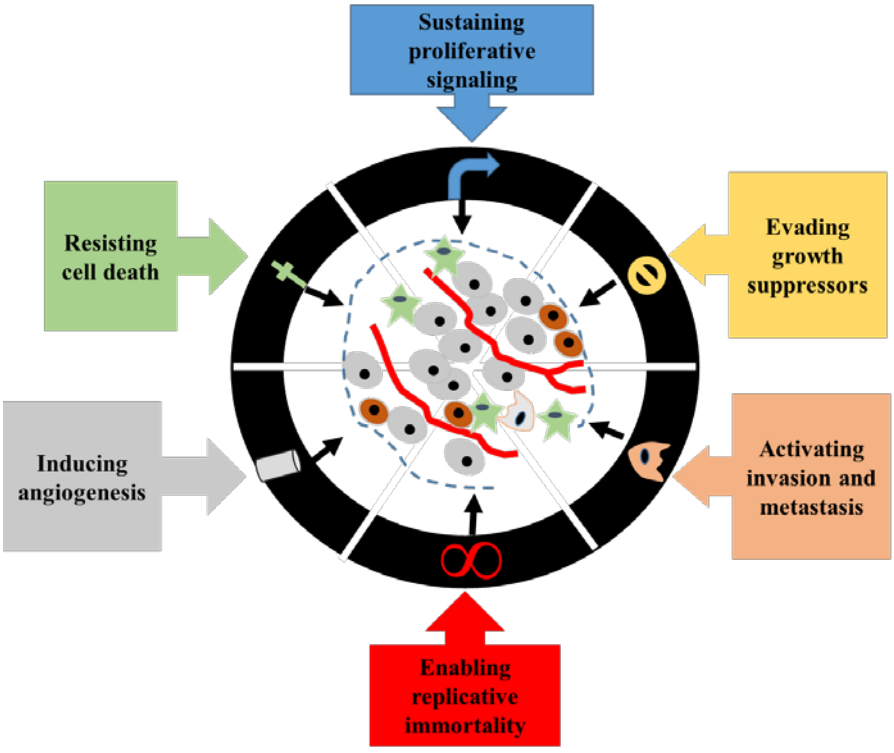


Figure 1.11: Hallmarks of Cancer.

The figure represents the 6 characteristic changes taking place in cancer cells for its growth and transformation. *[Adapted from Hanahan and Weinberg, 2011]*

1. Sustaining proliferative signalling

Mitogenic growth signals are required by normal cells to transform from a quiescent state to an active proliferative state. These growth signals are transmitted by diffusible growth factors, extracellular matrix components and cell-to-cell adhesion/interaction molecules which bind to transmembrane receptors on healthy cells. In the absence of these growth signals the normal cells perish and die (Hanahan and Weinberg, 2011). Changes in the internal environmental condition switch tumour cells to an autocrine production of growth signals (Herzig and Christofori, 2002). For example glioblastomas and sarcomas produce PGDF (platelet derived growth factor) and TGF α (tumour growth factor α) respectively to which they become responsive, creating autocrine stimulation. Alternatively, there are also mutations in the signal transduction pathway that lead to production of mitogenic signals. The growth receptors on the surface of the cells also undergo alterations, which lead to up-regulation of these receptors on tumour cells (Kroll *et al*, 2010).

2. Evading growth suppressors

Proliferation in normal cells are controlled by growth suppressors that force healthy cells into the quiescent (G_0) state from an active state or signals cells to permanently abandon their proliferative state by entering into a postmitotic state. Hanahan and Weinberg (2011) state that emerging tumour cells must evade anti-proliferative signals in order to drive malignant growth. Also inactivation of tumour suppressor genes in many human and animal cancers have been reported that stops cell growth and proliferation.

3. Resisting cell death

A stable rate of cell proliferation and cell death helps to maintain the growth of normal tissues. Apoptosis (programmed cell death) is the most common pathway followed by dying cells. A variety of physiological signals triggers a cascade of proteins that lead to the disruption of the cell membrane, breaks down the cytoplasmic and nuclear components, degrades chromosomes and fragments nucleus. Almost all tumour cells develop an ability to evade apoptosis by down regulating proapoptotic factors (such as Bax, Bim, and Puma), and increasing the expression of anti-apoptotic regulators (Bcl-2, Bcl-x_L) or that of survival signals (Igf1/2) (Hanahan and Weinberg, 2011).

4. Enabling replicative immortality

Normal cells have a definite potential to replicate but at some point they lose their ability to divide and enters senescence (Hanahan and Weinberg, 2011). It has been reported, tumours can prevent senescence by inactivating their tumour suppressor proteins (p53 and pRb)

which leads to uncontrolled cell division and growth of the tumour (Herzig and Christofori, 2002). In addition, a progressive shortening of telomeres after each cell cycle may also lead to cell death. In cancer cells, the overexpression of telomerase surmounts telomere shortening and ultimately leads to indefinite cell proliferation (Kim *et al*, 1994).

5. Inducing angiogenesis

The major problem confronted by a growing tumour mass is the supply of oxygen and nutrients (Kroll *et al*, 2010). Cells closer to the blood vessels grow, while cells further away from the blood vessels degrade. In order for a tumour mass to grow, increased blood supply is required. This is achieved by formation of new blood vessels from pre-existing vessels, a process known as angiogenesis (Otrock *et al*, 2007; Buschmann and Schaper, 1999). Angiogenesis is also required for metastasis of the tumour cells (Herzig and Christofori, 2002). Tumour angiogenesis begins with the secretion of angiogenic factors from the tumour cells, which proceed to stimulate the neighbouring tissues. This leads to production of proteins, which further encourage the formation of new blood vessels. Vascular endothelial growth factor (VEGF), angiopoietins and fibroblast growth factors are some of the angiogenic factors released by tumours. Inflammatory cells, which infiltrate the tumour site and the surrounding fibroblast cells, also add to the secretion of angiogenic factors. With the secretion of angiogenic factors and proteases like MMPs, the vascular basement membrane (VBM) of the blood vessels undergoes structural changes to form a provisional matrix. Proliferation and migration of endothelial cells occurs with the formation of this matrix. Production of growth factors leads to transition of the provisional matrix to intermediate and ultimately to mature new VBM. In presence of the pericytes and endothelial cells, VBM leads to formation of new blood vessels. Formation of new blood vessels supplies more blood, along with nutrients and oxygen to the tumour, resulting in growth and proliferation of the tumour cells (Carmeliet and Jain, 2000; Francavilla *et al*, 2009).

6. Activating invasion and metastasis

During the different stages of cancer, tumour cells start invading neighboring tissues and organs. This invasion can be due to mechanical as well as enzymatic process. In the mechanical process, growth of tumour mass increases pressure on the surrounding cells leading to invasion. In the enzymatic process, the growing tumour cells secrete some proteolytic enzymes [such as matrix metalloproteinases (MMPs)], which degrade the extracellular matrix (ECM) barrier and destroys the neighboring cells (Otrock *et al*, 2007).

Metastasis is the spread of the tumour from the site of origin to secondary sites. This usually occurs at the end-stage of the disease (Schedin and Elias, 2004). These metastatic cells

degrade the basement membranes (BM) of the primary site and starts invading the neighbouring cells and tissues. Likewise single tumour cells can also invade the blood vessels (or the lymphatic vessels), a process called intravasation. These cells are carried along with the flow of the blood (or the lymph) till they are blocked either by the bone marrow, the lymph nodes or the blood vessels of the secondary sites (Schedin and Elias, 2004; Herzig and Christofori, 2002). At the secondary sites, the cells attach to the vessel walls and by degrading the vessel wall, the tumour cells evade out from the BVs, a process termed extravasation. Tumour cells may immediately start growing or may remain in a state of dormancy for many months or years at the secondary site. When favourable stimuli are secreted, dormant tumours start proliferating again.

Four new additional hallmarks were stated by Hanahan and Weinberg in 2011 and these are specified below.

7. Deregulating cellular energetics

In normal respiring cells, the glucose is converted to pyruvate by glycolysis followed by the TCA cycle to produce ATP and carbon dioxide via the respiratory chain in the mitochondria. However, during anaerobic conditions, glycolysis alone is to reduce the oxygen usage. This results in the accumulation of lactic acid (Warburg metablism). This effects is observed in tumour cells even with the presence of oxygen resulting in around 18 fold reduction in ATP production. This deficit of ATP is compensated by tumour cells by upregulating glucose transporters (i.e. GLUT-1), and thus increasing the glucose uptake into the cell (Jones and Thompson, 2009; Hsu and Sabatini, 2008; DeBerardinis *et al*, 2008).

8. Avoiding immune destruction

Tumour cells create a microenvironment by which they escape the immune response (Kaufman and Disis, 2004). Tumour cells can regulate the secretion of cytokines/chemokines, the expression of growth factor receptors and the homing of regulatory cells. They can also release vesicular bodies which carry “molecular messages” of the tumour (Whiteside, 2009). They interact with dendritic cells (DC) and produce tumour signals (Bennaceur *et al*, 2009). The local DCs are usually immature, therefore incapable of processing the tumour cells for destruction. Moreover, regulatory T cells (T-regs) infiltrate the tumour sites and suppress the T-cell binding to antigens. In addition to this, the phenotype of helper CD4 is also directed towards Th2 pathways to inhibit cellular immunity. As a result of these changes, antigen presentation is hindered and expression of MHC molecules on the surface of tumour cells is altered, making them invisible for the immune system. Finally, tumour cells secrete cytokines such as IL-6, IL-10 and Transforming growth factor-beta

(TGF-β), which help in further suppressing the immune response. In some organisms, such as lower vertebrates, the above stages alone can lead to a cancerous form of tumour (cancerous forms are usually the end-stage tumours which are fatal to the organism). However, in higher organisms like humans, metastasis and angiogenesis are necessary for the tumour to become cancerous (Kroll *et al*, 2010).

9. Genomic instability and mutation

Mutations of cells can lead to selective subclones in a microenvironment and thus cause its dominance and outgrowth. Multistep tumour progression is believed to be a succession of clonal expansions, each triggered by the acquisition of a mutant genotype (Hanahan and Weinberg, 2011). DNA repair mechanisms detect and resolve any defects in the DNA to ensure that the rate of spontaneous mutations are kept low after every cell replication. In cancer cells there is increase rate of mutation either due to its sensitivity to mutagenic agents or the breakdown of the DNA repair mechanism which ultimately leads to tumour formation (Negrini *et al*, 2010; Salk *et al*, 2010).

10. Tumour promoting inflammation

Cells of the immune system infiltrate the tumour and lead to inflammations (Pages *et al.*, 2010). Most of the cells in the inflammation may contribute to some of the hallmarks of cancer by supplying growth factors, proangiogenic factors, matrix-metalloproteinases and survival factors (DeNardo *et al*, 2010; Karnoub and Weinberg, 2006; Grivennikov *et al*, 2010; Qian and Pollard, 2010).

1.3 Placentation vs. Tumorigenesis

Even though placenta is a normal temporary organ, the trophoblast especially in the first trimester gives the placenta ‘pseudo-malignant’ and ‘physiological metastasis’ characteristics. As seen in Figure 1.13 both the placentation and tumorigenesis starts with a highly invasive mass of cells.

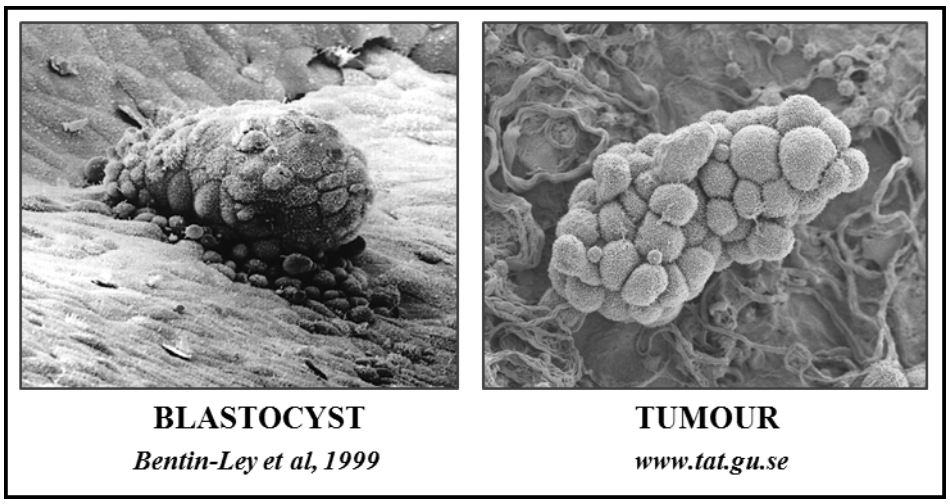


Figure 1.12: The close resemblance of the blastocyst and a tumour at their early stage of development.

1.3.1 Resemblance of placenta to tumour

There are certain cellular processes and molecular circuits, which are common to both tumour and placental cells. Some of these processes are summarised below.

1.3.1.1 Cell invasion

The tumour cells manipulate the extra cellular matrix (ECM) network at various stages and thus invade the normal cells. Cell invasion involves binding of cells to the ECM components (e.g. type II collagen, fibronectin and laminin) through integrin and/or non-integrin receptors; degradation/manipulation of ECM using enzymes [such as MMPs and urokinase type plasminogen activator (uPA)]. uPA is required for activation of some MMPs. Finally, migration of the cells through the degraded ECM takes place [which requires α5β1 integrin and asparagine (Asn) linked oligosaccharides on the cell surface] (Lala *et al*, 2002).

Placentation, like tumour invasion, is a multi-step process that involves the attachment of the trophoblast to the ECM, degradation of ECM and migration through the degraded ECM.

Trophoblast is the first epithelium that develops during placentation and acts as the prototype for the later epithelial developments. Trophoblast cells are often referred to as 'pseudo-malignant' due to their highly proliferative nature, their escape from the immune response, and their migration and invasion of neighbouring tissues (Ferretti *et al*, 2007). As explained in Section 1.1.1.3, a specific sub-population of the trophoblast called the "extravillous trophoblast" (EVT) is involved in the uterine endometrium invasion. Like any other invading / tumour cell, the EVT requires proteases to digest its surrounding environment, thus becoming invasive in nature (Bischof *et al*, 2003).

Matrix metalloproteinases such as MMP2 and MMP-9 and their inhibitors (tissue inhibitor of metalloproteinases - TIMP) play an important part in EVT invasion (Cohen *et al*, 2006). The expression of TIMPs regulates the invasion of trophoblasts by inhibiting the activity of MMPs. Moreover, trophoblast cells that are destined for further invasion of the neighbouring cells modulate their surface proteins (e.g. integrins like α6β4 and α1β1) which help migration through the degraded ECM (Bischof *et al*, 1995; Damsky *et al*, 1994).

1.3.1.2 High rate of cell proliferation

In cancer cells, the overexpression of telomerase surmounts telomere shortening and ultimately leads to indefinite cell proliferation (Kim *et al*, 1994). Unlike other somatic cells (and like cancer cells), human trophoblast cells also express telomerase and undergo undefined cell proliferation during the early stages of placentation (Chen *et al*, 2002). These high rates of proliferation are essential for normal functioning and development of placenta. However, the expression of telomerase is stopped once the placenta is established. This makes placentation a controlled phenomenon. There is also the presence of numerous growth factors and growth factor receptors in both trophoblastic and malignant cells (Ferretti *et al*, 2007). This suggests that an autocrine or paracrine loop may also assist the high rate of proliferation in these cell types. Some of the important loops are epidermal growth factor (EGF)/EGF Receptor (EGFR), VEGF/VEGF Receptor (VEGFR), Platelet derived growth factor (PDGF)/PDGF Receptor (PDGFR) and Colony Stimulating Factor1 (CSF1)/CSF1 Receptor (CSF1R). These loops further interrelate with the mitogen-activated protein kinase (MAPK) signalling pathways.

1.3.1.3 Avoiding apoptosis

The rapid growth of tumour and placenta is not only due to high cell proliferation, but also to avoidance of apoptosis. By balancing the expression of apoptotic detecting signals like growth factors and sensors, these cells maintain their integrity. Amongst the numerous important signals and detectors, the insulin-like growth factor-1 (IGF-1) and its receptors have come into the limelight. It has already been proved that the expression of these signals increases proliferation as well as the metastasis of malignant cells (Ferretti *et al*, 2007). However, the signals have shown only proliferative improvement in trophoblast cells. In addition to these signals, it has been discovered that like tumour cell, trophoblast cells express anti-apoptotic proteins (e.g. survivin) (Fest *et al*, 2008).

1.3.1.4 Escape from the immune system

As explained in Section 1.2, tumour cells cover up surface antigens or shed surface proteins that would lead to their detection by the lymphocyte T cells. This helps tumour cells to escape immune detection. In the same way, the embryo is semi-allogeneic to the mother. Despite the genetic differences between the mother and the developing embryo, the immune system of the mother does not reject the embryo. This is achieved by various hormones and proteins secreted during pregnancy that blocks the mother's immune response against the embryo (Beer and Billingham, 1974). The maternal blood and lymphatic system is also separated from the foetus by the placenta. The foetal trophoblast cells escape the maternal immune surveillance as they fail to express MHC molecules on their surface (Ferretti *et al*, 2007).

1.3.1.5 'Pseudo-Vasculogenesis' in placenta

Tumour cells and the embryo require new blood supply in order to grow. This is achieved by the process of angiogenesis. Angiogenesis switches to an invasive phase due to the secretion of proteins like basic Fibroblast Growth Factor (bFGF) and VEGF during tumour growth. Expression of these factors is also seen in human trophoblast cells (Hamai *et al*, 1998).

1.3.1.6 Oncogenes in human placenta

Oncogenes are responsible for undefined cell proliferation, which leads to cancer. These are altered forms of the proto-oncogenes, which include growth factors and their receptors, nuclear transcription factors, proteins involved in the control of the cell cycle and intercellular signal transducers (Bischof and Campana, 2000). Some of these factors and/or receptors are also expressed in placenta and are discussed as follows.

- **Growth factors**

PDGF is one of the products of oncogenes. The β chain of PDGF is encoded by the c-sis proto-oncogene which is expressed during trophoblast proliferation. Interestingly, it is also reported to be expressed by a variety of cancer cell lines (Goustin *et al*, 1985).

- **Growth factor receptors**

Cancer cells have several growth factor receptors. Receptors such as Macrophage-Colony Stimulating Factor Receptor (MCSF-R), EGFR and VEGFR are encoded by the proto-oncogenes c-fms, c-erb B and c-flt, respectively. These proto-oncogenes are also involved in trophoblast proliferation and invasion. Another proto-oncogene, HER-2/neu (c-erbB2), which belongs to a family of EGFR, has been found to be expressed in high levels in

placental, foetal epithelial cells and in human carcinomas (Quenby *et al*, 1998). Studies carried out on normal placenta, gestational choriocarcinoma and partial mole samples have shown the presence of c-erbB3 and c-erbB4 genes (Tuncer *et al*, 2000). In addition, c-met (which encodes for hepatocyte growth factor receptor) and c-kit (which encodes for mast cell growth factor receptor), also belong to the receptor tyrosine kinase family and are found to be present in both cancer cells and in placenta (Kauma *et al*, 1997; Doneda *et al*, 1997).

- **Transcription factors**

The proto-oncogenes c-jun and c-fos form a transcriptional complex called AP-1 complex (Activator Protein-1). Studies have demonstrated that AP-1 is necessary for the expression of MMP-9 during trophoblast invasion (Bischof *et al*, 2003). Fos and Jun are abundantly expressed by trophoblast cells. A binding site for Fos-Jun can be found on the promoters of MMPs (e.g. MMP-9). Thus, any changes in the expression of the Fos and Jun oncogenes alter the levels of MMPs and ultimately lead to changes in invasion by the trophoblasts (Soundararajan and Rao, 2004).

One of the most important proto-oncogenes is c-Myc. The product of c-Myc is a transcription factor which is required for cell cycle progression. c-Myc expression promotes the expression of hTERT, which in return helps with the undefined proliferation of cancer cells. Interestingly, c-Myc acts in similar fashion during trophoblast proliferation (Quenby *et al*, 1998). All of these proto-oncogenes, factors and proteins function as a complex network controlling proliferation, invasion and metastasis of neoplastic cells as well as trophoblast cells (Holtan *et al*, 2009). Figure 1.14 summarises the parallels between the molecular circuit of the maternal-foetal interface in placenta and the microenvironment of the tumour where the secretion and expression of various oncogenes, angioproteins and other factors maintain both the early pregnancy and the tumour growth.

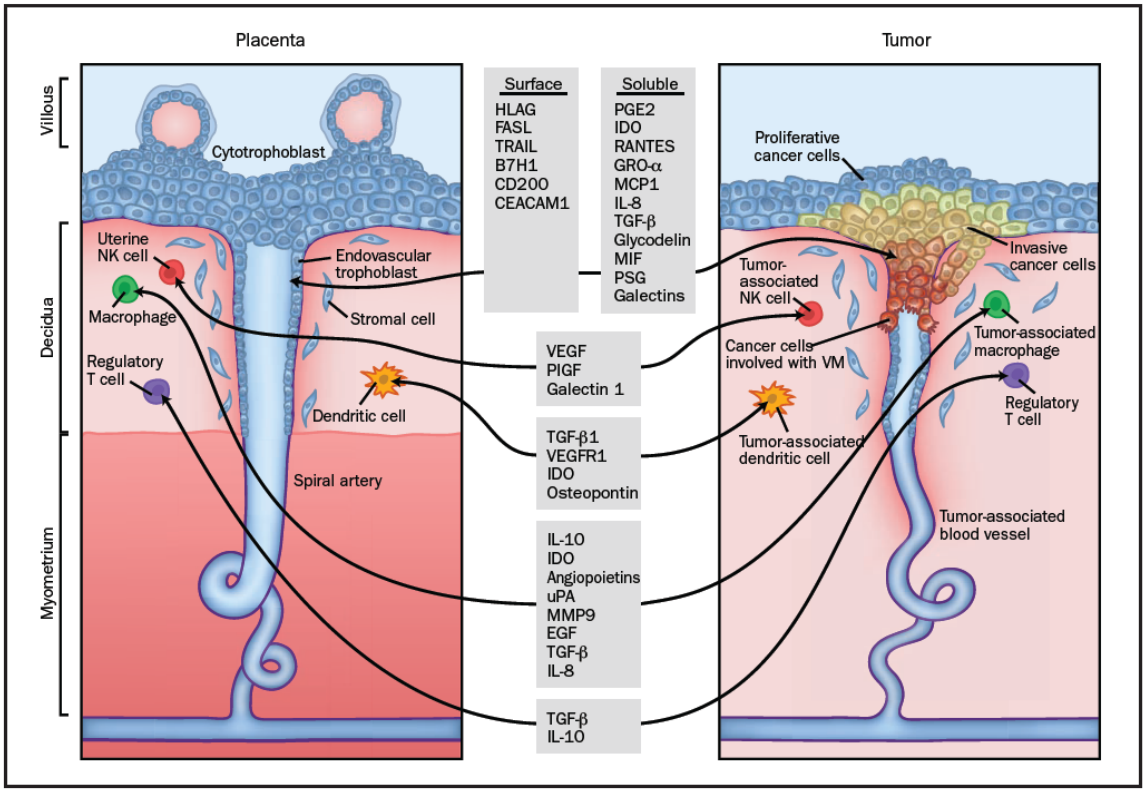


Figure 1.13: Resemblance of the microenvironment in placenta and tumour.
[Figure adapted from Holtan et al, 2009].

1.3.2 Differences between placenta and tumour

Like tumorigenesis, placentation follows the same processes of invasion and migration. However, placentation is a temporary malignancy, whereas tumorigenesis is an indefinite malignancy. In other words, the placenta "knows" exactly where and when to stop cell proliferation and its tumorigenic features are only temporary. It is for this reason that placenta is often described as a '*well-behaved*' tumour (Soundararajan and Rao, 2004).

Trophoblast cells are highly invasive in the first trimester of pregnancy (12 weeks p.c.). Nonetheless, they lose their invasiveness as the pregnancy progresses. Only one-third of the myometrium is invaded and this stops by the second trimester. Conversely, the tumour cells continuously invade their surroundings. Unlike cancer cells, EVT cells senescent after 5-15 passages (during cell culture) and become anchorage-dependent (Lala *et al*, 2002). The terminal trophoblast undergoes a programmed differentiation at the end phase of placental development. This programmed event is regulated by changes in gene expression and hormones secreted during pregnancy leading to apoptosis. The differentiation of trophoblast also leads to the loss in telomerase activity through suppression of hTERT by TGFβ1. This allows the cells to become mortal again. Some of the proto-oncogenes, which participate during trophoblast proliferation like c-Myc, are also regulated during the differentiation phase (Quenby *et al*, 1998).

Furthermore, the secretion of hormones such as oestrogen and gonadotropin releasing hormone control the differentiation of the trophoblast cells. Therefore, there are various intrinsic and extrinsic factors that control the transient nature of the trophoblast. There is thus a firm demarcation between tumour as a disease and placentation as a normal development by the end of the first trimester as variations in gene expression arise.

1.4 New avenues for exploration

The variations in gene expression in tumour and placenta provide a new avenue for exploration. Due to the multifunctional nature of placenta, it expresses more than 20,000 DNA sequences (Centlow *et al*, 2010). For this reason, placenta has been the target organ for investigating and identifying new genes. Genomic and proteomic studies are being performed on human placental samples to study the functions of the genes in placenta. Cancer-testis antigens and 5-hydroxytryptamine (serotonin) receptors are among the several genes expressed by placenta.

Cancer-testis antigen is a type of tumour antigen that is expressed only by cancer cells and reproductive tissues such as testis and placenta (e.g. NY-ESO-1) (Scanlan *et al*, 2002). In recent years, cancer-testis (C/T) antigens have become the target for designing therapeutic vaccines for cancer treatment. Hence, the study of these antigens in placenta could reveal their molecular actions and this may lead to identification of certain targets for vaccine production.

Similarly, researchers have pointed out that 5-hydroxytryptamine (5-HT) and its receptors may have a fundamental role in placental development and pregnancy maintenance (Oufkir *et al*, 2010). Bolte *et al*, 2001 have shown that 5-HT influences the early extravillous trophoblast (EVT) and endothelial layer of the spiral arteries *via* 5-HT_{1B} or 5-HT_{2A} receptors. However, there is not enough information on (a) the effects of 5-HT on early trophoblast (EVT) invasion and (b) the changes in the expression profiles of different 5-HT receptors produced by increased 5-HT during this invasion. Since 5-HT is important for migration and proliferation, it would also be interesting to investigate the effects of 5-HT on the expression profiles of different C/T antigens.

1.4.1 Expression of C/T antigens

The mRNA expression of C/T antigens is observed only in few normal tissues like ovary, placenta and testis. This restricted expression of C/T antigens suggests that there are some common mechanisms between these tissues. Testis is the only organ which shows expression

for all the C/T antigens. Moreover, the level of expression is higher in testis than in any the other somatic tissue. Due to this restricted expression, C/T antigens have been interesting targets for the development of cancer vaccines (Li *et al*, 2005).

The different mRNA expression studies of these C/T antigens have so far revealed the following (Scanlan *et al*, 2002):

- Individual C/T antigen shows variable frequencies of expression in different cancer histotypes with melanoma showing the highest frequency of expression.
- Different C/T antigens show variable frequencies in single tumour types.
- Certain tumours show expression for a group of C/T antigens, while others show no expression of the C/T antigens suggesting that the expression of C/T antigens is clustered.
- Different stages of tumour development show various expression frequencies for C/T antigens, thus the expression of C/T antigens correlates with tumour progression.

It should be noted that the expression patterns for mRNA cannot always be linked to protein expression patterns. However, due to the limited availability of monoclonal antibodies against C/T antigens, very few studies have demonstrated the expression of C/T antigens at the protein level (Miles *et al*, 2007; Mathieu *et al*, 2009; Linley *et al*, 2012).

1.4.1.1 Characteristics of C/T antigens

For a tumour antigen to be classified as C/T antigen, it should possess the following characteristics (Scanlan *et al*, 2002):

- Expressed only by gametogenic and cancer tissues.
- Heterogenous proteins are expressed due to multigene families.
- The coding gene often maps to Chromosome X.
- Produces an immune response in cancer patients.
- Expression is controlled by DNA methylation and histone modifications
- Expression is associated with tumour metastasis and progression.

1.4.1.2 Functions of C/T antigens

The functions of the C/T antigens in gametogenic tissues and cancer are still not understood, but are often allocated according to the sequence homology of a closely related protein (Kalejs and Erenpreisa, 2005). The proposed functions of these antigens are:

- as part of spermatozoa components,

- as part of synaptonemal complex components,
- to play a role in regulating transcription,
- to participate in the signal transduction,
- to perform functions of helicase,
- involved in cell-cell interaction
- involved in enzymatic reactions,
- and apoptosis inhibition.

The products of these genes are also believed to participate in various biological processes such as chromosomal recombination, signal transduction and translation (Linley *et al*, 2012; Mathieu *et al*, 2014). It is still not understood if the expression of these genes plays a role in tumorigenesis or if they are produced after the transformation of normal cells to tumour cells. Since cancer cells share features with placentation or gametogenesis, the C/T genes expressed in gametogenesis may give similar features to cancer cells.

1.4.1.3 CT antigens of interest

Although many different C/T antigens have been studied by various immunologists, with interesting results (Miles *et al*, 2007; Mathieu *et al*, 2009; Linley *et al*, 2012), some novel C/T antigens whose functions and expressions in different normal tissues have not yet been investigated. Fifteen C/T antigens were selected as C/T antigens of interest. These 15 antigens were selected according to their functions and importance in placenta (summarised in Table 1.1).

The techniques for identifying tumour antigens have dramatically changed over the years. The research has advanced from T-cell cloning and SEREX (serological identification of cDNA expression libraries) to more complex methods like representative difference analysis (RDA), differential display (DD), suppression subtractive hybridization (SH) and bioinformatics. Thus, it is difficult to clearly define the C/T antigen products as ‘antigens’, as some of these techniques are only based on mRNA expression and do not depend on the demonstration of immunogenicity.

Table 1.1: C/T antigens of interest for comparative studies in NT and PE placentae.

C/T ANTIGENS	ABBREVIATION	IMPORTANCE
Cancer-associated Gene	CAGE	Involved in Cell cycle
Cancer Antigen 1	CAGE1	Structural role in spermatozoa
Melanoma antigen family A, 1	MAGE A1	Expressed in placenta. Repress p53.
Melanoma antigen family A, 3	MAGE A3	Expressed in placenta.
Melanoma antigen family A, 4	MAGE A4	Expressed in placenta.
Per ARNT Sim domain containing 1	PASD1	Functions as transcription factor.
G antigen 1	GAGE1	Studied at protein level.
P antigen family, member 4 (prostate associated)	GAGE C1/PAGE 4	Expressed in placenta. Target of TGF-β1
P antigen family, member 5 (prostate associated)	GAGE E1/PAGE5	Expressed in placenta.
Fetal and adult testis expressed 1	FATE1	Expressed in placenta. Cell differentiation and proliferation
Helicase Antigen	HAGE	DEAD-box family of ATP-dependent RNA helicases
New-York Esophageal cancer antigen 1	NY-ESO-1	Highly immunogenic tumor antigen
Breast associated unigene cluster 11*	BUC11	Novel antigen
Prostate antigen T128*	T128	Novel prostate cancer associated antigen
Prostate antigen T21*	T21	Novel prostate cancer associated antigen

**antigens identified at John van Geest Cancer Research Centre, NTU*

[Data summarised from Jungbluth *et al*, 2007; Monte *et al*, 2006; Cho *et al*, 2002; Gnjatic *et al*, 2006; Mathieu *et al*, 2009; Miles *et al*, 2007]

Further information on these C/T antigens are given below.

- **CAGE (Cancer-Associated Gene)**

A novel cancer/testis antigen gene, CAGE (cancer-associated gene) was identified by Bomsoo Cho and colleagues using SEREX (Cho *et al*, 2002). CAGE protein was found mostly in the nucleus and was confirmed to be localised on chromosome X (Xp22.11). CAGE is also known as DDX53 as it encodes a DEAD box domain. It was also found to have no introns and exists as a single copy. CAGE protein is approximately 75 kDa (630

amino acids) in size. Among other helicase proteins, CAGE shows homology to p72, p68 and HAGE, out of which HAGE is also a C/T antigen.

- *Expression*

CAGE is expressed in testis and cancer cells with negligible expression in other normal tissues. Demethylation of its promoter activates its transcription, which is increased in tumour cells. However, methylation status does not always directly correlate with increased CAGE expression, suggesting that there are other factors involved in its expression. Although the expression of CAGE was studied in normal tissues by Cho *et al* (2002), placenta was not included in their study.

- *Function*

Proteins containing DEAD box domains are associated with a family of enzymes called helicases. These proteins play a major role in RNA metabolism, spermatogenesis and cellular growth. It has also been reported that CAGE activates the extra-cellular signal-regulated kinases ERK and p38 MAPK, therefore promoting cell motility (Kim *et al*, 2010). CAGE, therefore, enhances invasion potential whilst down-regulating the expression of p53, which hinders apoptosis.

- **CAGE1 (Cancer Antigen 1)**

Cancer antigen 1 (CAGE1) is different from the cancer-associated gene (CAGE). CAGE1 is also known as cancer/testis antigen 95 and cancer/testis antigen 3 (CTAG3/CT3). CAGE1 encodes a long coiled coil domain (Alsheimer *et al*, 2005). No signal peptide was found for the localisation of CAGE1 into the lumen of acrosomes. It is located in chromosome 6 (6p24.3).

- *Expression*

CAGE1 is expressed in cancer tissues and testis. There was nothing in the literature to confirm the expression of CAGE1 in placenta. CAGE1 was seen to be expressed during post-meiotic stages of spermatogenesis.

- *Function*

Due to its coiled coil domains, CAGE1 may function as a structural protein for acrosomes thus enhancing spermatogenesis.

- **Melanoma Antigen (MAGE-A) subfamily**

MAGE-A belongs to the MAGE-I family along with MAGE-B and MAGE-C groups. MAGE-A is the first group of C/T antigens to be cloned successfully by Van der Bruggen *et al* in the early 1990s from a melanoma patient; hence the annotation, melanoma antigen (Sang *et al*, 2011). MAGE-A also forms the largest subfamily with 12 members encoding a common domain (Mage Homology Domain; MHD). MAGE-A genes were found to be expressed in different forms of cancer and restricted expression to testis, ovary and placenta amongst normal tissues. The MAGE-A family forms a locus at q28 of chromosome X and contains one protein-coding exon with several noncoding exons preceding it (Artamonova and Gelfand, 2003).

- **MAGE-A1**

Melanoma antigen-1 (MAGE-1) was the first member of the MAGE-A family to be identified. It was isolated from a MZ-2 human melanoma cell line. It was later defined as MAGE-A1.

- *Expression*

MAGE-A1 is the most frequently expressed C/T antigen. The increase in MAGE-A1 protein was correlated with an increase in the clinical stages of cancer patients (Zhang *et al*, 2010). The MAGE-A1 is activated by DNA methylation.

- *Function*

MAGE-A1 protein is found to repress the transactivation function of p53 (Monte *et al*, 2006). It has also been found that the MAGE-A1 binds to the transcription regulator SKI-interacting protein (SKIP), which is involved in several signaling pathways. It is implicated in cell differentiation during spermatogenesis.

- **MAGE-A3**

MAGE-A3 expression was found to be correlated with elevated levels of plasmocytes proliferation in multiple myeloma patients (Jungbluth *et al*, 2007). MAGE-A3 has also been shown to bind to pro-caspase-2, thus hindering its activation and providing a protection to the cancer cells by inactivating the apoptotic pathway (Sang *et al*, 2011).

- **MAGE-A4**

The carboxyl terminal (107 amino acids in length) of MAGE-A4 was found to induce apoptosis by both p53-dependent and p53-independent mechanisms. Blocking of the up-regulation of p21 by MAGE-A4 leads to the inhibition of the p21-mediated cell cycle arrest.

However, no such changes were observed using intact MAGE-A4 protein (Sang *et al*, 2011). It is therefore believed that intact MAGE-A4 acts more like a tumour suppressor than an onco-protein.

- **Per ARNT Sim domain containing1 (PASD1)**

PASD1 is a diffuse large B-cell lymphoma (DLBCL)-associated C/T antigen thus showing a wide range of expression patterns in hematological malignancies. PASD1 contains a PAS domain, an aryl-hydrocarbon receptor nuclear translocator (ARNT) domain and a nuclear receptor recognition motif (LXXLL) (Campbell *et al*, 2011). Various isoforms of PASD1 have also been reported. PASD1 is also seen to be located on chromosome X position q28 like MAGEA family members.

- *Function*

PASD1 is assumed to act as a transcription factor, but nothing is known about its definite function. Due to the presence of three domains (PASD1, ARNT and LXXLL), it is predicted to mediate responses to environmental stimuli (Campbell *et al*, 2011).

- **GAGE (G melanoma antigen) Family**

G melanoma antigen (GAGE) family consists of 8 members and can be divided into three groups based on different features (Gjerstorff *et al*, 2006). The three groups include GAGE-1; GAGE-2, and -8; and GAGE-3, and -7. These genes encode highly identical acidic proteins (Gjerstorff *et al*, 2008). Like other C/T antigens, transcription of GAGE proteins is correlated with hypo-methylation of their promoters. Immunohistochemistry staining of normal tissues by monoclonal antibodies to GAGE reveals expression in oocytes and in primary spermatocytes (Gjerstorff *et al*, 2006). GAGE-1 is the only C/T antigen showing expression in oocytes of both the resting and mature ovary. GAGE expression is seen during the second trimester. However, its expression is down-regulated when the primary spermatocytes mature to secondary spermatocytes. Due to its localization in the nucleus of spermatocytes, it is seen as a regulator of germ line cells. GAGE genes may inhibit apoptosis, but the functions of GAGE genes are not clearly understood. It is believed that GAGE proteins have evolved recently by positive selection (Gjerstorff *et al*, 2008). This family is rapidly expanding, leading to many paralogous genes.

- **GAGE-1**

Among GAGE genes, only GAGE-1 antigen possesses a unique exon encoded C-terminal. GAGE-1 also lacks a phosphorylated tyrosine (Y9) which is present in other GAGE family

members. GAGE-1 is expressed in melanoma and lung adenocarcinomas. It is located in between p11.23-p11.22 of chromosome X.

- **Prostate Associated Antigen 4 (PAGE-4/ GAGE-C1)**

PAGE-4, also known as GAGE-C1 is a gene belonging to a gene family homologous to GAGE along with five other members (PAGE-1, -2, -2B, -3 and -5). PAGE family genes are small proteins with 102-146 amino acids revealing highly charged/ hydrophilic residues and hydrophobic residues [intrinsically disordered proteins (IDPs)] (Hellman *et al*, 2011). Due to divergence in expansion, PAGE-4 is significantly different from the GAGE family, although they have conserved introns and exons. GAGE and PAGE families show common ancestry, nonetheless the identity is only 55%. PAGE-4/ GAGE-C1 is also located on chromosome X in between p11.23-p11.22 like GAGE-1. It is characterized as a disordered protein with a nuclear localisation signal at its N-terminal.

- *Expression*

In addition to expression in tumour, testis and ovary tissues, PAGE-4 is also found to be expressed in placenta, prostate and uterus (Sampson *et al*, 2007). PAGE-4 is up regulated by TGFβ in prostatic cells. However, the TGFβ-mediated expression of PAGE-4 is not prostate cell-specific, as similar patterns were observed in JEG3 (a choriocarcinoma cell line).

- *Function*

Differences in the function of GAGE and PAGE protein families are expected due to differences in protein structures. The function of PAGE-4 is still not clearly understood. It can bind double stranded DNA (dsDNA). Anti-apoptotic properties have been highlighted recently for PAGE-4 (Sampson *et al*, 2007).

- **Prostate Associated Antigen 5 (PAGE-5/GAGE-E1)**

PAGE-5, also known as GAGE-E1, has recently been studied by NMR with a view to characterizing the protein and its function (Hellman *et al*, 2011). It consists of 130 amino acids, and the monomeric form of the protein is only 11kDa. Like PAGE-4, PAGE-5 is also a highly disordered protein, but with few predominant secondary structures. It is proposed that these secondary structures act as interaction sites for PAGE-5 in cancer and germ cells. PAGE-5 is located at p11.21 on chromosome X.

- **Foetal and Adult Testis Expressed 1 (FATE-1)**

FATE-1 was previously known as BJ-HCC-2 due to its high expression in hepatocellular carcinoma. It is mapped to chromosome Xq28. FATE-1 promoter encodes two binding sites for steroidogenic factor-1 (SF-1), which is a sex determining and differentiation factor.

- *Expression*

Immunohistochemical analysis has revealed high expression of FATE-1 in germ cells and sertoli cells present in the seminiferous tubules (Yang *et al*, 2005). Staining was stronger in spermatogonia and in primary spermatocytes and gradually decreased with maturation of the spermatogonia. This pattern of expression was also seen in the GAGE family. Very faint staining was observed in Langerhans' islet cells with practically no staining being apparent in other normal tissues.

The mRNA expression of FATE-1 was seen in testis and a few normal tissues like kidney, heart, lung and brain (Olesen *et al*, 2011). The expression in placenta was reported to be negative (Dong *et al*, 2003). Like MAGEA4 in transitional cell carcinoma and NY-ESO-1 in melanoma, FATE-1 is expressed in moderate and poorly differentiated HCC.

- *Function*

It has been reported that the expression of FATE-1 is correlated with the expression of the SRY (Sex determining region) gene (Olesen *et al*, 2011). It is thought to play some role in early testicular development. Wilms' tumor gene 1 (WT-1) is another sex-determining factor and seems to be co-expressed with FATE-1. Both SF1 and WT-1 are involved in tumour development thus suggesting a role for FATE-1 in tumorigenesis as well (Olesen *et al*, 2011).

- **Helicase Antigen (HAGE)**

The helicase antigen HAGE was identified by Martelange and colleagues in Boon's laboratory at the Ludwig Cancer Institute, Brussels in the year 2000 (Martelange *et al*, 2000). It was first identified together with sarcoma antigen (SAGE) by using representational difference analysis. HAGE is also known as DDX43. The HAGE gene is not located on the X chromosome, but has been mapped to chromosome 6 (6q12-q13) as is CAGE-1. It encodes for a putative 73 kDa protein (648 amino acids in length). Like CAGE, this protein belongs to the DEAD-box family of ATP-dependent RNA-helicases.

- *Expression*

Expression of HAGE was found to be significantly up-regulated in tumours of various histology with relatively low levels or negative expression in normal tissues apart from testis (Mathieu *et al*, 2009).

- *Function*

RNA helicases are involved in transcription, translation and other RNA functions. Their involvement in cancer development has already been proved, thus suggesting that HAGE expression may be linked with processes favouring tumour growth (Roman-Gomez *et al*, 2007). Hypomethylation of the HAGE gene is correlated with increased expression,

advanced disease and poor prognosis. Thus, HAGE is a potential target for immunotherapy vaccines (Mathieu *et al*, 2010). HAGE is thought to increase cellular proliferation. Studies by Linley *et al* (2012), have revealed that the HAGE gene enhanced expression of NRAS (neuroblastoma RAS oncogene), thus leading to regulation of AKT and ERK phosphorylation. The regulation of this pathway would enhance the cell proliferation in tumour cells.

- **NY-ESO-1**

NY-ESO-1, NY stands for New York City where it was discovered at the Ludwig Institute for Cancer Research and Weill Medical College of Cornell University in 1998 by Chen *et al*; ESO stands for oesophageal cancer (Gnjatic *et al*, 2006). This gene was identified during SEREX (Serological analysis of recombinant tumour complementary DNA (cDNA) expression libraries) analysis of oesophageal cancer. The protein coded by the NY-ESO-1 gene is known as CTAG1 and shows the characteristics of C/T antigens with an expression which is restricted to germ cells and cancer cells. According to the C/T nomenclature, NY-ESO-1 belongs to the CT6 family and according to the MAGE nomenclature, it is named LAGE-2. NY-ESO-1 was mapped at the telomeric end of the Xq28 region of chromosome X (Scanlan *et al*, 2002). NY-ESO-1 is an 18 kDa protein made up of 180 amino acids.

- *Expression*

Using monoclonal antibodies, the restricted expression of NY-ESO-1 in germ cells and cancer cells was confirmed. ES121 determined the expression of NY-ESO-1 exclusively in spermatogonia of the testis and oogonia of the ovary. NY-ESO-1 expression is gradually lost with sperm cell differentiation and shows rare expression in a few somatic tissues from ovary, uterus, placenta and normal breasts (Scanlan *et al*, 2004). NY-ESO-1 is the most frequently expressed amongst all the C/T antigens and in different histological cancer types.

- *Function*

Due to the lack of its homolog in a (non-human) animal model, the study of this gene is difficult and the function of NY-ESO-1 remains unknown. So far, NY-ESO-1 has been shown to be the most immunogenic C/T antigen with the activation of both the humoral and cellular immune responses in patients with tumours expressing this gene (Satie *et al*, 2002; Tureci *et al*, 2006). The antibody response differs depending on the tumour grade in melanoma patients, indicating that NY-ESO-1 can be used as a marker for advanced tumour stages (Goydos *et al*, 2001). For these reasons, NY-ESO-1 has been targeted for developing vaccines for immunotherapy in cancer patients.

- **BUC11**

BUC11 (Breast-associated UniGene cluster 11) is one of the members of an unpublished gene family. BUC6, BUC9, BUC10 are the other members of this family. This gene family was identified by Expressed Sequence Tag (EST) database mining technique at The John van Geest Cancer Research Centre, Nottingham Trent University.

- Expression

The mRNA expression of BUC11 was analysed in various normal and cancerous tissue samples. BUC11 could not be considered as a true C/T antigen, as it was found to be expressed in normal tissues. It was also found not to be tumour-specific as it is not expressed in all tumours. It is expressed at high levels in testicular cancer, 25% in prostate cancer and at low levels in melanomas (Personal communication with Dr M. Mathieu, The John van Geest Cancer Research Centre, Nottingham Trent University). Interestingly, BUC11 mRNA expression was detected in both normal and cancerous breast samples. The expression was seen in 97% of samples. By using a custom-made anti-BUC11 antibody, BUC11 was found to be not associated with breast tumour formation and progression of the disease. Antibodies against BUC11 were detected in 60.6% of the patients. However when *in vitro* silencing/induction were performed, BUC11 was seen to participate in cell proliferation.

- **T128**

T128 is another novel prostate antigen identified by SEREX from prostate cancer patients at The John van Geest Cancer Research Centre, Nottingham Trent University. The expression analysis of this gene depicted over-expression in malignant tissues and prostate cancer tissues, along with expression in testis. When the reactivity of this gene was investigated in patients' sera, the prostate cancer sera was seen to have reacted, but a few samples of normal sera also reacted with this gene. Thus, more studies on the characteristics and immunogenic properties of T128 are required.

- **T21**

Prostate cancer antigen T21 is a novel antigen identified in samples from patients with prostate cancer by modified SEREX by The John van Geest Cancer Research Centre, Nottingham Trent University and has been added to the C/T family (Miles *et al*, 2007). T21 is mapped at chromosome 12q21.33 encoding a protein of 534 amino acids. Another gene called centrosomal protein 290kDa (CEP290) is also located at the same region. Mutations of CEP290 cause Joubert syndrome (a rare genetic disorder that is characterised by the absence or underdevelopment of the cerebellar vermis) and nephronophthisis (an autosomal recessive disorder that starts as chronic tubulo-interstitial nephritis and progress to end-stage

renal failure). Thus, it is not clear whether T21 could be a splice variant of CEP290. DNA sequencing revealed a 694bp sequence with truncated 5′ and 3′ ends (Miles *et al*, 2007).

- *Expression*

T21 is expressed in various cancer tissue types, but is silenced in normal tissues, with the exception of testis and foetal brain. T21 protein levels remain relatively constant throughout the cell cycle. Several forms of cancer have been associated with antibodies against this protein. Antibodies against T21 have been detected in 50% of prostate cancer patients, but not in normal individuals (Miles *et al*, 2012).

- *Function*

Using *in silico* analysis, relevant motifs in T21 gene products were identified, suggesting it to be a transcription factor. However, the localization of the protein in the cell cytoplasm due to the presence of SPAN-X domain suggests that T21 not only acts as a transcription factor, but also participates in the transcription process.

1.4.1.4 The importance of expression of C/T antigens in placenta

Cancer/testis (C/T) antigens have been detected in placenta along with their presence in foetal and adult germ cells. Studies of these genes are scarce in placenta, mostly being related to mRNA expression; and protein expression studies are yet to be fully carried out. Jungbluth *et al* studied the protein levels of C/T antigens in 50 placental samples from different gestational stages and showed a pattern of protein expression that varied for different antigens (Jungbluth *et al*, 2007). No immunity was detected for placental stages older than 32 weeks. C/T antigens were mostly expressed in trophoblast cells which resemble cancer cells, and which are known to express these antigens. However, the expressions of C/T antigens in placenta are not exactly the same to its expression in germ cells of testis. Since placenta resembles tumour, C/T antigens may help in trophoblast development in a similar way to that in tumour formation. The expression of some of the novel genes have been studied in placenta. NY-ESO-1 has been seen in cytotrophoblast and syncytotrophoblast of the placenta (Scanlan *et al*, 2002). The origins of these C/T antigens (whether they are foetal, maternal or common) are not known (Gnjatic *et al*, 2006). HAGE was found to be expressed in placental samples, but the level of expression was shown to be lower than that in testis (Mathieu *et al*, 2010).

1.4.2 Expression of 5-HT receptor subtypes

5-hydroxytryptamine (5-HT), commonly referred to as serotonin, was first identified by Rapport in 1949 (Martin, 1994). Serotonin is a monoamine hormone and acts a neurotransmitter in brain. On the other hand, functions of 5-HT are not limited to the brain, and is also linked to responses in the gut, immune system activation and vaso-activation. The effects of 5-HT on blood vessels and its cardiovascular gastrointestinal responses have

been extensively studied. Almost 99% of 5-HT are synthesized in the enterochromaffin cells (enteroendocrine cells lining the lumen of digestive tract) (Mylecharane, 2009). The majority of this (90%) is stored in the same cells while the remaining 9% is carried by blood platelets. A small amount (around 1%) of serotonin is synthesized and stored in the neurons. The free 5-HT in the plasma ranges between 15-120 nM. During vascular injury, the platelets release serotonin at the site of injury. 5-HT can also activate the RhoA and ERK pathways (Matsusaka and Wakabayashi, 2005). The many aspects of 5-HT are correlated with its interaction with different serotonin receptor subtypes (Section 1.4.2.2) and the different actions of 5-HT can be brought about only by its interactions with the different 5-HT receptors. Therefore, the study of 5-HT receptors has been a target at both academic and pharmaceutical level.

1.4.2.1 Characteristic of 5-HT receptors

The 5-HT receptors are G-protein coupled receptors which span the transmembrane. Except for 5-HT_3 receptor subtype which is a ligand-gated ion channel. The general structure of 5-HT receptors is represented in Figure 1.15 below. The receptor consists of seven α-helices which span the transmembrane (Kroeze and Roth, 2006). These helices form three intercellular loops and three extracellular loops with an extracellular amino (N) terminal and an intracellular carboxyl (C) terminal. The transmembrane is known to bind 5-HT and the intracellular domains link the receptors to various actions. The functions of the extracellular domains are not defined. A review by Raymond *et al* (2001) mentions the presence of cysteine residues on the extracellular domain which form disulphide bonds to confirm the structure of the receptors.

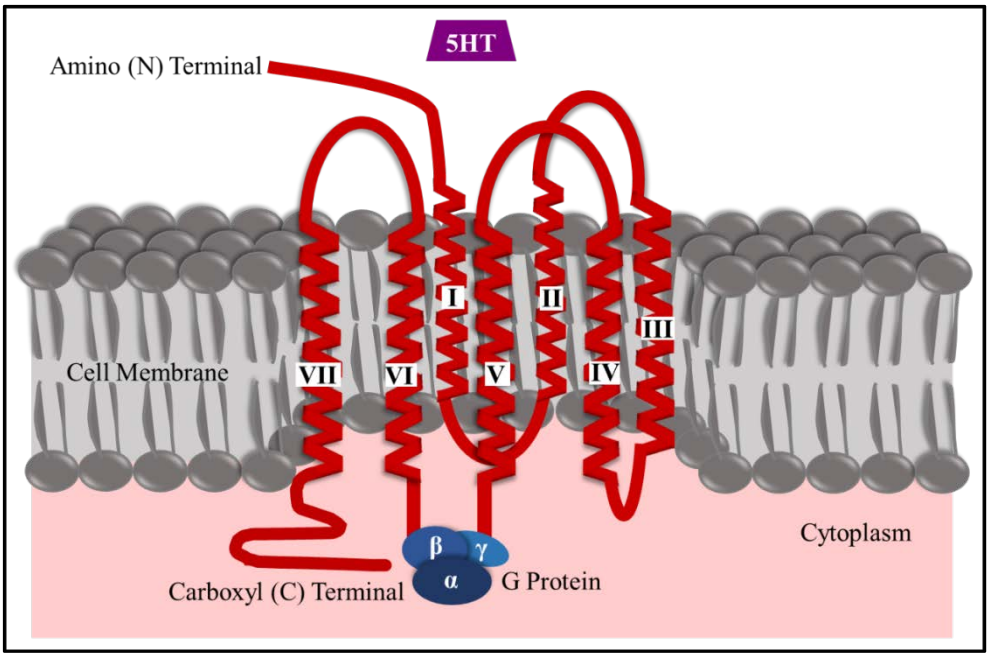


Figure 1.14: General structure of 5-HT receptors.

The G-protein coupled 5-HT receptor has seven α-helices which span the transmembrane beginning with an extracellular amino (N) terminal and an intracellular carboxyl (C) terminal in the cytoplasm. *[Figure adapted from CNS Forum]*

1.4.2.2 Different 5-HT receptor subtypes and their functions

The classification of 5-HT receptors began when Gaddum and Picarelli in 1957 found that the guinea pig ileum was partly blocked by morphine (M) and dibenzyline (D). They classified the receptors into M and D respectively. Since then, the 5-HT receptors have been studied and classified by various researchers and given different nomenclatures.

The Serotonin Club Receptor Nomenclature Committee proposed a system of nomenclature based on operation, structure and transduction information. These rules were then incorporated into the International Union of Basic and Clinical Pharmacology (IUPHAR). The nomenclature of 5-HT receptors followed in this study is incorporated from the IUPHAR database. According to this classification, 5-HT receptors form seven families and some of them divide into subunits. It is difficult to characterise the functions of each 5-HT receptor subtypes. Each receptor has a specific mode of action relying on the proteins they interact with. That is why the structure and functions of these 5-HT receptors are specified in detail below.

1.4.2.3 5-HT receptors of interest

As a result of the multiple interactions of 5-HT receptors and their functions, it was difficult to select just a few receptors of interest. Since there is limited availability of data for the status of 5-HT receptors in human placenta, additional investigations are essential. Therefore all seven families of 5-HT receptors were investigated, at least at the mRNA expressions. Table 1.2 gives a short overview of the functions mediated *via* the receptors.

Table 1.2: 5-HT receptors and their functions, which are of interest in the comparative study in NT and PE placentae.

5HT RECEPTORS	FUNCTIONAL EFFECTS MEDIATED VIA THE RECEPTORS
$5HT_{1A}$	• Secondary messenger responses • Electrophysiological responses • 5HT release • Acetylcholine release • Noradrenaline release
$5HT_{1B}$	• Secondary messenger responses • 5HT1B autoreceptors • 5HT1B heteroreceptors • Physiological responses
$5HT_{1D}$	• Secondary messenger responses • 5HT1D autoreceptors • 5HT1D heteroreceptors • Physiological responses
$5HT_{1E}$	Secondary messenger responses
$5HT_{2A}$	• Secondary messenger responses • Electrophysiological responses • Physiological responses
$5HT_{2B}$	Signal transduction; Involved in mitogenesis and migration
$5HT_{2C}$	• Signal transduction • Electrophysiological responses • Physiological responses
$5HT_{3}$	Physiological responses
$5HT_{4}$	• Transduction system • Interaction with the 5HT4 receptor
$5HT_{5A}$	• Transduction system • Secondary messenger responses
$5HT_{6}$	Transduction system
$5HT_{7}$	• Transduction system • Manipulation of receptor expression

*[*Adopted from *Barnes and Sharp, 1999]*

These receptors are further discussed in depth below.

- **5-HT_1 receptors**

There is a high homology of amino acids in the members of the 5-HT_1 receptor family. The 5-HT_1 receptors have the general seven transmembrane spanning regions which couple to G-proteins (Figure 1.15). There is negative coupling of adenylate cyclase to all of them *via* G-protein.

- ***5-HT_{1A} receptor***

The 5-HT_{1A} receptor has been fully characterised and sequenced. It is a 422-amino acid introns-less sequence located on chromosome 5 (5q11.2-q13) (Barnes and Sharp, 1999).

Functions mediated via 5-HT_{1A} receptor:

At the cellular level, 5-HT_{1A} receptor couples negatively to adenylate cyclase via G-Proteins (α_i). Alterations in intracellular Ca^{2+} and activation of phospholipase C have also been reported in 5-HT_{1A} receptor transfected cell lines. 5-HT_{1A} receptor activation causes hyperpolarisation through the opening of coupled K^+ channels.

The release of 5-HT is hindered by 5-HT_{1A} receptor activation and mediates the influence of 5-HT on both noradrenergic and cholinergic pathways. 5-HT_{1A} receptor activation, on the

other hand, increases the release of acetylcholine. Studies on both animal and human 5-HT_{1A} receptor have concluded that 5-HT_{1A} receptor activation increases the release of hormones such as adrenocorticotropic hormone (ACTH), corticosteroids, prolactin and growth hormones.

- ***5-HT_{1B} receptor***

The gene encoding the human 5-HT_{1B} receptor is located in chromosome 6 (6q13) (Barnes and Sharp, 1999). It has been estimated that the 5-HT_{1B} receptor may be synthesised in certain locations in the brain and then transported to different regions by cell bodies.

Functions mediated via 5-HT_{1B} receptor:

The 5-HT_{1B} receptor couples negatively to adenylate cyclase in transfected cell lines conditioned with forskolin. The 5-HT_{1B} receptor activation inhibits glutamate release, thus inhibiting depolarisation of synaptic nerves. At the nerve terminals, 5-HT_{1B} receptor acts as an autoreceptor, which inhibits the release of 5-HT. As mentioned before, it is speculated that in some regions of the brain, the 5-HT_{1B} receptor is transported to nerve terminals to act as a heteroreceptor. Upon activation, these receptors inhibit the release of acetylcholine (Barnes and Sharp, 1999).

- ***5-HT_{1D} receptor***

It is still a challenge to distinguish between the 5-HT_{1B} receptor and the 5-HT_{1D} receptor due to their sequence homology. In humans, these two receptors are located on different chromosomes (1p34.3-36.3 for 5-HT_{1D} receptor and 6p13 for 5-HT_{1B} receptor). When compared to other 5-HT receptor subtypes, the expression level of 5-HT_{1D} receptor is relatively low.

Functions mediated via 5-HT_{1D} receptor:

Although it was found that 5-HT_{1D} receptor coupled negatively to adenylate cyclase in transfected cells, the same could not be attributed to native tissues (Hamblin and Metcalf, 1991). In fact, the secondary response for 5-HT_{1D} receptor cannot be confirmed at native forms. Also, due to the close homology of 5-HT_{1B} and 5-HT_{1D} receptor and their ligand binding, it is difficult to attribute definite functions to 5-HT_{1D} receptors.

- ***5-HT_{1E} receptor***

The gene encoding the 5-HT_{1E} receptor is located on chromosome 6q14-q15. It is intron-less and encodes a 365 amino acid sequence.

Functions mediated via 5-HT_{1E} receptor:

A moderate inhibition by forskolin conditioned adenylate cyclase via 5-HT_{1E} receptor has been reported. However, very little is known about the mechanisms mediated *via* this receptor.

- ***5-HT_{1F} receptor***

The 5-HT_{1F} receptor is located on chromosome 3p12. It has the general structure of 5-HT receptors with seven transmembrane spanning regions. The physiological responses mediated via 5-HT_{1F} receptor are unclear.

- **5-HT_2 receptor**

The 5-HT_2 receptor comprises three subtypes: 5-HT_{2A}, $_{2B}$ and $_{2C}$. Unlike 5-HT_1 receptors which are intron-less, the 5-HT_2 receptors have introns in their coding sequence (two introns for 5-HT_{2A} and $_{2B}$; three introns for 5-HT_{2C}). These exons couple to phospholipase C and transport intercellular calcium ions (Ca^{2+}).

- ***5-HT_{2A} receptor***

The 5-HT_{2A} receptor encoding gene is located on chromosome 13q14-q21 and consists of sites for phosphorylation, glycosylation and palmitoylation.

Functions mediated via the 5-HT_{2A} receptor:

The 5-HT_{2A} receptor mediates secondary messenger responses by activating phospholipase C through G-protein coupling. This leads to increase in intercellular Ca^{2+} and inositol phosphates. The activation of 5-HT_{2A} receptor also mediates neuron depolarisation and inhibits noradrenaline release, but increases the secretion of ACTH, cortisol and prolactin.

- ***5-HT_{2B} receptor***

The 5-HT_{2B} receptor gene is located on 2q36.3-2q37.1, encoding 481 amino acids sequence.

Functions mediated via 5-HT_{2B} receptor:

5-HT_{2B} receptor activation mediates signal transductions like the other two members of the family and stimulates phosphatidyl inositol hydrolysis. 5-HT_{2B} receptor knockout studies in mouse by Choi *et al* (1997) have revealed mitogenic effects of 5-HT that are mediated via 5-HT_{2B} receptors.

- ***5-HT_{2C} receptor***

Amongst all the other 5-HT receptor subtypes, only 5-HT_{2C} receptor is X-linked, located at Xq24. Multiple isoforms of 5-HT_{2C} receptor have been reported due to mRNA editing and thus each isoform may have different functional consequences.

Functions mediated via 5-HT$_{2C}$ receptor:

At the cellular level, 5-HT$_{2C}$ receptor activation increases phosphatidyl inositol hydrolysis. It also mediates depolarisation of neurons and inhibits release of noradrenaline and dopamine.

- **5-HT$_3$ receptor**

5-HT$_3$ is the only ligand-gated receptor in the 5-HT receptor family. It is encoded by two genes, 5-HT$_{3A}$ and 5-HT$_{3B}$, located in the same locus 11q23.1 (Bockaert *et al*, 2010). These share high homology at the channel domains with the other Cys-Cys loop ligand gated channels like nicotinic, glycine and gamma-aminobutyric acid (GABA$_A$) receptors. Unlike the G-protein coupled receptors, both the N-terminal and C-terminals of 5-HT$_3$ are located outside the cell membrane and contain four transmembrane spanning regions. 5-HT binds to the extracellular domain to open the channel, which becomes permeable to Na^+ and K^+ (See Figure 1.16, Panel A). The native form of 5-HT$_3$ is pentameric (Figure 1.16, Panel B). Only the 5-HT$_{3A}$ subunit (α subunit) is functional and the 5-HT$_{3B}$ (β subunit) binds to 5-HT$_{3A}$ to form channels resembling the native forms. Clones similar to 5-HT$_{3C}$, $_D$ and $_E$ have also been reported, but are not functional (Niesler *et al*, 2007).

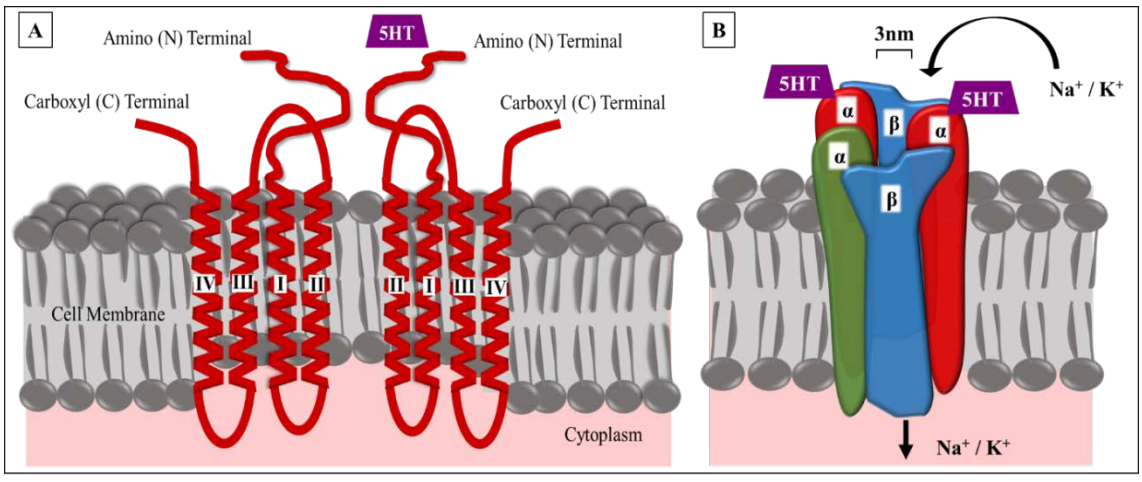


Figure 1.15: Structure of ligand gated 5-HT$_3$ receptor.

[**A**] Two α subunits of 5-HT$_3$ aligned close to one another with their extracellular N and C-terminals and four transmembrane regions. The transmembrane region II of the subunits forms the channel for the gates. [**B**] Both α and β subunits form a pentameric channel. The core is 3nm in diameter. Once the 5-HT ligand binds to the N-terminal extracellular domain, the channel opens up for ion exchange. *[Figure adapted from Nagradova, 2010 and Rammes et al, 2004]*

Functions mediated via 5-HT_3 receptor:

The activation of 5-HT_3 receptor opens the cation channels and the exchange of ions leads to depolarisation of the neurons. The activation of 5-HT_3 receptor also increases the release of some neurochemicals like 5-HT, ACTH, GABA, Cholecystokinin (CCK) and dopamine, but decreases acetylcholine release.

- **5-HT_4 receptor**

The 5-HT_4 receptor is encoded by a highly fragmented gene (at least five introns) located on chromosome 5 (5q31-q33) (Hannon and Hoyer, 2008). Due to the complexity of the gene, various splice variants have been identified. Most of the variants have a consensus sequence on the transmembrane region with an N-terminal glycosylation region and transmembrane and C-terminal phosphorylation regions. The expression of these isoforms has been found to be tissue-specific.

Functions mediated via 5-HT_4 receptor:

The 5-HT_4 receptor binds positively to adenylate cyclase. It is proposed that the 5-HT_4 receptor couples to potassium and voltage-gated channels. 5-HT_4 receptor activation stimulates the release of 5-HT, acetylcholine and dopamine.

- **5-ht_5 receptor**

5-ht_5 is the least understood receptor therefore it retains the small letter nomenclature as recommended by IUPHAR. Also, there is no evidence for expression of native 5-HT_5 receptor. Two subtypes have been identified: 5-ht_{5A} and $_{5B}$. The 5-ht_{5A} gene is located on chromosome 7 (7q36.1) and encodes a protein of 357 amino acids in length with consensus sequences predictive of phosphorylation and glycosylation. The 5-ht_{5B} subtype is located on chromosome 2 (2q11-13). However, due to the presence of stop codons in the sequence, a functional protein cannot be expressed from the 5-ht_{5B} subtype.

Functions mediated via 5-ht_5 receptor:

There is no data available for the functional role of 5-ht_5 in native cells and tissues. Therefore, the predictive functions are based on investigations using transfected cells and knock out models. In cells transfected with recombinant 5-ht_{5A}, it has been reported that the receptor coupled negatively to cAMP via G proteins to inhibit forskolin-stimulated production (Francken *et al*, 1998). Similarly, it was predicted that 5-ht_{5A} could couple to potassium channels.

- **5-HT_6 receptor**

The 5-HT_6 receptor is 440 amino acids long and its gene is located on chromosome 1 (1p35-36). The gene consists of two introns and consensus sequences (on N-terminal for glycosylation and on C-terminal for phosphorylation).

Functions mediated via 5-HT_6 receptor:

The 5-HT_6 receptor binds positively to adenylate cyclase like 5-HT_4 and $_7$. It also stimulates the intercellular accumulation of cAMP.

- **5-HT_7 receptor**

The gene for the 5-HT_7 receptor is located on chromosome 10 (10q21-24). The sequence consists of two introns. The first intron is located on the intracellular loops and the splice variants does not encode functional proteins. The second intron lies on the C-terminal and gives rise to four functional splice variants (5-$HT_{7A, B, C}$ and $_D$). Human tissues can express only three variants 5-HT_{7A} (448 a.a.), 5-HT_{7B} (435 a.a.) and 5-HT_{7D} (479 a.a.). They have the general G-protein coupled receptor structure (Figure 1.15).

Functions mediated via 5-HT_7 receptor:

Due to the variation in the phosphorylation site for each variant their functional role differs. In general, all the variants are capable of coupling and activating adenylate cyclase.

1.4.2.4 Importance of expression of 5-HT receptors in placenta

Researchers have pointed out that 5-HT and its receptors may have a fundamental role in placental development and pregnancy maintenance (Oufkir *et al*, 2010). Bolte *et al* (2001) showed that 5-HT influences the early extravillous trophoblast (EVT) and endothelial layer of the spiral arteries *via* 5-HT_{1B} or 5-HT_{2A} receptors. 5-HT induces several physiological effects, including vasoconstriction or vasodilation, depending upon which member of the 5-HT receptor family it interacts with (See Section 1.4.1.2). Several studies have reported 5-HT-mediated constriction of placental veins and the myometrium; specifically implicating 5-HT_1 and 5-HT_2 receptor (Cruz *et al*, 1998; Cordeaux *et al*, 2008; Ugun-Klusek *et al*, 2011). It is worth noting that circulating 5-HT is increased in PE (Cruz *et al*, 1998). Here, 5-HT is thought to be involved in PE because of its vasoconstrictory effects resulting in compromised blood flow to the placenta. The increased level of 5-HT seen in PE placenta could be due to low levels of its metabolising enzyme, monoamine oxidase (MAO) (Sivasubramaniam *et al*, 2002). Thus, the high levels of 5-HT in PE may lead to extensive vasocontriction and as a result limit the maternal blood supply into the placental circulation, causing it to be at a more hypoxic state.

Due to ethical constraints, it is impossible to study the status and the changes of 5-HT receptor expression in response to increased 5-HT during early stages of human pregnancy. A few studies have looked at the expression profiles of 5-HT receptors *in vitro* using term choriocarcinoma cell lines such as JEG3, JAR and BeWo (Sonier *et al*, 2005). However, there is not enough information on (a) the effects of 5-HT on early trophoblast (EVT) invasion and (b) the changes in the expression profiles of different 5-HT receptors produced by increased 5-HT during this invasion.

1.4.3 Hypoxia and its relationship to invasion

Oxygen is a vital molecule for the survival of multicellular organisms. The oxygen demand varies from organ to organ and depends mostly on their respective function. Hypoxia promotes cell survival in both placenta and tumour through many pathways as shown in Figure 1.17.

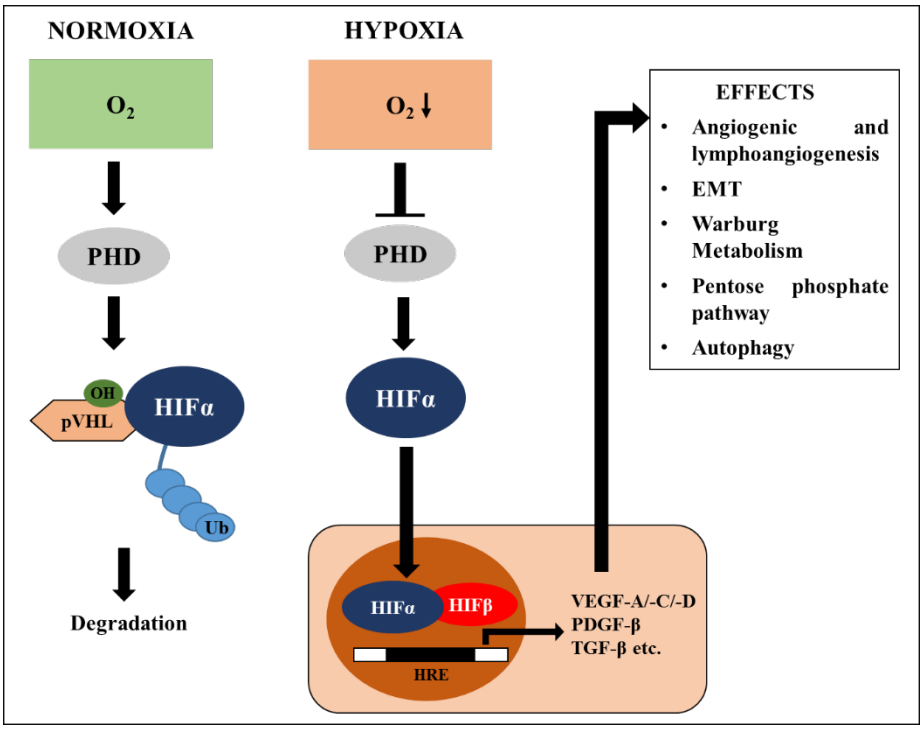


Figure 1.16: HIFα-related pathways under normal and hypoxic conditions.
In normoxic conditions the HIFα subunit undergoes degradation by PHD and pVHL. Under hypoxic conditions, HIFα escapes the hydroxylation and enters the nucleus of the cells. There, the HIFα and β subunits form a complex, which binds to the HRE regions of the genes to be regulated initiating various effects in the cells. *[Figure adapted from Taddei et al, 2013 and Zhang and Zen, 2014]*

Under hypoxic conditions, a range of transcription factors known as hypoxia-inducible factors (HIFs) are key regulators of cell survival. HIF is composed of three α subunits (HIF1α, 2α and 3α) and one β subunit (HIF1β); and their expression increases during hypoxic conditions (Ji, 2014; Zhang and Zen, 2014). In normoxic conditions, the HIFα

subunits are hydroxylated by a proly hydroxylase domain protein (PHD) and are recognised by von Hippel–Lindau protein (pVHL). The α subunit undergoes ubiquitination and degradation. However, when the oxygen levels fall, PHD loses its activity and HIFα becomes more stable. HIF1α then enters the nucleus where it binds to the β subunit to form a fully functioning transcription complex. All the genes targeted by HIF complex have hypoxia response elements (HRE) in their promoters. The binding of HIF complex onto this HRE activates the transcription of various genes involved in metabolism, signalling, proliferation, invasion, migration, apoptosis and angiogenesis.

1.4.3.1 Hypoxia during normal placentation

Although a hypoxic state is detrimental for most organs, placenta, like tumour cells, has the capacity to survive at extreme hypoxic conditions. In fact, the hypoxic condition during early placental development is crucial for embryogenesis. As discussed earlier in Section 1.1.1.3, the EVT cells form a plug on the spiral arteries during the first trimester (10^{th} week) (Figure 1.5). As a result of this, the blood supply along with its oxygen is cut off and a hypoxic environment is created. This hypoxic environment created by the EVT plug protects the developing embryo (and the primitive villi) when the levels of anti-oxidising enzymes (such as mitochondrial superoxide dismutase, glutathione peroxidase and reductase) are limited in foetoplacental unit (Hutter *et al*, 2010). The STB cells start secreting these enzymes only from 9-10^{th} week. At the end of the 10^{th} week, the plug disrupts and there is influx of maternal blood into the IVS. Hence, there is a significant increase in oxygen level during the second trimester in comparison to the first trimester. Burton and Jaunaiux (2001) recorded the oxygen tensions in 30 women who underwent pregnancy termination at 7-16 weeks due to psychosocial conditions. It was recorded that the peripheral oxygen tension in the maternal arteries was constant throughout. However the pO_2 concentrations in decidua increased from 64.3 mmHg (7-10 weeks) to 73.2 mmHg (11-16 weeks). Likewise, there was a two-fold increase of pO_2 in the IVS, from 25.6 mmHg (2-3% O_2) to 56.2 mmHg (~8% O_2).

The HIF pathway plays a crucial part in placental development. The genes regulated by HIF are classified as (a) those whose expression would help to maintain the energy levels for trophoblast under hypoxic conditions and (b) those which would lead to differentiation and invasion of trophoblasts. HIFα and β subunits, under hypoxic conditions, regulate the expression of these genes. The functional complex of HIF stimulates the Warburg metabolism by which oxygen use to produce ATP at the end of glycolysis is avoided. It is worth noting that HIF-knockout mice resulted in severe placental damage (Fryer and Simon, 2006).

The effects of hypoxia on trophoblast differentiation and invasion remain, however, debatable. Seminal studies confirmed that trophoblasts proliferate under hypoxic conditions and differentiate to become invasive at high oxygen levels (Caniggia *et al*, 2000; Esterman *et al*, 1997; Kilburn *et al*, 2000; Genbacev *et al*, 1996). Conversely, another group of investigators noticed that hypoxic conditioning improved trophoblast differentiation and invasion (Graham *et al*, 2000; James *et al*, 2006). These disputes may be due to the different experimental designs and trophoblast samples used and also because of the complex network of pathways connected to hypoxia.

Hypoxia-induced autophagy has also been determined to maintain the EVT functions and induce invasion (Saito and Nakashima, 2013). Autophagy is a process where the cells undergo self-digestion when the nutrient and energy supply is limited. The proteins and cytoplasmic contents of the dying cells accumulate in autophagosomes for degradation. The breakdown of these autophagosomes generates ATP to maintain the energy supply of the trophoblast (and tumour) cells even under low nutrient conditions.

Oxygen levels are also very important in the remodelling of spiral arteries (Cartwright *et al*, 2007). The iCTB cells are in a hypoxic state when they invade the placental decidua. Thus, they cannot produce pro-apoptotic factors [e.g. TNF-related apoptosis-inducing-ligand (TRAIL)], which could be detrimental to the surrounding cells. When the iCTB reaches the spiral arteries, it receives oxygen from the maternal blood which triggers the pro-apoptotic factors to express. These factors will induce apoptosis to the vessel cell lining the spiral arteries and help in the remodelling to enhance the blood supply (see also Section 1.1.1.2).

- **Hypoxia and PE**

Kingdom and Kaufmann have classified hypoxia in pregnancy into three categories:

(a) *Pre-placental hypoxia*: the mother and foetus are in an hypoxic state (high altitude)

(b) *Utero-placental hypoxia*: the oxygen level in the mother is normal, but the level in the foetus is reduced (e.g. PE)

(c) *Post-placental hypoxia*: hypoxia occurs only in the foetus

The utero-placental hypoxic conditions (in PE) are discussed in detail as follows.

As seen in Figure 1.10 and Section 1.1.5, in PE, the iCTB and eCTB do not completely remodel the spiral arteries. As a result of this, the diameter of the arteries is reduced leading to insufficient flow of maternal blood into the IVS. Consequently, the oxygen levels are lower even in the second trimester. There has been no direct measurement of oxygen tension recorded *in vivo* during PE. Due to the narrow arteries, there is reduced blood flow into the placental lobules, but the foetal blood keeps on extracting the oxygen in the IVS. When the

maternal blood flow is restored, there is a sharp increase in oxygen tension. This fluctuation in the oxygen tension indicates an ischaemia / reperfusion kind of injury (H/R injury) (Hung and Burton, 2006; Cartwright *et al*, 2007). The formation of free radicals during H/R injury is detrimental to placenta. There are very little to no oxygen molecules available to receive the free electrons from the respiratory chain. The accumulation of electrons forms superoxide, which leak to any oxygen available around them. However, if oxygen supply is restored before the cells die, then there is elevated production of superoxide.

1.4.3.2 Hypoxia in cancer

The rapid proliferation of tumour cells demands a profuse supply of blood with oxygen and nutrients. Tumours develop their own vasculature from the existing host blood vessels. This vascular formation cannot keep pace with the tumour cell proliferation resulting in primitive leaky vessels, as a result of which a hypoxic and highly acidic tumour microenvironment is created. The partial pressure of oxygen (pO_2) measured at the tumour microenvironment (TME) is around 2 mm Hg (0.3%) to 15 mm Hg (2.1%) depending on the tumour type (Byrne *et al*, 2014). Hypoxia plays a particularly important role in solid tumours. In hypoxic conditions, tumour cells become quiescent and secrete factors, which increase both vessel formation and survival rate of the tumour. If hypoxia is prolonged, tumour cells undergo apoptosis like normal cells. The tumour cell death through prolonged hypoxia have been investigated for cancer therapy (Alarcon *et al*, 2004; Byrne *et al*, 2014).

HIF up-regulates the expression of growth factors like VEGF and cytokines to enhance angiogenesis and lymphoangiogenesis (Figure 1.17). Hypoxia also plays a major role in the transition of epithelial cells to a mesenchymal state [epithelial-mesenchymal transition (EMT)] (Brahimi-Horn *et al*, 2011). EMT cells have stem-like properties and can invade surrounding tissues giving rise to tumour cells. Furthermore tumour cells, like early trophoblasts, follow Warburg metabolism under hypoxic conditions. NADPH levels are also elevated due to the anabolism of the pentose phosphate pathway (PPP), which leads to the synthesis of DNA and ribose to overcome the oxidative stress. As stated in Section 1.1.1, hypoxia also maintains the energy supply by generating ATP through autophagy.

Due to all the above mechanisms, tumour cells flourish better under hypoxic conditions. In fact hypoxia, a process which should be killing the cells, is actually enhancing their growth. Studies on both *in vitro* cell culture (Chinese Hamster cells) and *in vivo* tumour SCCVII carcinoma radiated under normal and hypoxic conditions have reported differences in cell survival (Nordsmark *et al*, 2014). Tumour cells under hypoxic conditions were shown to survive much higher doses of radiation. Hypoxia reduces the response of tumour cells to

radiotherapy. Therefore understanding the hypoxic regulation of the genes is the prime target for cancer therapy.

1.5 Aims and hypothesis

Literature review and preliminary results indicated differential gene expression patterns in normal and PE placentae. 5-HT have also been demonstrated to be involved in proliferation, migration and invasion of various cells. Increased 5-HT levels correlates to PE and activation of various signalling pathways through 5-HT receptors. Furthermore due to resemblance of placental cells to tumour, C/T antigens are also expressed in placenta.

Therefore, it was hypothesised:

- That the expression patterns of 5-HT receptors and C/T antigens in normal and PE placentae are different.
- The expression patterns also differ at different gestational stages. Thus, the first trimester placentae demonstrate differences in 5-HT receptors and C/T antigens when compared to normal and PE placentae.
- 5-HT is hypothesised to influence the behaviour of trophoblast cells; such as viability, proliferation, migration and invasion. Since trophoblast cells are habituated to low oxygen levels, the effects of 5-HT on these cells would also alter under hypoxic conditions.
- These changes in cell behaviour under 5-HT influence is mediated by altered expression of 5-HT receptors and C/T antigens.

This study aims to:

- Establish the status and the regional expressions of (a) all 5-HT receptor subtypes and (b) C/T antigens in pre-eclamptic (PE) placentae in comparison with normotensive (NT).
- Analyse the expression of 5-HT receptors and C/T antigens in the placentae from first trimester pregnancies.
- Investigate the effects of 5-HT on the cell behaviour of transformed early first trimester placental cells in relation to choriocarcinoma (cancer) cell lines under normoxic and hypoxic environment.
- Elucidate the effects of 5-HT on the expressions of C/T antigens in placental cell lines under normal and hypoxic conditions.

Chapter 2
Materials and Methods

2.1 Buffers and solutions

Table 2.1: Buffers and Solution

Reagent	Recipe
PBS 1X	10 Phosphate Buffered-Saline tablets (Fisher Scientific) in 1 litre ddH_2O
PBS-Tween20 (PBS-T)	1 litre 1X PBS, 5ml Tween 20
TBS 10X	24.23g Trizma HCl, 80.06g NaCl, 1 litre ddH_2O, pH 7.6
TBS-Tween20 (TBS-T)	1 litre 1X TBS, 5ml Tween20
Running Buffer 5X	15 g Tris Base, 72 g Glycine, 5.0 g SDS, 1 litre ddH_2O, pH 8.3.
Transfer Buffer 10X	60.4g Tris Base, 288g Glycine, 1.8 litres ddH_2O. Methanol added to 1X transfer buffer before use.
TAE Buffer 50X	242 g Tris Base, 57.1ml Acetic Acid, 100 ml 0.5M EDTA, 1 litre ddH_2O, pH to 8.5
Laemmli Buffer 4X	200mM Tris-HCl pH6.8, 400mM DTT, 0.8% w/v SDS, 0.4% w/v Bromophenol blue, 40% v/v glycerol, 5% v/v β- mercaptoethanol
Antibody Buffer	3% BSA, TBS-T, 0.02% Sodium Azide

2.2 Antibodies

Primary antibodies	**Catalogue Number and Supplier**	**Antibody dilutions**
CT Antigens		
Rabbit Anti-CAGE1	HPA029830 Sigma	IHC (1:200); IF (1:200); WB (1:1000)
Rabbit Anti-FATE1	HPA034604 Sigma	IHC (1:350); IF (1:350); WB (1:1000)
Rabbit Anti-GAGE1	H0002543-B01P Novus	IHC (1:300); WB (1:500)
Rabbit Anti-GAGEE1 (PAGE5)	sc-138503 Santa cruz	IHC (1:250); IF (1:250); WB (1:500)
Rabbit Anti-MAGEA1	AB21472 abcam	IHC (1:100); IF (1:100); WB (1:500)
Rabbit Anti-MAGEA4	LF-MA10178 AbFrontier	IHC (1:100); IF (1:100); WB (1:1000)
Rabbit Anti-PASD1	HPA011122 Sigma	IHC (1:250); IF (1:250); WB (1:500-Not detected)
5-HT Receptors		
Rabbit Anti-Serotonin 5-HT_{1B} Receptor	Sigma (IHC), abcam (WB)	IHC (1:200); IF (1:200); WB (1:500)

Rabbit Anti-Serotonin 5-HT_{2B} Receptor	Sigma (IHC), biorbyt (WB)	IHC (1:200); IF (1:200); WB (1:500-Not detected)
Controls		
Rabbit Anti-ACTB (loading control for WB)	AV40173 Sigma	WB (1:1000)
Mouse Anti-Vimentin (positive control for IHC)	NCL-L-VIM-572 Leica	IHC (1:400); IF (1:400)
Secondary Antibodies	**Catalogue Number and Supplier**	
Goat Anti-Rabbit Human IgG peroxidase conjugated	A0545 Sigma	WB (1:10,000)
Donkey Anti-Rabbit Human IgG H+L (HRP) conjugated	ab6802 abcam	WB (1:3000)
Fluorescein Anti-Rabbit IgG (H+L)	Vector Laboratories, Inc	WB (1:1000)
Biotinylated Anti-Mouse/Rabbit IgG provided with VECTASTAIN Elite ABC Kit	PK-6200 Vector Laboratories, Inc	IHC (1:10,000)

IHC-Immunohistochemistry; WB-Western blotting; IF- immunofluorescence

2.3 Human placental collection

Human placental tissue samples were previously collected by the Director of Studies (Dr. Sivasubramaniam) in accordance with the ethical approval by the Nottingham City Hospital Ethical Committee and stored at -80°C. Some of them were further preserved by paraformaldehyde fixation and embedded in paraffin for immunostaining protocols (see Section 2.9.1). The Ethical approval was extended to suit this study by the Health Research Authority – NRES Committee North East (REC: 12/NE/0112; See Appendix A.1); their demographic details are given in Section 3.2.1. First trimester placental explants and protein lysates were kindly provided by Prof. Berthold Huppertz, Medical University of Graz, Austria. These samples were collected under the local ethical guidance of the Medical University of Graz and stored at -80°C until further experimentation. Demographical details are given in Section 4.2.1.

2.4 Total RNA extraction

Total RNA was extracted from the placental samples using commercially available RNeasy® Plus Mini RNA extraction kit (Qiagen) to prepare cDNA templates for PCR amplification (Section 2.6.2). The samples were homogenized in liquid nitrogen and weighed in pre-cooled centrifuge tubes. Then 600µl of RLT buffer plus 6µl β-mercaptoethanol (Sigma Aldrich) was added to the sample. This homogenized lysate was transferred into a QIAshredder®

(Qiagen) and centrifuged at maximum speed (15,000 x *g*) for 2 minutes in microcentrifuge (Microcentaur Sanyo). The supernatant was transferred into a gDNA eliminator spin column. The column was centrifuged again at 10,000 x *g* for 30 seconds. The supernatant was mixed with 600µl 70% v/v ethanol (Fisher Scientific). This mixture was transferred to an RNeasy spin column placed in a collection tube. Centrifugation was repeated at 10,000 x *g* for 15 seconds. RNeasy spin column was retained and 700µl RW1 buffer was added and centrifuged again at 10,000 x *g* for 15 seconds. This was followed by 2x 500µl RPE buffer washes. Lastly the column was treated with 50µl RNase-free Millipore water and centrifuged at 10,000 x *g* for 1 minute. The supernatant was collected and stored at -80°C.

Due to the limited availability of first term placental samples, an AllPrep RNA/Protein Extraction kit® (Qiagen) was used. The kit allowed the simultaneous extraction of RNA and protein from same sample. The fresh first term placental samples were homogenised in APL buffer supplied in the kit and centrifuged in an All Prep column for 1 minute at ≥8,000 x *g*. The flow through was pipetted onto a Protein Cleanup spin column and centrifuged for 3 minutes at 240 x *g*. This flow through contains the protein and was further purified using ice-cold acetone (Fisher Scientific). The concentrations of purified protein samples were then estimated using the BCA assay, as detailed in Section 2.7.2. The AllPrep column was treated exactly as RNeasy spin column above. For quality control purposes, extracted RNA samples were electrophoresed in 1% w/v agarose gel (BIOzym group) to fractionate the two RNA subunits, 28S and 18S. New England, BioLabs® Inc 1kb ladder was used to identify the two subunits. The RNA samples extracted were also quantified using a Nanodrop® spectrophotometer.

2.5 cDNA template preparation by reverse transcription

A reverse transcription reaction was carried out using extracted RNA samples. SuperScript™ II Reverse Transcriptase kit (Invitrogen™) was used for this reaction. A 20µl reaction volume was prepared. The following 20µl reaction volume was used for total RNA concentrations of 10pg-5µg. The reaction mix contained the appropriate volume of RNA extracted and nuclease-free water, 1 µl Oligo (dT) 15 Primer (Promega), 1µl dNTP (Promega) and 1µl RNAsin (Promega) and heated to 65°C for 5 minutes followed by chilling on ice. 4µl of 5X First-Strand Buffer and 2µl of 0.1M DTT was added to the reaction and incubated at 42°C for 2 minutes. After this incubation, 1µl of SuperScript™ II Reverse Transcriptase was added and allowed to incubate at 42°C for 50 minutes. cDNA was also produced from commercially available human testis and brain total RNA (Clontech) as controls. This was used as a positive control in qRT-PCR. The cDNA produced was stored at -4°C.

2.6 Gene expression study

2.6.1 Primer designing and optimising

Coding sequences (CDS) for the genes of interest were selected from National Centre for Biotechnology Information (NCBI) website (**www**[7]). CDS were used to design the primers using Primer 3® Input version 4 (**www**[8]) and primer properties were checked by Integrated DNA Tool (**www**[9]). Common nucleotide regions for the genes with variants were selected using the ClustalW2 (**www**[10]). These common regions were then used to design the primers. The primer sequences were then checked for their specificity using BLAST (**www**[11]) and finalised.

Primers were selected according to the following primer designing guidelines:

1) **Sequence:**
   a) The following were avoided: (i) 3 or more runs of G or C at 3´ end, (ii) a T at the 3´ end, (iii) mismatches at the 3´end and (d) complementary sequences.
   b) 3´ end should end in G or C or CG or GC. This will increase the efficiency of priming.
2) **Length:** 18-30 nucleotides
3) **GC Content:** 40-60%
4) **Melting Temperature (T_{ms}) :** 55-80˚C
5) **Primer Concentration:** 0.1 and 0.5µM
6) **Product size** is less than 200 bps

The primer sequences with their annealing temperatures are given in Table 3.2. The annealing temperatures for the primers were first calculated using the following equations:

$$\boldsymbol{T_m} = 4\,(G + C) + 2\,(A + T)$$

$$\boldsymbol{T_A} = \frac{T_m\ Forward + T_m\ Reverse}{2} - 5℃$$

Where $\boldsymbol{T_m}$ is melting temperature for the primers, $\boldsymbol{T_A}$ is the annealing temperature for the gene of interest.

2.6.2 Gene amplification using conventional PCR and gradient PCR

Genes of interest were amplified using the primers designed in Section 2.6.1 by conventional TECHNE® Thermocycler TC3000 (Bibby Scientific Ltd.). PCR Master Mix from Promega was used for the reaction mixture. The reaction mixture was prepared according to company guidelines.

- **Reaction Mixture for 25µl**

Components	Volume	Final Concentration
PCR Master Mix, 2X	12.5µl	1X
Upstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
Downstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	25µl	N.A.

- **General program followed for PCR was as follows:**
  - Initial Denaturation at 95°C for 2 minutes
    - Denaturation at 95°C for 30 seconds
    - Annealing at X°C for 30 seconds
    - Extension at 72°C for 30 seconds

    } x 40 cycles
  - Final Extension at 72°C for 2 minutes

Note: Where X°C is the $\boldsymbol{T_A}$ calculated in Section 2.6.1.

TECHNE® ^{3}Prime temperature gradient PCR (Geneflow Ltd.) was also used to amplify genes for which the calculated $\boldsymbol{T_A}$ showed low/no products from genomic DNA. The PCR product sizes were then analysed on 1.5% w/v agarose gel using a 100bp ladder from New England Biolabs® Inc.

2.6.3 Agarose gel electrophoresis

The desired percentage of agarose gel was prepared in fresh 1X TAE buffer (1.5% and 1% w/v agarose gels were used to analyse PCR products and to detect the integrity of the RNA samples respectively). GelRed™ nucleic acid stain (Biotium Inc.) was used as a detection dye in the gel. The gel was electrophoresed at 50 Volts for 15 minutes followed by 80 volts for 30 minutes which were then imaged on the Gel Doc (InGenius, Syngene) under UV light.

2.6.4 Confirmation of the PCR products by sequencing

The PCR products were cleaned using Wizard® Genomic DNA Purification kit (Promega) according to manufacturers' guidelines. An equal volume of membrane binding solution was mixed with the PCR products. The mixture was then transferred into a minicolumn assembly and incubated for 1 minute at room temperature. The minicolumn was centrifuged at 16,000 x *g* for 1 minute; washed twice with membrane wash solution and centrifuged again at 16,000 x *g* for 1 minute. Purified DNA was eluted out from the column using nuclease-free water. The products were then sent for sequencing to the Functional Genomics, Proteomics and

Metabolomics Facility in University of Birmingham for confirmation. The sequences were analysed using SnapGene software for accuracy.

2.6.5 Relative expression of genes of interest by Real-Time quantitative PCR

Genes of interest were also amplified by the Corbett® quantitative PCR (qPCR) thermo cycler (Corbett Lifesciences). A reaction mixture of 12.5µl was prepared containing 6.75µl SYBR green mix (Biorad Laboratories, Inc.), 4.25µl millipore water, 0.5µl Forward Primer, 0.5µl Reverse Primer and 0.5µl cDNA template. The primer concentration was made to 10 pM/µl. All the reactions were prepared in duplicates. Their calculated annealing temperatures are given in Table 3.2. The melting curve for the genes were analysed after the reaction using Rotor Gene-6000 Series 1.7 software and the delta Ct values ($2^{-\Delta\Delta Ct}$) were calculated. $2^{-\Delta\Delta Ct}$ is a method to calculate relative quantitative expressions by comparing Ct values (Livak and Schmittgen, 2001). The amount of target is normalised to endogenous reference gene (house-keeling gene) and relative to calibrator (positive control). The house-keeping genes used in this study are GAPDH, HPRT1 and TBP1; primers for these can be found in Table 3.2. Calibrator for C/T antigen expression was testis and for 5-HT receptor was brain. All real time PCR experiments were performed twice.

2.7 Protein expression studies

2.7.1 Tissue lysate preparation for protein analysis

Placental samples were homogenised in liquid nitrogen and transferred into centrifuge tubes. RIPA buffer (10 ml) (Sigma Aldrich) was added to approximately 1g tissue powder for lysis. Protease inhibitor cocktail (Roche) 0.2% v/v and 1mM Na_3VO_4 (Sigma Aldrich) were added to the samples. Samples were vortexed on ice for 40 minutes. After incubation, the tubes were centrifuged at 27,000 x *g* for 20 minutes at 4°C (Harrier 15/80 refrigerated centrifuge, Sanyo). The supernatant (i.e. the native form of the tissue lysate) was collected and stored at -80°C.

2.7.2 Estimation of protein concentration by Bicinchoninic Acid (BCA) Assay

The Bicinchoninic Acid (BCA) assay (Sigma Aldrich) was performed according to the manufacturers' guidelines to estimate the protein concentrations in the tissue lysates prepared in Section 2.7.1. The BCA working reagent was prepared by mixing 50 parts of reagent A (BCA) with 1 part of reagent B (4% w/v Copper (II) sulphate solution; Sigma Aldrich). BSA (Bovine Serum Albumin) (Fisher Scientific), in concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml was used to obtain a standard curve. The placental tissue lysates, BSA

standards and blank (RIPA buffer) were loaded into a 96 well plate and mixed with 8 parts of BCA working reagent. The plates were sealed and incubated at 37°C for 30 minutes and cooled down for 5 minutes. Finally, the absorbance was read at 570nm by using a microplate reader (Biochrom Asys UVM340) and concentrations were calculated from the standard curve.

2.7.3 Protein separation by SDS-PAGE

Protein samples were denatured for SDS-PAGE separation by adding Laemmli Buffer (4X) at 70-80˚C for 10 minutes on a heating block. Equal concentrations of the protein samples were loaded into 10% or 12.5% w/v SDS-PAGE along with molecular weight markers (Biorad Laboratories, Inc.). ProtoGel® 30% w/v, ProtoGel® 4X Resolving and ProtoGel® stacking buffers were used to prepare SDS-PAGE gels according to manufacturers' guidelines (National diagnostics). The SDS-PAGE was run for 50 volts for 15 minutes followed by 80 volts for 90 minutes in running buffer. Finally, they were visualised by staining with Coomassie Brilliant Blue (BioRad Laboratories, Inc.).

2.7.4 Protein analysis by immunoblotting

Immunoblotting was carried out by transferring separated proteins from SDS-PAGE onto 0.2µm nitrocellulose paper (Sigma Aldrich) by wet-transfer over-night in cold room. The nitrocellulose membrane was incubated in blocking solution (3% w/v BSA in TBS-Tween20) for 1 hour. After incubation, the membrane was washed three times with Millipore water followed by TBS-Tween20 wash. The membrane was then incubated in primary antibody, at dilutions 1:500-1:1000, overnight on a shaker at 4ºC. Following incubation the membrane was washed and incubated in secondary antibody (1:10,000 dilution) for 1-2 hours. Following this incubation the membrane was washed by TBS-Tween20, three times for 5 minutes each, to remove excess secondary antibody and developed using RapidStep™ ECL Reagent (Calbiochem®) or Westar Supernova for weak signal (Cyanagen Srl).

The image capture was carried out by using a Gel Doc, InGenius (Syngene) and Gel Doc™ XR+ System (BioRad Laboratories, Inc.). Antibodies were prepared at appropriate dilutions using antibody buffer.

2.7.5 Analysis of western blot results

New GeneSys Image analyser software was used for blots captured by InGenius and Image lab software was used for blots captured by the BioRad imaging system. The expression levels of the genes of interest were normalised against the house keeping proteins (β-actin). After normalisation with β-actin, the expression levels in each sample were compared to the

expression levels in positive controls (testis for C/T antigens and brain for 5-HT receptors). Human brain and testis protein medley were obtained from Clontech. Thus, the expression levels in positive controls are arbitrarily set at 1.

2.8 *In vitro* cell culture

2.8.1 Maintenance of cell lines

All cell lines, specifications and culture media are mentioned in Table 2.2. The growth media were supplemented by 10% v/v FBS (foetal bovine serum) (Lonza); 2mM L-Glutamine (Lonza) and 25 U/ml penicillin/streptomycin (Lonza). Cells were initially grown in T25 flasks (Sarstedt); incubated at 37˚C in a humidified atmosphere of 95% v/v air/5% v/v CO_2. Daily checks were performed and the cell lines were sub-cultured when they reached 70-95% confluence.

Table 2.2: Cell Lines

Cell line	Cell type	Provider	Culture media
TEV-1	Human first-trimester extravillous trophoblast	Dr Mei Choi Choey University of Hong Kong, China	RPMI-1640
HTR-8/SVneo	Human first-trimester villous trophoblast transfected with Simian Virus 40 large T antigen	Dr Charles Graham University of Kingston, Canada	RPMI-1640
JEG3	Human placenta choriocarcinoma	ECACC*	EMEM
BeWo	Human placenta choriocarcinoma	ECACC	Ham's F12
HT1080	Human fibrosarcoma	ECACC	EMEM

*European Collection of Cell Cultures

Sub-culturing was carried out by first aspirating the growth media and washing with respective wash media. Cells were detached from the flasks by adding 2.5% v/v trypsin (Lonza). The flask was replaced back into the incubator for 5-10 minutes. An inverted light microscope (Motic AE2000, Scientific Laboratory Supplies Ltd.) was used to check detachment of the cells; the flasks were gently tapped at the sides to improve detachment. Then respective growth media were added to inactivate the trypsin. The cells were collected into Sterilin® tubes (Sarstedt) and centrifuged at 1,000 x *g* for 5 minutes in Harrier 15/80 centrifuge (Sanyo). The supernatant was decanted and the cell pellets were re-suspended in 1ml of growth media. Viable cells were counted by using 0.4% v/v trypan blue (Sigma Aldrich). 100µl of cell suspension was mixed with 300µl trypan blue. A small volume (10µl)

of this mix was used to count in a haemocytometer. The cell numbers in four large grids were counted to estimate the total cell count using the following calculation.

$$\boldsymbol{Cell\ Numbers/ml} = Average\ cell\ number \times 10^4 \times 4\ (dilution\ factor)$$

These cells were either bulked up into T75/T175 flasks (Sarstedt), used for further experiments or frozen down for future use.

2.8.2 Cryopreservation of cells

For continuous supply of cell lines, cells were cryopreserved in liquid nitrogen. The cell pellet after centrifugation was re-suspended in freezing media (Media 6.4 ml, Penicillin/ Streptomycin 0.1 ml, FBS 2.5 ml, DMSO 1.0 ml). The cells with freeze media were then transferred to cryovials placed on ice and immediately transferred to -80˚C freezer. The following day the cryovials were then transferred into liquid nitrogen for extended storage.

2.8.3 Revival of cryopreserved cells

Cryovials containing the cells were removed from liquid nitrogen and immediately thawed in warm water. The cells were then transferred into Sterilin® tubes (Sarstedt) containing 5ml of fresh growth media and mixed thoroughly. The tubes were then centrifuged at 1,000 x g for 5 minutes. The pellets were then re-suspended in 1ml growth media and grown in T25 flasks. The flasks were maintained as in Section 2.8.1.

2.8.4 Determining cell seeding density by MTT Assay

The correct cell seeding density was determined by performing cell proliferation estimations using an MTT assay. MTT assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) to violet formazan crystals by both mitochondrial and extra-mitochondrial dehydrogenases (Cobb, 2013; Liu *et al*, 1997). Viable and proliferating cells contain higher ratios of NADH/NAD ratios which acts as intermediates during MTT reduction. Likewise succinate and NADH-linked mitochondrial substrates (malate, pyruvate or glutamate) support the MTT reduction (Slater *et al*, 1963). Thus the formazan formation is the direct representation of viable cells and determines the optimum cell seeding density. Different cell densities (serial dilution from 10 to 0.3125x 10^4) were transferred into 24 well plates and grown for 24, 48 and 72 hours. Growth media were removed from the wells and 50µl of 5mg/ml MTT solution (Sigma Aldrich) was added to the wells, followed by incubation for an hour. After incubation 500µl DMSO (Sigma Aldrich) was added to the plates, which were then gently shook in a plate shaker to dissolve

the crystals. A small volume of this solution (200 µl) was transferred into a 96 well plate and the absorbance was read at 570 nm. This experiment was performed for all the cell lines and repeated for three different passage numbers .

2.8.5 Estimation of 5-HT cytotoxicity by MTT Assay

The cell seeding density determined in Section 2.8.4 ($5x10^4$ for placental cell lines and $2.5x10^4$ for HT1080) was used to grow cells in 96 well plates. In one set of experiments, the cells were grown along with different doses of 5-HT (Sigma Aldrich) for 24 and 48 hours (i.e. cells were plated in the presence of 5-HT). In another set of experiments, the cells were treated with 5-HT after 24 hours of plating for 24 and 48 hours duration (i.e. 5-HT treatment was after cell attachment). After treatment, MTT assays were performed, following the same principle as in Section 2.8.4. The growth media was removed and 25µl 5mg/ml MTT was added to the wells and incubated for an hour. DMSO (250µl) was then added and plates were gently shaken in a plate shaker. Then 200µl of the solution was transferred into a fresh 96 well plate for reading and absorbance was read at 570 nm using a plate reader. This experiment was performed for all the cell lines and repeated for three different passage numbers.

2.8.6 Estimation of 5-HT cytotoxicity by CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega)

The CytoTox 96® Non-Radioactive Cytotoxicity assay on the other hand measures the release of lactate dehydrogenase (LDH) from the disintegrated membrane of unviable cells (Cobb, 2013). The LDH then converts lactate to pyruvate with reduction of NAD^+ to $NADH/H^+$. The H^+ from $NADH/H^+$ is transferred to INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) which reduces it to a red formazan dye. Thus CytoTox 96® Non-Radioactive Cytotoxicity assay represents the cell death exclusively. The 96 well plates were seeded with $5x10^4$ for placental cell lines and $2.5x10^4$ for HT1080 cells. An additional set of wells were plated for Maximum LDH Release (positive) Control. The assay was performed according to the manufacturers' guidelines. After 5-HT treatment, Lysis (10X) Solution was added to the maximum LDH release control wells. 45 minutes later, the plates were centrifuged at 250 x *g* for 4 minutes. After centrifugation 50µl of the supernatant from each well was mixed with 50µl of substrate mix and incubated for 30 minutes on a shaker, protected from light. After 30 minutes of incubation the reaction was stopped by adding 50 µl of stop solution. The absorbance was read at 490 nm. This experiment was performed for all the cell lines and repeated for two passage numbers for each cell line for 24 and 48 hours.

The sensitivity of the assays are often cell and drug dependent and can show variations (Fotakis and Timbrell, 2006). Hence both MTT and CytoTox 96® Non-Radioactive Cytotoxicity assay were performed in this study.

2.8.7 Estimation of 5-HT on cell proliferation by ^{3}H thymidine incorporation

A proliferation assay was performed to analyse the effect of 5-HT on the DNA synthesis of the cells. The increase in DNA synthesis as a result of 5-HT treatment was measured by adding [^{3}H] Thymidine. The plates were seeded with $5x10^{4}$ for placental cell lines and $2.5x10^{4}$ for HT1080 cells, and treated with 5-HT after 24 hours of cell recovery. The cells were incubated with [^{3}H] Thymidine (1µ Ci per well) for 18 hours before termination of the assay. The cells were harvested onto a 96 well UniFilter GF/C plate after the incubation period using a Filtermate Harvester (Packard). These plates were allowed to dry at room temperature before addition of Microscint O to assist in cell counting. The newly synthesised DNA was measured using TopCount NxtYM (Packard). Furthermore, the effect of 5-HT on cell proliferation recovery were also examined. The cells were treated with 10µg/ml of 5-fluorouracil (5-FU) (Sigma Aldrich) for 24 hours to inhibit proliferation. 5-FU is a pyrimidine analogue with a fluoride atom attached to the base of pyrimidine ring. 5-FU is metabolised to Fluorodeoxyuridine monophosphate (FdUMP) and Fluorouridine triphosphate (FUTP) (Wyatt and Wilson, 2009). The first metabolite FdUMP inhibits the activity of the enzyme thymidylate synthase (TS). TS in normal conditions converts uracil to thymine by the addition of a methyl group at the 5^{th} carbon position. Inhibition of TS causes nucleotide imbalance in the cells eventually blocking the DNA synthesis. Furthermore FdUMP is converted to Fluorodeoxyuridine triphosphate (FdUTP) which gets incorporated into the DNA triggering additional damage. Second metabolite Fluorouridine triphosphate (FUTP) is incorporated into RNA leading to translation impairments.

2.8.8 RNA extraction and protein fractionation

After the confirmation of the 5-HT doses to be used, the cells were treated with 5-HT and extracted for gene expression and protein analysis. RNA extraction was followed as in Section 2.4. Similarly cell lysate were prepared as in Section 2.7.1.

2.8.9 Determination of cell migration by wound healing assay

An investigation on effects of 5HT on human aortic endothelial cells by Matsusaka and Wakabayashi in 2005 have highlighted 5HT as inducer for migration. As mentioned in Section 1.1.1, trophoblast migration and invasion are the key steps in regulating the formation of placenta. Also tumour cell migration and its regulation are crucial to control

tumour metastasis. This raised interests in investigating the effect of 5-HT on trophoblast and choriocarcinoma cell migration. Therefore a wound healing assay often known as scratch assay was performed using fluorescent (confocal; Leica) microscopy after staining with a plasma membrane (FM1-43FX) fluorescent stain (Molecular Probes™). 96 well black/clear imaging plates (BD Falcon) were coated with 50µl of 0.01% w/v poly-l-lysine (Sigma Aldrich) and incubated for an hour at room temperature to make the surface adherent. Excess poly-l-lysine was removed prior to plating. Cells were plated with optimum cell densities ($5x10^4$ for placental cell lines and $2.5x10^4$ for HT1080 cells) obtained from MTT/LDH assays (see Sections 2.8.5 and 2.8.6) and allowed to grow until confluent. Upon confluency cells were treated with 10µg/ml of 5-fluorouracil (5-FU) (Sigma Aldrich) for 24 hours to inhibit proliferation. The cells were stained for 15 minutes before scratching using 10µM FM1-43FX fluorescent stain. Then a straight scratch was made on the monolayer using a two microlitre pipette tip. The wells were washed gently with 1X DPBS (Lonza) to remove the debris after the scratch. The plates were then cultured with serum free media (SFM) (media with 1% w/v L-glutamine and 1% v/v penicillin/streptomycin) containing different doses of 5-HT (and untreated control). The scratches were imaged at 30 minutes intervals under the confocal microscope for 48 hours. This experiment was performed in duplicates for all the cell lines and repeated twice.

- **Calculating cell migration**

The migration assays were analysed by using WimScratch automated analysis by Wimasis Quantitative Image Analysis (**www[12]**). This software detects the edge of the scratch and measures the gap area. The analysed results include graphic representation of the assay; for summary file and image readout see Appendix A.2.

2.8.10 Invasion Assay

It is also known that the formation of placenta and tumour requires the cells to both migrate as well as invade simultaneously. It was apparent to investigate the effect of 5-HT on cell invasion after confirming its effect on migration. Thus, an invasion assay was performed by using BD Falcon™ BioCoat tumour invasion systems (BD Falcon) with FluoroBlok™ 96 well insert plates (8µm pore size) coated with BD matrigel Matrix. This was compared with migration of cells through uncoated BD Falcon™ FluoroBlok™ 96 well insert plates. The assay was performed according to the manufacturers' guidelines. The protocol followed was as follows:

- **Rehydration:** BD Falcon™ BioCoat tumour invasion systems was removed from -20˚C freezer and allowed to stand at room temperature. Once the plates reached room

temperature the apical chambers were filled with 75µl warm media and allowed to rehydrate for 2 hours at 37˚C at 5% v/v CO_2.

- **Pre-staining the cells:** The cells were grown to 70% confluency and collected after trypsinisation. Collected cells were incubated with 10µM CellTrace™ CFSE dye (Molecular Probes®) for 15 minutes. After incubation the cells were centrifuged and re-suspended in warm media.
- **Cell suspension:** Cell density of $2x10^4$ cells were prepared in SFM.
- **Assay set-up:** After rehydration of BD Falcon™ BioCoat tumor invasion plates the media from the apical chambers was removed and replaced with 50µl of cell suspension. Similarly 50µl of cell suspension was also added to the apical chambers of the BD Falcon™ FluoroBlok™ 96 well insert migration plates. The bottom chambers were filled with growth media containing 5% v/v FBS, which acts as a chemo-attractant. The apical as well as the bottom chambers were treated with different concentrations of 5-HT (See Table 2.3 and Figure 2.1 for illustration of the assay plate set up).

Table 2.3: Treatment of invasion assay plates

CONDITION	TREATMENT	APICAL CHAMBER		LOWER CHAMBER		
		1mM 5-HT	Cells in SFM	1mM 5-HT	Growth Media	SFM
NORMAL 20% 0_2	-VE CONTROL	-	50.0 µL	-	-	200 µL
	+VE CONTROL	-	50.0 µL	-	200 µL	-
	10µM 5-HT	0.5 µL	49.5 µL	2 µL	198 µL	-
	20µM 5-HT	1.0 µL	49.0 µL	4 µL	196 µL	-
	30µM 5-HT	1.5 µL	48.5 µL	6 µL	194 µL	-
	40µM 5-HT	2.0 µL	48.0 µL	8 µL	192 µL	-
HYPOXIC 2% 0_2	-VE CONTROL	-	50.0 µL	-	-	200 µL
	+VE CONTROL	-	50.0 µL	-	200 µL	-
	20µM 5-HT	1 µL	49.0 µL	4 µL	196 µL	-
	40µM 5-HT	2 µL	48.0 µL	8 µL	192 µL	-
	60µM 5-HT	3 µL	47.0 µL	12 µL	188 µL	-
	80µM 5-HT	4 µL	46.0 µL	16 µL	184 µL	-

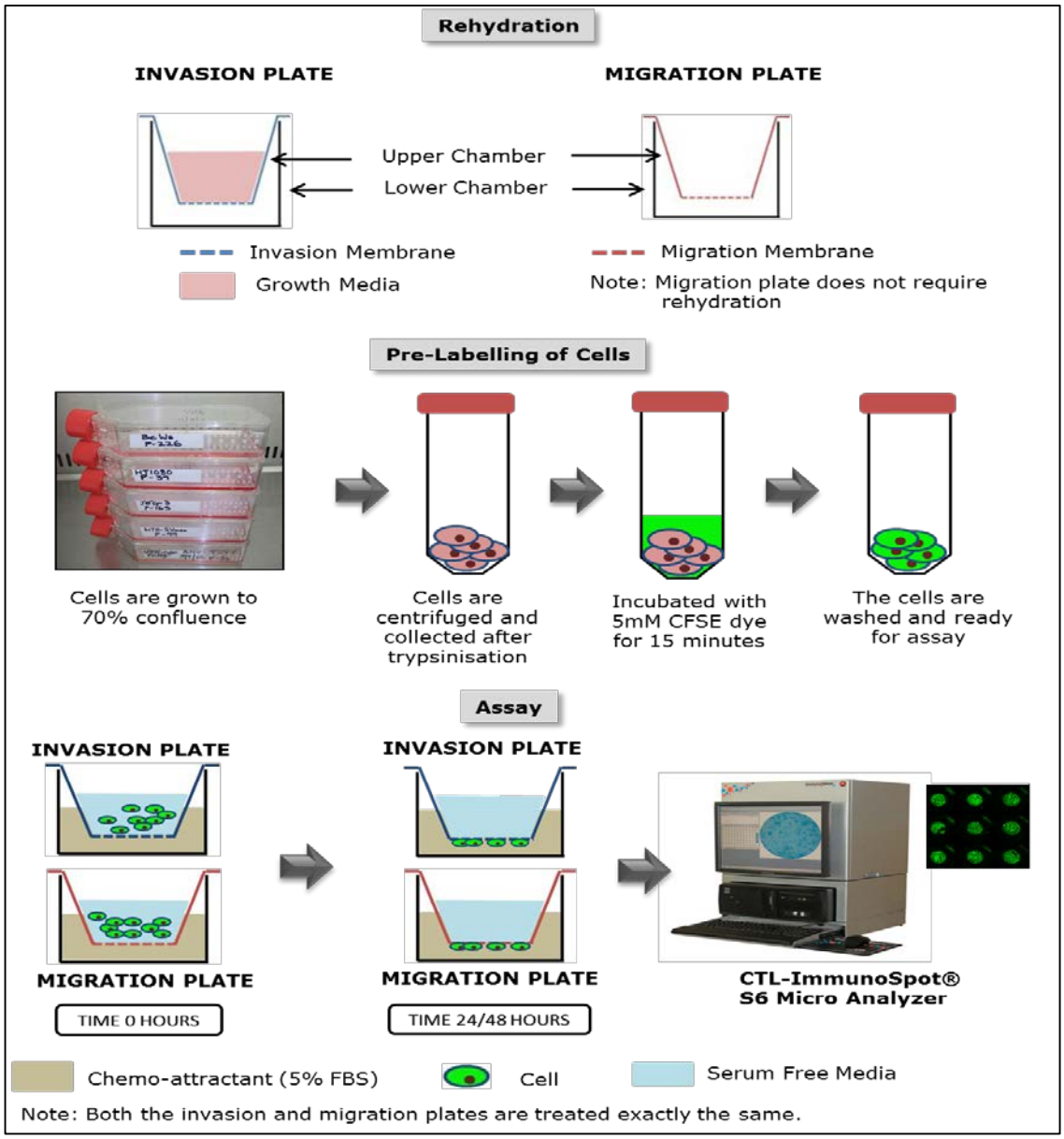


Figure 2.1: Diagrammatic representation of the Invasion Assay.

- **Image capture:** The images of the plates at time zero were immediately taken with a top plate imaging system using ImmunoSpot® analyser [Cellular Technology Ltd. (CTL)]. CFSE has an excitation/emission of 492/517nm. The plates were then incubated at 37˚C at 5% v/v CO_2 and images taken at 48 hours. At the end of the assay, the membranes were removed from the inserts and mounted on slides. These were further imaged using a fluorescent microscope (Olympus BX51) with Olympus camera.
- **Cell counting:** The images scanned by the CTL analyser were counted using the CTL software. The images taken by Olympus were analysed and counted using the Image J software. The cell counts by the two methods were then compared.
- **Calculating percentage invasion:** This was calculated by taking the migration plate to have 100% of cells coming through the membrane. Since not all cells will pass

through the matrigel matrix, the percentage invasion is calculated as a fraction of the migration.

The equation is as follows:

$$Invasion\ \% = \frac{Number\ of\ cells\ invaded}{Number\ of\ cells\ migrated} \times 100$$

2.9 Staining protocol

In order to analyse the human placental sections the following staining protocols were performed.

2.9.1 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was performed to investigate the localisation of the proteins of interest in the tissue sections. The paraffin embedded sections were taken at 5µm thickness and mounted on poly-l-lysine coated slides (Thermo Scientific). The sections were baked at 60ºC for 1 hour.

- **Deparaffinising**: The sections were deparaffinised in xylene (Fisher Scientific) twice for 10 minutes and 2 minutes respectively.
- **Hydration:** The sections were hydrated through an alcohol gradient as follows:
    - 2x in Absolute alcohol for 2 minutes each
    - 2x in 95% v/v ethanol for 2 minutes each
    - 1x in 75% v/v ethanol for 2 minutes
- **Blocking endogenous peroxide:** The sections were then immersed in 3% v/v hydrogen peroxide (H_2O_2) (Fisher Scientific) in methanol for 30 minutes. Followed by wash with distilled water.
- **Heat induced Epitope Retrieval (HIER):** HIER involved unmasking the epitopes in the tissue sections by immersing the sections in retrieval buffers (10mM Tris-1mM EDTA buffer, pH 9 or 10mM Citrate Buffer, pH 6) and microwaving at high temperature for 20 minutes. This was followed by wash with PBS.
- **Blocking:** The sections were blocked for unspecific binding with horse serum for 30 minutes or longer.

NOTE: Negative controls were left in blocking solution until remaining slides were incubated in primary antibodies.

- **Primary antibody incubation:** After blocking the sections were incubated in primary antibody, at dilutions of 1:200-1:500, for 1 hour at room temperature. This was followed by washing with PBS 3 x 5 minutes each.

NOTE: The sections were stained for the expression of vimentin to check for loss of signal due to long-term fixation (Dabbs 2006).

- **Secondary antibody incubation:** The sections were then incubated in biotinylated secondary antibody (1:10,000 dilution) for 1 hour at room temperature followed by brief wash with PBS.
- **Avidin/Biotinylated enzyme Complex (ABC):** The sections were developed in ABC reagent for 30 minutes at room temperature. The sections were briefly washed with water. The ABC reagent from Vectastain® was prepared according to manufacturers' guidelines.
- **3,3'-Diaminobenzidine** (**DAB) :** DAB substrate from Vectastain® was prepared according to manufacturers' guidelines and added to the sections until brown staining was observed, followed by a brief wash with water. In presence of antibody conjugated to peroxidase enzyme and peroxide the DAB is oxidised to give brown colour.
- **Counterstain:** The slides were counter-stained using Gills Haemotoxylin for 15-30 seconds. Followed by wash with water and 1% w/v Di-potassium tetraborate (blueing agent) for few seconds.
- **Dehydration:** The sections were dehydrated through an alcohol gradient as follows:
    - 1x in 75% v/v ethanol for 2 minutes
    - 2x in 95% v/v ethanol for 2 minutes each
    - 2x in Absolute alcohol for 2 minutes each

  Followed by soaking in xylene for 3x5 minutes.
- **Mounting:** The sections were mounted with coverslip using Entellen®.

2.9.2 Visualisation and semi-quantification of IH

The slides were visualised at 200X and 1000X magnification and images were captured using an Olympus camera DP73. The images captured were taken approximately at the same locations in comparative slides. Slides prepared from the consecutive tissue sections were compared. The staining in the images were then semi-quantified using a point scale immuno-reactivity scoring (IRS) system (as shown in Table 2.4). These scorings were carried out compared to the positive staining for vimentin IRS (Scored as 3) and negative stain (secondary antibody only; 0) in the same sample. Multiple images of the slides were scored independently by three observers.

Their individual scores were then compared and tallied for final scores. The most frequently repeated score between the three scorers (mode) was taken as the final score for the gene of interest in overall pool of samples (NT, PE or FT). A weighted kappa statistical analysis was also performed with the scores provided individually by the three scorers to analyse the inter-observer error. The values for the kappa were interpreted as mentioned by Viera and Garrett (2005). If the values were: < 0, there was less chances of agreement; 0.01–0.20, indicated slight agreement; 0.21– 0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement and 0.81–0.99, practically perfect agreement.

Table 2.4: IRS for semi-quantification of IHC slides.

Immunoreactivity score (IRS)	Staining Intensity
0	No or Very Low Positive Staining
1	Low Positive Staining
2	Medium Positive Staining
3	High Positive Staining

2.9.3 Haemotoxylin and Eosin (H&E) Staining

To study the structural features of the various placental tissue types, haemotoxylin and eosin (H&E) staining was performed. The deparaffinisation and hydration steps were repeated as in Section 2.9.1. The tissue was then stained in Gills Haematoxlylin for 2 minutes. The excess stain was removed under running tap water and followed by a quick dip in 1% w/v Di-potassium tetraborate (blueing agent). The slides were then rinsed and counter stained in 1% v/v Eosin (Fisher Scientific). Excess stain was rinsed and followed by dehydration and mounting as in Section 2.9.1.

2.9.4 Immunocytochemistry staining

Immunocytochemistry protocol from abcam® was observed to investigate the localisation of the antibodies of interest in the cell lines studied. The cells were first grown on GG-18-PLL (neuVitro), poly-l-lysine coated coverslips. The coverslips were then rinsed with 1X DPBS and air dried. The general procedure followed for immunocytochemistry staining is given below:

- **Fixation:** The cells were fixed in either ice cold acetone for 5 minutes, methanol for 10 minutes or in 4% w/v paraformaldehyde in PBS pH 7.4 for 15 minutes at room temperature, followed by washing twice in PBS.
- **Permeabilisation:** For staining intracellular proteins the cells fixed with paraformaldehyde and methanol were permeabilised in 0.25% v/v Triton X-100 in PBS for

10 minutes. This was followed by washes in PBS for 3 x 5 minutes. The permeabilisation step was not required for acetone fixed cells and for detection of membrane proteins.

- **Blocking:** The cover slips were then incubated in 1% w/v BSA in PBST for 30 minutes-1 hour. For paraformaldehyde-fixed coverslips, 0.3M Glycine was added to the blocking buffer.
- **Primary Antibody Incubation:** The coverslips were then incubated in primary antibody diluted in 1% w/v BSA in PBST for 1 hour at room temperature or overnight at 4ºC in a humidified dark chamber. After incubation the coverslips are washed again in PBS 3 x 5 minutes.
- **Secondary Antibody Incubation:** The coverslips were then incubated in secondary antibody diluted in 1% w/v BSA in PBST for 1 hour at room temperature in dark followed by 3 x 5 minutes wash with PBS.
- **Counter staining:** The coverslips, if required, were counter-stained with VECTASHIELD® HardSet™ mounting medium with DAPI.

Once dried, the edges of the coverslips were sealed with colourless nail varnish to avoid it from moving while taking images. The images were captured using an Olympus camera at different magnifications (Objective magnification: 10, 20, 40 and 100X).

2.10 Hypoxic conditioning

Placenta can be considered hypoxic only when compared to other organ environments, in which the metabolic demand for oxygen is much higher, but the supply is low. Interestingly, studies using isolated trophoblast from term placenta have indicated that these cells are tolerant to prolonged periods of hypoxia (Esterman *et al*, 1997). Therefore the *in vitro* experiments were repeated at hypoxic conditions (2% v/v O_2, 5% v/v CO_2 balanced by nitrogen) using modular incubator chambers MIC-101. The cells were pre-conditioned with hypoxic conditions before the experiments for 24 hours. The modular incubator chambers maintained a hypoxic environment with no fluctuations. For confocal imaging of the wound healing assay at hypoxic conditions, the entire microscopic stage was kept under hypoxic conditions (see Figure 2.2). Hypoxia was monitored by an O_2 sensor throughout the experiment.

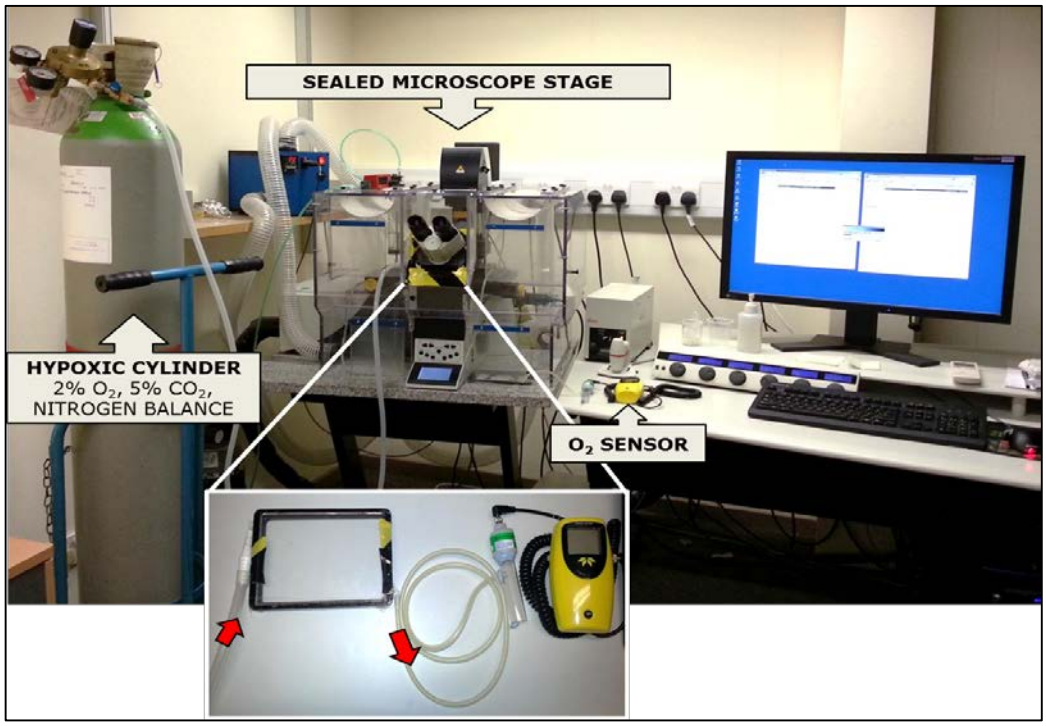


Figure 2.2: Confocal Imaging of wound healing assay at hypoxic conditions.
The bold red arrow heads indicate the flow of gas through the system.

Chapter 3
Term placental study

Comparative expression analysis in Normotensive and Pre-eclamptic Placentae

3.1 Introduction

Despite the fact that between 2-3% of pregnancies are affected by pre-eclampsia (PE) and result in approximately 15% of these babies being born prematurely, the aetiology of this syndrome is not well established. Conversely, several causative factors have been identified, including genetic predisposition, reduced invasion of the trophoblast cells (during placental development), ethnicity, parity etc. A number of genetic differences have been identified between pre-eclamptic and normal placental tissues. Differential gene expression techniques such as microarray have identified several candidate genes involved in signalling pathways (Centlow *et al*, 2008, 2010; Enquobahrie *et al*, 2008). A limited number of studies have also investigated differential protein expression in PE (Centlow *et al*, 2009). However, it is difficult to identify mutant or ameliorated protein(s) expression involved in the progression of PE because the human genome has in excess of 25,000 genes. Due to splicing events these genes can then give rise to over 1,000,000 proteins, which can undergo post-translational modifications such as phosphorylation, glycosylation etc. Proteomics chip technology would be an ideal way to study altered or mutant protein expression. This technology is still in its infancy and thus does not have the power to fully identify pre-eclamptic proteins. Therefore the protein detections are carried out by conventional methods like 1-D and 2-D gel analysis.

Several circulating factors, such as 5-hydroxytryptamine (5-HT; serotonin), have been reported to increase in PE pregnancies (Cruz *et al*, 1998). Here, 5-HT is thought to be involved in PE because of its vasoconstrictory effects resulting in compromised blood flow to the placenta. The increased level of 5-HT seen in PE placenta could be due to low levels of its metabolising enzyme, monoamine oxidase (MAO) (Sivasubramaniam *et al*, 2002). 5-HT induces several physiological effects, including vasoconstriction or vasodilation, depending upon which member of the 5-HT receptor family it interacts with (See Section 1.4.1.2). Several studies have reported 5-HT-mediated constriction of placental veins and the myometrium; specifically implicating 5-HT_1 and 5-HT_2 receptor (Cruz *et al*, 1998; Cordeaux *et al*, 2008; Ugun-Klusek *et al*, 2011). Whilst these studies have focused mainly on 5-HT_1 and 5-HT_2 receptors the involvement of other 5-HT receptor subtypes in PE placentae is not fully understood. Therefore the first aim of this chapter was to investigate the differential expression of various members of the 5-HT receptor family during normal and PE placental development.

Cancer testis (C/T) antigens are a large family of tumour-associated antigens, whose function is unknown. They are usually only found in human tumours, with the exception of healthy testis and placental tissue. In 2007, Jungbluth and co-workers used immunohistochemistry, to show the cellular localisation of MAGE family, GAGE and NY-ESO-1 antigens in 50

placental samples ranging between weeks 5 to 42. From the few studies that have been carried out, these antigens are known to be involved in invasion, tumorigenesis, and apoptosis (Olesen *et al*, 2011; Sang *et al*, 2011; Gjerstorff *et al*, 2006). Furthermore, a few microarray (and other) studies have shown differences in expression of some of C/T antigens in PE placentae where the invasion is shallow (Founds *et al*, 2009; Old, 2001). However there are no detailed studies on the status of C/T antigens in PE placentae. Therefore, the second aim of this chapter was to comparatively analyse the expressions of C/T antigens between normal (NT) and PE placentae.

3.2 Results

Initially using NT and PE placentae, the mRNA expressions of 5-HT receptors together with all know C/T antigens were compared to identify the receptors and antigens that show a highly significant change in PE placentae. Then the faithful translation of these identified protein were compared semi-quantitatively by Western blotting and qualitatively by immunohistochemistry. By this way, the study only focussed on the proteins that are differentially expressed in PE placentae. It is a well-known fact that the human brain express all the know 5-HT receptors (Mendelsohn and Paxinos, 1991). Therefore, human brain was used as a positive control for 5-HT receptor comparisons. Likewise, C/T antigens are abundantly expressed in human testis (Simpson *et al*, 2005), hence human testis was used as the positive control for C/T expression analysis.

3.2.1 Human placental samples

The demographic details of NT and PE samples are given in Table 3.1 (Ethical approval REC: 12/NE/0112). Individual patient data can be found in the Appendix A.3.

Table 3.1: Demographic details of the pre-eclamptic patients and normotensive subjects

Parameter	Normotensive (*n=13*)	Pre-eclamptic (*n=12*)	P value §
Maternal age (years)	34.2 ± 1.4 (28-41)	28.5 ± 2.2 (19-39)	0.075
Gestational age (weeks)	38.9 ± 0.1 (38.4-39.3)	37.5 ± 0.7 (33-39.4)	0.036
Systolic blood pressure (mmHg)	122.2 ± 3.5 (109-140)	153.8 ± 3.4 (140-168)	**<0.0001**
Diastolic blood pressure (mmHg)	70.6 ± 3.4 (60-90)	98.5 ± 1.9 (91.5-108)	**<0.0001**
Protein in urine (g / lt)	N/D	1.149 ± 0.328	
(g / 24 hour)	N/D	2.687 ± 0.852	
Placental weight (g)	657.4 ± 37.3 (527-855)	506.3 ± 35.8 (360-620)	**0.015**
Mode of delivery			
Parity	1.9 ± 0.3 (1-4)	0.3 ± 0.3 (0-2)	**0.005**
Gestational Weight (Kg)	3.4 ± 0.1 (3.03-4.24)	2.6 ± 0.2 (1.66-3.59)	0.015

The details are expressed as mean ± SD, N/D= none detected, n=number of women in each group. § Mann – Whitney U test.

3.2.2 Histology of Normotensive (NT) and Pre-eclamptic (PE) placentae

The histological differences between NT and PE placental sections were studied using haematoxylin and eosin staining as mentioned in Section 2.9.3. The sections were analysed and graded by Mr Roy Stewart (histopathologist) and the director of study (placentologist) at Nottingham Trent University. The staining patterns were reproducible for the different sample sections within the two groups (NT and PE) and only representative data is given below. In NT sections regular villous system were observed with a single outer layer of syncytiotrophoblast cells (See Figure 3.1.A). The cytotrophoblast cells were scattered into the maternal decidua and also lining the sprial arteries. Some of the syncytiotrophoblast cells undergo apoptosis and form blebs called syncytial knots that disintegrate and are removed by circulating blood. The number of syncytial knots in PE sections were found to be higher than in NT (See Figure 3.1.B) suggesting increased apoptosis in PE placentae. There were distinctive fibrinoid depositions observed in PE sections. The villous capillaries of PE sections are lined with necrotic fibrinoid and is filled with plaques. In PE cytotrophoblast cells also showed vacuolation and cytomegaly (not shown in figure).

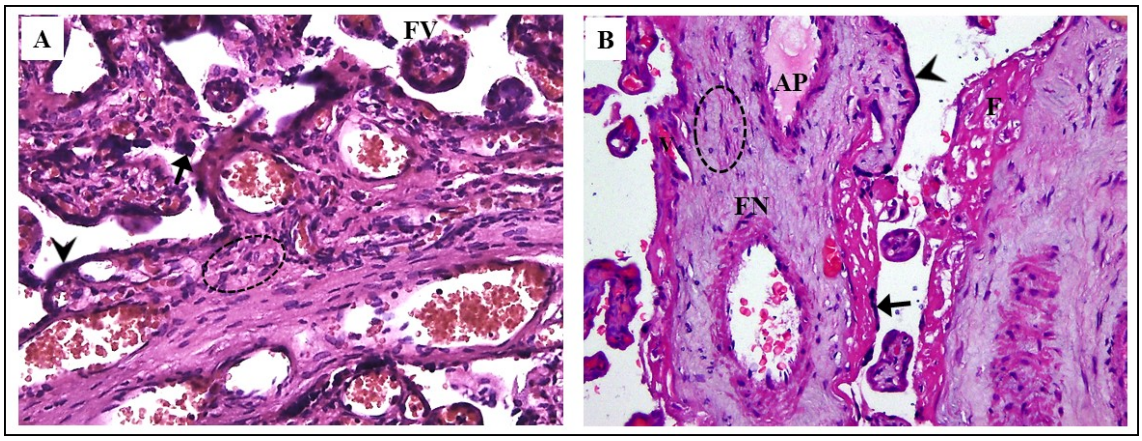


Figure 3.1: Histological differences between NT and PE placentae.

[A] NT placental section showing the villous system with a single syncytiotrophoblast outer layer (arrow head) and an inner cytotrophoblast cell (dotted circle) network. [B] Fibrinoid (F) deposits are seen in PE sections. Atheromatous plaque (AP) is formed in the villous capillaries. Fibrinoid necrosis (FN) is also observed lining the capillaries. FV- Floating villi; Black Arrow- Syncytial knot; (A-B: H&E staining; Magnification: X200).

3.2.3 Gene expression studies

The varying expression patterns of 5-HT receptor subtypes and C/T antigens of interest in NT and PE placental samples were studied. Total RNA was extracted and reverse transcribed according to the protocol in Section 2.4 and 2.5. The purity and integrity of RNA samples was high because the absorbance ratio of 260/280nm ranged from 1.9-2.3 and after

separation on a 1% w/v agarose gel, two clear bands corresponding to 18S and 28S were evident.

Primers for the genes of interests were designed in accordance with Section 2.6.1. Conventional and gradient PCR were performed using the primer sets. Optimised annealing temperatures and sequence details of C/T antigens and 5-HT receptors together with housekeeping genes are summarised in Table 3.2. Human testis and brain cDNA templates were used as positive controls to optimise the T_A for C/T antigens and 5-HT receptors respectively. Primer dimers and product dimers were eliminated by amending the $\boldsymbol{T_A}$. The accession numbers for target genes are listed in Appendix A.4. All PCR products were confirmed by DNA sequencing carried out by Source Bioscience (Nottingham) Ltd. Examples of DNA product confirmation gels and sequencing can be found in Appendix A.5.

Table 3.2: Primer Sequences and annealing temperatures (T_A)

GENES	PRIMER DIRECTION	SEQUENCES (5´-3´)	PRIMER LENGTH (Nucleotides)	T_A
HOUSE KEEPING GENES				
GAPDH	Forward	ACCACCAACTGCTTAGCACC	20	58°C
	Reverse	CCATCCACAGTCTTCTGGGT	20	
HPRT1	Forward	TGACACTGGCAAAACAATGCA	21	55°C
	Reverse	GGTCCTTTTCACCAGCAAGCT	21	
TBP1	Forward	TGCACAGGAGCCAAGAGTGAA	21	56°C
	Reverse	CACATCACAGCTCCCCACCA	20	
GENES OF INTEREST				
5-HT Receptors				
5-HT_{1A}	Forward	GAGCTTTCTACATCCCGCTG	20	58°C
	Reverse	CCCGACTCTCCATTCACACT	20	
5-HT_{1B}	Forward	CTCCCGGATTTTGAAACAGA	20	55°C
	Reverse	TGATCCCTAGGGTCTTGGTG	20	
5-HT_{1D}	Forward	GAGGGAAGGCTCTCTGGTCT	20	58°C
	Reverse	CACGGACAACTGGGAGAGAT	20	
5-HT_{1E}	Forward	TCCACCTCAGACCCTACCAC	20	55°C
	Reverse	GGCAGCCAGGATAAAATGAA	20	
5-HT_{2A}	Forward	AGCTTCCTCCCTCAGAGTTCTTT	23	58°C
	Reverse	GGGCACCACATCACCACAAA	20	
5-HT_{2B}	Forward	GAACGTTTTGGCGATTTCAT	20	53°C
	Reverse	AACCATGTTAGGCGTTGAGG	20	
5-HT_{2C}	Forward	ATGGTGAACCTGAGGAATGC	20	55.8°C
	Reverse	AATTTGAAGCGTCCACCATC	20	
5-HT_{3}	Forward	ACTGCCTGTAGCCTCGACAT	20	57°C
	Reverse	AACTCCCACTCTCCCTGGTT	20	
5-HT_{4}	Forward	GGTTTCGGGTCAGTGGAGAAG	21	57°C
	Reverse	AGATCCGCAAAAGCAAGAGA	20	
5-HT_{5A}	Forward	CTGTGTGGGCTCTTCGTGT	19	55.6°C
	Reverse	GCTGTTTGGCAGAGTCCTTC	20	
5-HT_{6}	Forward	GGGCATGTTCTTTGTGACCT	20	56°C
	Reverse	GGGGTTCATGGTGCTGTTAC	20	
5-HT_{7}	Forward	CCTCCATCACCTTACCTCCA	20	59°C
	Reverse	TGTGTTTGGCAGCACTCTTC	20	
C/T Antigens				
BUC11	Forward	CTTGCCACCTCCCAGTAAAA	20	58°C
	Reverse	CTTGGTTTCCAGCTCTTTGC	20	
CAGE1	Forward	GGTTGCAAGGAGTGAAGAGC	20	56°C
	Reverse	CAATCCGGGAGCAGATTAGA	20	
FATE1	Forward	CGCAGACTAGCCTGGAAGAG	20	58°C
	Reverse	ATGATCAGGGTCTCCCTGTG	20	
GAGE1	Forward	CTCAAGTCTCCTCAAGGCTGT	21	59°C
	Reverse	GGGATAGGTACCTGGAGCTG	20	
GAGE C1/ PAGE 4	Forward	CCACCAACTGACAATCAGGA	20	56°C
	Reverse	CGCTCACTCCGAGTCTTTTC	20	
GAGE E1/ PAGE5	Forward	GCACCTAGTGGGGAGATCAA	20	56°C
	Reverse	TGACATCAGGACCATCTCCA	20	
HAGE	Forward	GGAGATCGGCCATTGATAGA	20	64.5°C
	Reverse	GGATTGGGGATAGGTCGTTT	20	
MAGE A1	Forward	GTCAACAGATCCTCCCCAGA	20	55°C
	Reverse	GGAGCAGAAAACCAACCAAA	20	
MAGE A3	Forward	CTCCAGCAACCAAGAAGAGG	20	55°C
	Reverse	CAGCATTTCTGCCTTTGTGA	20	
MAGE A4	Forward	TCCTTGTTCCGAGAAGCACT	20	55°C
	Reverse	CAGCATTTCTGCCTTTGTGA	20	
NY-ESO-1	Forward	GGCTGAATGGATGCTGCAGA	20	64°C
	Reverse	CTGGAGACAGGAGCTGATGGA	21	
PASD1	Forward	GCAAGTGCTCAGCCATTACA	20	56°C
	Reverse	GCTGCTTCTGAAGGTGATCC	20	
T128	Forward	GACGTTCAAGGTCTCCAAGG	20	57°C
	Reverse	TAGTCGCTGGCGTTCTTTCT	20	
T21	Forward	CAAAGAATGAAATCATAGCACAGG	24	59°C
	Reverse	TTCTGTTCTGCCTGGCTTCT	20	

3.2.3.1 Relative mRNA expression of 5-HT receptors in NT and PE

Real-time PCR was used to determine expression levels of the various 5-HT receptor transcripts in normal (NT) and PE placenta. The $2^{-\Delta\Delta Ct}$ values were calculated as described in Section 2.6.5. Scatter-plots (Figure 3.2) show that in general all the NT and PE placentae tested expressed most of the 5-HT receptor subtypes. However, NT and PE samples showed high variability of expression. Expression levels for 5-HT_3 and 5-HT_4 receptors transcripts in both NT and PE were negligible and therefore no further analysis performed.

- **5-HT_1 Receptor Subtypes:**

Relative expression of 5-HT_1 receptors is shown in Figure 3.2. There was no significant difference in the levels of 5-HT_{1A} receptor mRNA expression between NT and PE samples. However, expression of the other receptor subtypes, 5-HT_{1B}, $_{1D}$ and $_{1E}$, were significantly different ($p<0.05$). The expression patterns of individual subtypes are reported below:

- **5-HT_{1A} Receptor:**

5-HT_{1A} receptor mRNA expression was higher in PE when compared to NT placentae (Figure 3.2). Yet, the data were highly variable and therefore not statistically significant. Few samples that show expression were high, but most of the samples (NT as well as PE) aligned at the x-axis showing negligible levels of expression.

- **5-HT_{1B} Receptor:**

Relative mRNA levels of expression for the 5-HT_{1B} receptor was significantly reduced in PE compared to NT placentae ($p<0.05$). Therefore, the protein expression and localisation of 5-HT_{1B} receptor were investigated further (See Sections 3.2.3.2 and 3.2.3.3).

- **5-HT_{1D} Receptor:**

Although the data for the 5-HT_{1D} receptor were variable, the relative mRNA expression in PE was significantly higher than NT placentae (*$p<0.05$). The expression in NT samples were uniformly distributed, but expression in a few of the PE samples were negligible.

- **5-HT_{1E} Receptor:**

Overall, mRNA levels for the 5-HT_{1E} receptor was significantly reduced in PE placentae ($p<0.05$). Most of the PE samples showed no expression of this receptor, whereas NT placenta had a maximum reading of approximately 8.

- **5-HT_2 Receptor subtypes:**

Only one of the three 5-HT_2 receptor subtypes studied showed a significant difference in mRNA expression in NT compared to PE placental tissue; 5-HT_{2B} ($p<0.001$) (Figure 3.2). These expression patterns are elaborated individually below.

- **5-HT_{2A} Receptor:**

There were no changes in mRNA expression of the 5-HT_{2A} receptor in NT compared to PE samples.

- **5-HT_{2B} Receptor:**

Maximum levels of mRNA for the 5-HT_{1B} receptor were approximately 8,300 in NT placental tissues. However, in PE placental tissues, they were significantly reduced to 700 ($p<0.001$) and represented a 12-fold decrease in the expression of 5-HT_{2B} in PE placenta (See Figure 3.2). Further analysis for protein estimation and localisation were carried out on this receptor subtype due to this significant reduction in its levels of mRNA.

- **5-HT_{2C} Receptor:**

There was a slight down-regulation in mRNA expression for the 5-HT_{2C} receptor in PE when compared to NT which was not statistically significant. The mRNA expression of 5-HT_{2C} receptor was the lowest among the other 5-HT_2 receptor subtypes, as well as other members of 5-HT receptor family (See Figure 3.2).

- **5-HT_3 and 5-HT_4 Receptors:**

Levels of mRNA expressions for the 5-HT_3 and 5-HT_4 receptor subtypes were negligible in placental samples. Therefore no further analysis was performed. (Data not shown)

- **5-HT_{5A} Receptor:**

The relative mRNA expression of 5-HT5A receptor in placental tissues were very low to negligible. Only three PE samples showed elevated levels, but due to high variability in samples it was not statistically different to NT.

- **5-HT_6 Receptor:**

The level of mRNA expression for the 5-HT_6 receptor was comparable to 5-HT_{1B} receptor (See Figure 3.2). The expression was lower in PE when compared to NT, but this difference was not statistically significant.

- **5-HT_7 Receptor:**

Expression levels were similar between NT and PE placentae, although PE samples showed slightly higher expression compared to NT (See Figure 3.2).

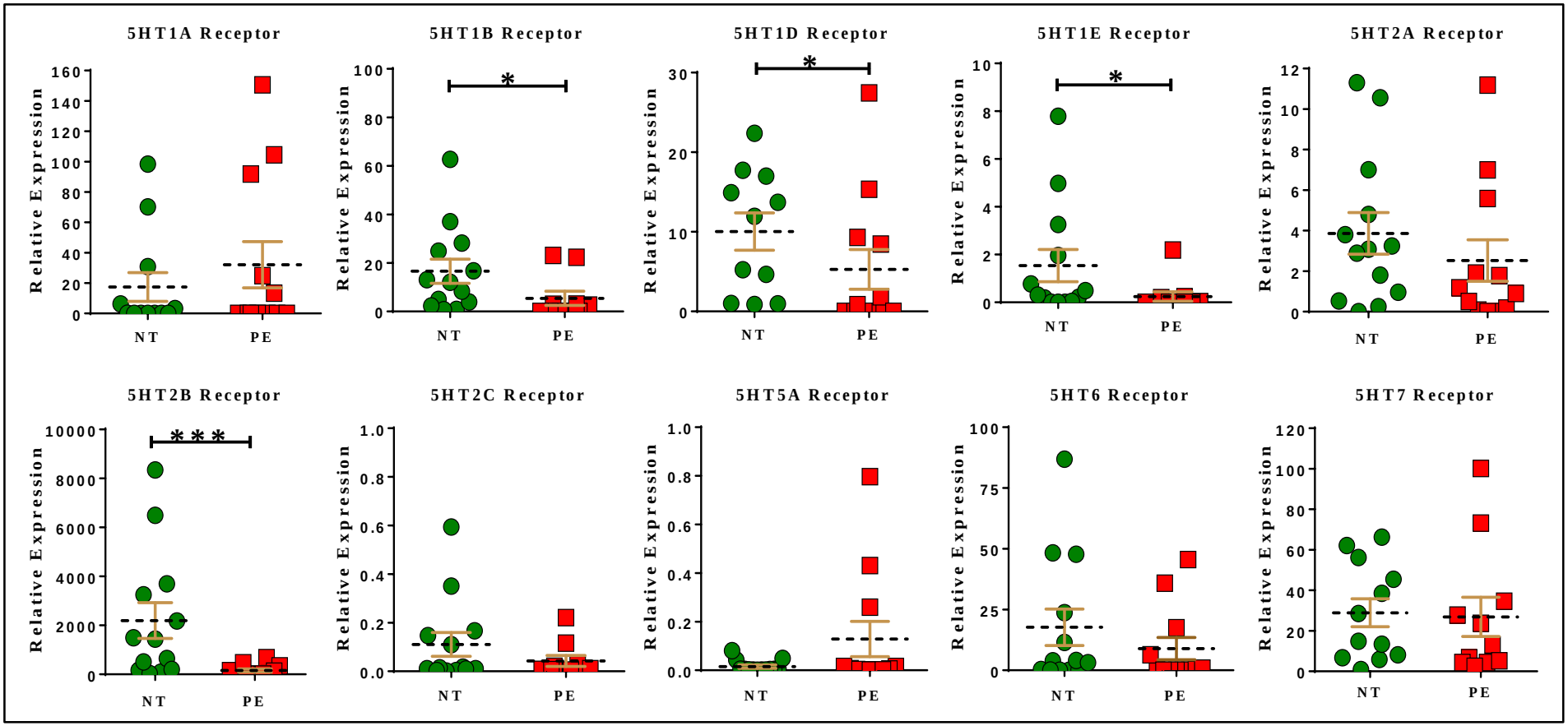


Figure 3.2: Expression levels of 5-HT Receptor transcripts in NT and PE placentae.

Statistical significance was determined using the Mann – Whitney U test (n = 12-13; *p<0.05; ***p<0.001). The error bars (SEM) are represented with brown lines. Values are arbitrary units relative to expressions in brain (positive control; not shown in figure) set to 1. Therefore the scales (y-axis) vary with expression levels in placental samples relative to brain.

3.2.3.1.1 Collective expression analysis of 5-HT receptor subtypes

A major problem with studying gene expression of the 5-HT family of receptors is determining whether a change in the expression profile of a particular subunit is real. That is, if a low expressing subtype is altered it may be considered insignificant in relation to an abundantly expressed family member. For this reason, a tissue that expresses all 5-HT transcripts, the brain, was chosen to normalise expression levels against. So since expression levels of individual transcripts were ultimately compared to their counter parts in the brain, it is possible to determine whether the alteration in expression in placental tissue is due to pre-eclamptic conditions *per se.*

Since the relative mRNA expression of 5-HT_{1B} and 5-HT_{2B} receptors mRNA levels were significantly reduced in PE placentae, further protein expression studies were conducted only on these receptors. Although the 5-HT_{1D} and 5-HT_{1E} receptor mRNA levels are also significantly reduced in PE placentae, their expression was generally low in placentae, they were not explored in protein expression analysis.

3.2.3.2 Protein expression of 5-HT receptors

The expression of 5-HT_{1B} and 5-HT_{2B} receptors was investigated in NT and PE samples using western blot analysis to check the consistency between mRNA and protein expressions; see Section 2.7 for methods. Levels of protein expression were first normalised against β-actin levels and then levels of 5-HT_{1B} or $_{2B}$ in human brain tissue. The scatter-plots for the relative protein expression of 5-HT_{1B}R is represented in Figure 3.3 below. The β-actin for the same blot confirmed equal loading of the samples (70µg) and were used as internal controls for normalisation. The loading amount was reduced for positive control (brain) to avoid over-saturation of the blot. Brain samples (20µg) are separated by dotted lines in the blot. Examples of wholes blots can be found in Appendix A.6.

Unfortunately the antibody available for immunoblotting of 5-HT_{2B}R (biorbyt) were unable to detect proteins in placental samples as well as positive control (brain), even at the highest concentrations.

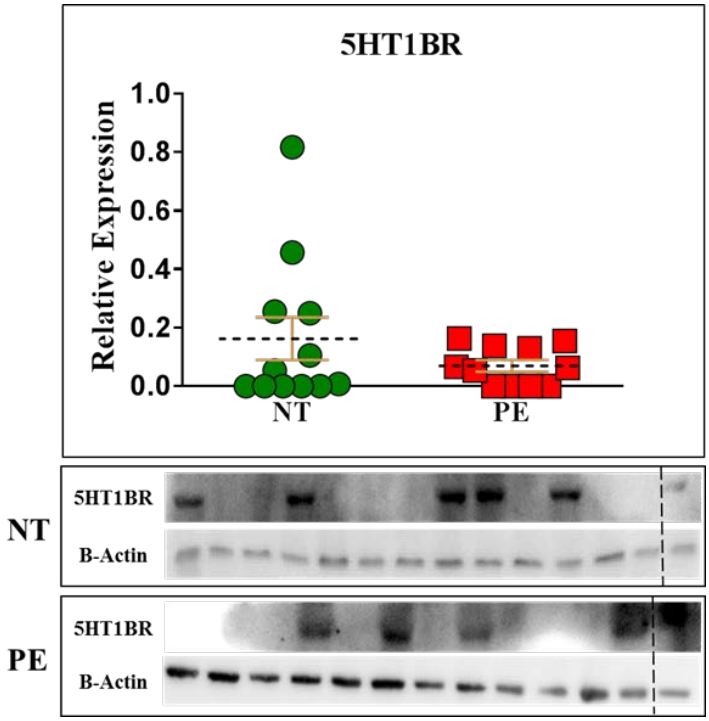


Figure 3.3: The expression of 5-HT$_{1B}$R protein in NT and PE placentae.

5-HT$_{1B}$ receptor expression was normalised to both an internal control (β-actin) and a positive control (5-HT$_{1B}$ levels in human brain). Scatter-plots are derived from 12-13 samples and relative expressions were determined from two independent blots. Mann – Whitney U test was performed. The error bars (SEM) are represented with brown lines. Below are representative Western blots showing the band detected by the anti- 5-HT$_{1B}$ and β-actin antibodies.

- **5-HT$_{1B}$R**

The anti-5-HT$_{1B}$ receptor antibody recognised a single band that migrated at approximately 33kDa in 10% w/v SDS-PAGE gel. This is slightly less than the predicted molecular weight which was 47kDa. Among all the placental samples, only few samples (five NT and four PE) showed expression of 5-HT$_{1B}$R receptor at protein level. Furthermore the level of expression in PE was found to be slightly lower when compared to NT after analysis of the bands. However, this difference in expression was not significant. Overall the placental samples expressed lower 5-HT$_{1B}$R when compared to levels in the brain.

3.2.3.3 Cellular localisation of the 5-HT$_{1B}$R and 5-HT$_{2B}$R

The status and cellular localisation of 5-HT$_{1B}$ and $_{2B}$ receptors was investigated by immunohistochemistry (IHC). The brown staining with 3,3'-Diaminobenzidine (*DAB*) substrate were considered as immuno-positive and scored according to the intensity of staining (See Section 2.9.1). The scoring was carried out blindly by three different scorers (two trained histopathologists and the author) and the final IRS can be found in Table 3.3. The Fleiss' Kappa Statistics performed on this IRS data provided a kappa value of 0.47 suggesting a moderate level of attribute agreement between the scorers. The values for the

kappa were interpreted as mentioned by Viera and Garrett (2005). If the values were less than 0, there was less chances of agreement; 0.01–0.20, indicated slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement and 0.81–0.99, practically perfect agreement.

Table 3.3: Immuno-reactivity score (IRS) of 5-HT$_{1B}$R and 5-HT$_{2B}$R in NT and PE

5-HT RECEPTORS	NT (n=5)	PE (n=5)	P VALUE	SIGNIFICANCE
5-HT$_{1B}$R	3 ± 0.3	3 ± 0.4	> 0.9999	ns
5-HT$_{2B}$R	3 ± 0.2	3 ± 0.2	> 0.9999	ns

The intensity of staining was represented by the mode (the most frequently repeated score) ±SEM of the IRS from 5 individual samples in each group. Unpaired t-test was then performed on the IRS modes of NT and PE (ns=no significance).

- **5-HT$_{1B}$R**

The location of 5-HT$_{1B}$R can be observed from compiled images for NT and PE tissues (Figure 3.4 and 3.5 respectively). Distinct staining was observed around the CTB and STB layer in NT tissues (thin arrows) suggesting immuno-positivity for the receptor. In addition, the CTB and STB layer of the placental villi (represented by *) showed intense staining. The 5-HT receptor in PE tissues showed immuno-reactivity at similar locations when compared to NT. The IRS scores showed no statistical difference in staining between NT and PE samples (Table 3.3).

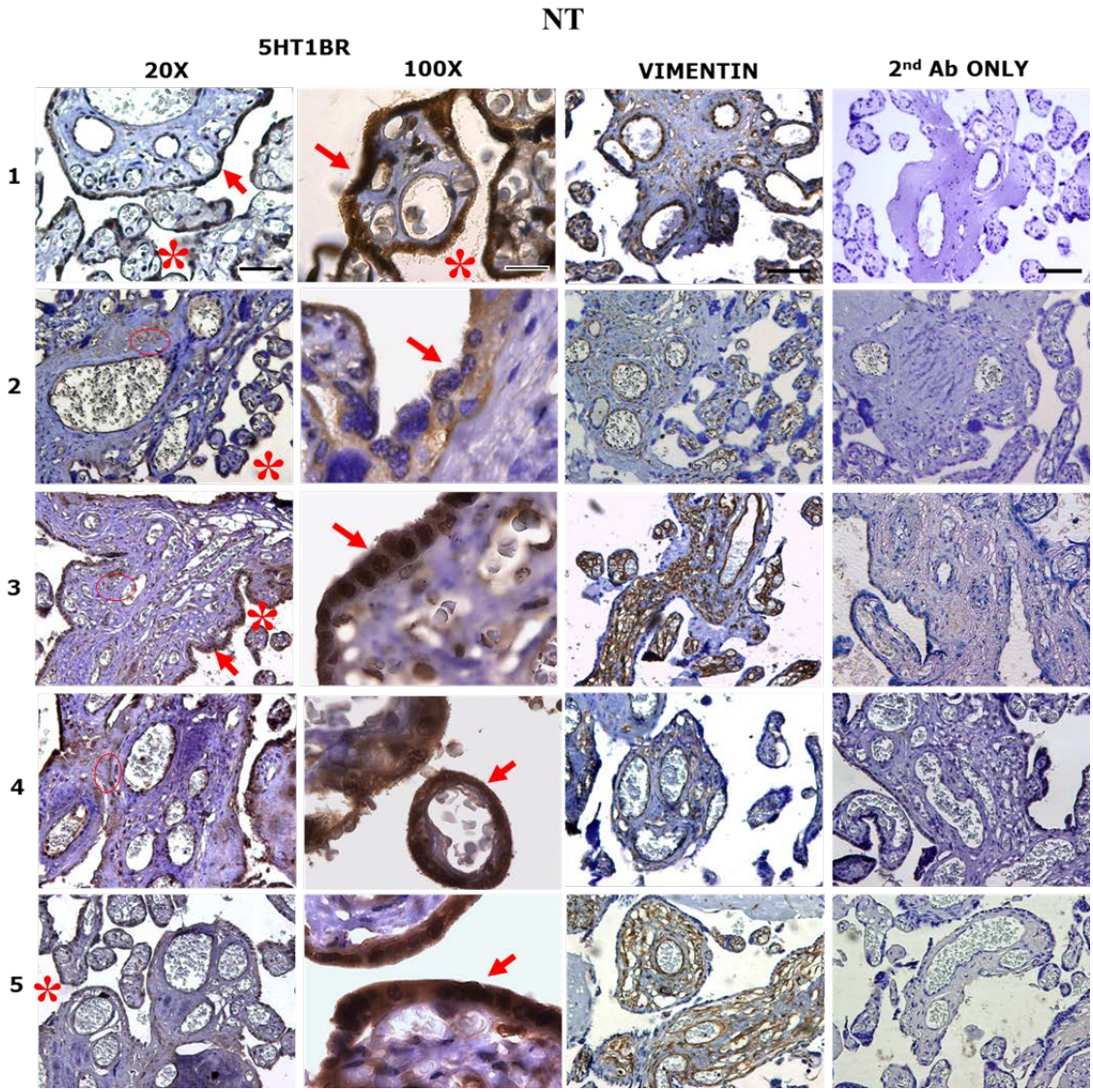


Figure 3.4: 5-HT$_{1B}$ Receptor localisation in NT placentae, determined by immunohistochemistry.

5-HT$_{1B}$R reactivity for each sample was scored against immuno-reactivity for Vimentin (+ve) and secondary (2nd) Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Circled area = EVT cells; Asterisk (*) = placental villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

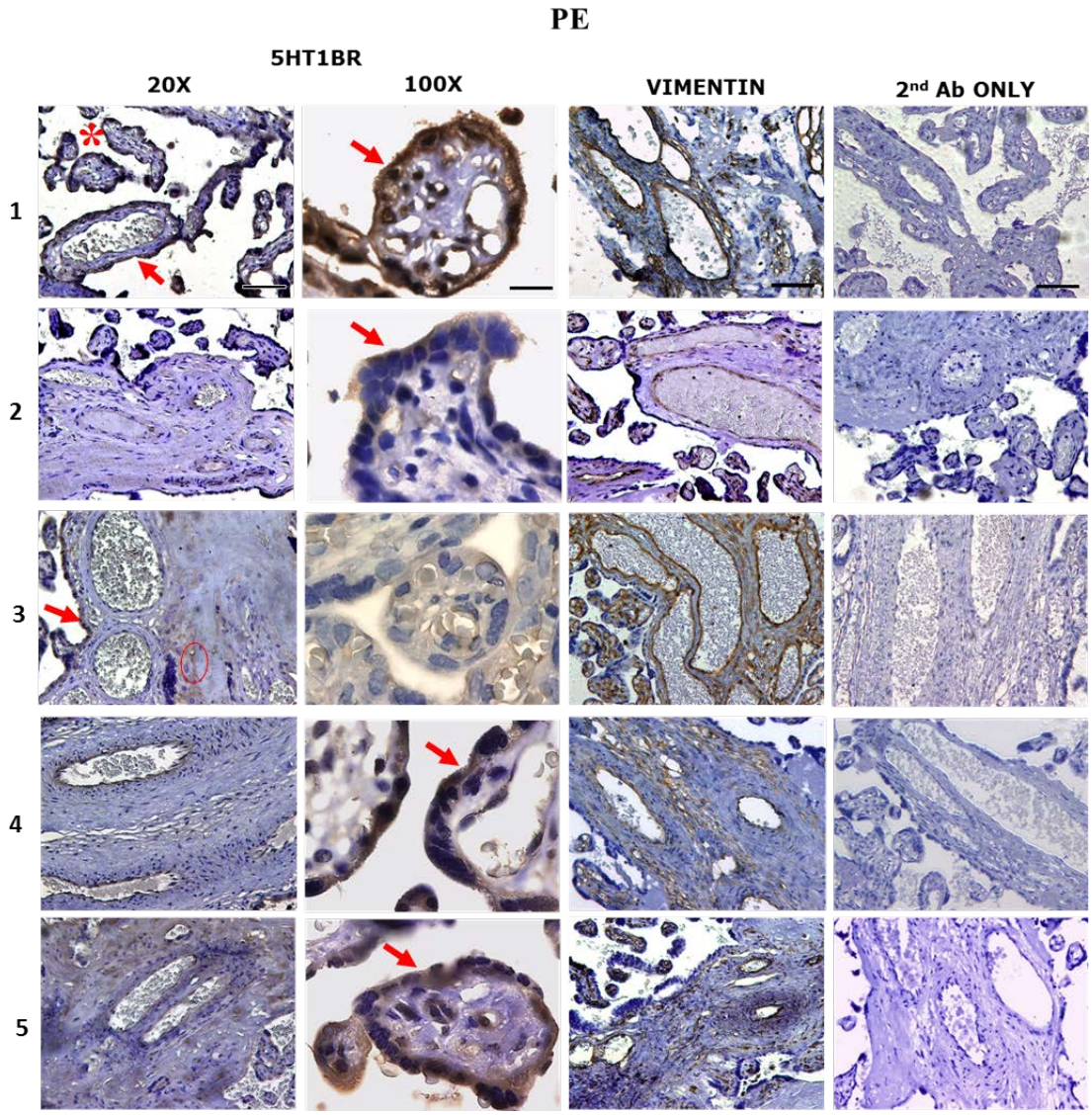


Figure 3.5: 5-HT$_{1B}$ Receptor localisation in PE placentae determined by immunohistochemistry.

Five placental samples for each NT and PE groups are represented here. 5-HT$_{1B}$R reactivity for each sample was scored against immuno-reactivity for Vimentin (+ve) and secondary (2nd) Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Circled area = EVT cells surrounding the arteries; Asterisk (*) = placental villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **5-HT$_{2B}$R**

Both NT and PE tissues showed high immuno-reactivity for 5-HT$_{2B}$R (Figure 3.6 and 3.7 respectively). The STB, CTB layers (thin arrow) and the lining of the spiral arteries (arrow head) were distinctly stained. The stromal cells (double arrow) surrounding the spiral arteries showed immuno-positivity in NT (image 5). The villi sections (*) were intensely stained in NT tissues when compared to PE. The STB layer in PE showed even deeper staining than the CTB layer. Focal positivity was observed in decidual sections of PE placenta (image 5) with staining concentrated around the syncytial knot (+ in Figure 3.7). The IRS confirmed no significant differences in staining between NT and PE tissues (Table 3.3).

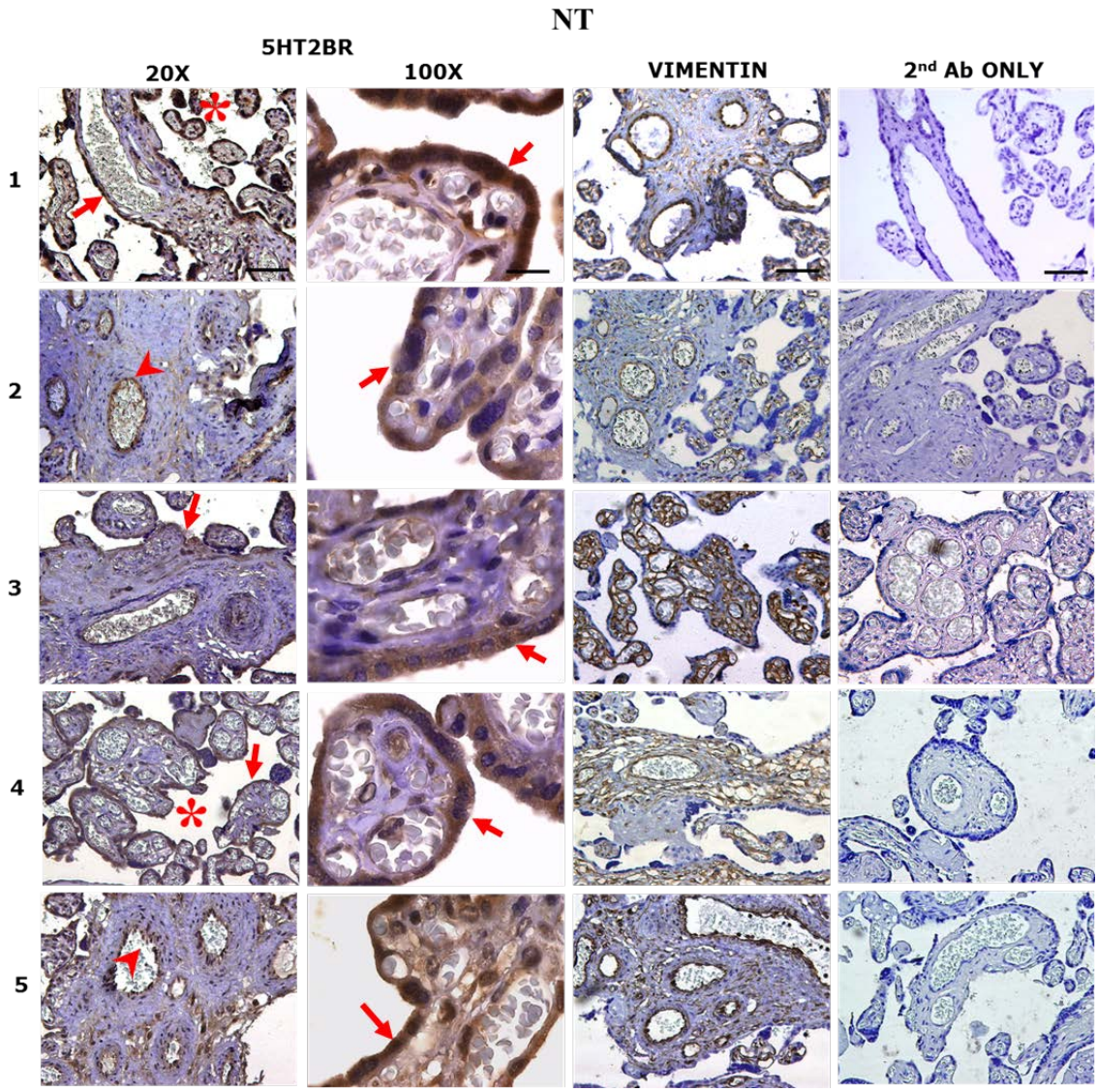


Figure 3.6: 5-HT$_{2B}$ Receptor localisation in NT placentae determined by immunohistochemistry.

5-HT$_{2B}$R reactivity for each sample was scored against immuno-reactivity for Vimentin (+ve) and secondary (2nd) Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = the lining of the spiral arteries; Circled area = EVT cells surrounding the spiral arteries; Asterisk = placental villi; Plus = syncytial knots. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

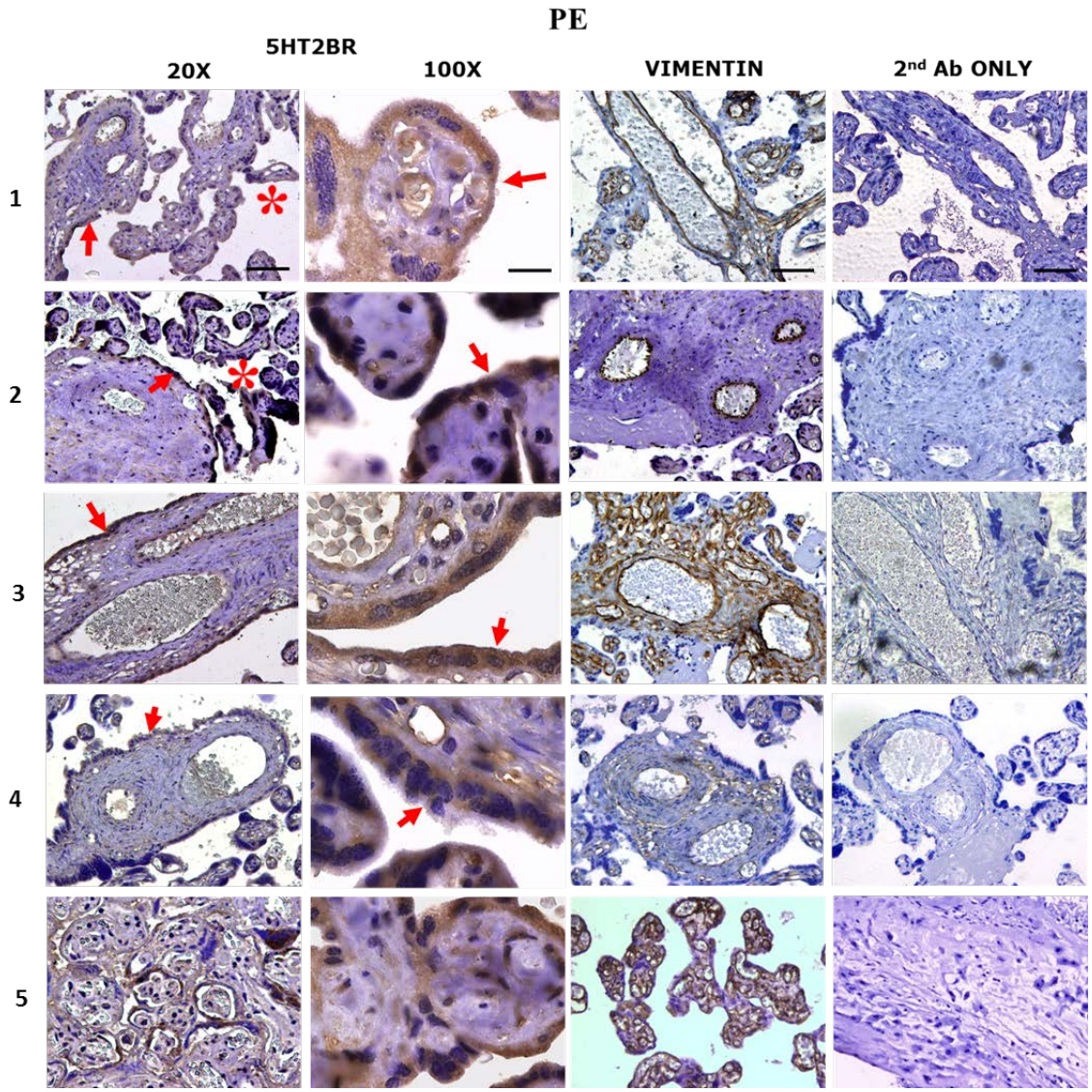


Figure 3.7: 5-HT$_{2B}$ Receptor localisation in NT placentae determined by immunohistochemistry.

5-HT$_{2B}$R reactivity for each sample was scored against immuno-reactivity for Vimentin (+ve) and secondary (2nd) Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = the lining of the spiral arteries; Circled area = EVT cells surrounding the spiral arteries; Asterisk = placental villi; Plus = syncytial knots. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

3.2.3.4 Relative mRNA expression of C/T antigens in NT and PE placenta

All C/T antigens studied showed differential expressions patterns in NT and PE placentae. Some show increased expression in NT placentae (e.g. CAGE1), whereas others show increased expression in PE placentae (e.g. MAGEA1). In contrast, some show very low expression in both (e.g. FATE1). However, amongst the 14 C/T antigens studied, seven showed statistical significant difference at the mRNA level. The expression of C/T antigens were analysed in relation to human testis (as positive control). Unlike the 5-HT receptors family, all the C/T antigens studied do not belong to the same family so an overall representation of their expressions could not be performed. The scatter plots comparing the expression of these genes in NT and PE are provided in Figure 3.6.

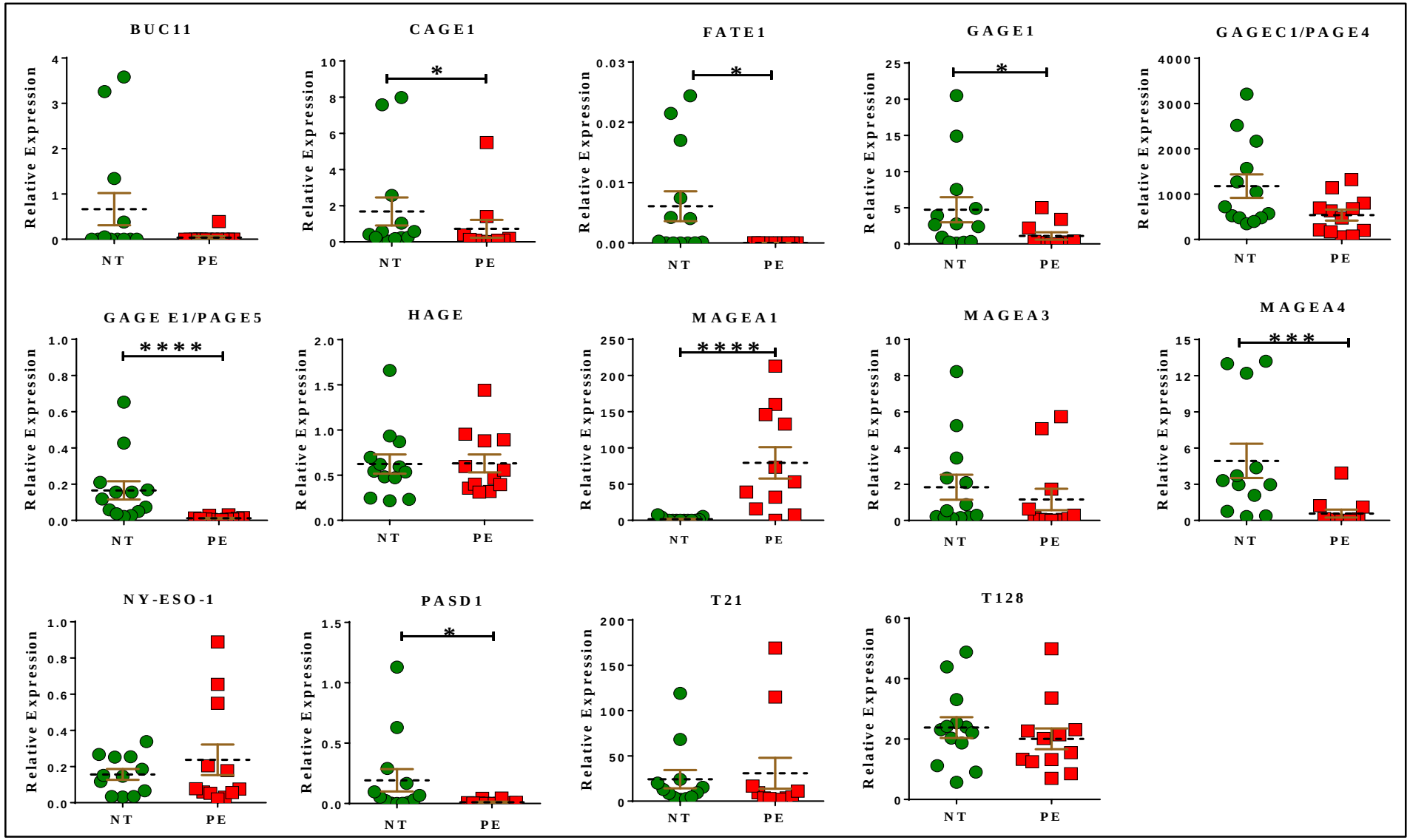


Figure 3.8: Relative expressions levels for C/T antigens mRNAs

Statistical significance was determined using the Mann – Whitney U test (n = 12-13; *p<0.05; ***p<0.001; ****P<0.0001). The error bars (SEM) are represented with brown lines. Values are arbitrary units relative to expression in testis (positive control; not shown in figure) set to 1. Therefore the scales (y axis) vary with expression levels in placental samples relative to testis.

The expression patterns for each of the C/T antigens are elaborated below:

- **BUC11**

The majority of placental samples showed lower levels of expression of BUC11 compared to human testis. The expression of BUC11 was lower in PE placentae when compared to NT (Figure 3.6).

- **CAGE1**

Interestingly, mRNA expression for CAGE1 was significantly down-regulated in PE compared to NT placentae ($p<0.05$) (See Figure 3.6).

- **FATE1**

Although the level of expression of FATE1 was seen to be lower in all placental samples, the expression was significantly lower in PE when compared to NT ($p<0.05$).

- **GAGE Family:**

The relative expression of GAGE family genes was lower in PE placentae when compared to NT samples (Figure 3.7). Among the three members of the GAGE family, GAGEC1/PAGE4 showed the highest expression in all placental samples. However, it was the only member which did not show any significant different in expression between NT and PE placentae. On the other hand, whilst GAGE1 and GAGEE1/PAGE5 showed relatively lower levels of expressions; they were both significant difference in patterns for NT and PE ($p<0.05$ and $p<0.0001$).

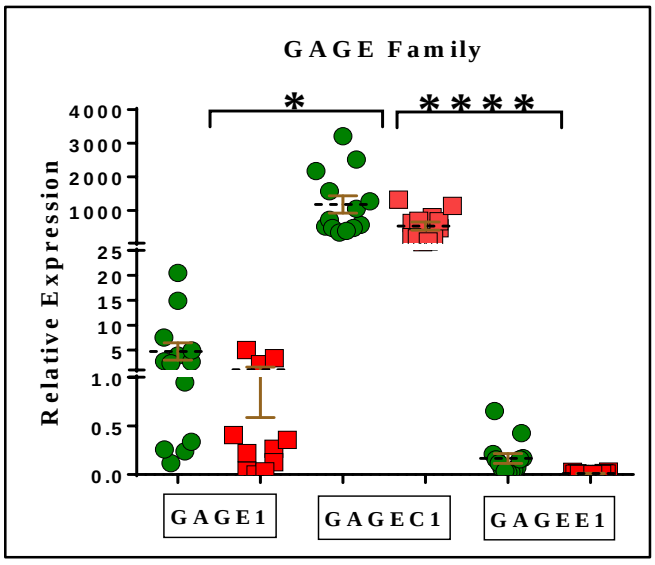


Figure 3.9: Overall expression patterns of GAGE family members.
NT and PE placental samples are represented in green dots and red squares respectively.

➢ **HAGE**

There was no change in mRNA expression for HAGE between NT and PE placentae (Figure 3.6).

➢ **MAGE Family:**

- **MAGEA1**

MAGEA1 was significantly up-regulated in PE placentae compared to NT ($p<0.0001$; Figure 3.6).

- **MAGEA3**

Whilst MAGEA3 appears to have a higher level of expression in NT placenta, the difference is not significant. This is probably due to high variability in individual data points (Figure 3.6).

- **MAGEA4**

The level of expression of MAGEA4 was statistically lower in PE compared to NT placentae ($p<0.001$; Figure 3.6).

The comparative expression patterns of MAGE family members are represented in Figure 3.8. The MAGEA1 is significantly up-regulated in PE, thus resulting in significant difference in expression levels to other MAGE family, MAGEA3 ($p<0.05$) and MAGEA4 ($p<0.01$).

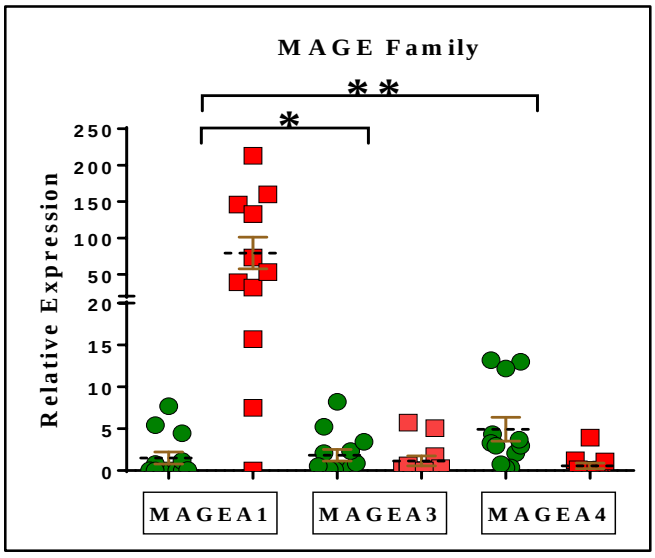


Figure 3.10: Overall mRNA expression patterns of MAGE family members.
NT and PE placental samples are represented in green dots and red squares respectively. Variances were analysed by Kruskal-Wallis test followed by Dunn's multiple comparison between the three members (*$p<0.05$; **$p<0.01$±SEM).

➢ **NY-ESO-1**

Approximately 25% of the PE placenta showed elevated NY-ESO-1 levels compared to NT (Figure 3.6) with the rest of the PE samples having similar levels to NT placenta. The level of expression was less in all placental samples relative to testis.

- **PASD1**

This mRNA was of low abundance and hardly expressed in placental tissue. Nevertheless, a few of the NT samples (~25%) did show relatively high levels of expression when compared to PE samples, resulting in statistical significance ($p<0.05$). The level of PASD1 mRNA expression was statistically lower in PE compared to NT placentae ($p<0.05$; Figure 3.6).

- **T21**

Although two samples in both NT and PE placenta did show high levels of T21 expression, generally levels were not elevated (Figure 3.6).

- **T128**

Expression levels for T128 were highly variable in both NT and PE samples suggesting factors unrelated to placental development/homeostasis are playing a role in their expression (Figure 3.6).

3.2.3.5 Protein expression of C/T antigens

The scatter-plots for protein expression of C/T antigens are shown in Figure 3.9. Equal amounts of placental samples (35-70µg) were loaded, as confirmed by the internal controls (β-actin and GAPDH) on the same blots. Only 20µg of the positive control (testis) were loaded to avoid over-saturation of the blot. The last lane containing the testis samples are separated by dotted lines (See Figure 3.9). Of the seven C/T antigens that show significant differences in mRNA expression, only six were checked for protein expressions as there were no reliable, commercially available, antibodies against PASD1. The protein expressions of the rest, namely CAGE1, FATE, GAGE and MAGE families were compared between NT and PE samples. Examples of whole blots can be found in the Appendix A.6.

- **CAGE1**

The anti-CAGE1 antibody yielded three distinct bands in three of the minority of the NT and PE placental samples. The molecular weight of these bands were approximately 100, 150 and 175kDa respectively (indicated by red arrows in Figure 3.9). This molecular weight of the bands is within the range (95-230kDa) of detected bands declared by the supplier (Sigma). The three visible bands were analysed in all the samples as the positive control also displayed these bands, making it difficult to predict the correct band. The expression levels in PE samples were comparatively lower than in NT placentae. Although only 20µg of

positive control (testis) was loaded along with 70µg of placental samples, the intensity of the band was still stronger in testis.

- **FATE1**

The predicted molecular weight for FATE1 protein declared by the supplier (Sigma) was 17-28 kDa. The two bands detected for FATE1 in positive control and placental samples were within the range of 20kDa to 25kDa (indicated by red arrows in Figure 3.9). Almost all the NT samples showed expression of FATE1. On the other hand, only few of the PE samples showed expressions of FATE1, but the intensity of the bands was higher when compared to NT.

- **GAGE Family**

The two members of GAGE family that were investigated for protein expression showed significant differences in expression between NT and PE placentae.

  - **GAGE1**

Two bands were observed for GAGE1 immunoblotting in both testis and placental samples that migrated between 30-40kDa (indicated by red arrows in Figure 3.9), which is within the predicted range of between 25-37kDa. Both the bands were analysed, since these were also visible in positive control (testis). Few placental samples showed very close level of expressions to that in testis (approximately 0.8). Additionally a significant difference in expression was observed between NT and PE placentae ($p<0.05$). The levels were significantly lower in PE when compared to NT, which could be as a result of 6 PE samples showing negligible expression.

  - **GAGEE1/PAGE5**

GAGEE1 (also known as PAGE5) was one of the few C/T antigens which exhibited very intense bands on the immunoblots (Figure 3.9). Therefore, of all the C/T antigens studied, GAGEE1/PAGE5 showed the greatest expression in placental samples. As the scatter plot shows, GAGEE1/PAGE5 expression is lower in PE placenta ($p<0.001$). Whilst the expected molecular weight of the band was 14kDa, the antibody detected a band at ~22kDa in placental samples and testis (positive control).

- **MAGE Family**

  - **MAGEA1**

The commercially available antibody for MAGEA1 (Abcam) was not able to detect MAGEA1 expressions in placental samples, even after increasing loading amounts of protein (70-140µg). Since a band was detected in testis (positive control) that migrated at ~34 kDa, it confirms that this protein was not expressed in the placental tissue studied (data not shown).

- **MAGEA4**

Almost all the samples showed expression of MAGEA4 protein (See Figure 3.9). The bands were detected at 37kDa for placental, as well as testis samples. Interestingly, when the expression levels were compared between NT and PE placentae, a significant reduction was observed in PE samples ($p<0.001$).

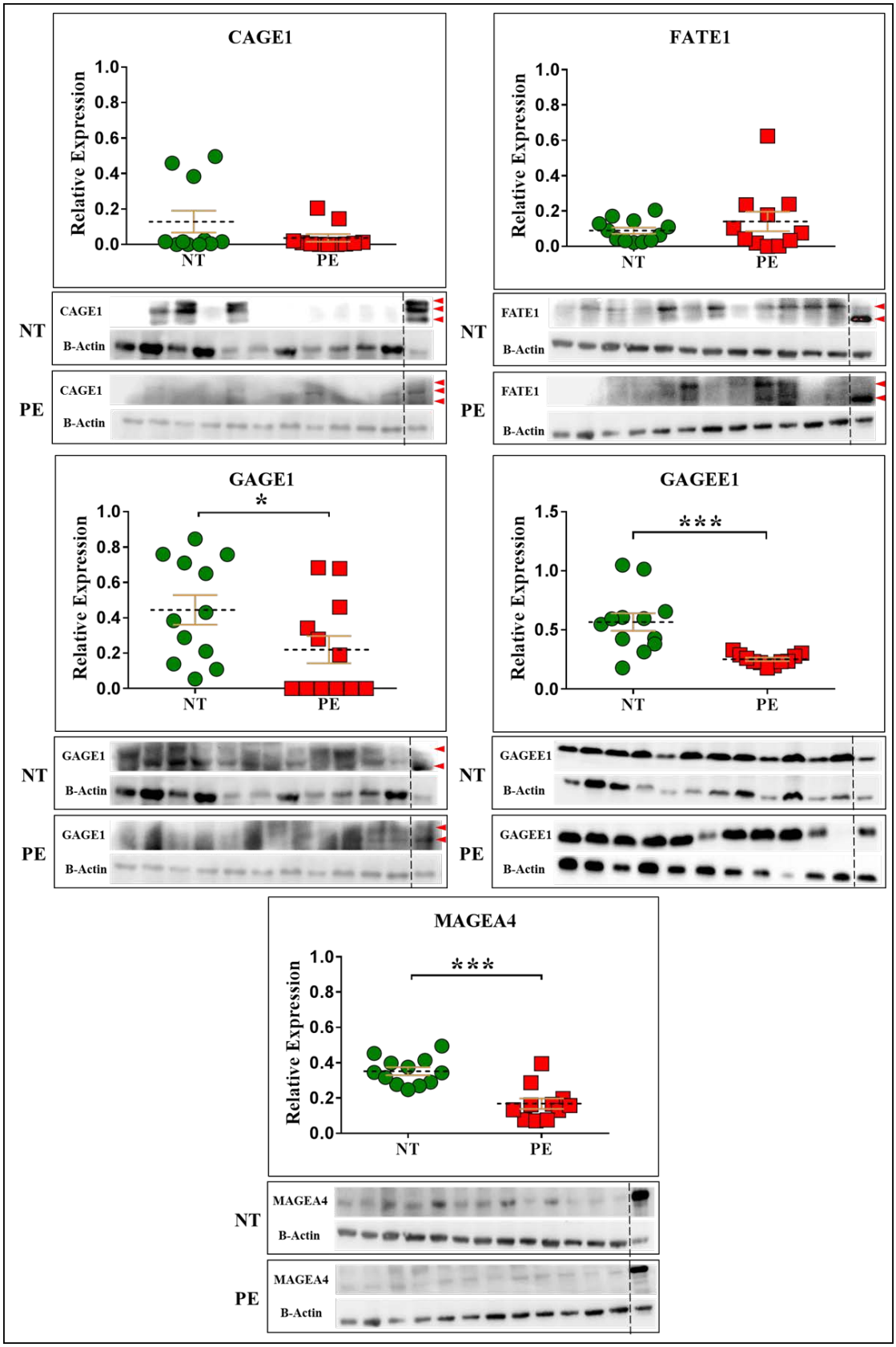


Figure 3.11: C/T antigen protein expression – Comparative scatter plots.

The relative expressions are arbitrary units of the values calculated from two independent blots normalised firstly to internal control (β-actin) and secondly to a positive control (human testis). Mann – Whitney U test was performed (*p<0.05; ***p<0.001). The error bars (SEM) are represented with brown lines.

As a result from the protein investigations of these six C/T antigen, three showed significantly lower protein levels in PE samples compared to NT. Hence, the protein study supports the differential expression patterns of these genes in NT and PE placentae.

3.2.3.6 Cellular localisation of significant C/T antigens

The expression of C/T antigens in placental samples was also investigated by IHC in order to identify their cellular localisation. Most of the C/T antigens showed granular positive staining of the cytoplasm with a few exceptions. For example, the GAGE family for which both nucleus and cytoplasm were stained. The IRS semi-quantified data between NT and PE are shown in Table 3.4. As before (Section 3.2.3.3), the Fleiss' Kappa Statistics was performed on this IRS data and a kappa score of 0.468 was obtained indicating the agreement between the scorers were moderate.

Table 3.4: Immuno-reactivity score (IRS) of C/T antigens

C/T ANTIGENS	NT (n=5-6)	PE (n=4-5)	P VALUE	SIGNIFICANCE
CAGE1	2 ± 0.5	2 ± 0.2	> 0.9999	ns
FATE1	1 ± 0.6	0 ± 0.2	0.1761	ns
GAGE1	3 ± 0.4	3 ± 0.0	> 0.9999	ns
GAGEE1/PAGE5	3 ± 0.1	2 ± 0.5	0.1231	ns
MAGEA1	3 ± 0.4	1 ± 0.3	0.0094	**
MAGEA4	1 ± 0.2	1 ± 0.3	> 0.9999	ns
PASD1	3 ± 0.2	3 ± 0.4	> 0.9999	ns

The intensity of staining was represented by the mode±SEM of the IRS from 5 individual samples in each group. Unpaired t-test was then performed on the IRS modes of NT and PE (ns=no significance).

- **CAGE1**

Moderate immuno-staining was observed for CAGE1 in both NT and PE samples (Figure 3.12 and 3.13). Staining was visible in the cytoplasm of the cells that were immunopositive. The STB and CTB layers of the placental villi were deeply stained for CAGE1 in NT tissues (thin arrows). The lining of the spiral arteries also showed similar staining intensities (arrow head). The EVT cells, present around the villous columns and in the placental beds, also revealed distinct immuno-positivity (encircled). Intense staining was also observed in the collagen and fibrous layers surrounding the placental arteries (bold arrows; image 5 of Figure 3.12). However, in PE tissues, the staining of CAGE1 was reduced, relatively around the placental villi, but was more concentrated on the decidual placental beds. Various cells, including EVT cells, in the placental bed showed moderate immuno-reactivity to CAGE1

(PE; image 1 and 4). The CAGE1 staining intensities for NT and PE showed no significant differences (Table 3.4).

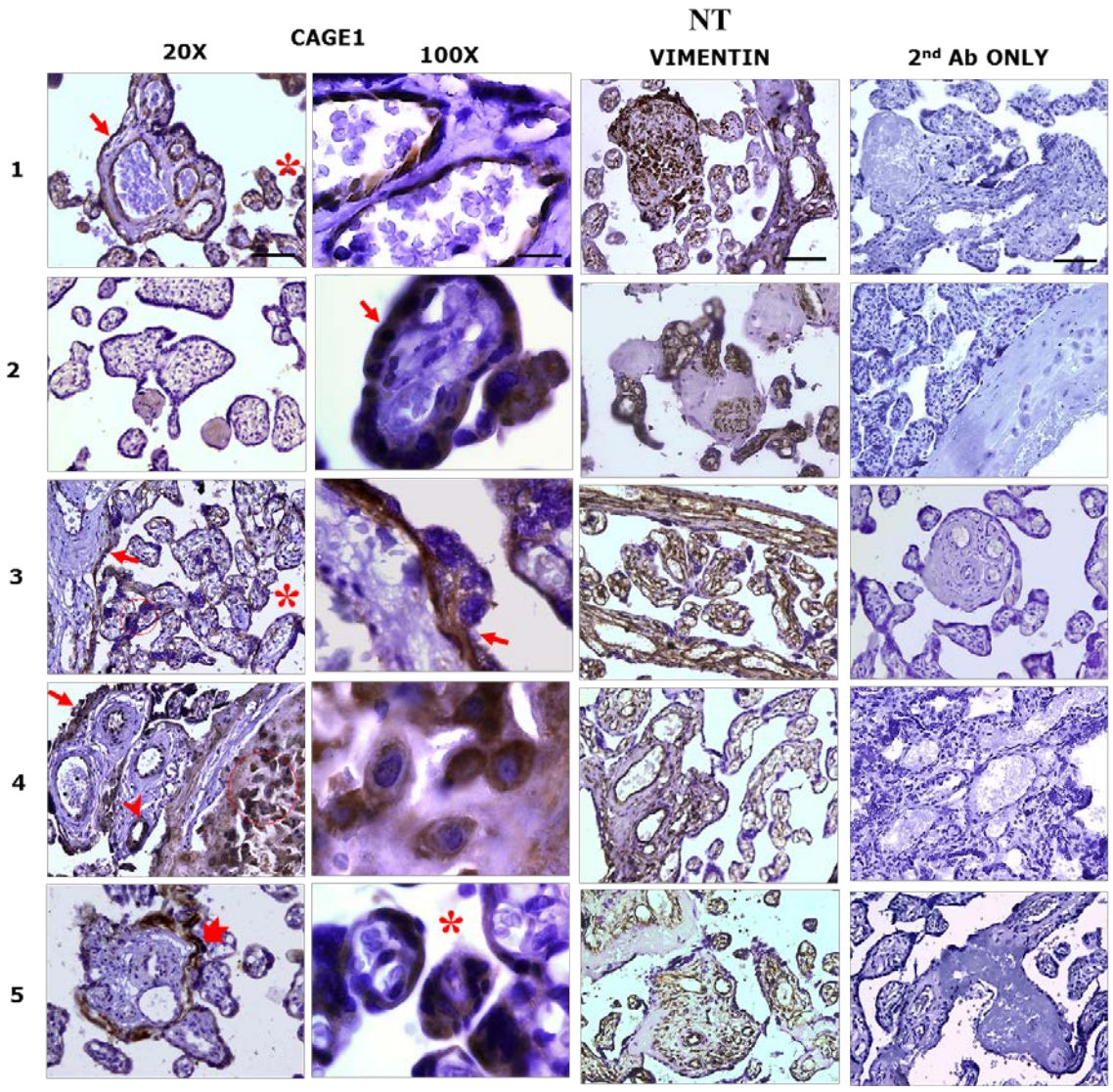


Figure 3.12: CAGE1 protein placental localisation in NT placentae determined by immunohistochemistry.

CAGE1 reactivity for each sample was scored against immuno-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = spiral arteries encircled area = villous column; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

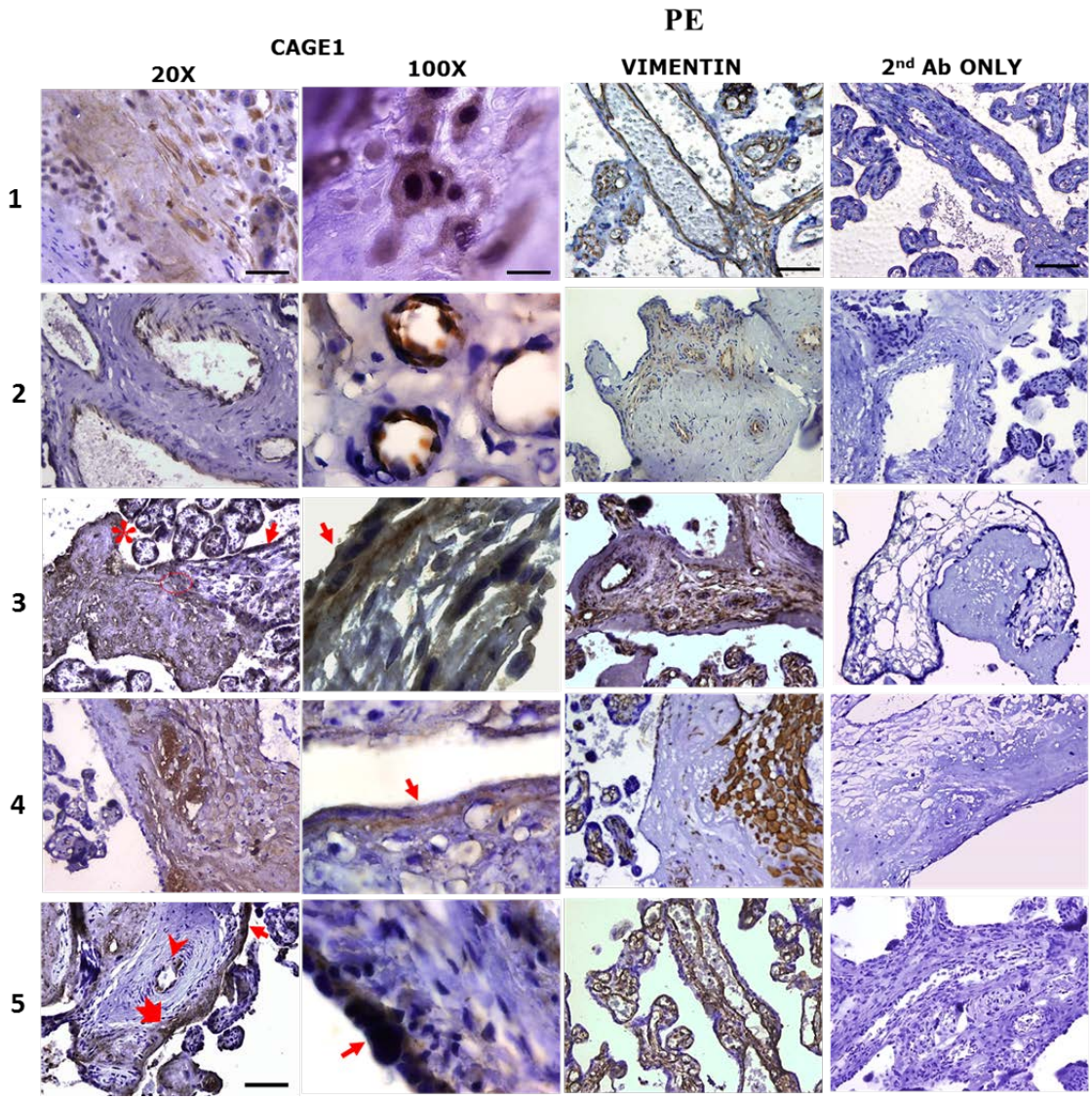


Figure 3.13: CAGE1 protein placental localisation in PE placentae determined by immunohistochemistry.

CAGE1 reactivity for each sample was scored against immuno-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = spiral arteries encircled area = villous column; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **FATE1**

Compared to other C/T antigens studied, the overall IRS for FATE1 was very low. In NT tissues, granular cytoplasmic staining was observed in the CTB and STB layers (thin arrows). The EVT cells of the placental villous column showed more intense staining than the remaining tissue sections (encircled in Figure 3.14; NT, image 1). Fibrin and collagen layers around the arteries were also immuno-reactive to FATE1 (bold arrows). Interestingly, few cells in the stroma of the villi were also stained (double arrows), which were more focal in some sections (NT, image5). Furthermore, the FATE1 staining in PE samples was almost

negligible, with only few focal staining of the EVT in the decidua and stroma (Figure 3.15). It was not statistically significant (Table 3.4).

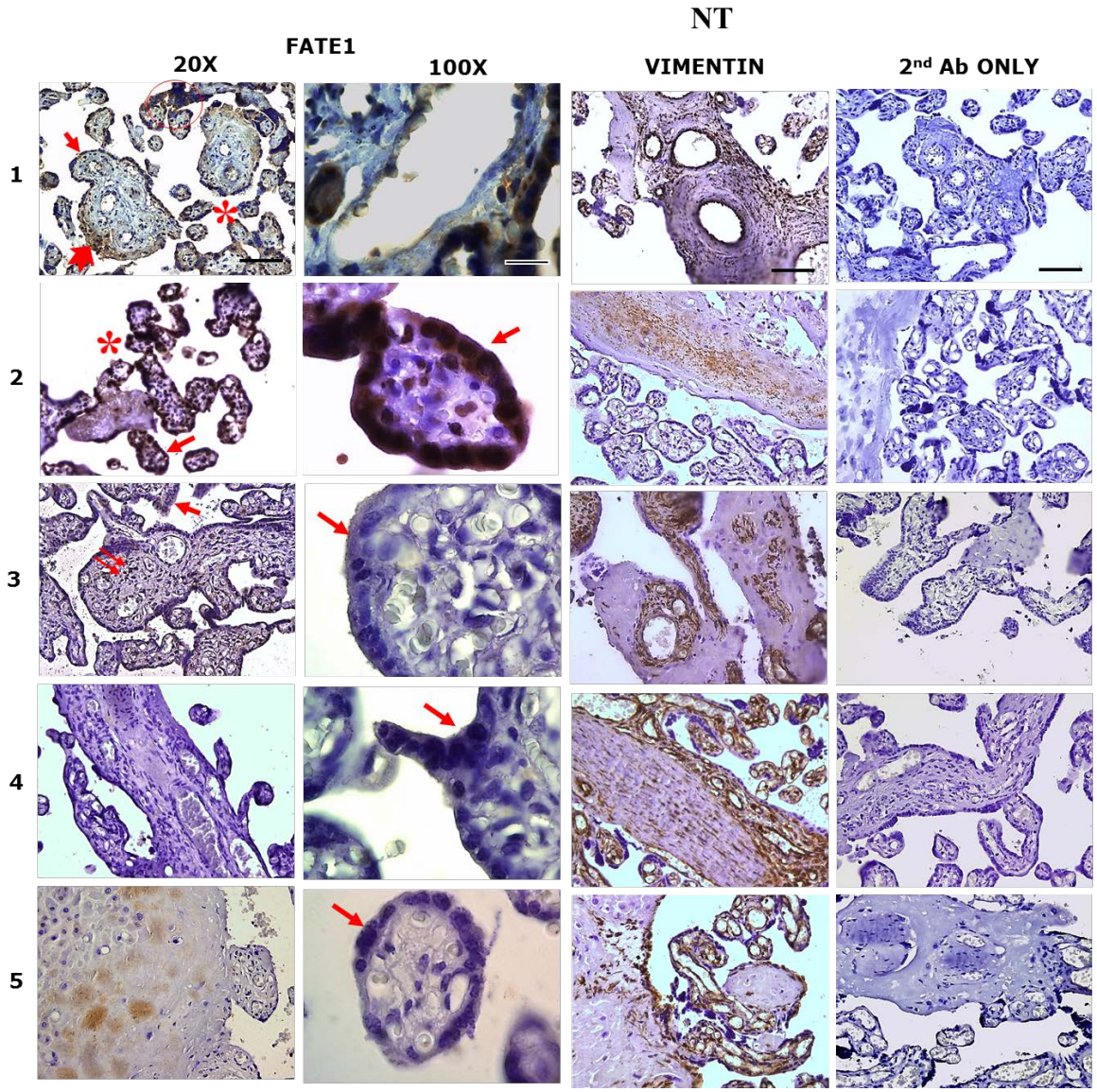


Figure 3.14: FATE1 protein placental localisation in NT placentae determined by immunohistochemistry.

FATE1 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; encircled area = villous column; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

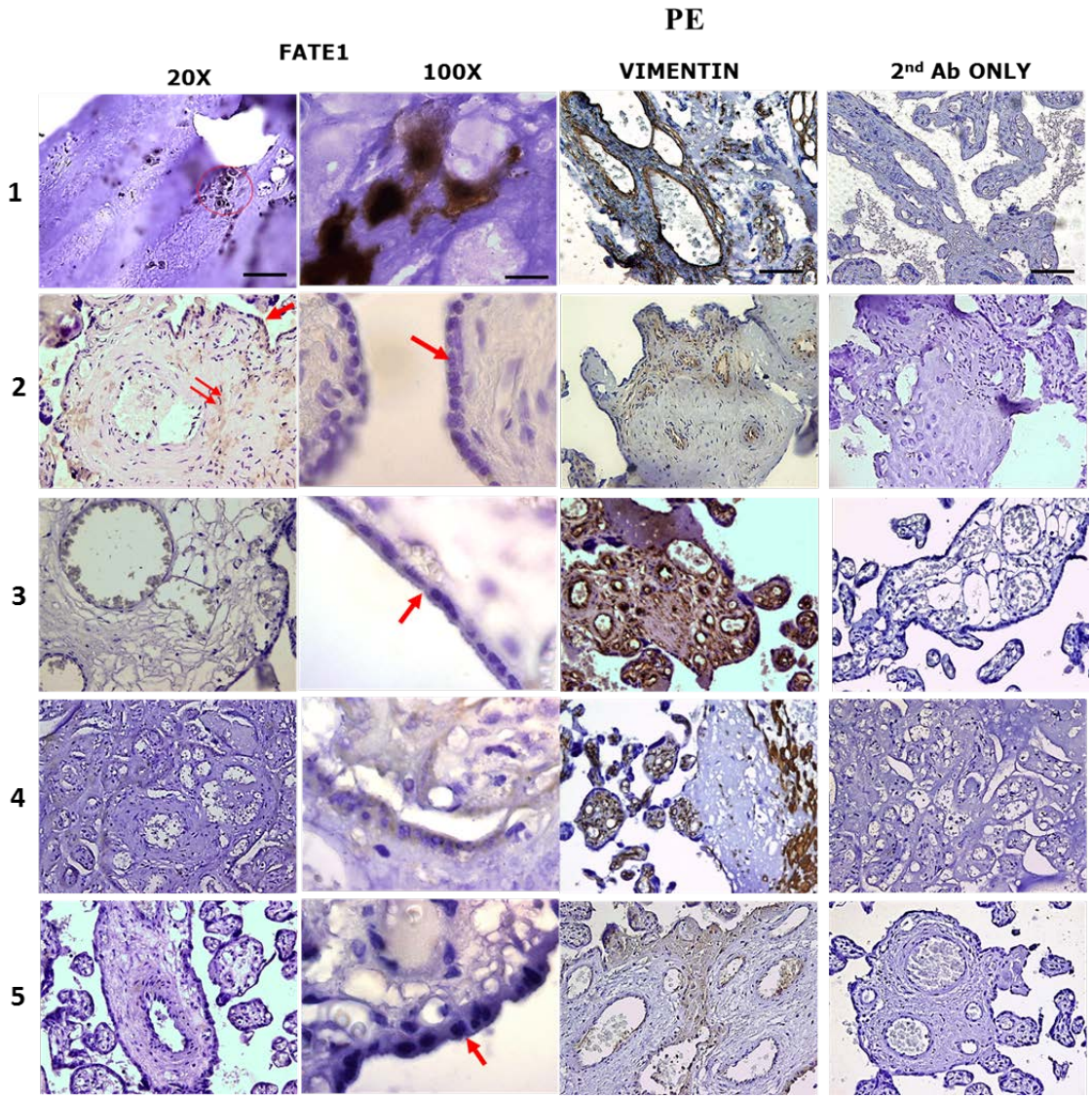


Figure 3.15: FATE1 protein placental localisation in PE placentae determined by immunohistochemistry.

FATE1 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; encircled area = villous column; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **GAGE Family**

Immunohistochemistry was performed on two members of the GAGE family, GAGE1 and GAGEE1/PAGE5.

- **GAGE1**

The IRS data for GAGE1 in NT and PE inferred high staining intensity with no significant difference between the two groups (Table 3.4). The CTB and STB layers of the placental villi (asterisk) as well as the villous tree (thin arrows) were deeply stained in both NT and PE tissues (Figure 3.16 and 3.17 respecitvely). Staining was also distinctly visible around the inner lining of the spiral arteries in NT tissues (represented with arrow heads in NT, image 3). Moreover, the cytoplasm and nucleus of the EVT in the placental beds of NT tissues exhibit a higher IRS when compared to the other stained areas of the same tissue (NT, images 4 and 5). Conversely, in PE tissues, in addition to staining of the CTB, STB and EVT, the fibrin and collagen deposits as well as few stromal cells were stained (Represented with bold arrows in PE, image 1).

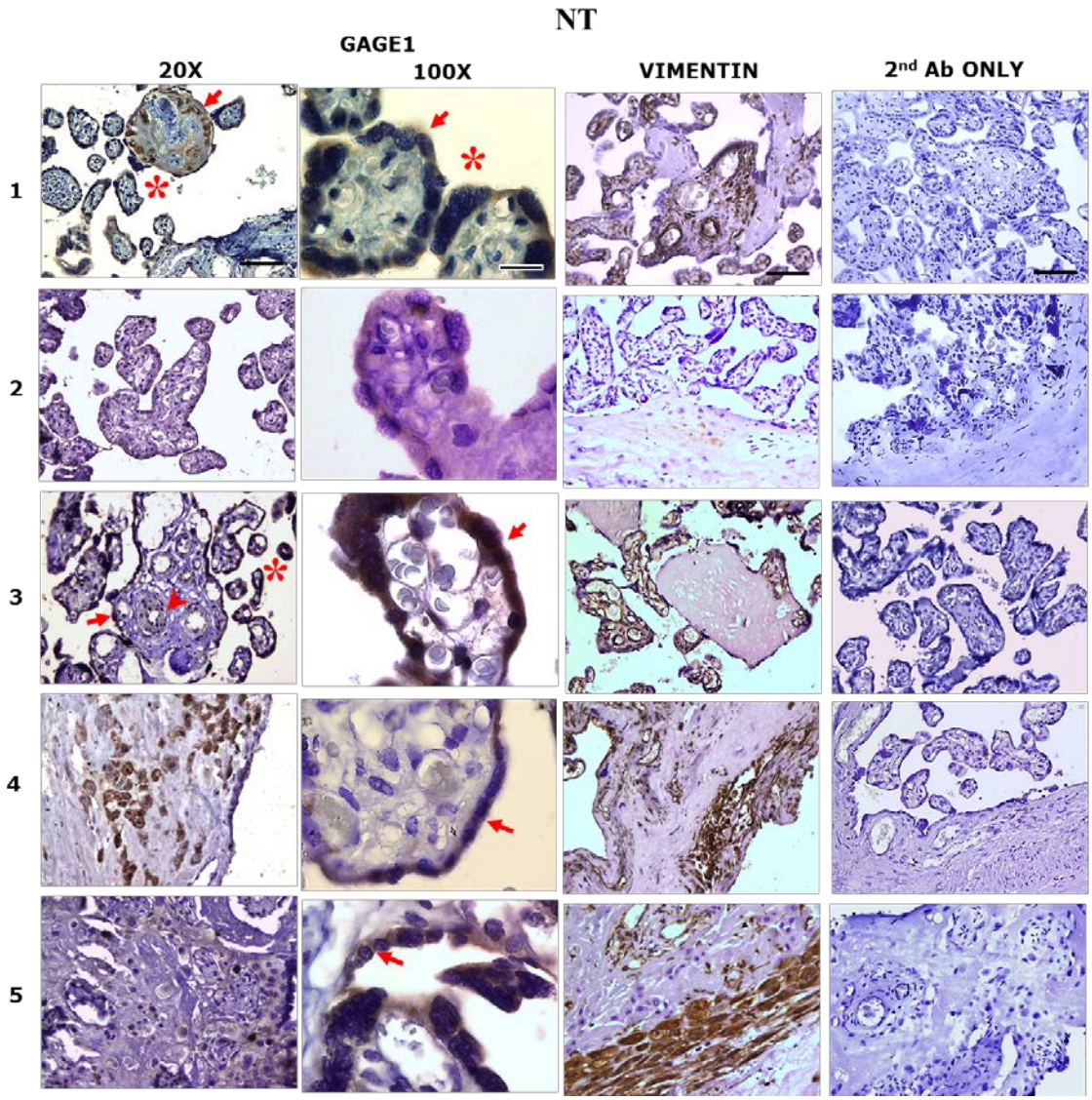


Figure 3.16: GAGE1 localisation in NT placentae determined by immunohistochemistry.

GAGE1 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = spiral arteries; encircled area = villous column; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

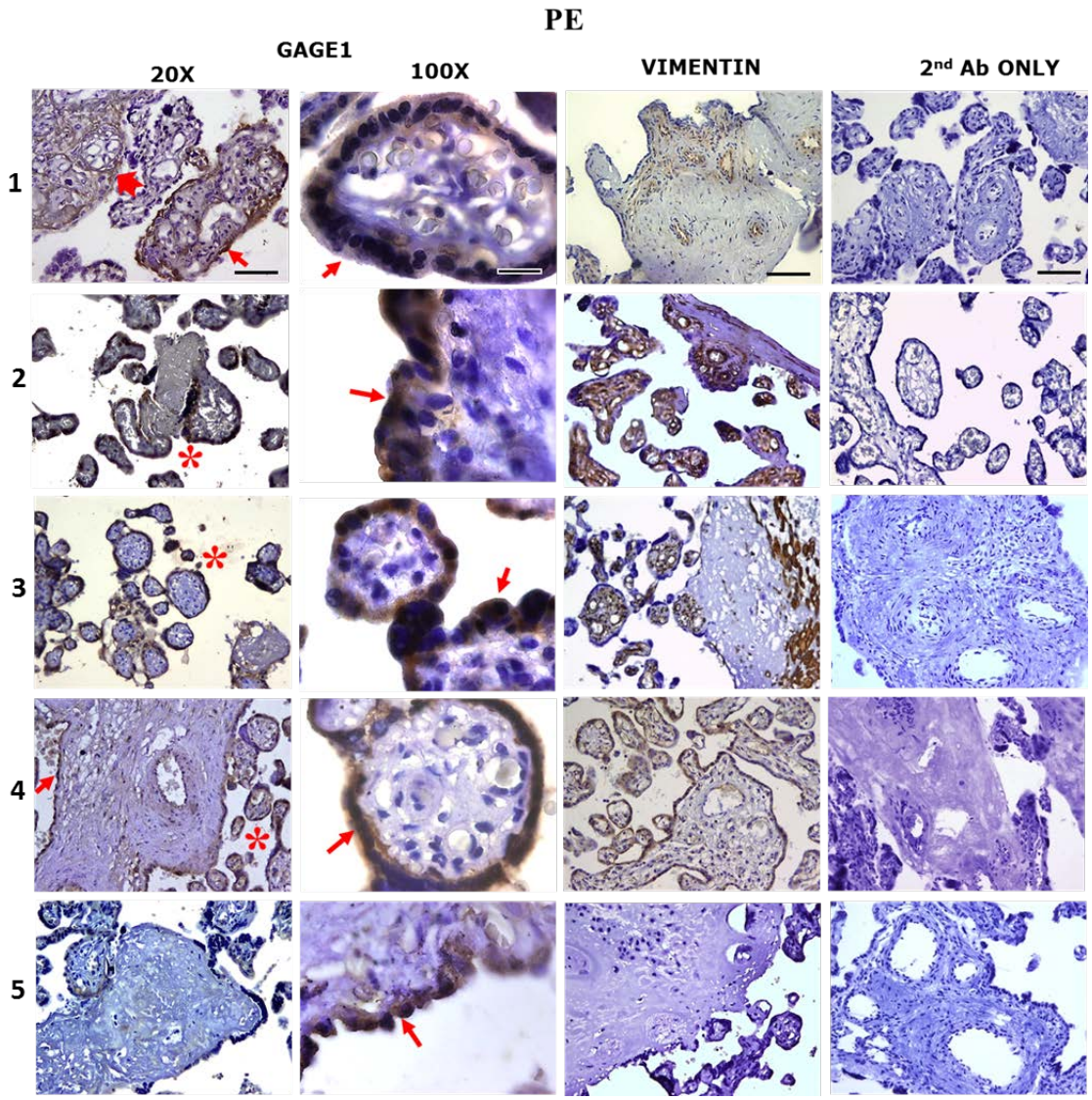


Figure 3.17: GAGE1 localisation in PE placentae determined by immunohistochemistry.

GAGE1 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = spiral arteries; encircled area = villous column; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **GAGEE1/ PAGE5**

Amongst all the C/T antigens investigated for IHC, GAGEE1/PAGE5 IRS exhibited the maximum intensity (See Figure 3.18 and 3.19). The entire NT sections were completely stained; with higher staining intensities around the spiral arteries (bold arrows). These regions comprised of fibrin, collagen layers and stromal cells (double arrows). The immuno-reactivity of the CTB and STB layers were relatively low. Futhermore, the EVT cells in the villous column as well as the villi showed moderate staining (encircled). On

the other hand, the staining in PE tissues were more dispersed and concentrated at few regions on the placental bed. In these regions, the cytoplasm of various cells were observed to have granular staining, especially EVT (encircled) and stromal cells (double arrows). Although the IRS score was lower for PE staining when compared to NT, the difference was not statistically significant (Table 3.4).

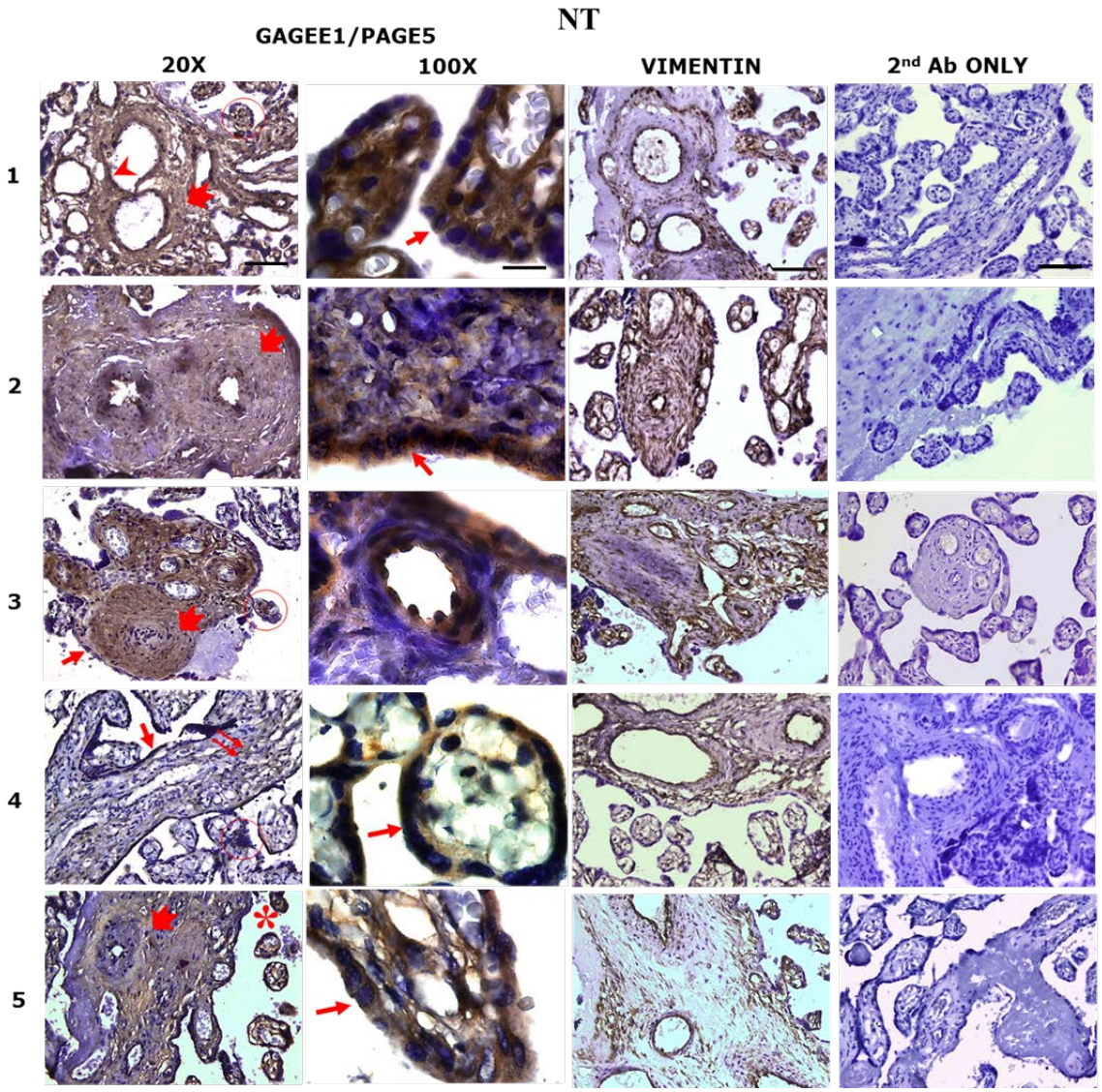


Figure 3.18: GAGEE1 localisation in NT placentae determined by immunohistochemistry.

GAGEE1 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = spiral arteries; encircled area = villous column; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

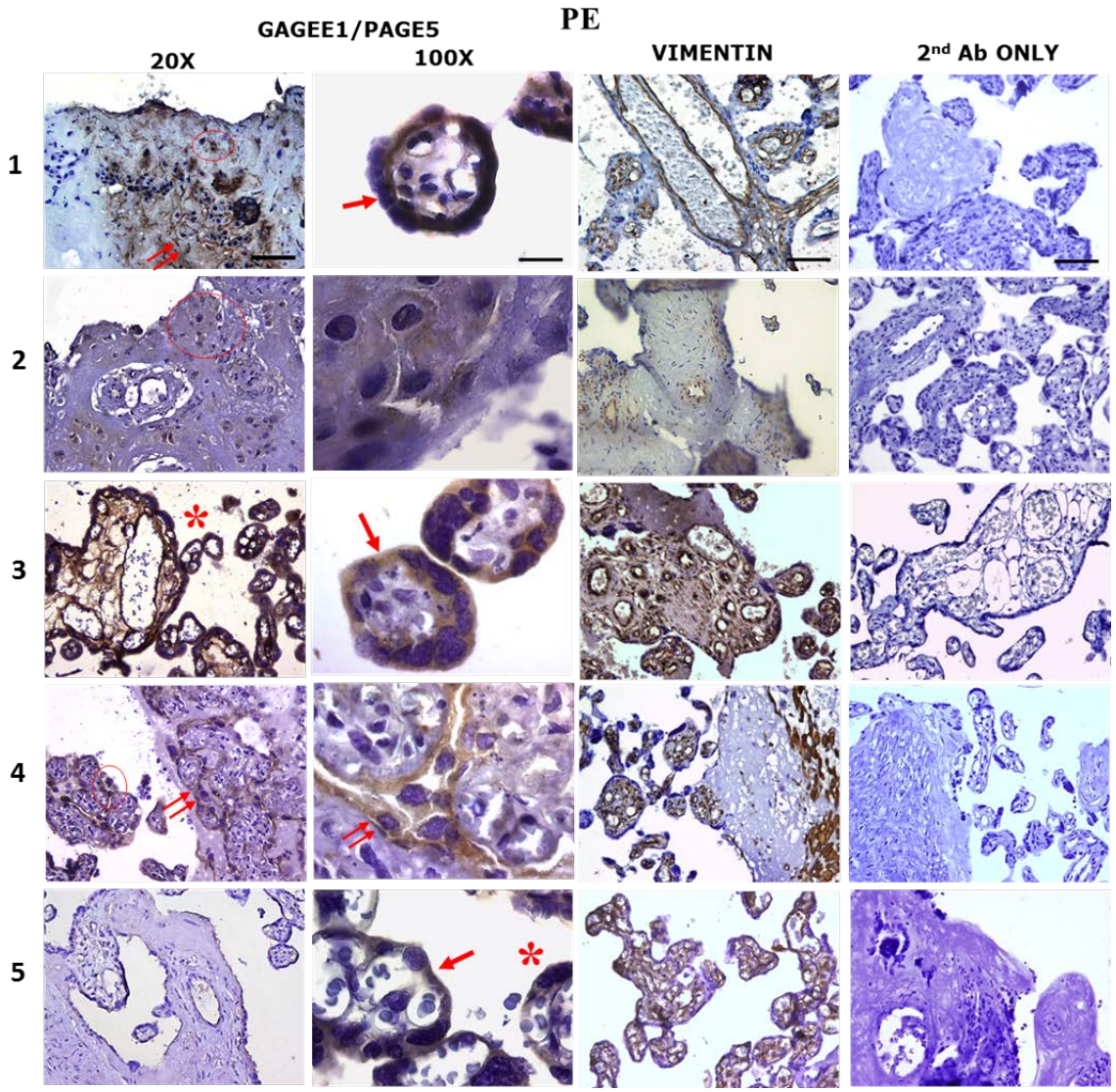


Figure 3.19: GAGEE1 localisation in PE placentae determined by immunohistochemistry.

GAGEE1 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = spiral arteries; encircled area = villous column; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

➢ MAGE Family

MAGEA1 and MAGEA4, members of the MAGE family were investigated for their localisation in NT and PE tissues by IHC.

• MAGEA1

The primary location for MAGEA1 staining in NT tissues was the placental villi (asterisk) with intense staining detected in the STB and CTB layers (thin arrows) and a few stromal cells (double arrows; Figure 3.20). In PE, IRS for MAGEA1 was observed in the decidual regions with more focal staining of the EVT cytoplasm in the placental beds (encircled). Patchy staining patterns were also observed in PE decidua (represented by double arrows in PE, image 4 of Figure 3.21). Overall, the staining intensity in PE tissues was lower when compared to the high staining levels in NT tissues. These were confirmed by the IRS data which showed statistically significant differences for MAGEA1 staining in NT and PE samples ($p<0.01$) (Table 3.4).

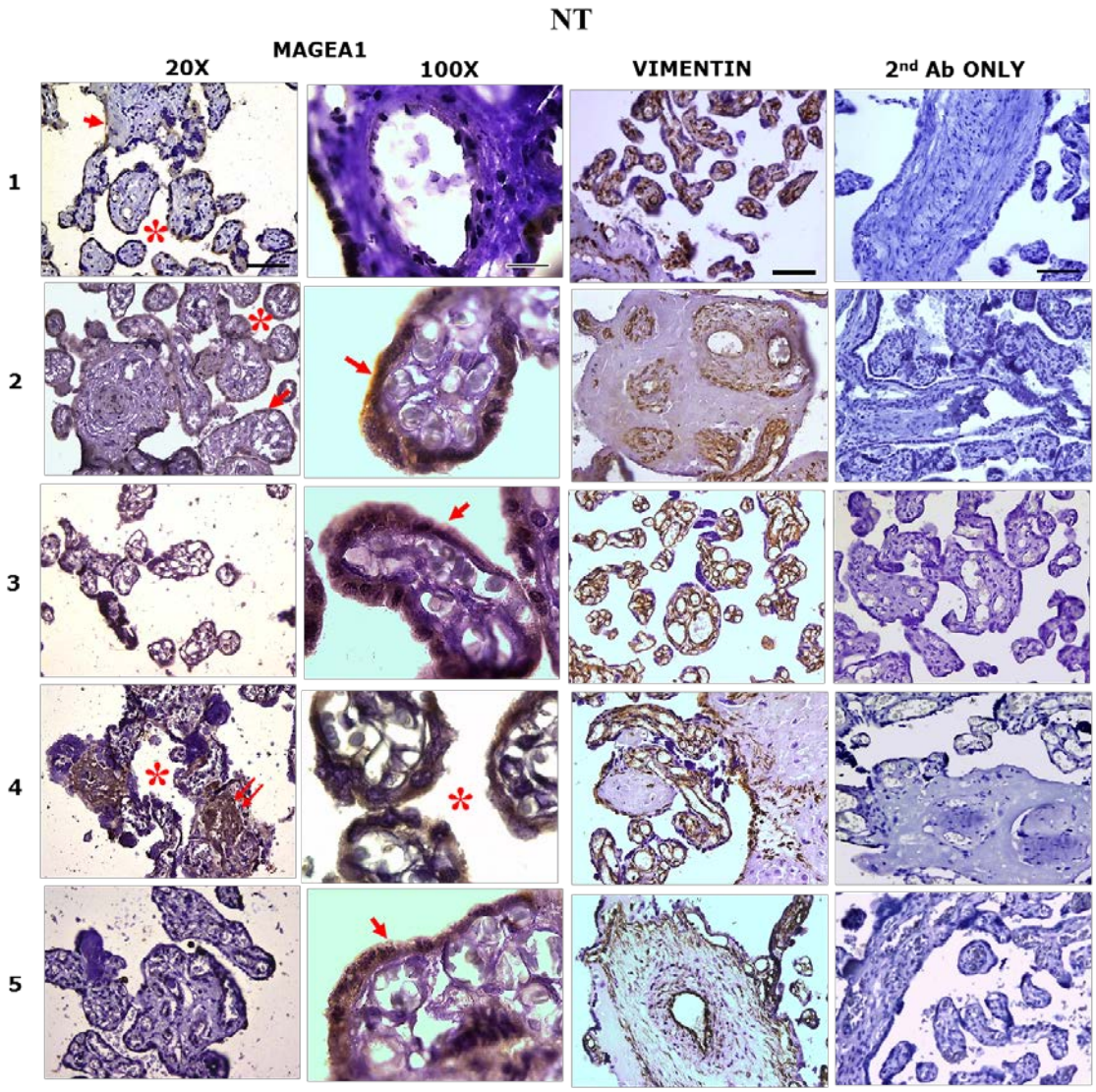


Figure 3.20: MAGEA1 localisation in NT placentae determined by immunohistochemistry.

MAGEA1 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; encircled area = villous column; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

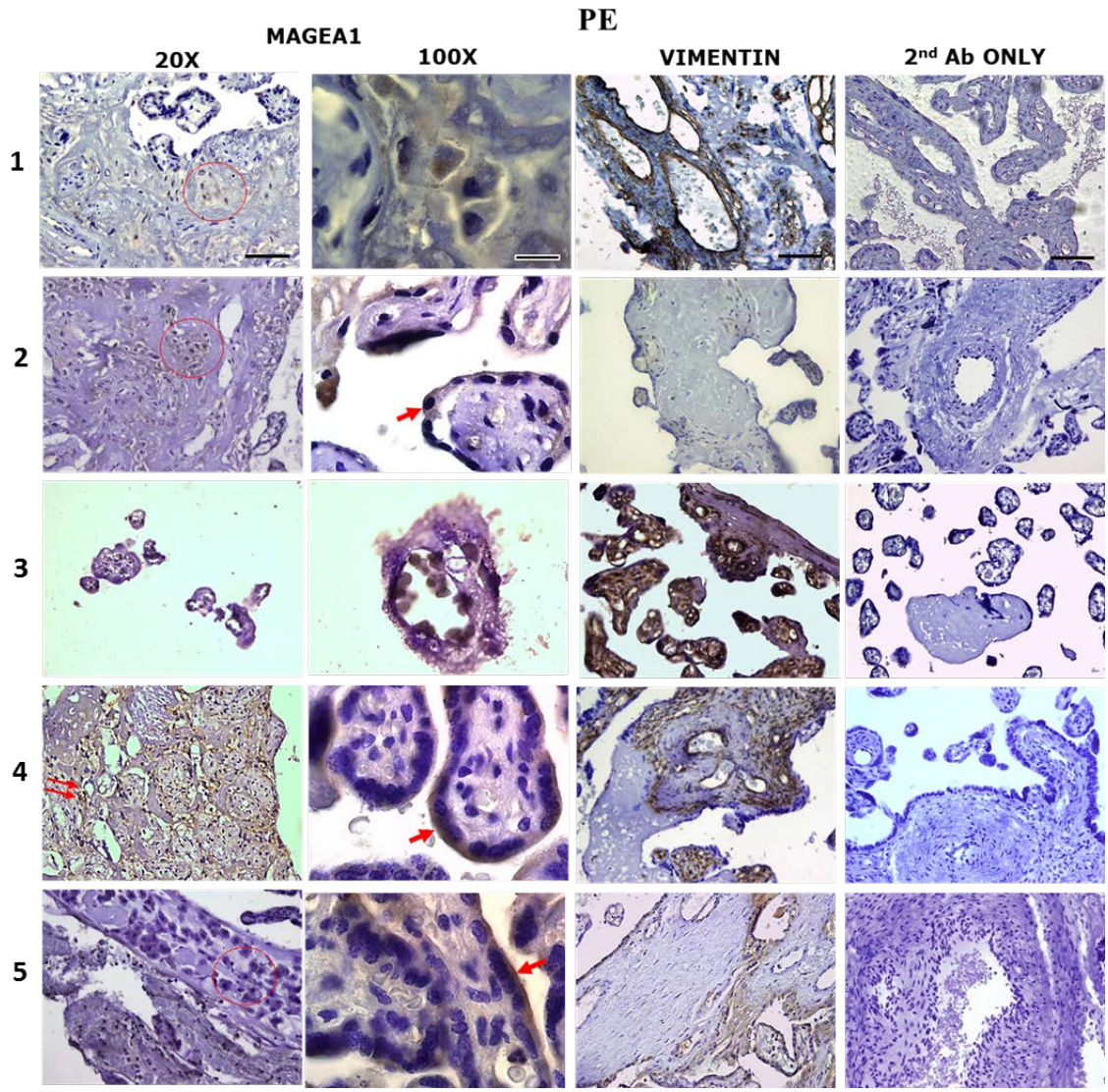


Figure 3.21: MAGEA1 localisation in PE placentae determined by immunohistochemistry.

MAGEA1 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; encircled area = villous column; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **MAGEA4**

The staining intensity for MAGEA4 was very weak in both NT and PE tissues, as can be seen in Figure 3.22 and 3.23. The staining around the CTB and STB layers were almost negligible. A small number of EVT cells in the placental beds showed granular staining of the cytoplasm. Weak staining was also observed in the fibrin deposits around the placental bed (bold arrow). Overall, there was no significant difference in staining intensity of MAGEA4 in NT and PE (Table 3.4).

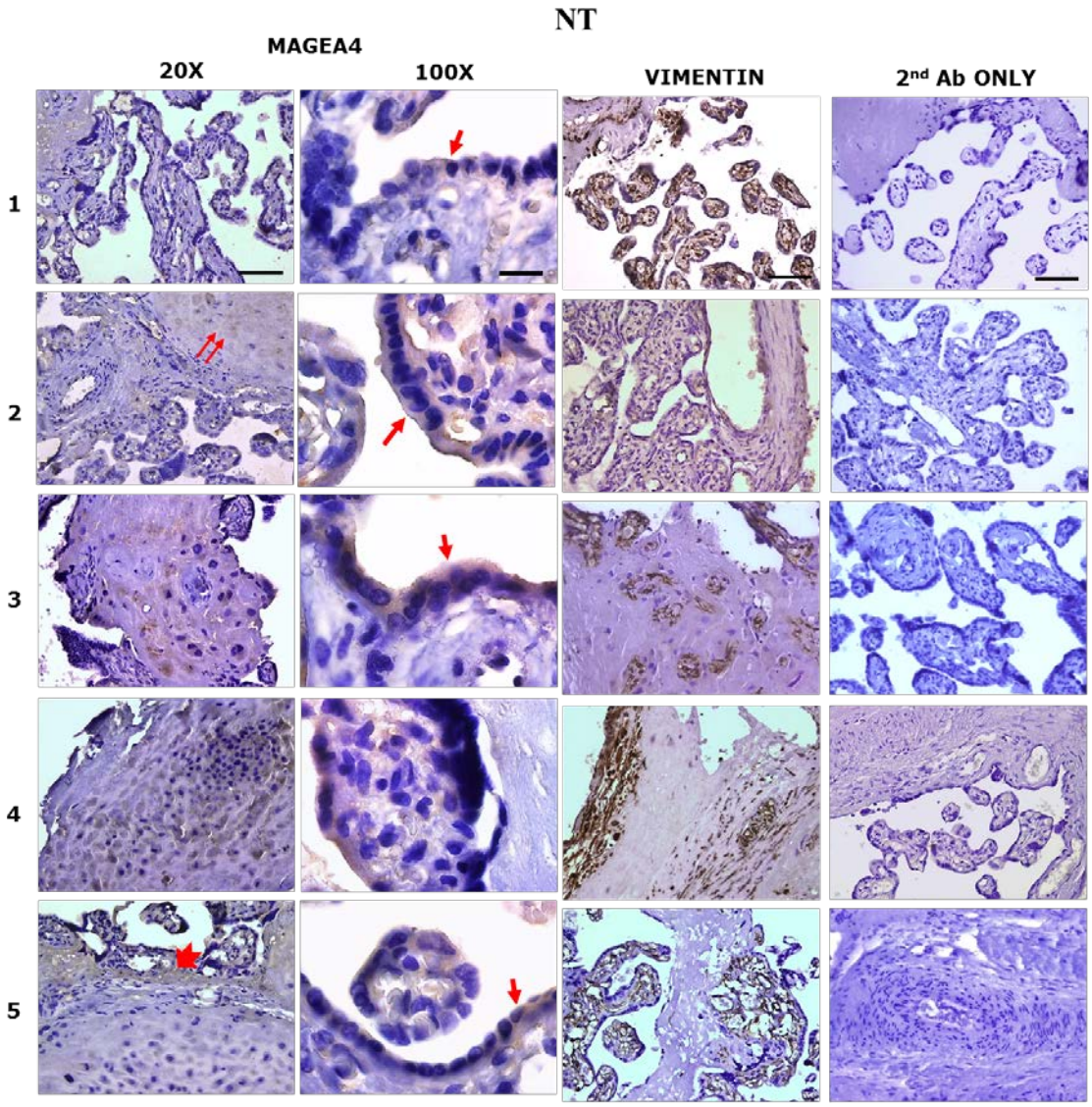


Figure 3.22: MAGEA4 localisation in NT placentae determined by immunohistochemistry.

MAGEA4 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = spiral arteries; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

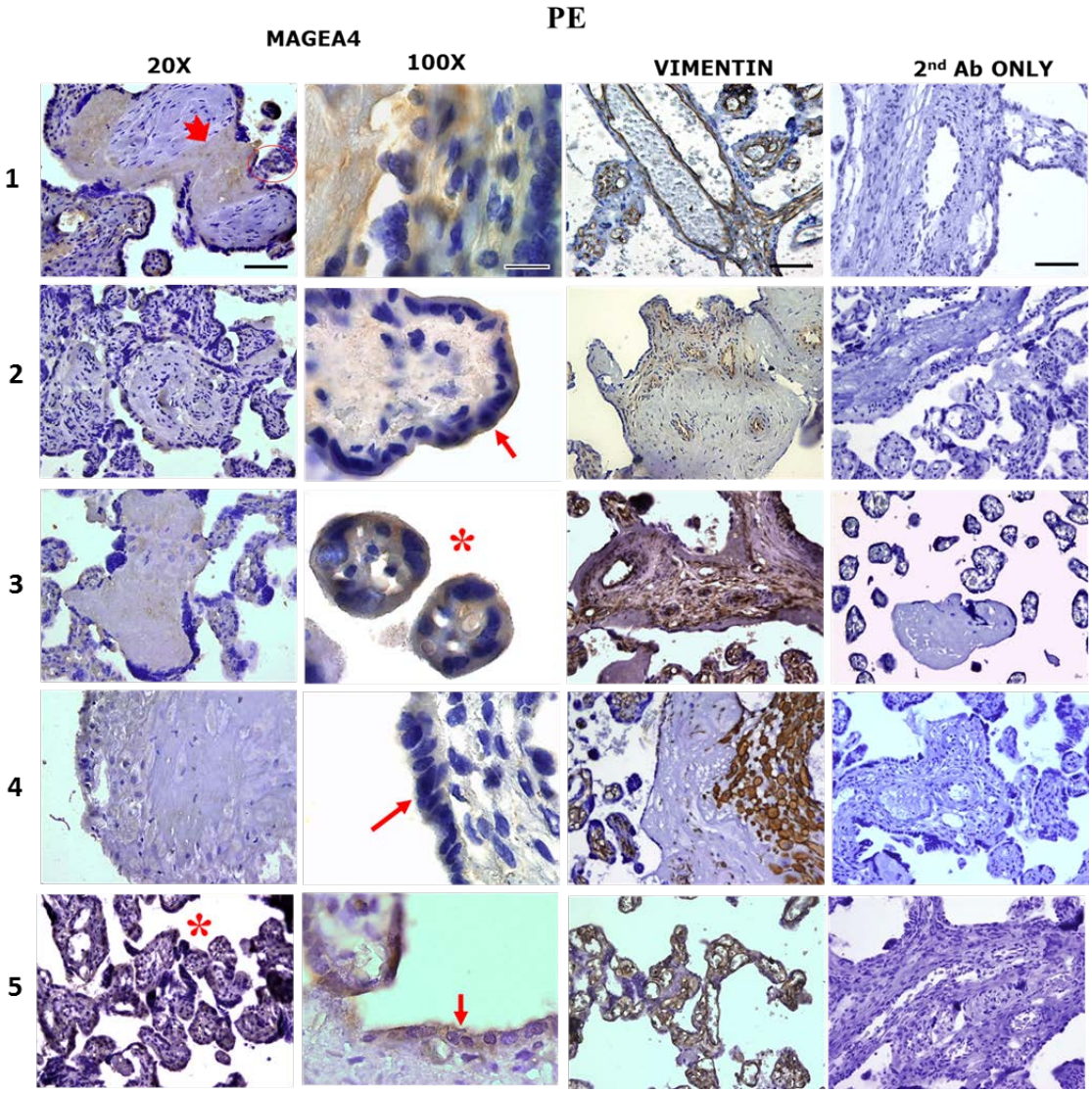


Figure 3.23: MAGEA4 localisation in PE placentae determined by immunohistochemistry.

MAGEA4 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = spiral arteries; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **PASD1**

NT and PE tissues showed relatively high IRS for PASD1. In NT tissues, the CTB and STB layers were distinctly stained (thin arrows in Figure 3.24). The cytoplasmic staining of the stromal cells (represented by double arrows; NT, image 3) as well as the EVT cells (encircled in NT, image 4) were also stained. Apart from staining of the CTB and STB layers, in PE the decidual sections showed intense staining around the EVT cells (bold arrows in PE, image 1, Figure 3.25). The IRS data showed equal intensities of staining in NT and PE tissues (Table 3.4).

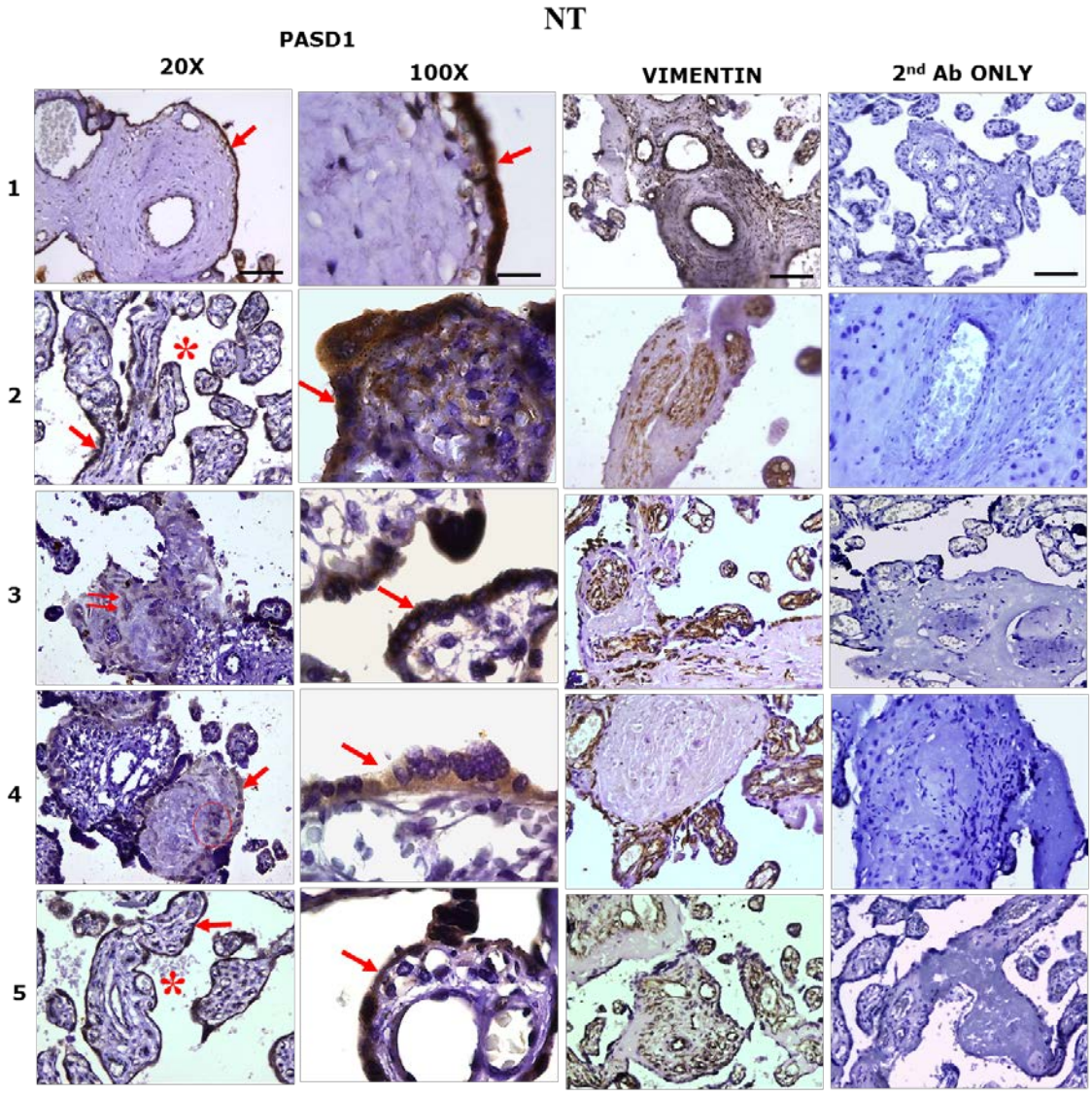


Figure 3.24: PASD1 localisation in NT placentae determined by immunohistochemistry.

PASD1 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = spiral arteries; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

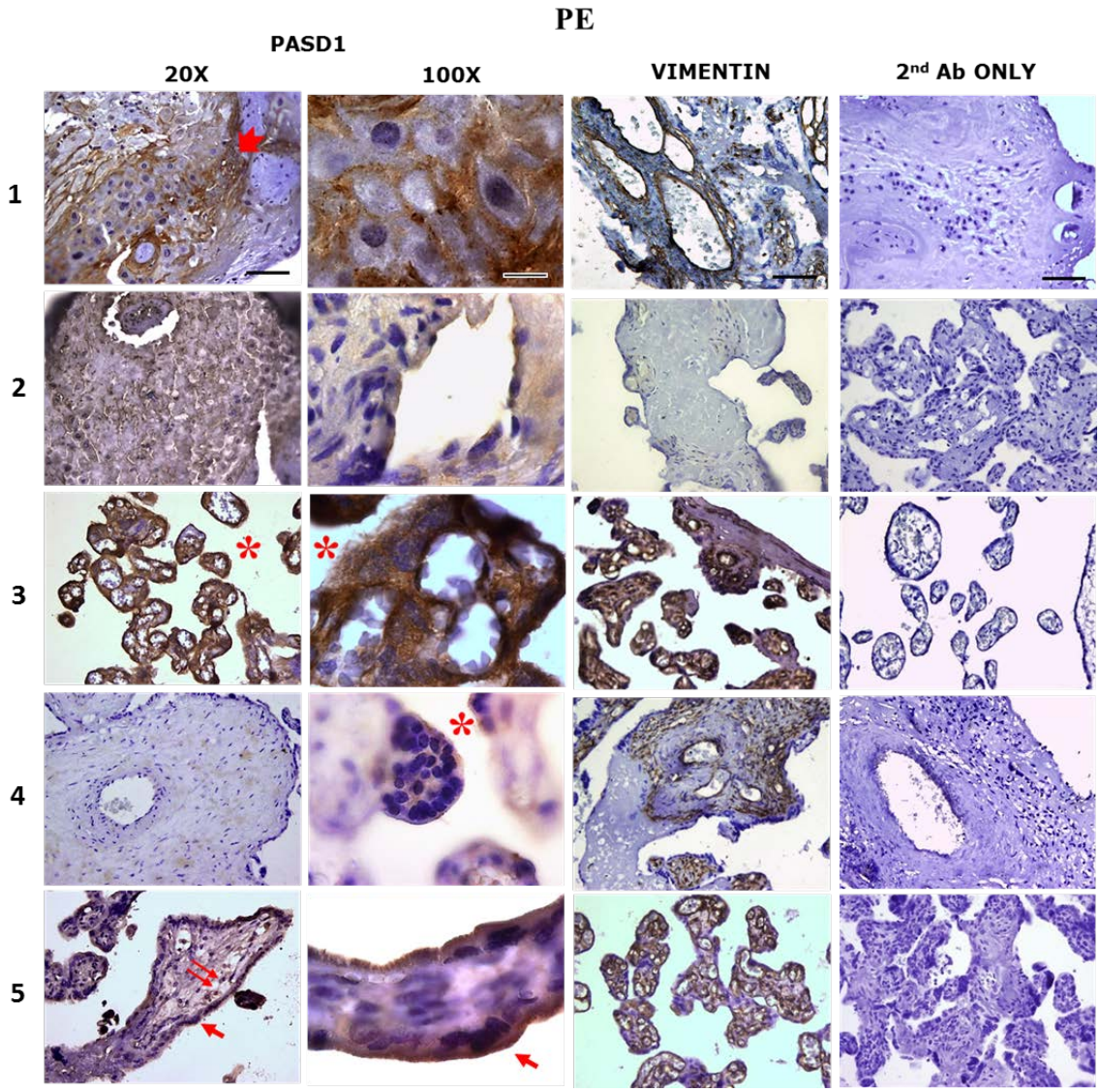


Figure 3.25: PASD1 localisation in PE placentae determined by immunohistochemistry.

PASD1 reactivity for each sample was scored against immune-reactivity for Vimentin (+ve) and 2nd Ab only (-ve) for the same sample. Thin arrows = CTB and STB layers; Arrow heads = spiral arteries; Bold arrows = collagen and fibrous layers surrounding the placental arteries; Asterisk = villi. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

3.3 Discussion

The aim of this chapter was to determine the levels of mRNA and protein expression of 5-HT receptors and C/T antigens as well as their cellular locations in NT and PE placentae.

Although there is no established direct link between 5-HT and the expression of C/T antigens, studies have highlighted that 5-HT promotes tumour growth and invasion (Soll *et al*, 2010). In fact the metabolism of 5-HT is affected by cancer chemotherapeutic agents was highlighted as early as 1992 (Cubeddu *et al*, 1992); in which the authors have suggested that the change in the 5-HT metabolism might be the cause of drug induced nausea and vomiting in cancer patients. Interestingly, breast cancer cells have shown to increase their biosynthetic capacity of 5-HT (Pai *et al*, 2009). It was argued that increased 5-HT, together with the changes in the expression of its receptors, would result in rapid proliferation of these cells into malignancy. The fact that the homeostatic regulatory mechanisms of 5-HT are changed during tumorigenesis, opens a new avenue for identifying the link between 5-HT, its receptors and any diagnostic and prognostic tumour markers.

As explained in Chapter 1, C/T antigens are mainly expressed in tumours; hence they are considered as tumour markers. The only two non-tumour tissues that show the expressions of C/T antigens are testis and placentae. Interestingly in PE, where the trophoblastic invasion is found to be low, the circulating level of 5-HT is increased (Bolte *et al*, 2001; 2001a; Sivasubramaniam *et al*, 2002; Salas, 2007). Also this increase affects the expression of 5-HT_{2A} receptors in chorionic arteries and veins (Ugun-Klusek *et al*, 2011). Trophoblast and tumour invasion share common characteristics and they both express C/T antigens. Together with the fact that invasion is low in PE, coupled with an increase in 5-HT, it is interesting to compare the expressions of (a) 5-HT receptors and (b) C/T antigens between NT and PE placentae. Therefore, the chapter compared the levels of mRNA and protein expression of 5-HT receptors and C/T antigens as well as their cellular locations in NT and PE placentae. This is then linked to (a) their status in first trimester trophoblast (Chapter 4) and (b) the *in vitro* effects of 5-HT on its receptors and C/T antigens using placental origin cell lines in (Chapters 5 and 6).

Since gene transcription does not guarantee faithful translation, the expression of both mRNA and protein in NT and PE placentae were studied. In addition, identification of any mRNA that was up-regulated, but not translated, is suggestive of another role in cell function, for example control of gene expression.

The summary of results for this chapter can be found in Table 3.5 below.

➢ **5-HT**

There are 13 known members of the 5-HT receptors family which is split into 7 main classes; 5-HT_{1-7}. The 5-HT_{1F} receptor has only been identified by comparative studies in the human genome and was therefore excluded from this study. Both mRNA and protein levels for 5-HT_{1B}R were decreased in PE placentae compared to NT tissue, although only mRNA levels were significant ($p<0.05$). On the other hand, there was no overall alteration in 5-HT_{1B}R cellular distribution. Interestingly, 5-HT_{2B}R mRNA levels were significantly decreased in PE tissue ($p<0.001$) to such a level that no protein was detected on immunoblots. This appears to contradict the immunohistochemistry data that clearly shows 5-HT_{2B}R protein in PE placenta. The reduction of 5-HT_{2B}R mRNA confirms previous study carried out by the author and co-workers using placental arteries and veins (Ugun-Klusek *et al*, 2011).

Table 3.5: Overall correlation amongst mRNA, protein expression and IRS in PE in comparison to NT placentae.

Genes Investigated	Significant	PE				
		mRNA (compared to NT)	Protein (compared to NT)	IHC		
				IRS	Cell type	Cellular Compartment
12 5-HT receptors	5-HT_{1B}R	(-) *	(-) ns	=	CTB; STB; EVT; SA	Cytoplasm
	5-HT_{2B}R	(-) ***	ND	=	CTB; STB; SA; D	Cytoplasm
14 C/T antigens	CAGE1	(-) *	(-) ns	=	CTB; STB; EVT; SA; C&F; D	Cytoplasm
	FATE1	(-) *	(+) ns	(-) ns	EVT; DS	Cytoplasm
	GAGE1	(-) *	(-) *	=	CTB; STB; EVT; C&F	Cytoplasm
	GAGEE1	(-) ****	(-) ***	(-) ns	EVT; DS	Cytoplasm
	MAGEA1	(+) ****	ND	(-) **	EVT	Cytoplasm
	MAGEA4	(-) ***	(-) ***	=	CTB; STB; EVT; C&F	Cytoplasm
	PASD1	(-) *	ND	=	CTB; STB; EVT; C&F	Cytoplasm

*The mRNA and protein expressions of C/T antigens which were in agreement are encircled in red. (-) down regulated; (+) upregulated; = - No change (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001); ns= no significance; ND= Not detected.*
C&F- Collagen & fibrin; CTB- Cytotrophoblast; D-Decidua; DS-Decidual stroma; EVT-Extravillous trophoblast; SA-Spiral arteries; STB- Syncytiotrophoblast.

➢ **C/T antigens**

The result for FATE1 is unexpected because even though the mRNA levels in PE tissue were significantly reduced its protein levels were actually elevated. Although the reasons for this inverse correlation is not clear, one explanation could be that high levels of the protein have shown to down regulate mRNA transcription as a compensatory effect. Another reason could be due to the half-life of some transcripts being extremely low and hence any mRNA expressed has already been degraded. The converse argument can be made for studies showing relatively high levels of mRNA compared to their protein counterpart; some transcripts have extremely long half-lives. In fact, Schwanhausser *et al* (2013) argued there is a poor correlation between some mRNAs and proteins, especially amongst those factors involved in the regulation of cell division and differentiation. They have suggested that the correlation between transcript and protein levels can be as little as 40% for factors/antigens that have regulatory functions (Vogel and Matcote, 2012; Guo *et al*, 2008). Since FATE1 is proposed to have steroidogenic factor 1 (SF-1, a protein that controls sexual development in the embryo and in tumorigenesis) binding sites in its promoter region. (Olesen *et al*, 2011) Although the functions of FATE1 in placenta are yet to be understood, this would seem a reasonable explanation for its differing transcript and protein expression profiles.

However, in some cases (such as CAGE1 and GAGE1), there were more than one protein bands visible on the blots. Since these bands were also consistently shown in positive controls, the intensities of all these bands were comparatively analysed. Although this may be a limitation of this study, it was unfeasible, within the study period, to extensively analyse whether these extra bands actually represent the original protein. This is one of the very few studies that used CT antigen specific antibodies in Western blots. In fact to author's knowledge, this is the first study that comparatively analysed the expression of CT antigens between NT and PE placentae. Since all the antibodies used were polyclonal in nature, these additional, but faint, bands may be due to non-specific binding.

CAGE1, GAGE1, GAGEE1/PAGE5 and MAGEA4 levels of both mRNA and protein expression were decreased in PE samples; although there appears to be no alteration in their cellular expression. Most of these C/T antigens are localised in CTB and STB layers of both NT and PE placentae. The cytoplasm of these cells showed granular staining. In human placentae, the cytotrophoblast layer function as stem cells for syncytiotrophoblasts. They continually differentiate into syncytiotrophoblasts during villous development. Since these cells are capable of invading uterine spiral arteries and involved vascular remodelling, they are believed to be "*pseudo-malignant*" (Sounderajan and Rao, 2004).

The expression of C/T antigens by these cells is an indication of their similarity to invading tumour cells. The fact that CTB cells continuously express these tumour markers even at

term suggests that they retain their proliferative capacity throughout the pregnancy. This agrees with the theory *"placenta can be regarded as a physiological counterpart of highly invasive tumours"* recently summarised by Novakovic and Saffery (2013).

The GAGE family is expressed primarily in germ cells, such as primary spermatocytes and oocytes (Gjerstorff *et al*, 2006). Since proteins for members of this family are found in the nucleus their role in germ cell regulation through apoptosis has been speculated. Since the IHC results (Figure 3.12) clearly shows the expression of GAGE1 in the nucleus, as well as the cytoplasm, of trophoblast cells this would suggest a nuclear role in the placenta. The IHC staining intensity was very high for GAGEE1/PAGE5 making it difficult to differentiate between nuclear and cytoplasmic staining. It has also been reported that the expression levels of GAGE family varied with the developmental stage of the germ cells. Although in oocytes, it has been shown that expression levels did not change during the maturation phase (Gjerstorff *et al*, 2006). On the other hand, expression levels of GAGE decreases during maturation of spermatocytes. Thus, it was important to study the expression of the GAGE family in first trimester placental tissues in order to understand expression patterns in trophoblast cells (this is given in Chapter 4).

MAGEA1 had a similar profile to 5-HT_{1B}R; no protein levels were detected even though mRNA expression were identified. This could be due to the fate of the proteins after extraction and processing. Therefore, care should be taken to extrapolate the data from the immunoblots. It is also worth noting that the IRS for MAGEA1 was reported to be significantly reduced in PE when compared to NT. Thus, the extremely significant increase in the mRNA expression of MAGEA1 could be a compensatory effect for the low levels of the protein which were almost undetectable in immunoblots. Although MAGEA1 is reported to be involved in cell differentiation during spermatogenesis (Monte *et al*, 2006), its function in placenta is not clear.

MAGEA4 is a member of the MAGE family that was also suggested to induce apoptosis by both p-53 dependent and independent pathways (Sang *et al*, 2011). So it is intriguing as to why four of the C/T antigen genes studied (GAGE1, GAGEE1/PAGE5 and MAGEA4) that are involved in apoptosis, were significantly reduced in PE placentae. These results contradict reports that indicate increased apoptosis rates during PE (Ishihara *et al*, 2002), and suggest a different function for these genes during placental development. This would be confirmed by functional studies using cell lines (Section 7.4: Future studies).

Even though the IRS scores from IHC is only semi-quantitative, it was very valuable to identify the cellular localisation between the two placental types (NT and PE). Staining for

most of the receptors/antigens were localised around the CTB/STB layers, placental villi and the lining of the spiral arteries in NT tissues. These regions contain cells that constantly undergo differentiation and division. Also the STB layers are undergoing constant apoptosis by forming syncytial knots. However in PE tissues the staining was frequently between low to moderate intensity around these areas. There were few exceptions like PASD1 which showed strong staining around the CTB/STB layers and placental villi. On the contrary the staining was more concentrated in the EVT as well as fibrin and collagen layers in the placental beds of PE which do not undergo further differentiation. Although it has been suggested that PASD1 acts as a transcription factor its definite function is still not known.

Conclusions:

The collective data from mRNA, protein and IHC studies suggest the expression patterns of many C/T antigens and at least two 5-HT receptors are altered in PE placentae. Since placental invasion resembles tumorigenesis, and the fact that expression of the putative tumour marker antigens are altered in PE placentae, which has low invasion it may be an interesting avenue to explore. Therefore it is important to study the functions of these antigens during placental development (see Section 7.4: Future Studies).

Chapter 4
First trimester placental study
Expression of 5-HT receptors and C/T antigens

4.1 Introduction

Due to the interesting data on the expression of C/T antigens observed in Chapter 3, it was decided to compare the status of common 5-HT receptors and C/T antigens in the first trimester (FT) and full term placentae. The development of FT placenta is summarised in Section 1.1.2.1. During the first trimester only two villous types are formed (Huppertz, 2008); (a) mesenchymal villi: rich in mesenchymal cells and weakly organised stroma and (b) immature intermediated villi: mesenchymal cells are organised in a mesh with placental macrophages (Hofbauer cells).

The villous CTB is present as a complete layer under the STB during the FT, behaving as stem cells for the outer STB. However, with progress in pregnancy the CTB numbers are reduced. The villous tree is then covered only with STB, with few patches of CTB. On the 35^{th} day p.c., the CTB differentiates into interstitial (extravillous) CTB (invades the decidua) and the endovascular CTB (eCTB; remodels the spiral arteries of the uterus) (James *et al*, 2005; Gude *et al, 2004;* Red-Horse *et al,* 2004*).*

The EVT invades the uterus up to the inner third of the myometrium (reviewed by Norwitz *et al*, 2001). This invasion takes place only in the first half of the pregnancy until a fully functional placenta is formed. Thus, the two different CTB cells are highly invasive during the first trimester and their invasive properties decline with pregnancy progression to full term. The invasive properties of the CTB cells earlier in the pregnancy could determine the fate of the pregnancy. The unregulated invasion of the CTB leads to complications like placental accrete (firm attachment to the myometrium), increta (invasion into the myometrium) and percreta (penetrations through the myometrium into the uterine serosa and other organs) (reviewed by Norwitz *et al*, 2001).

A number of microarray studies using first trimester CTB in comparison with term placentae have reported differences in gene expression patterns. Some of the major differences in expression in FT are as follows:

(1) Difference in gene expression between FT and term placentae.

A 25% difference in gene expression was reported by a microarray study involving 16 samples from FT and 21 samples from term placentae (Sitras *et al*, 2012). This study showed there was an up-regulation of genes involved in cell proliferation, differentiation, migration and angiogenesis in FT. Conversely, in term placentae, an up-regulation of genes involved in signal transduction and receptors were reported.

(2) Difference in gene expression with gestational age in FT placentae.

Khan *et al* (2010; 2004) reported that there were differences in gene expression with increasing gestational age (6 to 8 weeks) even within the first trimester. Three distinct expression clusters were observed in this study, with genes involved mostly in programmed cell development, immunity, adhesion and epithelial-to-mesenchymal transition.

(3) Difference in gene expression in eCTB and EVT.

Another microarray study by Apps *et al* (2011), showed differential expression patterns of 3000 transcripts in eCTB and EVT differentiated from the CTB stem cells. Most of these genes were involved in novel pathways for cell migration, invasion and immune modulation. The molecular mechanisms of isolated eCTB and EVT from the FT were also investigated by Tarrade *et al* (2001).

(4) Difference in gene expression in villous and decidua of the FT.

A high expression of 75 genes in chorionic villi and 25 genes in decidua of early gestational age placentae were reported by a human cDNA microarray study involving 9600 clones (Chen *et al*, 2002). This study reported the importance of expression of genes encoding proteins (such as IGFBP-1, 3, 4 and 5) in decidualisation of the uterus for the implantation and the invasion of the EVT.

It has been well investigated that growth factors and cytokines act in an auto-paracrine function to maintain the development of the placenta and foetus (Hofmann *et al*, 1993; Chen *et al*, 1991). Similarly, serotonin (5-HT) in the trophoblast may be involved in placental development (Deroy *et al*, 2013). An immunohistochemical staining of 5-HT and its receptors in human placenta reported their localisation in the cytoplasm of the cells (Huang *et al*, 1998). In the study it was reported the STB was immuno-positive for both 5-HT and its receptors. The staining intensities in STB were reported to be higher than in CTB, but reduced with increasing gestational age for 5-HT only. The CTB, on the other hand, was reported to express only the 5-HT receptors. The staining intensities of 5-HT and its receptors in maternal decidua were reported to be high. The antibodies used for the detection of the 5-HT receptors were specific only for the 5-HT_{1A} receptor but that used for 5-HT_2 receptors detection recognised common epitopes of all the 5-HT_2 receptors. Therefore, a generalised localisation for the 5-HT_2 receptor was reported (significant expression pattern observed in Chapter 3) and an antibody recognising a unique epitope of 5-HT_{2B} receptor is required for the specific identification of this receptor. The 5-HT_{2B} receptors are reported to mediate cell differentiation and it has been reported to be expressed in the maternal myometrium (Choi *et al*, 1997; Kelly and Sharif, 206).

Although the expression of C/T antigens have been reported in placenta, only two detailed studies could be found to have investigated a subset of the C/T antigens in FT placentae (Jungbluth *et al*, 2007; Khan *et al*, 2014). Jungbluth *et al* reported the cellular localisation of MAGE-A and C, NY-ESO-1 and GAGE in week 5 to 42 placental samples and the importance of gestational age in the expression of C/T antigens. The importance of gestational age was also reflected in the expression of CAGE1 in week 6 to 8 samples, in which expression increased with progression of gestational age (Khan *et al*, 2014).

Keeping in mind all these variations in gene expressions linked to the gestational age of placenta, this chapter aimed to investigate,

- The status of 5-HT receptors and C/T antigens in the first trimester (FT) and normal term placentae.
- The localisation of these receptors and antigens in FT chorionic villi and maternal decidua.
- The expression patterns in FT samples with different gestational age (weeks).

4.2 Results

This study was performed on the basis of the results obtained from previous Chapter 3, in which significant differences in expression of 5-HT receptors and C/T antigens were observed between NT and PE placentae. Two 5-HT receptors and seven C/T antigens which showed interesting differences in expression between NT and PE were analysed in this chapter. Although initially it was intended to perform a complete expression analysis of these receptors and antigens, due to limited availability of samples, only mRNA expression and cellular localisation studies were possible. The qRT-PCR was performed using brain (5-HT receptors) and testis (C/T antigens) as the positive controls as detailed in Section 2.6.5. The cellular localisation of these receptors and antigens was investigated by immunohistochemical (IHC) staining, as detailed in Sections 2.9.4.

4.2.1 First trimester human placental samples

The human first trimester placental samples were collected from the Medical University of Graz, Austria under the supervision of Dr. Martin Gauster (Institute of Cell Biology, Histology and Embryology). The fresh FT placental samples were used for RNA and protein extraction simultaneously using the AllPrep RNA/Protein Extraction kit® (Qiagen, UK). Due to the small size of the samples, the protein concentrations were not appropriate for Western blot detection. The integrity of the RNA samples were analysed by 1% w/v agarose gel

electrophoresis before qRT-PCR investigations. The gestational ages of the samples collected are detailed in Table 4.1. The samples are arbitrarily numbered as F1-15.

The samples collected were between 7 to 11 weeks of gestational age (8.3 ±0.4) (Mean ± SEM). As can be seen from Figure 4.1 (Section I), placental villous trees (trophoblast cells) are only developed during week 7. The membranous tissue formations start appearing from week 8 onwards, along with larger villous trees. Samples for RNA and protein investigations were collected only from the villous tree.

Table 4.1: Details of the FT samples collected.

SAMPLE ID	GESTATIONAL AGE (Weeks + Days)	SONOGRAPHIC SIZE OF FT PLACENTA (mm)	ANALYSIS
F1	7+0	-	mRNA
F2	7+0	3	mRNA; IHC
F3	7+0	5	mRNA; IHC
F4	7+0	-	IHC
F5	8+0	-	mRNA
F6	8+0	-	mRNA
F7	8+0	10	mRNA; IHC
F8	8+0	-	mRNA
F9	8+0	-	IHC
F10	8+5	-	mRNA
F11	9+0	-	IHC
F12	9+3	21	mRNA; IHC
F13	9+3	23	mRNA; IHC
F14	11+0	37	mRNA; IHC
F15	11+0	-	IHC

The histology of placental villous and decidual regions during FT is shown in Figure 4.1 (Section II). The immature intermediate villi during FT are represented distinct thick STB and CTB layers surrounding the stromal mass (S). A small number of mesenchymal cells (represented with arrow heads) and foetal blood vessels with blood cells (enclosed in square) can be found in the stroma. The decidual region contained vessels and distinct uterine glands with epithelial lining. Spiral arteries were also a common sight, which can be seen in other immunohistostaining images (not shown in Figure 4.1).

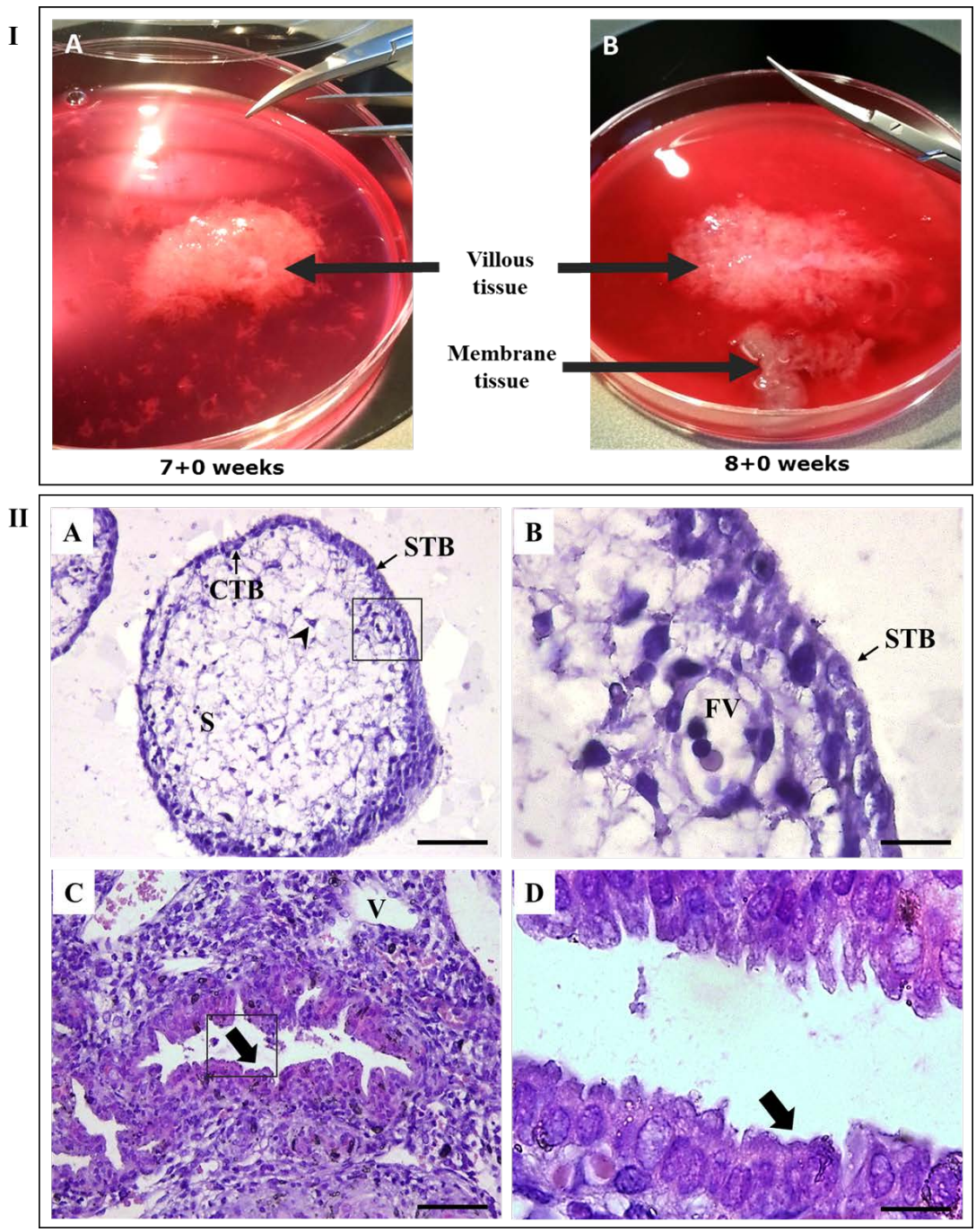


Figure 4.1: Structural features and histology of first trimester placentae.

Section I. Structural features of first trimester placentae. Panel A: Only villous tissue was visible in 7 week old placenta. The membrane tissue was visible in 8 week old placenta (**Panel B**) depicting the rapid development of the placenta within one week.

Section II. H&E staining showing the histology of a representative FT sample. [**A**] FT villi section showing an outer uniform STB and inner CTB layers. Mesenchymal cells (Arrow head) are scattered in the stroma (S). The box encloses a foetal blood vessel (FV) which is enlarged in Panel [**B**]. [**C**] Membrane and decidual section of an FT sample, showing distinctive uterine glandular epithelium (Bold arrows) and vessels (V). [**D**] Shows the enlarged section of glandular epithelium [the box in Panel **C**]. *Magnification: A, C- 200X (Scale bar=100µm); B, D- 1000X (Scale bar= 20µm).*

4.2.2 Expression analysis of 5-HT receptors

The relative mRNA expression and cellular localisation of 5-HT_{1B} and 5-HT_{2B} receptors were investigated as follows.

4.2.2.1 Relative mRNA expression of 5-HT receptors

To check for successful qRT-PCR performance, human brain samples (positive control for 5-HT receptors; Clontech, UK) were analysed along with FT samples. The relative mRNA expression is presented in the form of arbitrary units with positive set to 1 (data not shown). Figure 4.2 shows the differential mRNA expression patterns of 5-HT_{1B} and 5-HT_{2B} receptors between FT and term placentae. There were no differences in mRNA expressions of 5-HT_{1B} receptor observed. The 5-HT_{2B} receptor on the other hand showed a statistically significant difference in mRNA expression. The expression of 5-HT_{2B} receptor mRNA was dramatically up-regulated in term placentae when compared to FT ($p<0.01$).

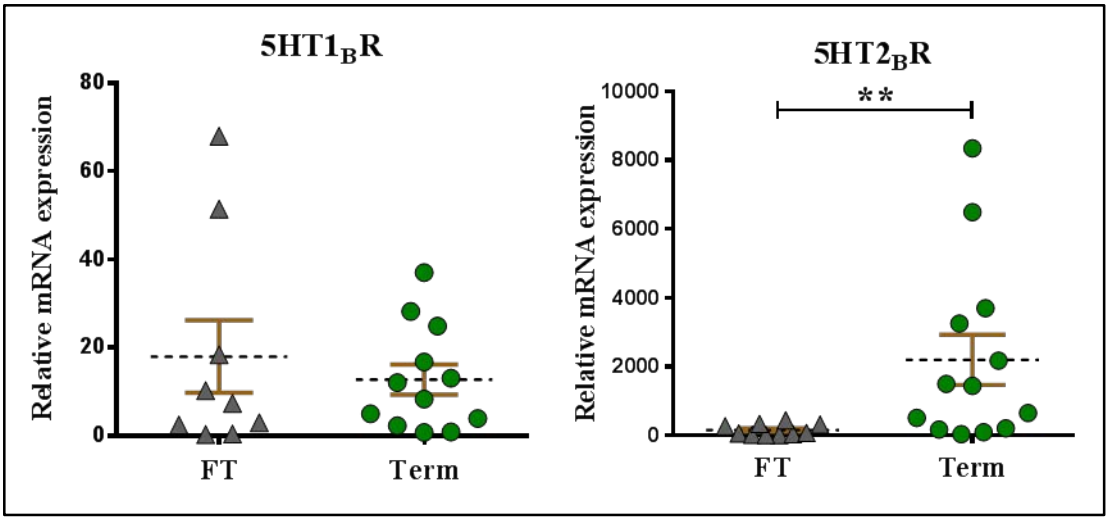


Figure 4.2: Comparative mRNA expression analysis of 5-HTR between FT and term placentae.

The mean gestational age (weeks) for FT and Term placentae are 8.3 ±0.4 and 38.9 ±0.1 respectively (Mean ±SEM). The relative mRNA expressions were compared by Mann-Whitney U test (±SEM; **$p<0.01$).

4.2.2.2 Cellular localisation of 5-HT receptors

The status and cellular localisation of 5-HT_{1B} and 5-HT_{2B} receptors were investigated by immunohistochemistry (IHC) and scored as previously described in Section 2.9.1. Positive 3,3'-Diaminobenzidine (*DAB*) staining was scored according to the intensity of staining. No brown stains were visible with just secondary antibody staining, confirming the specificity of the antibodies and DAB stain. The Fleiss' Kappa Statistics performed on this IRS data provided a kappa value of 0.55, suggesting a 50% agreement between the scorers. The aggregate scores for 5-HT receptor scoring are given in Table 4.2 below.

Table 4.2: Summary of staining intensity of 5-HT receptors

FT Placentae		**5-HT Receptors**	
Region	**Cell type**	**5-HT_{1B}R**	**5-HT_{2B}R**
Villous	CTB	3±0.0	3±0.4
	STB	3±0.4	3±0.4
	Mesenchymal cells	2±0.8	0±0.3
Decidua	Uterine epithelium	2±0.4	1±0.5
	Decidual stromal cell	2±0.5	2±0.5
	Spiral arteries	2±0.4	2±0.4

Staining intensity: 0-negative; 1-low; 2-moderate; 3-high (Mode±SD).

- **5-HT_{1B} Receptor:**

The STB (thin arrows) and CTB (bold arrows) layers of the placental villous tissue showed intense staining for 5-HT_{1B} receptor when compared to the stromal region (See Figure 4.3, Panel F1-5). The staining was mostly cytoplasmic and granular. The primary villi, which show dense CTB and STB layers, are intense in staining (represented with * in Figure 4.3, Panel F4). A number of mesenchymal cells in the stroma were also stained. The decidual regions also showed immuno-positivity to 5-HT_{1B} receptors (See Figure 4.3, Panel D1-3). However, the staining was more specific to the epithelial lining of the uterine glands (enclosed in box), spiral arteries (arrow heads) and the stromal cells of the decidua. Overall, the staining was high (score 3) in villous regions and moderate (score 2) in decidual regions (See Table 4.2).

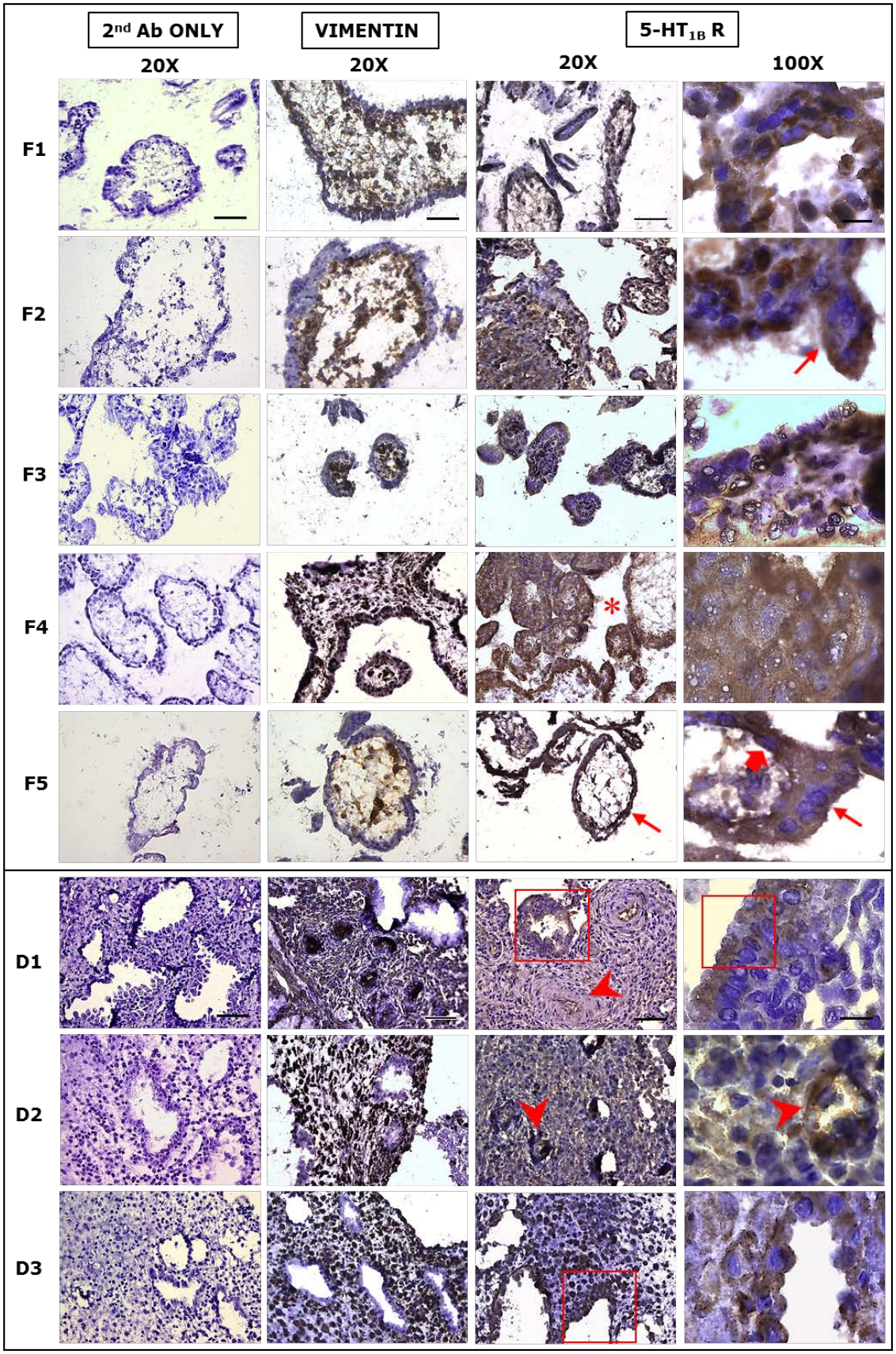


Figure 4.3: Localisation of 5-HT$_{1B}$ Receptor in FT placentae.

5-HT$_{1B}$ receptor localisation is shown on the right (at both 20X and 100X magnification), whilst the negative (no primary Ab) control images are shown in the left hand panels and the positive control (vimentin) images are in the centre. **Panel F1-5** show images for placental villous sections from 5 representative FT samples**. Panel D1-D3** represents the decidual sections from 3 representative FT samples. Thin arrows: STB; Bold arrows: CTB; *: primary placental villi; Arrow head: Spiral arteries; Uterine glandular epithelium enclosed in box. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **5-HT$_{2B}$ Receptor:**

Similar to 5-HT$_{1B}$ receptors, both the STB (thin arrows) and the CTB (bold arrows) showed high staining intensities (score of 3) for 5-HT$_{2B}$ receptor in the villous sections (See Figure 4.4, Panel F1-5; Table 4.2). Staining was also visible around the syncytial sprouting (represented with +) in the mesenchymal villi (rich in mesenchymal cells) (Figure 4.4, Panel F1). However, unlike the 5-HT$_{1B}$ receptor, the mesenchymal cells were negative for 5-HT$_{2B}$ receptor staining. The staining of 5-HT$_{2B}$ receptor in the maternal decidual regions were concentrated around the spiral arteries (arrow heads), epithelial cell lining of the glands/vessels (enclosed in box) and the stromal cells (see Figure 4.4, Panel D1-3). The staining appeared more granular in the epithelial cells lining the glands which are usually secretory in behaviour. The staining intensity was generally high (score 3) in villous sections and moderate (score 2) in decidua (See Table 4.2).

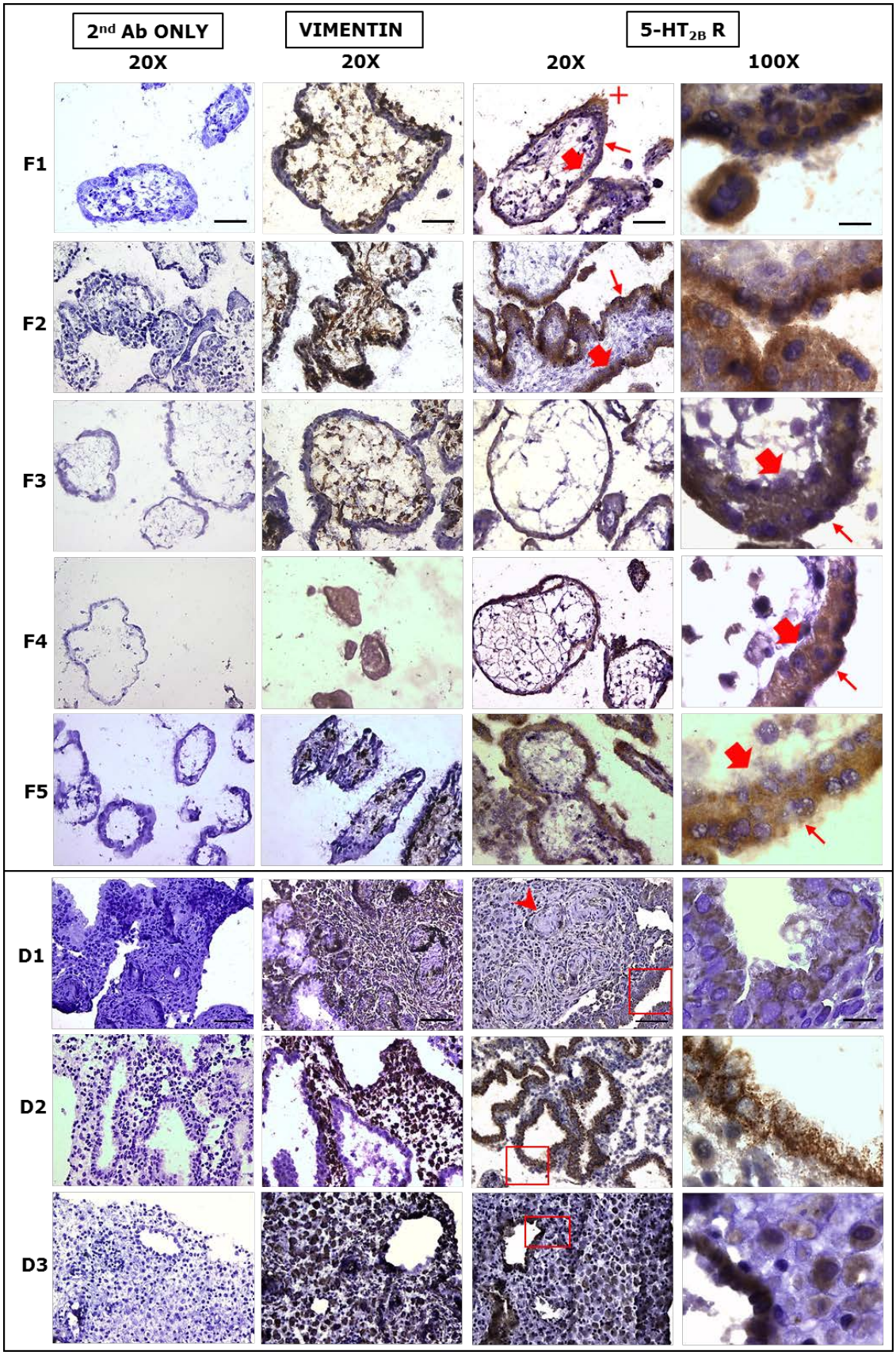


Figure 4.4: Localisation of 5-HT_{2B} Receptor.

5-HT_{2B} receptor localisation is shown on the right (at both 20X and 100X magnification), whilst the negative (no primary Ab) control images are shown in the left hand panels and the positive control (vimentin) images are in the centre. **Panel F1-5** show images for placental villous sections from FT**. Panel D1-D3** represents the decidual sections from FT as well. Thins arrows- STB; Bold arrows- CTB; + Syncytial knot; Arrow head- Spiral arteries; Uterine glandular epithelium enclosed in box. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

4.2.3 Expression analysis of C/T antigens

Seven C/T antigens (CAGE1, FATE1, GAGE family (GAGE1 and GAGEE1/PAGE5), MAGE family (MAGEA1 and MAGEA4) and PASD1 were investigated as follows.

4.2.3.1 Relative mRNA expression of C/T antigens

The mRNA expression of C/T antigens in FT was investigated along with expression in human testis (positive control; data not shown). The relative mRNA expression is calculated by normalising sample expression to the positive control (set to an arbitrary value of 1). With the exception of CAGE1 and GAGE1, statistically significant differences in expression were observed between FT and term placentae for C/T antigens (See Figure 4.5). Further details are given below:

- **CAGE1**

In general, the mRNA expression of CAGE1 was low in six out of 11 FT samples (Figure 4.5). The other five FT samples show higher expression. In contrast, most term samples show a very low expression of CAGE1 mRNA. Although not significant, mRNA expression in term samples showed a trend towards being lower than in FT placentae.

- **FATE1**

There were three distinguishable groups of mRNA expression seen amongst the FT group. Five out of 11 samples show negligible expression. Three samples showed moderate expression and a further 3 samples had relatively high expression. In term placentae, half of the samples showed negligible expression. Therefore, a significant up-regulation in FATE1 mRNA expression was observed in FT placentae compared to term placentae ($p<0.05$).

- **GAGE Family:**

In accordance with Chapter 3, only two of the GAGE family members were investigated in FT placentae. These were GAGE1 and GAGEE1 (PAGE5).

  - **GAGE1**

Two distinct groups were seen in GAGE1 mRNA expression in FT placentae. One group included samples with lower expression closely resembling the expression in term placentae and the second showed much higher mRNA expressions than term samples. Due to this scattered distribution of expression in FT samples, there was no significant difference found between the two groups.

  - **GAGEE1**

The mRNA expression of GAGEE1, also known as PAGE5, in FT samples was more consistent. Therefore, a highly significant down-regulation of expression was observed in FT placentae when compared to term ($p<0.01$). However, the expression levels of GAGEE1 was relatively lower when compared to the other member of GAGE family (GAGE1). Interestingly, GAGEE1 was the only C/T antigen that showed down-regulation in FT when compared to up-regulation of other antigens in FT.

- **MAGEA Family:**

MAGEA1 and MAGEA4 were investigated from the MAGE family in FT placentae.

- **MAGEA1**

Similar to other C/T antigen expression profiles, clustering into sub-populations was observed for MAGEA1 expression amongst different FT placentae. Overall, the FT placentae showed a higher mRNA expression than the term placental group; showing a significant up-regulation of MAGEA1 mRNA expression in the FT vs term placental groups ($p<0.05$).

- **MAGEA4**

MAGEA4 showed similar expression patterns to MAGEA1. Compared to the term placental group, the mRNA expression in FT placentae showed significant up-regulation ($p<0.05$).

- **PASD1**

The mRNA expression of PASD1 was almost negligible in term placentae. On the other hand, in FT two distinct groups were observed: five samples showed negligible mRNA expression, similar to term placentae; the remaining samples showed a scattered pattern of mRNA expression. Overall, there was a statistically significant up-regulation of PASD1 mRNA expression in FT, relative to term placentae ($p<0.05$).

Overall, apart from GAGEE1, the mRNA expression of the remaining C/T antigens were up-regulated in FT placentae, illustrating differences in expression patterns between different pregnancy stages (see Discussion).

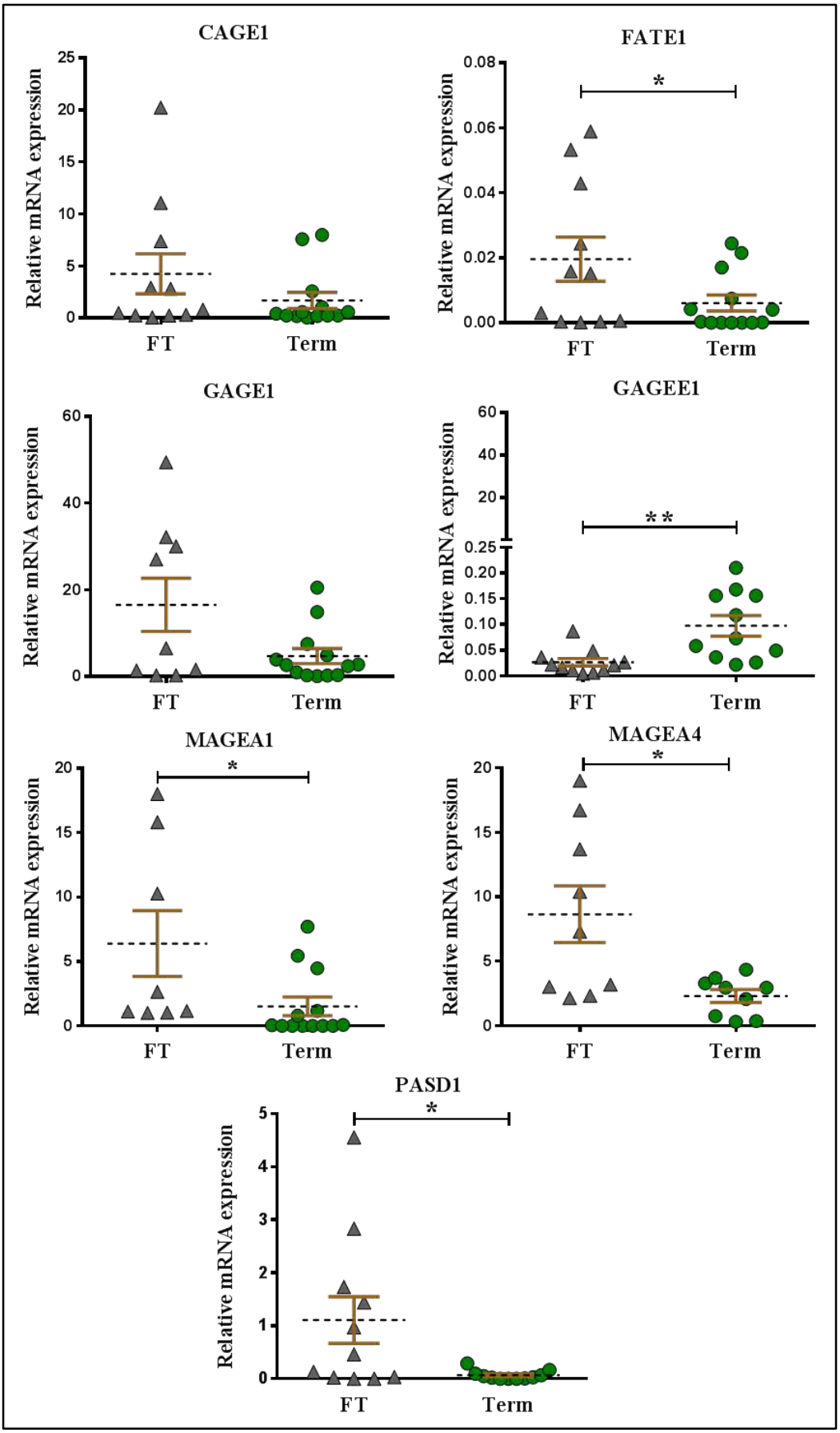


Figure 4.5: Comparative mRNA expression analysis of C/T antigen between FT and term placentae.

The mean gestational age (weeks) for FT and Term placentae are 8.3 ±0.4 and 38.9 ±0.1 respectively (Mean ±SEM). Relative mRNA expression compared by Mann-Whitney U test (±SEM; *$p<0.05$; **$p<0.01$).

4.2.3.2 Cellular localisation of C/T antigens

The cellular localisation of C/T antigens were investigated as explained previously in this chapter (for 5-HT receptors). The aggregate scoring for C/T antigens are given in Table 4.3 below.

Table 4.3: Summary of staining intensity of C/T antigens

FT Placentae		C/T antigens						
Region	**Cell type**	**CAGE1**	**FATE1**	**GAGE1**	**GAGEE1**	**MAGEA1**	**MAGEA4**	**PASD1**
Villous	CTB	2±0.5	3±0.4	2±0.6	3±0.0	3±0.0	2±0.7	2±0.6
	STB	2±0.5	3±0.5	2±0.6	2±0.9	3±0.4	2±0.5	2±0.6
	Mesenchymal cells	0±0.5	2±0.7	0±0.4	0±0.9	0±0.4	0	0±0.5
Decidua	Uterine epithelium	2±0.6	2±0.6	2±0.5	3±0.0	3±0.0	3±1.1	3±0.0
	Decidual stromal cell	0±0.6	2±0.6	1±0.6	2±0.6	0±1.2	1±0.6	2±0.6
	Spiral arteries	1±0.5	1±0.6	2±0.0	3±0.0	N/A	1±0.0	1±0.6

Staining intensity: 0-negative; 1-low; 2-moderate; 3-high (Mode±SD). N/A- not available

- **CAGE1**

Cytoplasmic regions of the STB (thin arrow) and CTB (bold arrow) in the placental villi were moderately (score 2) stained with CAGE1 (Figure 4.6, Panel F1-5; Table 4.3). The staining in the stromal mesenchymal cells was negligible. The epithelial lining the uterine glands (enclosed in box) and the lining of spiral arteries (arrow heads) of the decidua were moderate to low in CAGE1 staining (Figure 4.6, Panel D1-3). The surrounding stromal cells showed no positive CAGE1 staining.

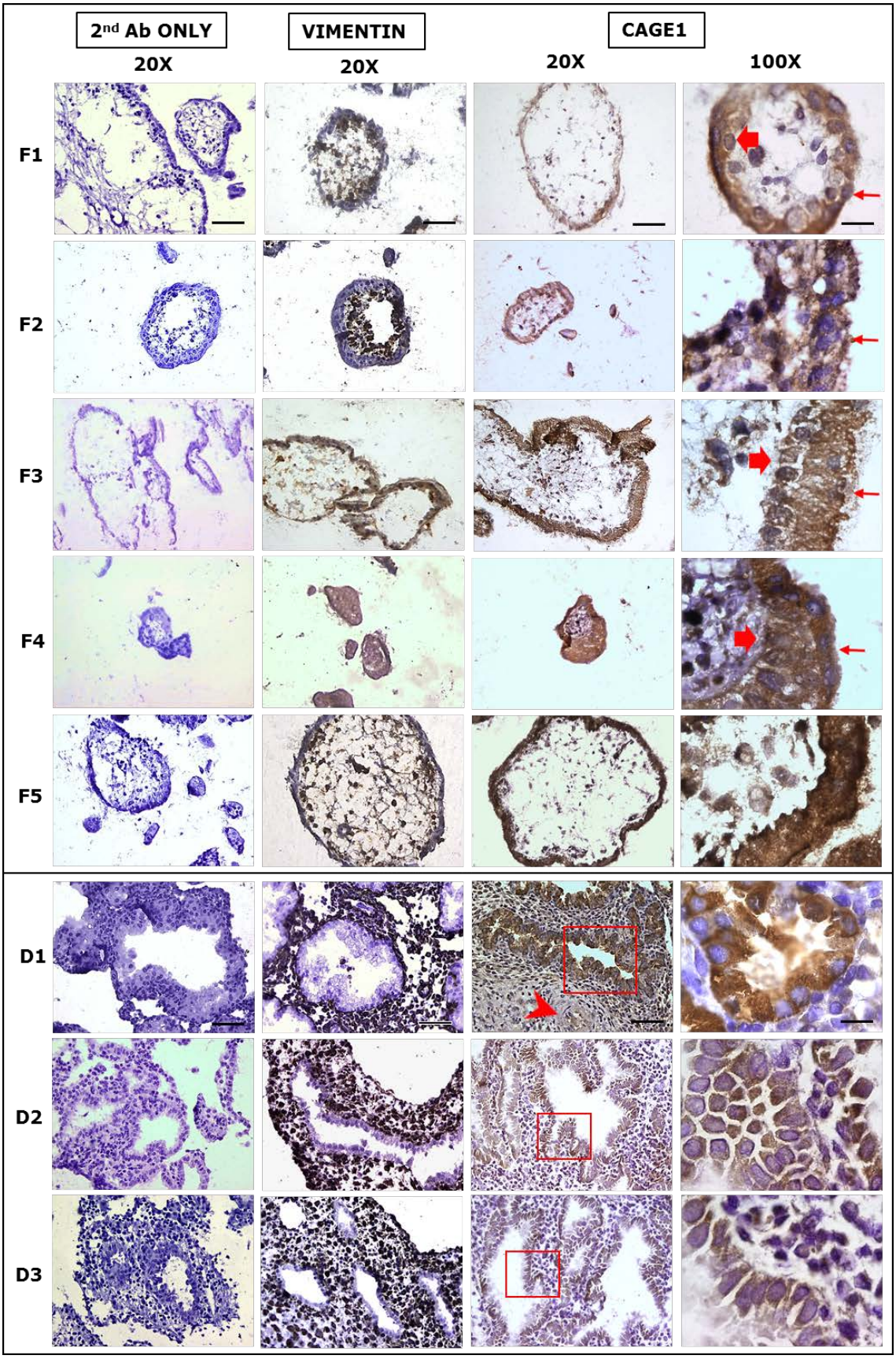


Figure 4.6: Localisation of CAGE1.

CAGE1 localisation is shown on the right (at both 20X and 100X magnification), whilst the negative (no primary Ab) control images are shown in the left hand panels and the positive control (vimentin) images are in the centre. **Panel F1-5** show images for placental villous sections from FT**. Panel D1-D3** represents the decidual sections from FT as well. Thin arrows- STB; Bold arrows- CTB; Uterine glandular epithelium enclosed in box. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **FATE1**

The trophoblast cells (CTB and STB) of the placental villi were highly stained for FATE1 (See Figure 4.7, Panel F1-5). Although in most of the samples the staining appeared to be mostly cytoplasmic, under higher magnification staining also visible in the nuclear region in some samples (Figure 4.7, Panel F2). The few mesenchymal cells within the stromal layer were also positive to FATE1. The staining in the maternal endometrium was localised around decidualised regions and spiral arteries (Figure 4.7, Panel D1). Moderate staining was also observed in the epithelial linings and in the stromal cells of decidua. The staining was higher in villous than in maternal sections.

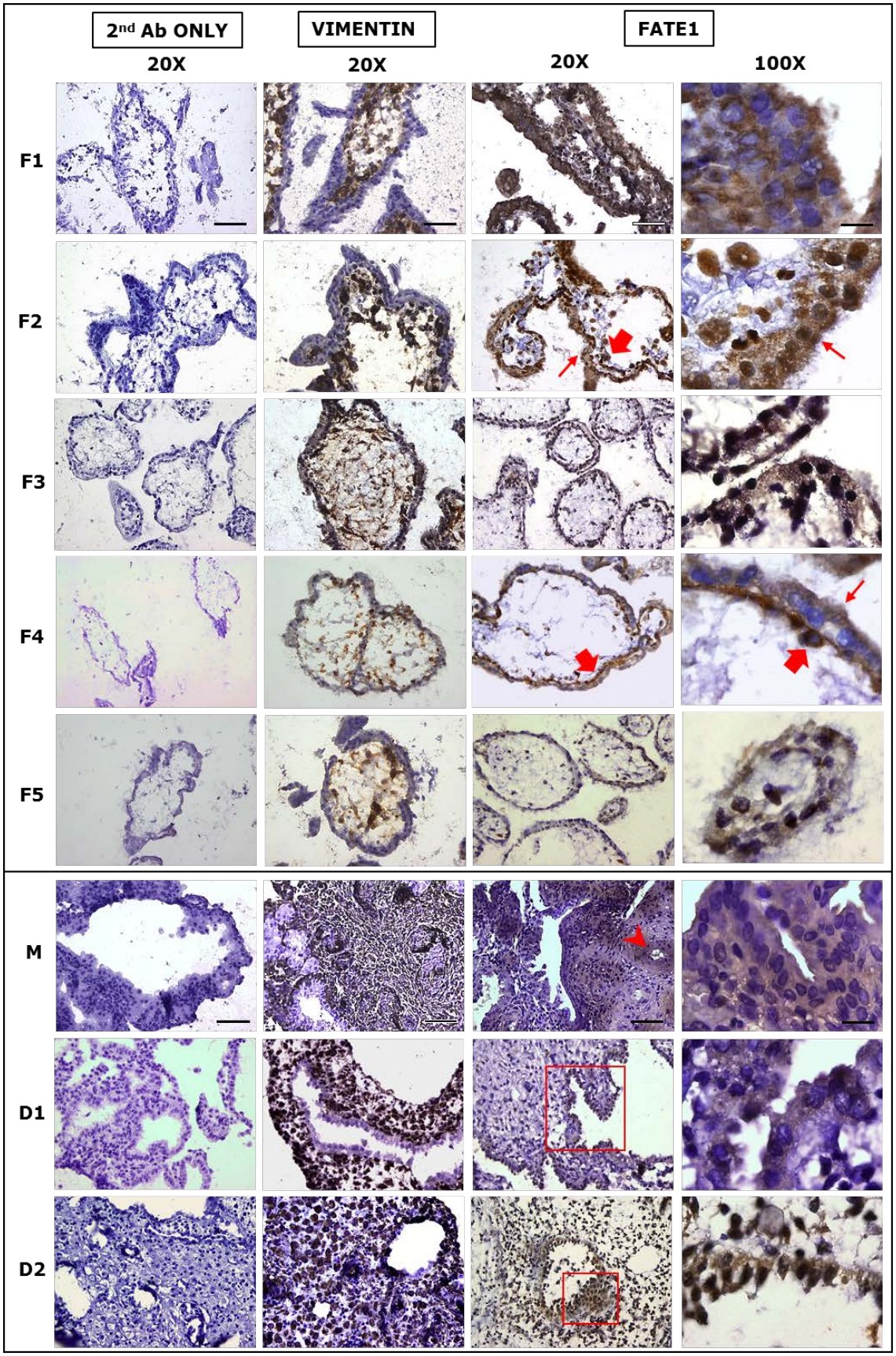


Figure 4.7: Localisation of FATE1.

FATE1 localisation is shown on the right (at both 20X and 100X magnification), whilst the negative (no primary Ab) control images are shown in the left hand panels and the positive control (vimentin) images are in the centre. **Panel F1-5** show images for placental villous sections from FT**. Panel D1-D3** represents the decidual sections from FT as well. Thin arrows- STB; Bold arrows- CTB; Arrow head- Spiral arteries; Uterine glandular epithelium enclosed in box. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **GAGE family**

The cellular localisation of two members of the GAGE family, namely GAGE1 and GAGEE1, was investigated. Both the placental villi and maternal decidua showed immuno-positivity for these two antigens (Figure 4.8 and 4.9). The staining intensities of the trophoblast cells (CTB and STB) were moderate (score 2) for GAGE1. However, the GAGEE1 antibody showed high staining intensity in the CTB (score 3) and moderate in STB (score 2) (Figure 4.9, Panel F1). The mesenchymal cells were negative for both antigens. The staining in maternal decidua was concentrated in the inner lining of the spiral arteries and glands. The staining of the stromal cells in the decidua was low for GAGE1 and moderate for GAGEE1. In general, the staining intensity of GAGEE1 was higher than GAGE1 in both placental villi and decidua.

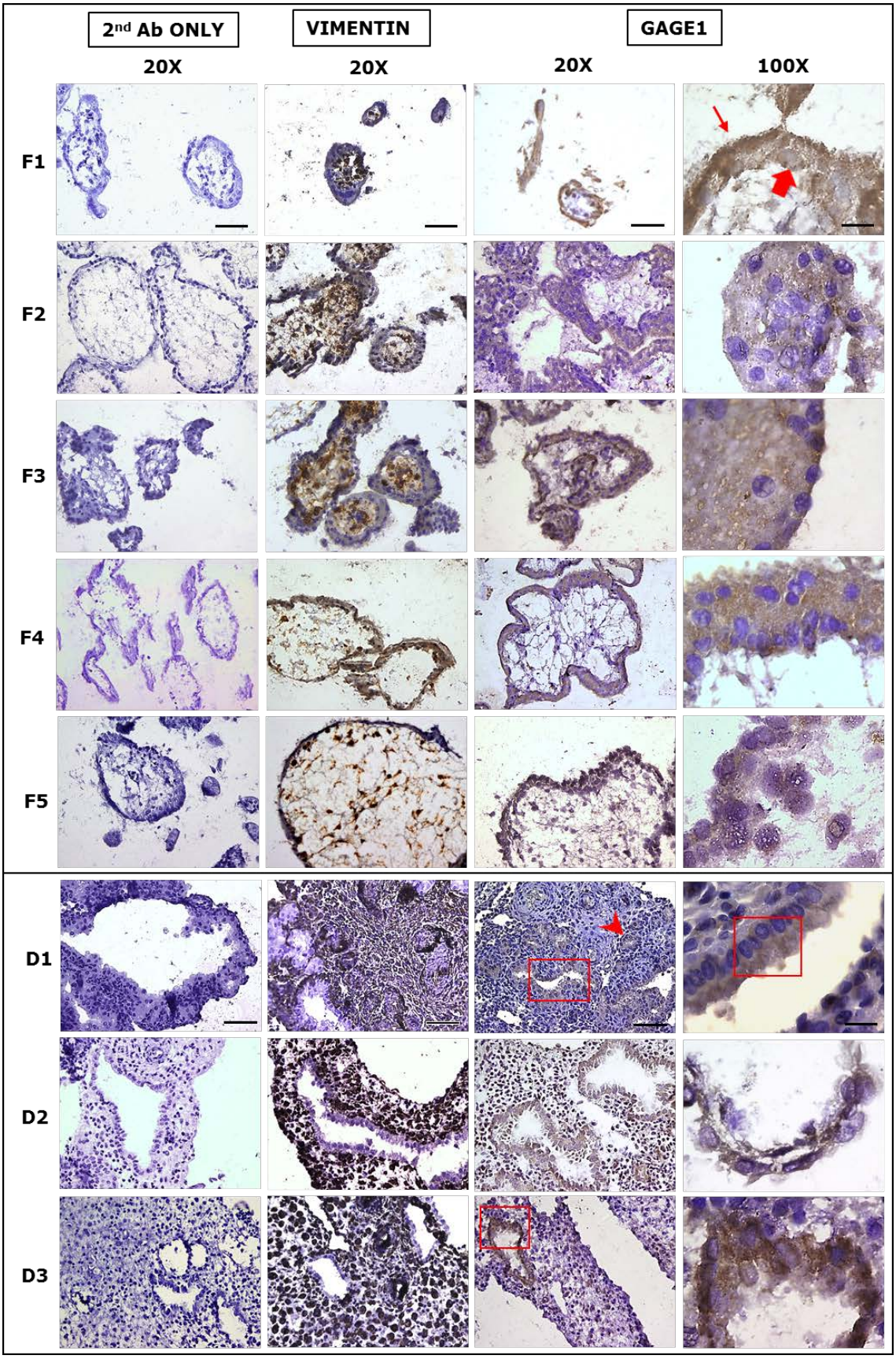


Figure 4.8: Localisation of GAGE1.

GAGE1 localisation is shown on the right (at both 20X and 100X magnification), whilst the negative (no primary Ab) control images are shown in the left hand panels and the positive control (vimentin) images are in the centre. **Panel F1-5** show images for placental villous sections from FT**. Panel D1-D3** represents the decidual sections from FT as well. Thin arrows- STB; Bold arrows- CTB; Arrow head- Spiral arteries; Uterine glandular epithelium enclosed in box. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

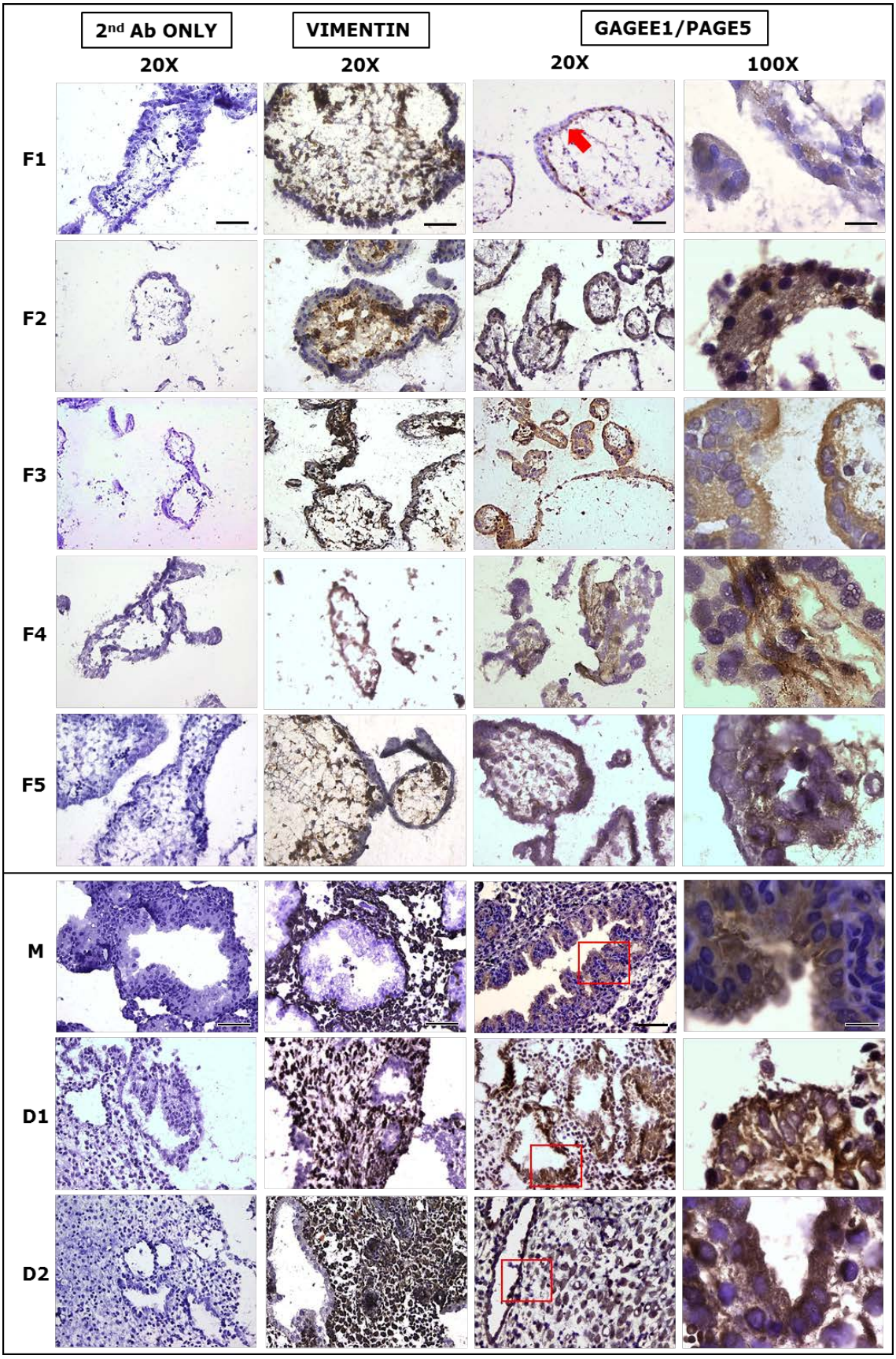


Figure 4.9: Localisation of GAGEE1.

GAGEE1 localisation is shown on the right (at both 20X and 100X magnification), whilst the negative (no primary Ab) control images are shown in the left hand panels and the positive control (vimentin) images are in the centre. **Panel F1-5** show images for placental villous sections from FT**. Panel D1-D3** represents the decidual sections from FT as well. Bold arrows- CTB; Uterine glandular epithelium enclosed in box. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **MAGE family**

The cellular localisation of the two members of the MAGE family, MAGEA1 and MAGEA4, was investigated.

- **MAGEA1**

Intense cytoplasmic staining was observed in the STB and CTB layers of the villi (See Figure 4.10, Panel F1-5). The syncytial spout (represented by +) of the mesenchymal villi was also immuno-positive (Panel F4). However, the mesenchymal cells in the stroma were negative for MAGEA1 staining. The epithelial layer lining the glands and vessels in the decidua was stained with the same intensity as trophoblast cells (Figure 4.10, Panel D1-3; Table 4.3). No staining was found on the decidual stromal cells or the lining of the spiral arteries.

- **MAGEA4**

The cellular localisation of MAGEA4 in the placental villi was similar to MAGEA1 (see Figure 4.11, Panel F1-5). The intensity of staining was only moderate (score 2) when compared to the high (score 3) intensity of MAGEA1 (Table 4.3). In the decidual regions, apart from staining of glandular epithelium, low levels of immuno-reactivity for MAGEA4 were also observed on the lining of the spiral arteries and the surrounding stromal cells (Figure 4.11, Panel D1-3).

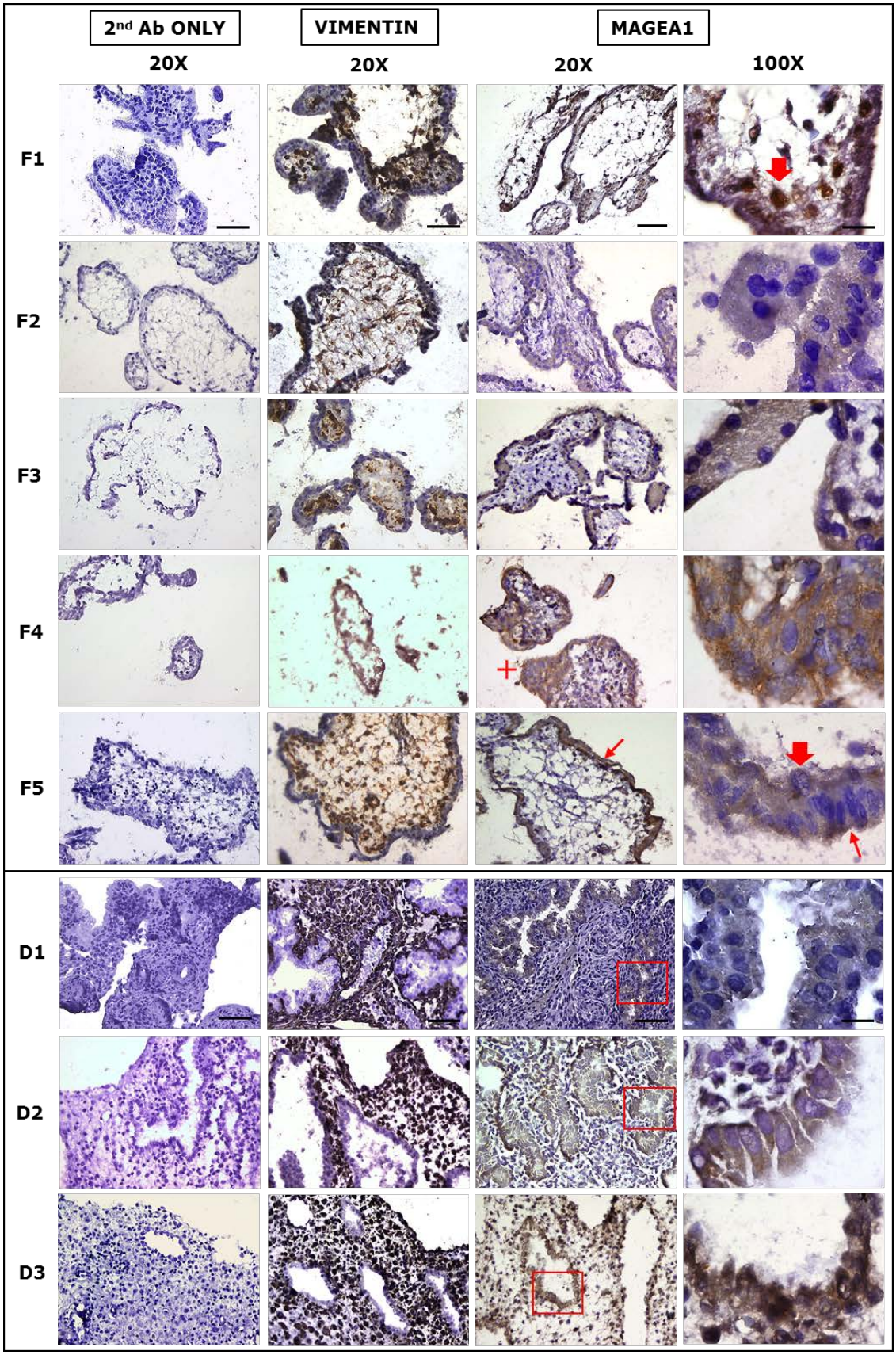


Figure 4.10: Localisation of MAGEA1.

MAGEA1 localisation is shown on the right (at both 20X and 100X magnification), whilst the negative (no primary Ab) control images are shown in the left hand panels and the positive control (vimentin) images are in the centre. **Panel F1-5** show images for placental villous sections from FT**. Panel D1-D3** represents the decidual sections from FT as well. Thin arrows- STB; Bold arrows- CTB; + Syncytial knot; Arrow head- Spiral arteries; Uterine glandular epithelium enclosed in box. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

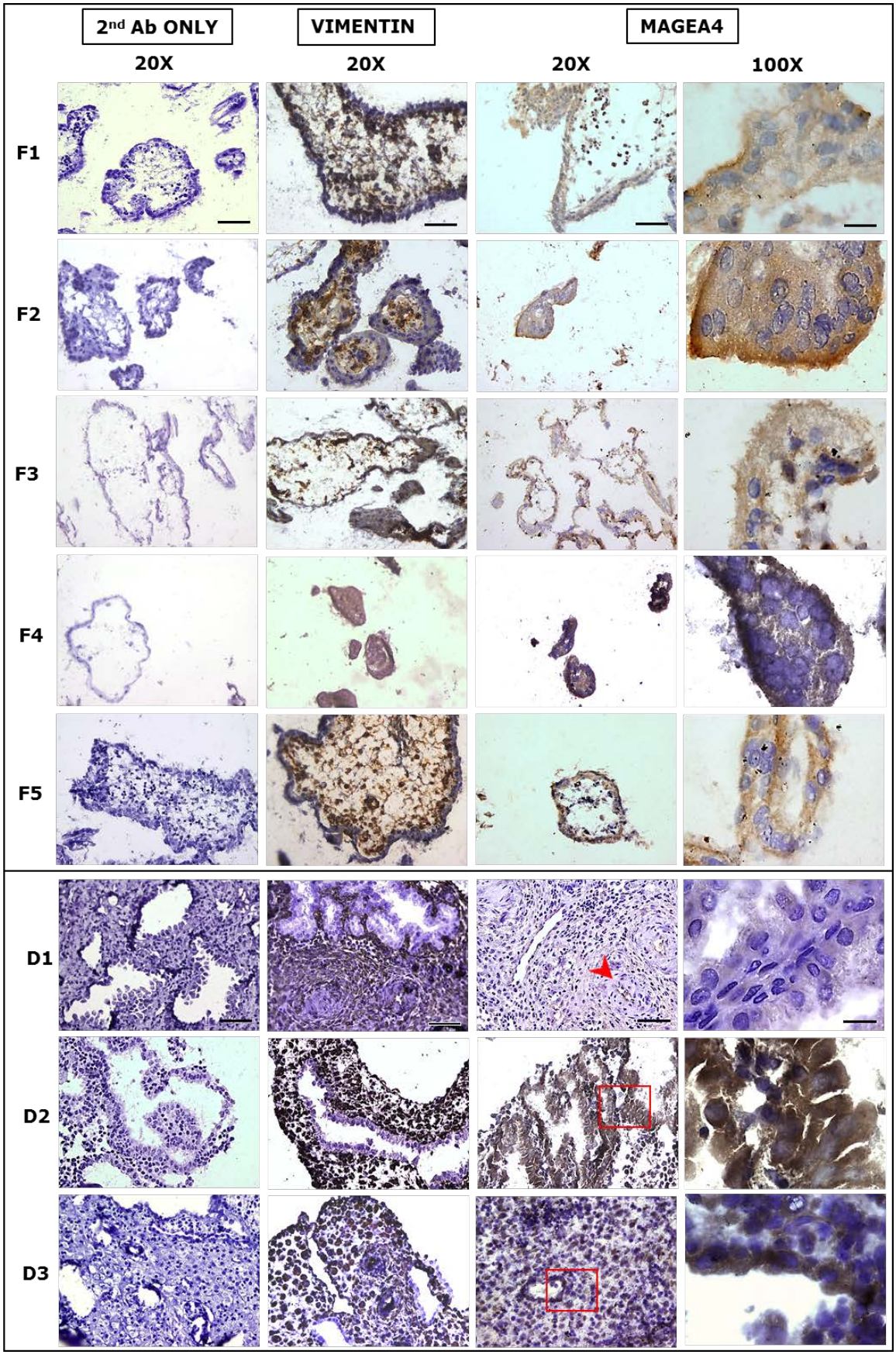


Figure 4.11: Localisation of MAGEA4.

MAGEA4 localisation is shown on the right (at both 20X and 100X magnification), whilst the negative (no primary Ab) control images are shown in the left hand panels and the positive control (vimentin) images are in the centre. **Panel F1-5** show images for placental villous sections from FT**. Panel D1-D3** represents the decidual sections from FT as well. Arrow head- Spiral arteries; Uterine glandular epithelium enclosed in box. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

- **PASD1**

Moderate levels of staining for PASD1 were observed in the cytoplasmic region of STB and CTB layers of the villous region (Figure 4.12, Panel F1-3 and 5; Table 4.3). However, nuclear positivity for PASD1 was observed in the CTB cells (Figure 4.12, Panel 4). The mesenchymal cells were negative. The uterine glandular epithelium showed high immuno-positivity in the decidua (Figure 4.12, Panel D1-D3). The decidual stromal cells and the inner lining of the spiral arteries showed moderate to low levels of staining.

On the whole, trophoblast (STB and CTB) and epithelial (decidua) cells showed immuno-positivity for most of the C/T antigens investigated. The staining in the mesenchymal cells decidual stromal cells and spiral arteries were antigen-dependent. Although most of the staining for the antigens was observed as granular brown stains in the cytoplasm of the cells, FATE1 and PASD1 showed nuclear staining, especially in certain of the villous CTB cells.

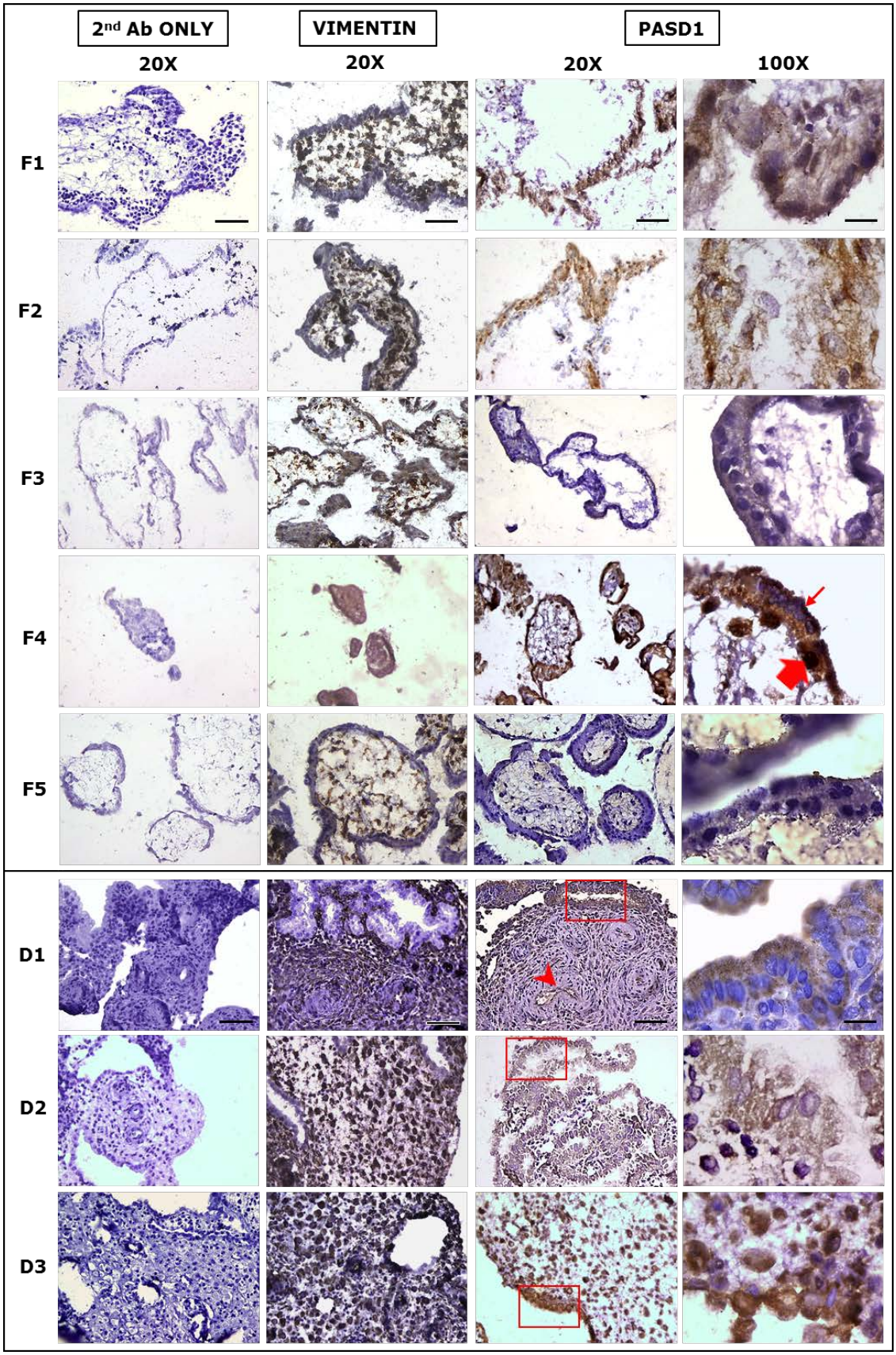


Figure 4.12: Localisation of PASD1.

PASD1 localisation is shown on the right (at both 20X and 100X magnification), whilst the negative (no primary Ab) control images are shown in the left hand panels and the positive control (vimentin) images are in the centre. **Panel F1-5** show images for placental villous sections from FT**. Panel D1-D3** represents the decidual sections from FT as well. Thin arrows- STB; Bold arrows- CTB; Arrow head- Spiral arteries; Uterine glandular epithelium enclosed in box. *Objective magnification: 20X (Scale bar=100µm); 100X (Scale bar= 20µm).*

4.2.4 Differential mRNA expression patterns within FT placentae

Interestingly, there was a heterogeneous distribution of C/T antigen mRNA expression levels amongst FT placenta. This may be due to the range in gestational age (7-9 weeks) of the samples. Therefore, the FT samples were grouped according to their different gestational ages and the expression patterns were investigated within groups (weeks 7, 8 and 9; Represented in Table 4.4 below).

Out of all the receptors and antigens investigated, four C/T antigens showed significant differential expression patterns between gestational ages. A significant down-regulation of FATE1 and GAGE1 mRNA expressions were observed in week 8 when compared to week 7 ($p<0.05$). On the other hand, a significant up-regulation of CAGE1, FATE1 ($p<0.01$), GAGE1 and MAGEA1 mRNA expressions were noticed in week 9. It should be noted that these effects are observed only using a small sample size (n=3 in each group).

Table 4.4: Differential mRNA expression patterns between gestational age (weeks).

Genes	Difference in mRNA expressions (between weeks)		
	7 and 8	7 and 9	8 and 9
5-HT Receptor			
5-HT_{1B} R	ns	ns	ns
5-HT_{2B}R	ns	ns	ns
C/T Antigens			
CAGE1	ns	$p<0.05$ $(+)^{\#}$	$p<0.05$ $(+)^{\#}$
FATE1	$p<0.05$ $(-)^{*}$	$p<0.01$ $(+)^{\#}$	$p<0.01$ $(+)^{\#}$
GAGE1	$p<0.05$ $(-)^{*}$	ns	ns
GAGEEE1/PAGE5	ns	ns	ns
MAGEA1	ns	ns	$p<0.05$ $(+)^{\#}$
MAGEA4	ns	ns	ns
PASD1	ns	ns	ns

$(-)^{*}$ Down-regulated in week 8; $(+)^{\#}$ up-regulated in week 9; ns= not significant.

4.2 Discussion

The placenta shows a rapid growth during the first trimester (FT) of pregnancy. This rapid growth is regulated by various factors like hormone secretion, gene expression and protein translation (See Section 1.1.1). These factors regulate the properties and functions of the cells involved during placental development. These cells also show variations in their functional properties with gestational age (Tarrade *et al*, 2001). The STB cells are highly invasive during the first trimester but their invasive properties reduce towards the end of the pregnancy. Differential expression patterns of proteins/factors involved in proliferation and invasion have been reported between the FT and term placenta (Sitras *et al*, 2012). Therefore this study was carried out to investigate the expression patterns of the main 5-HT receptors and C/T antigens in FT compared to term placentae. Also, the differences during the early gestational age (weeks 7 to 9) was studied. The cellular localisation and IHC staining intensities of these receptors were compared between villous and decidua of first trimester.

- **5-HT receptors:**

The comparative mRNA expression of the 5-HT receptors differed between FT and term. The relative mRNA expression of 5-HT_{1B} receptor was up-regulated in FT when compared to term placentae. 5-HT_{1B} receptors have been reported to mediate proliferation of other cells (Sibella-Argüelles, 2001; Banasr *et al*, 2004; Gurbuz *et al*, 2014). Therefore, it can be postulated that the high mRNA expression of 5-HT_{1B} receptor during FT would be an indication of 5-HT mediated proliferation of the trophoblast cells. Since invasion is no longer a necessity in fully developed term placentae, the expression of 5-HT_{1B} may have reduced.

The 5-HT_{2B} receptor however, was significantly down-regulated in FT placenta when compared to term. The mediation of contraction of chorionic vessels by 5-HT through this have been well documented (Cruz *et al*, 1998; Ugun-Klusek *et al*, 2011; Cordeaux *et al*, 2008). Moreover, studies using tumour-derived cell lines such as breast and hepatocellular cancer have confirmed the pro-migratory effects of 5-HT via 5-HT_{2B} receptor subtype (Pai and Horseman, 2008; Soll *et al*, 2012). Therefore, the observed down-regulation of 5-HT2B receptor in the FT placentae during the time of high levels of neo-vascularisation is surprising.

As mentioned in Chapter 3, the up/down-regulation of mRNA expression may not be a true indicator of changes at the protein level. The mRNA level does not usually predict its protein level, nor its activity (Wang *et al*, 2002; Yang *et al*, 2003). Therefore, the changes in 5-HT receptor mRNA levels in FT can only be taken as a guide to identify the interesting proteins. Although attempts were made to carry out immuno-blotting, it was not possible to quantify the protein expression due to very limited protein sample availability.

Previously, it has been shown that the activation of these two receptors are 5-HT concentration-dependent (Soll *et al*, 2012; Sonier et al, 2002; See also Chapter 5 and 6). A review by Moiseiwitsch (2000) has summarised that the migration of neural crest cells reduced and differentiation increased in response to higher 5-HT levels. Also, immunohistochemical staining of the placenta for 5-HT localisation have shown its presence only in first trimester STB, with a decrease observed with gestational age (Huang *et al*, 1998). This suggests that the high levels of 5-HT during FT may activate the 5-HT_{1B} receptor for migration of trophoblast cells. However, the reduced levels of 5-HT in term placenta may have activated the 5-HT_{2B} receptor and induce differentiation of the cells. Therefore 5-HT_{2B} receptor might have anti-migratory effects in STB of FT. It is worth noting the mRNA expression of 5-HT_{2B} in term placentae is 10000 fold higher than that of 5-HT1B, (Figure 4.2); which suggests an important role of this receptor subtype in the events related to parturition.

- **C/T antigens:**

The mRNA expression of C/T antigens were relatively higher in FT than term placentae, except for GAGEE1/PAGE5. The function(s) of these C/T antigens in general, and particularly in the placenta, are not fully understood. Only two studies to date have reported the expression profile of MAGE-A and C, NY-ESO-1, GAGE and CAGE1 (Jungbluth *et al*, 2007; Khan *et al*, 2014). The present study represents the first of its kind to compare many identified C/T antigens by both mRNA expression analysis and immunohistochemistry in FT, term as well as in PE placentae (Chapter 3). The proposed function of the antigens up-regulated in FT are as follows: linked to tumorigenesis (FATE1) (Olesen *et al*, 2011), structural protein (CAGE1) (Alsheimer *et al*, 2005), cell differentiation and signalling (MAGEA1) (Monte *et al*, 2006) and apoptosis (GAGE1 and MAGEA4) (Gjerstorff *et al*, 2006; Sang *et al*, 2011). Functions such as tumorigenesis, cell differentiation and signalling are required for the tumour-like behaviour of the trophoblast (enhanced proliferation, growth and invasion), especially during the FT. The high mRNA expression of C/T antigens GAGE1 and MAGEA4 (which have apoptotic functions) in FT could be due to constant proliferation, differentiation and apoptosis cycles within trophoblast cells. This cycle is required for the rapid expansion of the villous tree during the FT.

The STB cells constantly undergo apoptosis and the apoptotic nuclei of these cells are packed into syncytial knots which are released into the maternal blood stream (Huppertz, 2008). The STB layer is constantly maintained by the differentiation of the CTB which are located below the multinucleated STB. Therefore, the increased mRNA expression of GAGE1 and

MAGEA4 observed in FT could be an indication of such a proliferation/apoptosis cycle. In contrast, the mRNA expression of GAGEE1/PAGE5, which also belongs to the GAGE family and shares apoptotic functions, was interestingly the only antigen which was significantly lower in FT. This suggests that there may be some other additional functions for this antigen. The PASD1 antigen also showed significant differences in mRNA expression between FT and term. This antigen has three domains which respond to environmental stimuli and the function of PASD1 is thus stimuli-dependent (Campbell *et al*, 2011).

The differential expression of these antigens between FT and term placenta suggests that their functions are dependent upon, or changing with, pregnancy progression. If the mechanism of control of these changes is known, then it may be possible to demarcate the period at which the highly invasive placenta transforms into physiologically functioning organ. This might open new avenues for investigating the causes of PE, where the invasion is shallow.

It is worth noting that FT samples consisted of a heterogeneous group, with different gestational ages (in weeks). Although the sample numbers were small, this study attempted to investigate the difference in the mRNA expressions between: (a) 7 and 8 weeks, (b) 7 and 9 weeks, and (c) 8 and 9 weeks. Remarkably, significant differences in mRNA expression of four C/T antigens (CAGE1, FATE1, GAGE1 and MAGEA1) were observed with different gestational age in FT. The increase in CAGE1 antigen with gestational age (week 6 to 8) in FT was earlier reported by Khan *et al* (2014). The expression of FATE1 and GAGE1 showed an increase in week 7, but decreased at week 8; followed by an increase at week 9. MAGEA1 shows an increase in expression at week 9. These differences in expression with gestational age confirm and extend the findings by Khan *et al* (which also used small sample numbers) (2010). On the whole the oscillatory changes in the mRNA expressions of these antigens may be linked to some developmental process.

- **Cellular localisation of 5-HT receptors and C/T antigens in villous and decidua of FT:**

The IHC staining of 5-HT_{1B} and 5-HT_{2B} receptor in this study revealed a cytoplasmic localisation in the CTB and STB layers of the villi. This confirms data from an earlier study by Huang *et al* (1998). The staining intensities were comparable for both the receptors in villous as well as decidual tissue of first trimester. However, the mesenchymal cells of the villi were immuno-positive for 5-HT_{1B} receptor, but negative for 5-HT_{2B} receptor. The mesenchymal cells are progenitor cells for several cell types including fibroblast, endothelial

cells, smooth muscle cells, myofibroblasts, blood cells and macrophages (Huppertz, 2008). The expression of 5-HT_{1B} receptor in mesenchymal cells suggests differentiation was also mediated through the activation of the receptor similar to monocyte differentiation (Katoh *et al*, 2006). The expression of these receptors on the decidua of the first trimester were localised primarily to the stromal cells of the endometrium, epithelial lining of the uterine gland and spiral arteries.

Although a detailed study on the cellular localisation of MAGE-A and C, NY-ESO-1, GAGE was carried out by Jungbluth *et al* on placental samples from week 3 to 42 (2007), the present study localised additional C/T antigens (CAGE1, MAGE-A4, GAGEE1, PASD1) in the villous and decidual cells of FT. Furthermore, the antibodies used by Jungbluth's group were all monoclonal antibodies (M3H67, AE1/AE3 and 4H84) produced by their group. Although the antibodies used in this study were all commercially available polyclonal antibodies the expression patterns were comparable to Jungbluth *et al*. High staining intensities were observed in the trophoblast cells of the villi which is comparable to studies by Jungbluth *et al*. The cytoplasmic regions showed granular staining for most of these antigens except for FATE1 and PASD1. FATE1 showed intense cytoplasmic as well as nuclear staining for the STB. PASD1 on the other hand stained the cytoplasm and nucleus of the CTB in some of the villous samples. Interestingly FATE1 was the only antigen which showed distinct intense staining of the mesenchymal cells in the stroma. These cells also have numerous cisternae of the rough endoplasmic reticulum. The cellular localisations of these antigens were investigated through various online databases. Uniprot suggests that FATE1 is localised in the endoplasmic reticulum (Uniprot ID: Q969F0). Structures of cisternae continuous with the endoplasmic reticulum and the nucleus have also been reported in the STB (reviewed by Jones and Fox, 1991). The FATE1 expression in the endoplasmic reticulum of STB suggests a high biosynthetic activity of these cells. Uniprot suggests PASD1 is located in the nucleus, but expression was observed in both cytoplasm and nucleus (Uniprot ID: Q8IV76). However, the function of PASD1 is predicted to be stimuli-dependent (Campbell *et al*, 2011).

The cellular localisation of the C/T antigens were also investigated in the decidua of FT. The uterine glandular epithelium, decidual stromal cells and the spiral arteries were immuno-positive for the C/T antigens. The intensity of staining was highest in the epithelium followed by moderate staining in decidual stromal cells and weak in the spiral arteries for most of the antigens. Only the cytoplasm of the cells showed granular staining, even for FATE1 and PASD1. The expression of these antigens on the cells of the uterine endometrium can be linked to the maintenance of the decidualisation of the endometrium for the implantation of

the foetus. The stromal cells in the decidua function to regulate the epithelial cell growth (Arnold *et al*, 2001). The epithelial cells in the uterus determine the fate of the trophoblast cell adhesion (Thie *et al*, 1998). As discussed earlier, the initial attachment of the trophoblast onto the uterine epithelium is a critical stage that decides the development of the placenta and foetus (reviewed by Norwitz et al, 2001). Although the cellular localisation of the antigens were investigated in both the villous and decidua of FT, conclusions cannot be made about the expression of these proteins based on the staining intensities alone (due to variations in cell morphology). Therefore, further analysis of protein expression, such as Western blotting would be necessary to fully investigate their relative expression (See Chapter 7).

Conclusion:

The results from this study suggest that the mRNA expression of the 5-HT receptors and C/T antigens were different in FT and term placentae. Variances were also observed with gestational age within FT samples. These changes may be linked to the functions of the 5-HT receptors in the migration/differentiation of trophoblast cells and constriction of the placental blood vessels. On the other hand, the functions of the C/T antigens in placenta remain unknown.

Chapter 5
Effect of 5-HT on cell behaviour

Alterations in cell morphology, viability, proliferation, migration and invasion under normoxia and hypoxia.

5.1 Introduction

As explained in Chapter 1, placenta ensures the exchange of nutrients and gases to the foetus for its healthy development. Due to this critical function it is impossible to carry out *in vivo* studies during the developmental stage of the human placenta. On the contrary, the full term placentae are relatively easy to obtain after delivery with consent from the patients. These samples can be used to set up placental perfusion studies and be used as *ex vivo* models to study the materno-foetal transport and communication. However, this model is incapable to monitor functional changes during the early developmental stages of placenta. Furthermore, the placenta is one of the most variable organ in the animal kingdom and are classified by various characteristics such as shape, maternal-foetal barrier interface etc. The most commonly used classification is based on the histology of the placenta and are divided into three main groups: epitheliochordial, endotheliochordial and hemochordial (Furukawa *et al*, 2014). Epitheliochordial placenta are the least invasive causing no distruction of the maternal uterine layers. Such placentation takes place in horses, swines and ruminants. In endotheliochordial placenta, the maternal uterine epithelium and connective tissue disappear after implantation but the endothelium is still intact e.g. dogs and cats. The hemocordial is third and the highly invasive placentation, where maternal epithelium, connective tissue and the endometrium are displaced. The placentation in humans and rodents belong to this group. Since rodents and humans belong to the same placentation group, mouse models are extensively being used to investigate therapeutic drugs for human placental conditions (pre-eclampsia) (Faas *et al*, 1994). However, mouse has a very short gestational period when compared to the nine months period in humans. Therefore, mouse models are restricted to molecular level of studies (Reviewed by Mess, 2014). Although animal models are required for the restricted access to human placenta, investigators have to bare in the mind the limitations of the experimental models being used and the unique developmental and endocrine functions of the placenta (Redman and Sargent, 2001). The data obtained from these models have to be critically reviewed for its applications in humans.

Human placenta is made of heterogeneous group of cells mainly the trophoblasts, mesenchymal, and stromal fibroblasts with surrounding connective tissues. These different cell types display varying morphology, function and gene expressions. Therefore to study the specific processes in placental development *in vitro* it is more appropriate to include all these different cell types. By this way any cell specific changes can constantly be monitored. There are two options for *in vitro* studies; (a) primary cells from term and/or first trimester aborted pregnancies and (b) placental derived cell lines. Primary trophoblast cells isolated from term placenta are not suited to study the early developmental stages and the isolated cells are appropriate only for short term examination. Likewise, due to ethical constraints,

obtaining primary cells from the early trophoblast is difficult. Placental cell lines on the other hand partially behave in a similar manner to cells they were derived from the original tissue (Novakovic *et al*, 2011). The use of these placental derived cell lines dates back to 1968 when Patillo and Gey successfully established BeWo cell line from placental choriocarcinoma tissues. Following this, many placenta derived cell lines were established (Fogh *et al*, 1977; Graham *et al*, 1993; Feng *et al*, 2005). Furthermore, these cell lines are immortalised by various means and therefore they are useful for long time monitoring. However it should be noted that the physiological functions of these cell lines may be altered due to their modes of immortalisation. Therefore any *in vitro* investigations should use as many different cell lines as possible to obtain meaningful results.

Among the four placental cell lines used in this chapter, BeWo and JEG3, were derived from gestational choriocarcinoma and the other two transformed (non-tumour) placental cell lines, TEV-1 and HTR8/SVneo, were produced by respective immortalisation of first trimester EVT using retroviral vector with HPV16 E6/E7 gene (Feng *et al*, 2005), and SV-40 large T antigen (Novakovic *et al*, 2011). The origins and functions of the different cell lines are summarised in Table 5.1. Both choriocarcinoma as well as EVT cells show invasiveness, however the EVT cells invade only after proliferating whereas the choriocarcinoma cells can both proliferate and invade at the same time (Frank *et al*, 1999).

Therefore, it was evident that there are differences in mechanisms, gene expressions and functions between the two groups. Thus, two different groups of cell lines were used in this study. A fibrosarcoma-derived cell line which shows epithelial morphology like placental cell lines, HT1080, was also used as a positive control for migration and invasion studies (Rasheed *et al*, 1974).

- **5-HT- new potential for an old monoamine:**

As mentioned earlier (Chapter 1) increased levels of 5-HT have been reported during pregnancy, and especially in pre-eclampsia (Middelkoop *et al*, 1993; Cruz *et al*, 1998). Increased levels of 5-HT is believed to be coincide with craniofacial and cardiovascular development during embryogenesis (Choi *et al*, 1997). This led to suggestions that 5-HT might play some function during placental development. Previous studies have shown that 5-HT influences the early extravillous trophoblast (EVT) invasion (Sonier *et al*, 2005) and vasoreactivity of the spiral arteries *via* 5-HT_{1B} and/or 5-HT_{2A} receptors (Bolte *et al*, 2001). Futhermore, the influence of 5-HT on the contractility of chorionic arteries and veins have been reported by many authors (Cruz *et al*, 1998; ; Cordeaux *et al*, 2008; Ugun-Klusek *et al*, 2011). In fact, 5-HT not only induces cell proliferation, but also activates differentiation

of cells such as monocyte and T lymphoblastic cells (Katoh *et al*, 2006; Argüelles, 2000). The most important physiological effect of 5-HT in early embryogenesis is enhancing the migration of several cell types to develop into functional organs (Choi *et al*, 1997; Matsusaka and Wakabayashi, 2005).

On the tumour side, a very early study by Burtin *et al* (1982) reported a decrease in tumour size with direct serotonin injection. Subsequently, several studies have investigated the effects of 5-HT and its receptor activation pathways on tumour growth (Manda *et al*, 1998; Soll *et al*, 2010, 2012; Pai *et al*, 2009); some authors reporting a decrease in tumour growth with 5-HT, whereas others have shown survival of the tumour cells by the influence of 5-HT. These disparities could be due to the variations in 5-HT doses, cell types used, and the origin of the cells. Conversely, very few studies have concentrated on the effects of 5-HT on early placental cell lines (Sonier *et al*, 2005; Klempan *et al*; 2011). Many reporting the production and storage of 5-HT in the placenta and specifically in trophoblast cells have been postulated (Deroy *et al*, 2013; Huang *et al*, 1998). However, the cell lines investigated in these studies originated from placental choriocarcinoma (BeWo and JEG3).

Thus, it is important to investigate the various physiological effects of 5-HT on transformed trophoblast cell lines (TEV-1 and HTR8/SVneo) which have closer resemblance to early first trimester trophoblast and thereby explore any new potential for this old monoamine.

- ***In vitro* study under hypoxic conditions:**

As mentioned in Section 1.5 (Chapter 1), hypoxia is a condition that occurs when the demand for oxygen is more than the supply. It may be caused due to abnormal inspired oxygen, scarce blood supply or the inability of the tissue to extract/utilise oxygen from the blood. None of these happen during placental development; in fact in normal pregnancy the blood supply for the developing foetus and placenta is increased. Therefore, regarding the placental environment as being hypoxic is fundamentally incorrect. It can be considered hypoxic only when compared to other organ environments, in which the metabolic demand for oxygen is much higher, but the supply is low. Interestingly, studies using isolated trophoblast from term placenta have indicated that these cells are tolerant to prolonged periods of hypoxia (Esterman *et al*, 1997). The study also reported changes in cytoplasm during hypoxic condition with a reduction in microvilli on the surface of cytotrophoblasts. There were no significant differences in trophoblast proliferation in normoxic and hypoxic conditions.

Several studies have now confirmed that hypoxia inhibits the differentiation of cytotrophoblast cells and also has detrimental effects on differentiated syncytiotrophoblast

cells which appear during later placental development (Koklanaris *et al*, 2006; Genbacev *et al*, 1996; Burton and Jaunaiux, 2001). However, whether hypoxia stimulates or reduces the trophoblast invasive properties has been a controversial topic (James *et al*, 2006). One group of investigators consider hypoxic conditioning improves the trophoblast invasion (Graham *et al*, 2000), whereas others have made contradictory observations (Genbacev *et al*, 1996; Kilburn *et al*, 2000). Therefore this topic still remains open for further investigations. Additionally, further hypoxic conditions occurring during later stages of pregnancy have been considered to cause PE (Hung and Burton, 2006). However, this has not been confirmed by direct measurement of the dissolved oxygen in PE.

Thus, the aim of this chapter was to investigate the effects of 5-HT on transformed early trophoblast cell lines and compare them to placental cancerous cell lines (placental choriocarcinoma) under normoxic and hypoxic conditions. This first half of the study is performed under 20% v/v oxygen (normoxia). The same studies were then performed under hypoxic conditions (2% v/v O_2).

Table 5.1: Features and functions of the different cell lines used in this study.

	TEV-1	HTR8/SVneo	BeWo	JEG-3	HT1080
Origin	First trimester placental explant	HTR8 (First trimester villous explants)	Choriocarcinoma	Choriocarcinoma	Fibrosarcoma
Method of immortalization	Retroviral vector with HPV16 E6/E7 gene	Electroporation with pSV3neo containing early region of SV40 encoding the SV40 Tag	Naturally occurring	Naturally occurring	Naturally occurring
Morphology	Epithelial	Epithelial	Epithelial	Epithelial	Epithelial
Tissue	Placenta	Placenta	Placenta	Placenta	Connective
Migration		+			+
Invasion	+	+	+	+	+
Syncytialisation			+		
References	Feng et al 2005	Graham et al 1993	Patillo and Gey 1968	Kohler and Bridson 1971	Rasheed et al 1974

Table adapted from **Novakovic** *et al*, 2011

5.2 Results

5.2.1 Determination of cell seeding density for each cell line

Since most of the assays and experiments needed up to 48 hour incubation, it was essential to determine the cell seeding densities for each of the cell lines. Moreover, since these cell lines are relatively new only a few studies were available for comparative purposes. The accurate seeding density was determined by comparing the cell activity at different time points. MTT assay was carried out to measure the cell viability and mitochondrial dehydrogenase activity of the cells as mentioned in Section 2.8.4. The cell metabolism was then used as guide for seeding the suitable cell densities.

Compared to 24 hours, the cell activity of TEV-1 (2.5 $x10^4$) cells showed a 0.33 fold increase at 48 hours and 0.72 fold increase at 72 hours (See Figure 5.1, Panel A). However, the increase in activity was higher with 5 ($x10^4$) cells. A half fold increase was observed at 48 hours whereas 72 hours showed approximately one fold increase in cell activity (compared to 24 hours). The cell activity gradually decreased when the seeding number was increased to 10 ($x10^4$) cells. Both 48 and 72 hours incubation increased the activity by 0.6 fold. Since 5 ($x10^4$) number of cells showed the maximum activity up to 72 hours this density was used as seeding density for TEV-1 for further experimentations. The second transformed trophoblast cell line HTR8/SVneo, showed a reduction of activity after 48 hours incubation when compared to 24 hours (See Figure 5.1, Panel B). However, the activity increased with 72 hours of incubation. Cell numbers 0.625 ($x10^4$) and 5.0 ($x10^4$) showed the half fold increase of activity at 72 hours. Seeding with 10.0 ($x10^4$) cells, the activity was reduced at 48 and 72 hours. Although the differences in activity were not significant, 5.0 ($x10^4$) was selected as the seeding density for further experimentations.

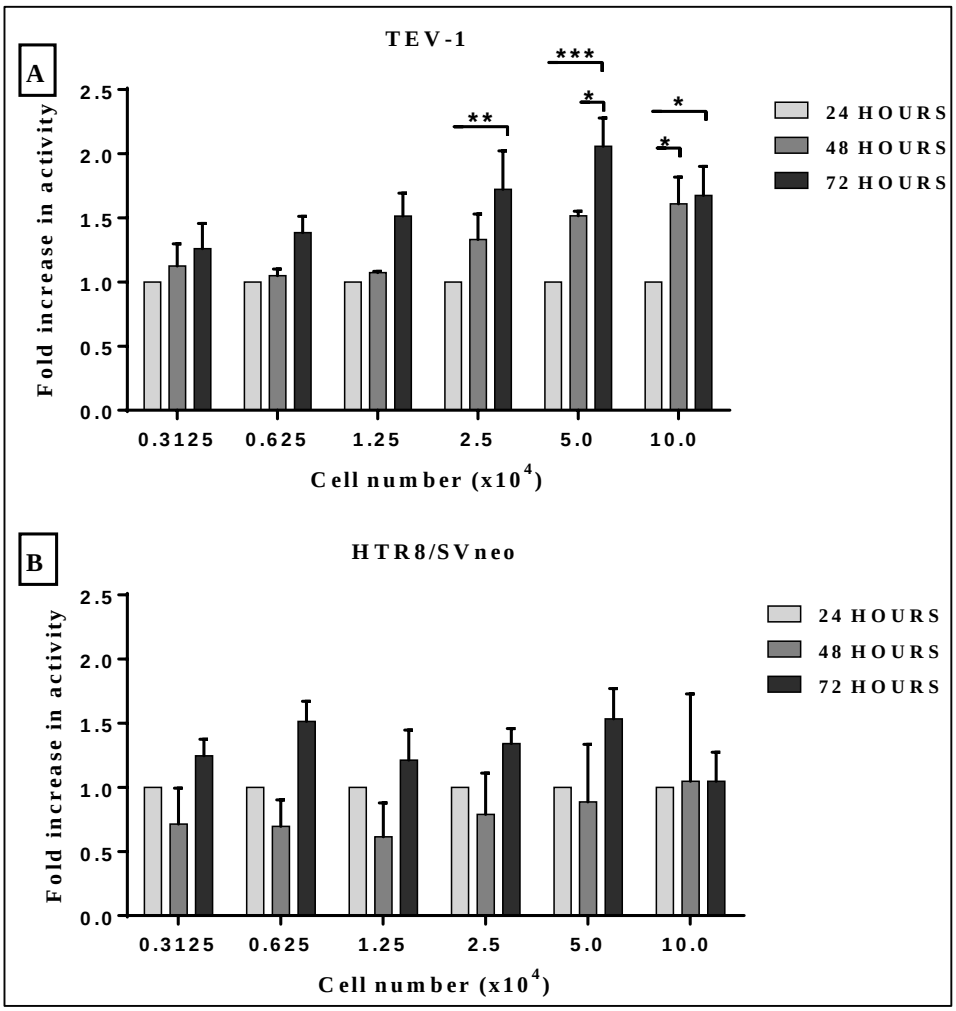


Figure 5.1: Cell seeding densities for transformed placental trophoblast cell lines.

Panels A and B represents the fold increase in activity of TEV-1 and HTR8/SVneo respectively. Statistical analysis was carried out using ANOVA. The results are a representation of the mean values for data for each time point for 3 independent experiments performed in triplicates ± SEM; *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$.

In the case of choriocarcinoma cells BeWo, from Figure 5.2.A, it can be seen that seeding with higher cell densities for BeWo (5-10 $x10^4$) hindered its activity. At 72 hours incubation there was 0.3 fold reduction in activity with 10($x10^4$) cells. Although there were no significant differences in activity, cell seeding densities below 5($x10^4$) were selected for further experiments. In case of JEG3 compared to 24 hours incubation, more than 0.7 fold increase in cell activity was observed at 48 hours incubation with cell densities of 2.5, 5.0 and 10($x10^4$) cells (Figure 5.2.B). Interestingly 72 hours showed a highly significant increase in activity (over 2 fold) when compared to 24 hours. Therefore 5.0 ($x10^4$) cells were used as ideal seeding density for JEG3.

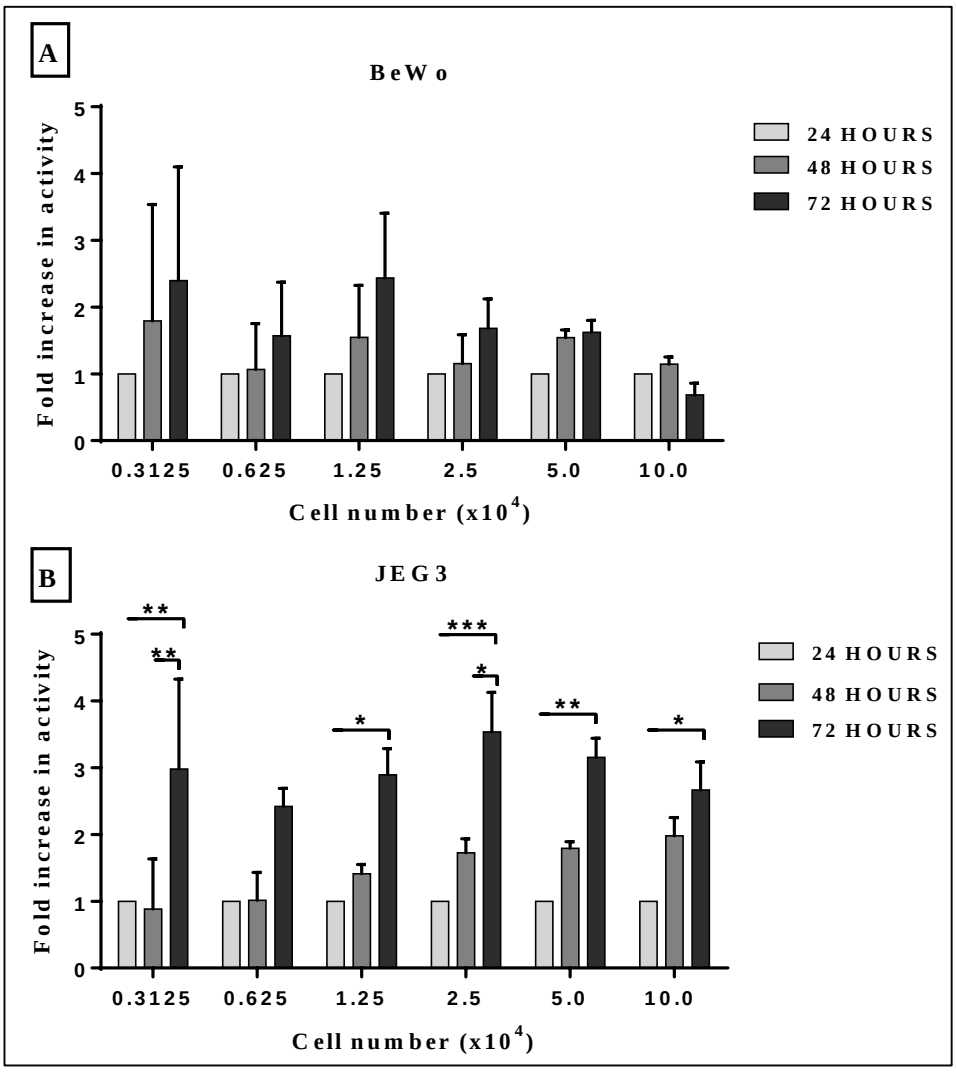


Figure 5.2: Cell seeding densities for placental choriocarcinoma cell lines.

Panels A and B represents the fold increase in activity of BeWo and JEG3 respectively. Statistical analysis was carried out using ANOVA. The results are a representation of the mean values for data for each time point for 3 independent experiments performed in triplicates ± SEM; *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$.

HT1080, a fibrosarcoma cell line which is used as a positive control for most of the experiments, showed significant increase in activity with cell seeding densities of 2.5 and 5.0 ($x10^4$) cells at 48 and 72 hours as seen from Figure 5.3. Almost one fold increase in 72 hours incubation was observed when compared to 24 hours. Therefore 2.5 ($x10^4$) cell number were used for further experimentations.

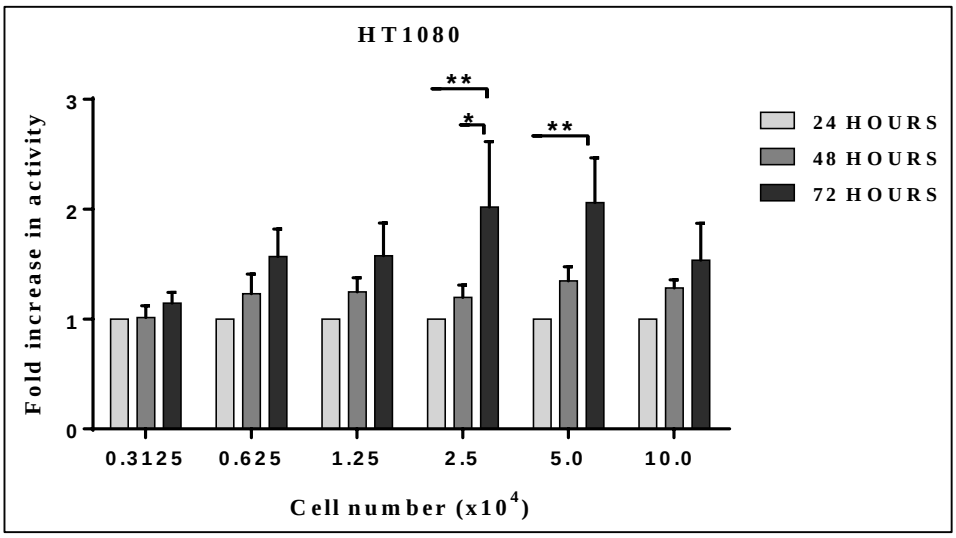


Figure 5.3: Cell seeding densities for fibrosarcoma cell line HT1080.

Statistical analysis was carried out using ANOVA. The results are a representation of the mean values for data for each time point for 3 independent experiments performed in triplicates ± SEM; *, $p<0.05$ and **, $p<0.01$.

5.2.2 Effect of 5-HT in *in vitro* under normoxia

The effect of 5-HT on cells were investigated under normal oxygen conditions of 20% v/v O_2 at 37ºC. The cells were maintained at this conditions for further investigations.

5.2.2.1 Effect of 5-HT on cell morphology

As predicted, H&E staining of cells treated with 5-HT for 24 hours revealed the changes in morphology. The 5-HT treated cells showed differences in their morphology. As the concentration of the 5-HT treatment increased there were increased numbers of necrotic cells with condensed and fragmented nuclei. Interestingly in some cells, such as HTR8/SVneo apoptotic blebbing and apoptotic bodies could be clearly seen. The 5-HT treatment also induced migration indicated by membrane ruffling, extended lamellipodium and filopodium. At higher concentrations (40µM and 80µM) 5-HT was cytotoxic as indicated by apoptotic and necrotic cells (cell swelling and disintegrated membranes). The presence of large multinucleated giant cells (which had anaplastic features) can also be seen at these high doses. It was noticed the cell numbers per field were lower at 80µM 5-HT treatment. Please refer to Figures 5.4 to 5.6 for further details on individual cell lines. Thus the cytotoxic effects of 5-HT could be observed by studying the morphological features of the cells.

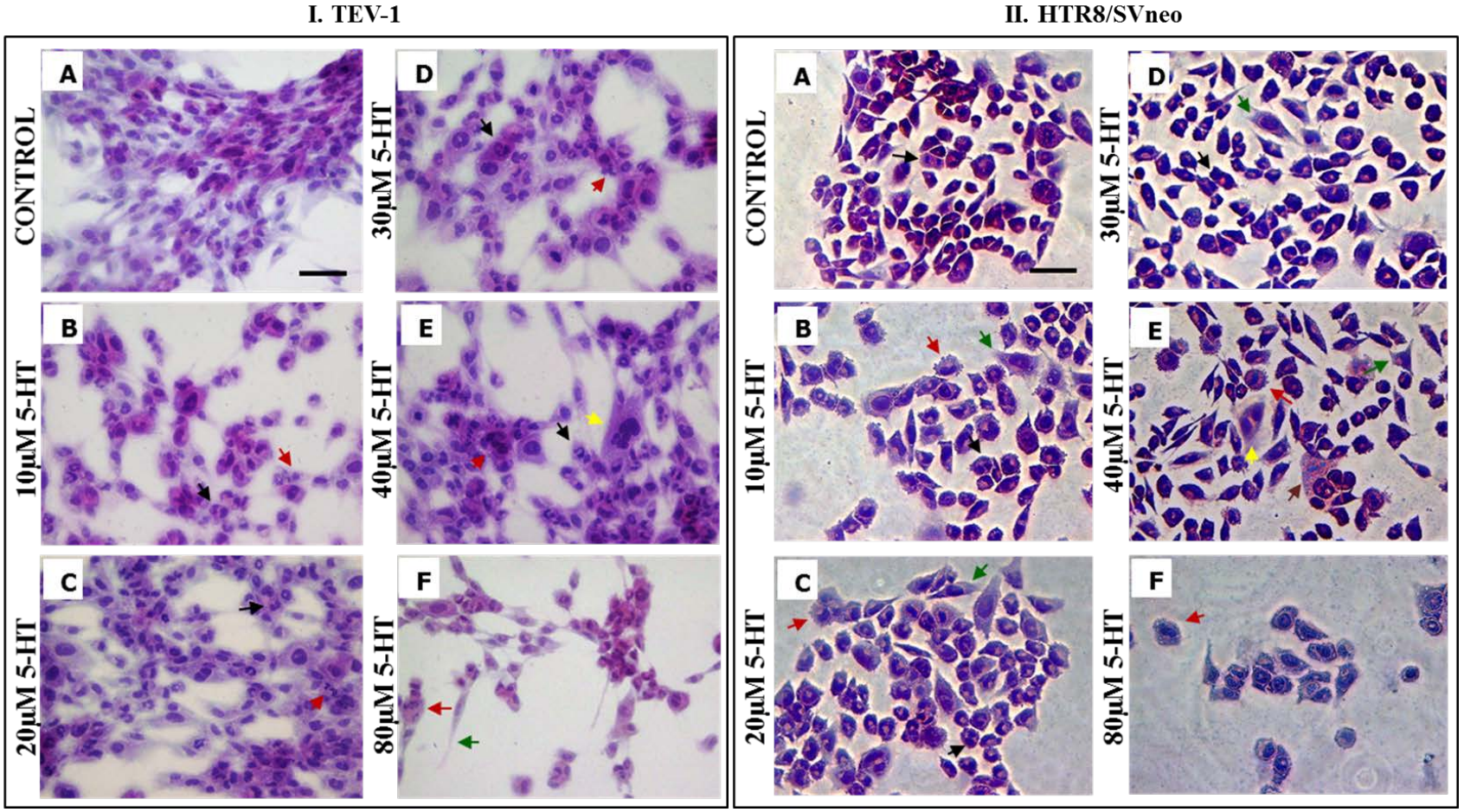


Figure 5.4: H & E staining for transformed trophoblast cell lines after 24 hours of 5-HT treatment.

Section I. and II. Shows images for **TEV-1** and **HTR8/SVneo** cells respectively. **Panel A** represents control cells and **Panels B-F** represents 5-HT treated cells (10-80µM 5-HT respectively). The mitotic cells undergoing division with two daughter chromatin structures were seen in all panels (indicated by black arrows). The red arrows points to cells undergoing apoptosis with characteristic features like blebs on the cell surfaces. These increase in number at higher 5-HT concentrations. The cells treated with 5-HT also induced formation of extended structures, membrane ruffles and lamellipodium indicated in green arrows. The yellow arrows show large anaplastic cells with undistinguished features (Sections I and II, Panel E). Necrotic cells were also seen with disintegrated membranes and faded nucleus (Brown arrows). *Scale bar= 100µm. Images taken at 200X magnification.*

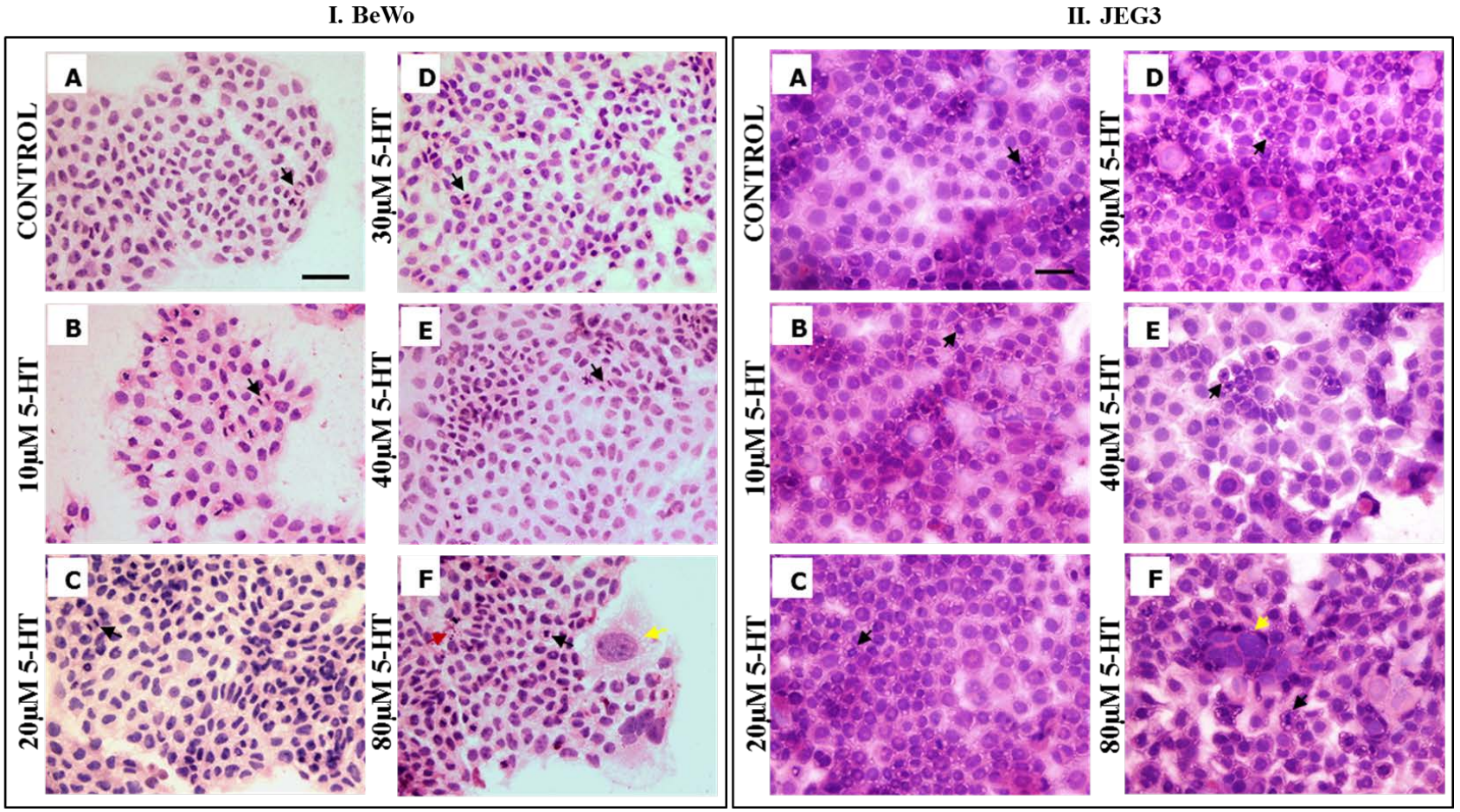


Figure 5.5: H & E staining for placental choriocarcinoma cell lines after 24 hours of 5-HT treatment.

Section I. and II. Shows images for **BeWo** and **JEG3** cells respectively. **Panel A** represents control cells and **Panels B-F** represents 5-HT treated cells (10-80µM 5-HT respectively). The nucleus and the chromatin structures were clearly distinguished (deep blue). The cytoplasmic surroundings formed uniform sheets (pink) with disruptions at higher 5-HT concentrations (Panel D-F). The black arrows indicate cells undergoing mitotic divisions with condensed chromatin structures in control as well as treated cells. These features increased with 5-HT treatment when compared to control. Disintegrated cell debris after apoptosis were more frequent at Panel F (represented by red arrows). The yellow arrow points to giant anaplastic cells with no distinctive features at Panel F. *Scale bar= 100µm. Images taken at 200X magnification.*

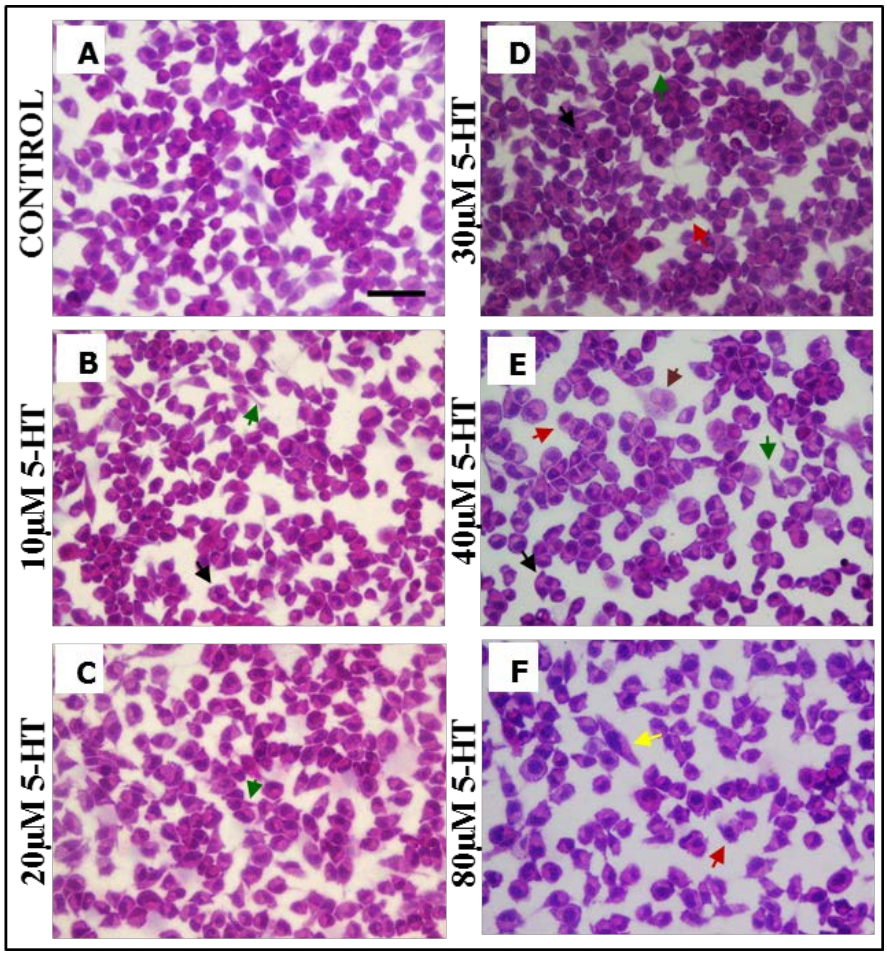


Figure 5.6: H & E staining for HT1080 after 24 hours of 5-HT treatment.

Panels A= Control, B, C, D, E, F= 10, 20, 30, 40, 80µM 5-HT treatments respectively. There were more extended features from the cells and mitotic cells in Panel C and D (green and black arrows respectively). A spike formation was seen on cells undergoing apoptosis indicated by red arrows (Panel D, E and F). The cell pointed by yellow arrow in Panel F varies from any of the other cells in the population thus distinguished as an anaplastic cell. Scale bar= 100µm. Images taken at 200X magnification.

5.2.2.2 Effect of 5-HT on cell viability and toxicity

To investigate the effect of 5-HT on viability or cytotoxicity of the cells, MTT and CytoTox 96® Non-Radioactive Cytotoxicity assays were performed respectively. These two assays are mostly the first choice of cell viability and cytotoxicity assays because of straight forward protocols, speed and requirement of non-radioactive reagents. The effect of 5-HT were investigated on (a) *plating cells along with 5-HT*- to investigate any immediate effects of 5-HT on vulnerable cells and well as on cell attachment (b) *treating cells after 24 hours of recovery*- to investigate effects on already viable and adherent cells. The data are represented as percentage in comparison to Control (no treatment) for both assays.

- **Plating cells along with 5-HT**

The cells were seeded into plates with different doses of 5-HT to check if there were immediate effects of 5-HT on cell viability and adherence.

- **Effect of 5-HT on transformed trophoblast cells**

The transformed trophoblast cell lines, TEV-1 and HTR8/SVneo, both show an increase in cell viability when treated with 5-HT.

In TEV-1, 30µM 5-HT treatment for 24 hours showed over 50% increase in cell viability when compared to control (no treatment - See Figure 5.7, Panel A). However, the viability reduced at higher doses of 5-HT (40 and 80 µM), also viability at 48 hours was lower than at 24 hours. From Figure 5.7, Panel B it can be seen that there were no significant changes in cell toxicity with 5-HT when compared to control.

HTR8/SVneo cells also showed similar results with 30µM 5-HT treatment showing 50% increase in cell viability and 8% decrease in cell toxicity when compared to control (Figure 5.7, Panel C and D). However, unlike TEV-1, 5-HT treatment for 48 hours in HTR/SVneo showed higher viability in comparison with 24 hours duration. Overall, 30µM 5-HT treatment increased the viability of the transformed trophoblast cells with the least cytotoxic effect.

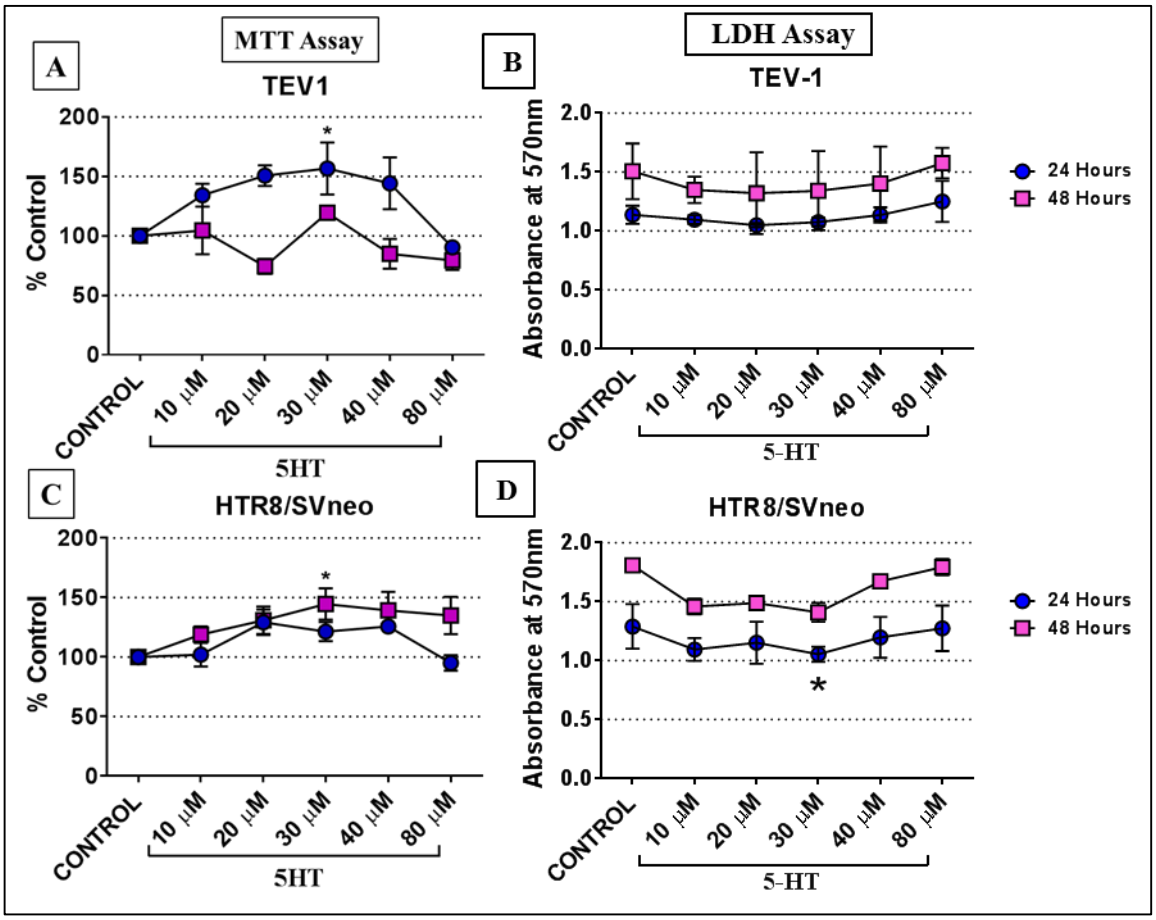


Figure 5.7: Dose-response curve for transformed trophoblast cell lines treated with 5-HT.

Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *p<0.05.

- **Effect of 5-HT on placental choriocarcinoma cells**

The effect of 5-HT on placental choriocarcinoma cell lines showed similar results to transformed trophoblast cell lines. A 77% increase in cell viability was observed in 30µM 5-HT treated BeWo cells when compared to control (See Figure 5.8, Panel A). The viability of the cells reduced with increasing dose concentration and duration. However no conclusive results could be inferred for 5-HT toxicity to the cells (See Panel B of Figure 5.8). No significant changes were observed with 5-HT treatment in JEG3 cells (See Figure 5.8, Panel C and D).

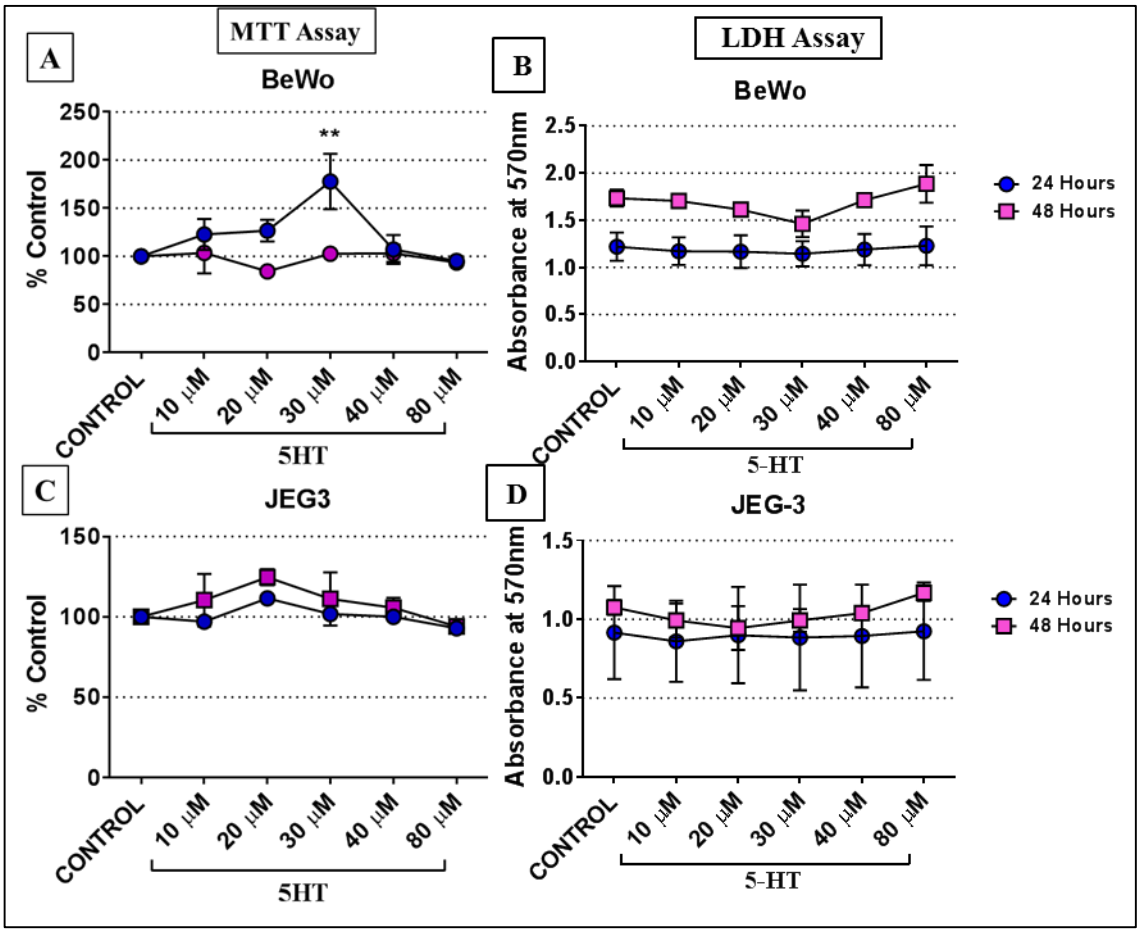


Figure 5.8: Dose-response curve for placental choriocarcinoma cell lines treated with 5-HT.

Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; **, $p<0.01$.

- **Effect of 5-HT on fibrosarcoma cells**

5-HT treatment (20 and 30µM) of fibrosarcoma HT1080 cell line enhanced cell viability at 48 hours (approximately 30%); compared to control (See Figure 5.9, Panel A). However, similar to the placental derived cell lines (stated above), a bell shaped curved was observed in HT1080. From Figure 5.9, Panel B it can be seen that 30 µM 5-HT treated cells showed significantly reduced LDH release when compared to the control.

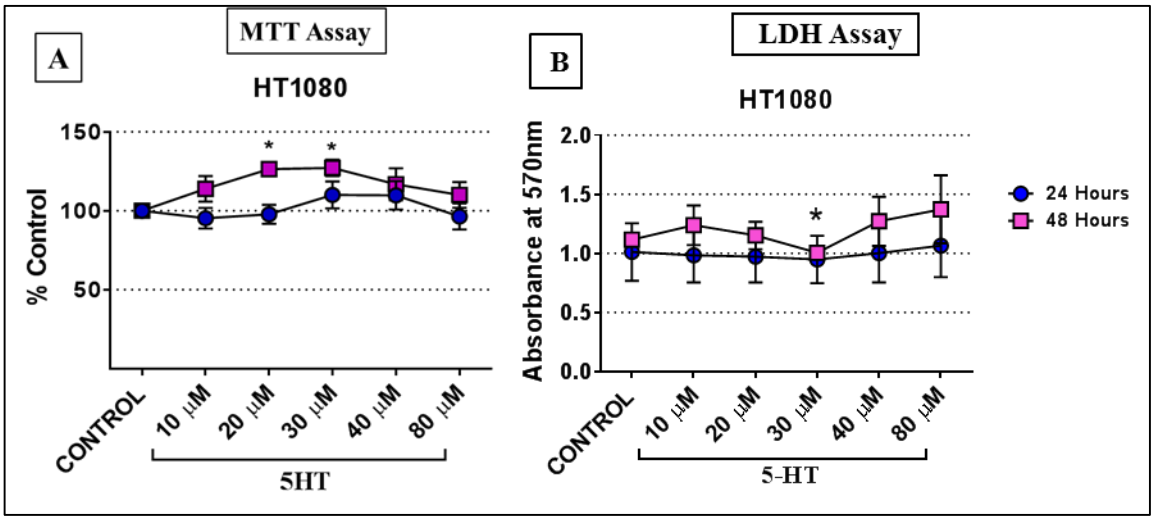


Figure 5.9: Dose-response curve for fibrosarcoma cell line HT1080 treated with 5-HT. Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *, $p<0.05$.

In summary, seeding the cells along with 5-HT improved the viability of the cells. However, higher doses of 5-HT were toxic to the cells and showed increased LDH release. The 20 and 30µM 5-HT were optimal concentrations for further investigations.

- **5-HT treatment after 24 hours of recovery**

The five cell lines used in this study are all adherent cells. Therefore, a trypsinisation step is required before the cells are ready for seeding. This causes stress to the cells and reduces the metabolic activity of the cells. Most cells require 24 hours of recovery from stress to function as viable cells again. Therefore, to investigate the effect of 5-HT on viable cells different doses of 5-HT were added to cells after 24 hours of seeding.

- **Effect of 5-HT on transformed trophoblast cells**

An 82% increase in cell viability was observed in 30µM 5-HT treated TEV-1 cells when compared to control for 24 hours of treatment (See Figure 5.10, Panel A). This increase is higher than cells seeded along with 5-HT. It may be due to more viable cells after 24 hours of seeding. Likewise, for 48 hours of treatment, 30µM 5-HT shows higher viability of cells, although this effect was not significant. No conclusive results could be drawn from LDH assay (See Figure 5.10, Panel B).

In Figure 5.10, Panel C, an increase of 27% and 17% in viability was observed with HT8/SVneo cells treated with 30µM 5-HT for 24 and 48 hours respectively. The HTR8/SVneo cells treated with 5-HT for 24 hours showed lower release of LDH when compared to control (See Figure 5.10, Panel D).

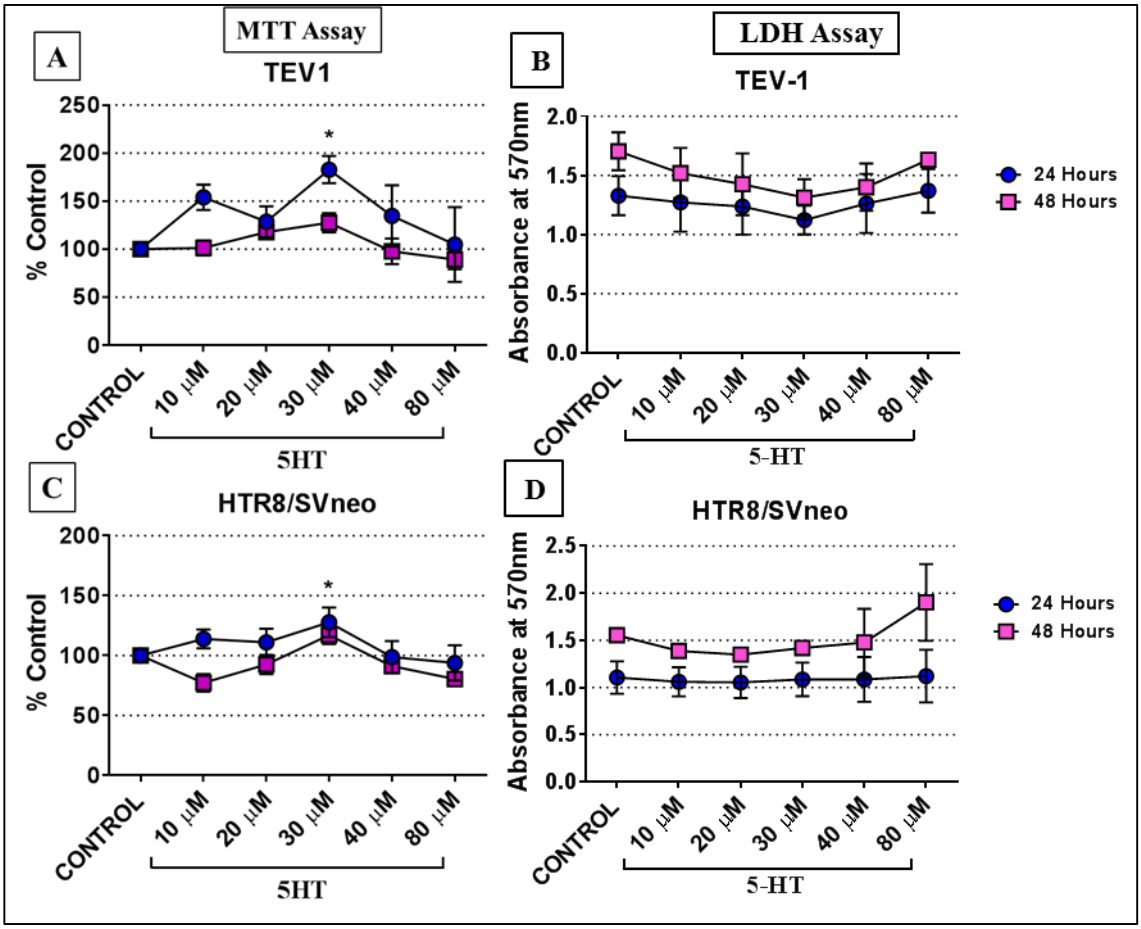


Figure 5.10: Dose-response curve for transformed trophoblast cell lines treated with 5-HT after 24 hours of recovery.

Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *p<0.05.

- **Effect of 5-HT on placental choriocarcinoma cells**

The increase in BeWo cell viability by 20µM 5-HT was 30% and 56% for 24 and 48 hours respectively when compared to control. Moreover, the 48 hours treatment showed higher cell viability when compared to 24 hours. The second choriocarcinoma cell line JEG3 also showed similar pattern of cell viability with 5-HT treatment as represented in Figure 5.11, Panel C. However, only the 24 hour treatment exhibited an increase in cell viability when compared to control. In contrast, at 48 hours of treatment the viability of the cells reduced in comparison to control. No conclusive results were drawn from the LDH assay.

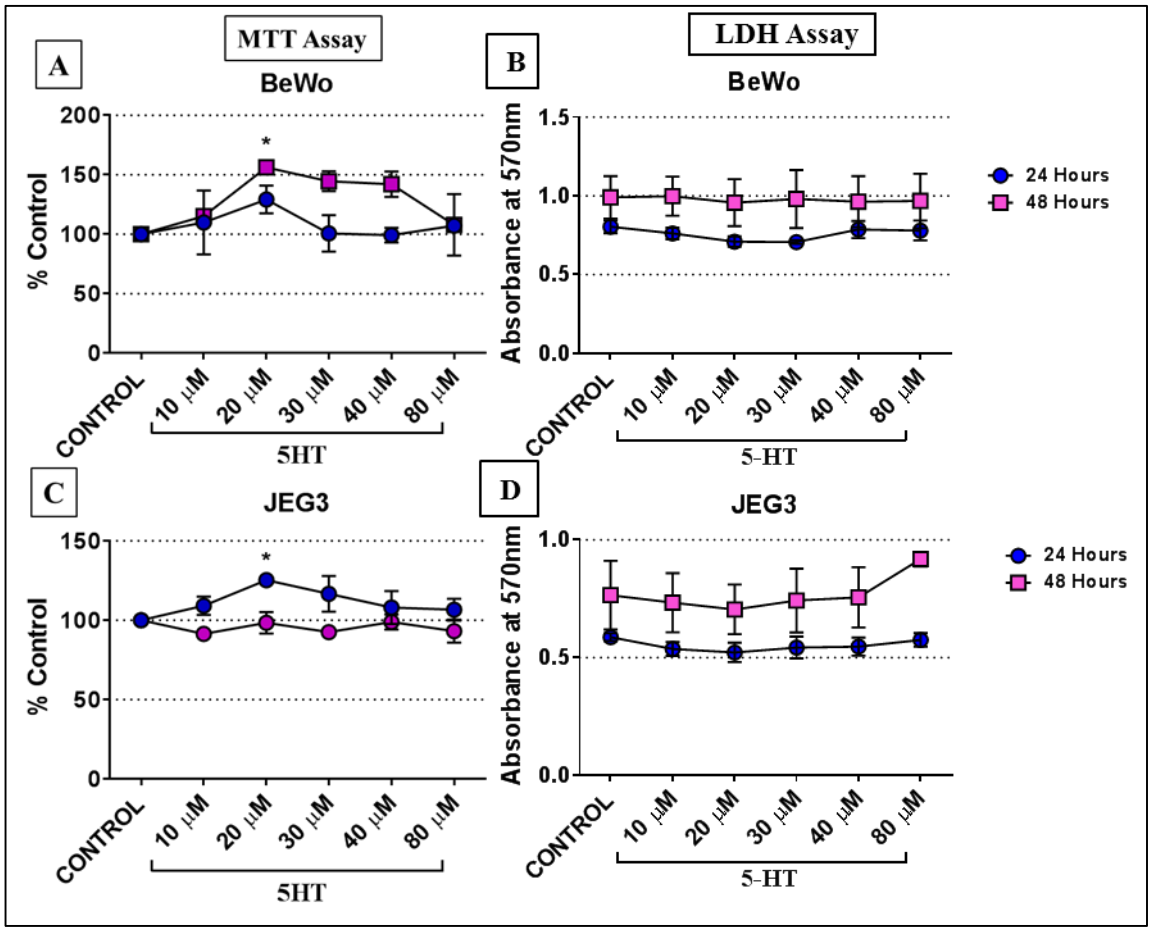


Figure 5.11: Dose-response curve for placental choriocarcinoma cell lines treated with 5-HT after 24 hours of recovery.

Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *, $P<0.05$.

- **Effect of 5-HT on fibrosarcoma cells**

The fibrosarcoma cell line HT1080 treated with 30µM 5-HT exhibited an approximately 30% increase in cell viability at 24 hours treatment (See Figure 5.12, Panel A). The toxicity of 5-HT on HT1080 cells could not be inferred from LDH assay (Refer to Figure 5.12, Panel B). Lower doses of 5-HT were less toxic to the cells leading to lower levels of LDH release.

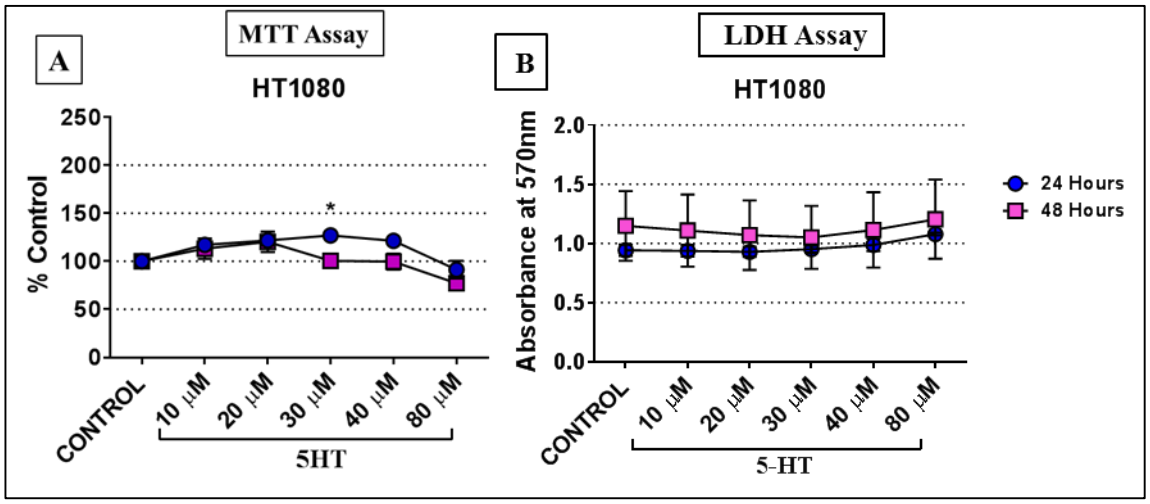


Figure 5.12: Dose-response curve for fibrosarcoma cell line HT1080 treated with 5-HT after 24 hours of recovery.

Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *$p<0.05$.

The effects of 5-HT after 24 hours of seeding were almost similar to the cells seeded along with 5-HT. Therefore, for further investigations, the cells were allowed to recover for 24 hours before treatment with 5-HT.

5.2.2.3 Effect of 5-HT on cell proliferation

In order to prove the data obtained from MTT and LDH assays are due to cell proliferation (not from increased metabolic activity), a tritium labelled (^{3}H) thymidine incorporation method was performed, as explained in Section 2.2.6.7. Furthermore, the effect of 5-HT on cell proliferation recovery were also examined. The cells were treated with 5-Fluoro-Uracil (5-FU) for 24 hours before 5-HT treatment. 5-FU is a pyrimidine analogue with a fluoride atom attached to the base of pyrimidine ring causing nucleotide imbalance and thus blocking DNA synthesis (Section 2.8.7).

- **Effect of 5-HT on transformed trophoblast cell proliferation**

There was no significant effect of 5-HT on TEV-1 proliferation at both 24 and 48 hours treatment (See Figure 5.16, Panel A). Furthermore, 5-HT had no recovery effect on the TEV-1 cells inhibited with 5-FU. On the other hand, in HTR8/SVneo, 5-HT treatment further reduced the proliferation of 5-FU inhibited cells at 24 hours. A significant decrease of 32% was observed in 80µM 5-HT treatment when compared to controls.

Overall, no significant increase in cell proliferation of transformed trophoblast cell lines were observed with 5-HT treatment. Moreover, the proliferation was further reduced in 5-FU inhibited cells confirming no recovery effects with 5-HT.

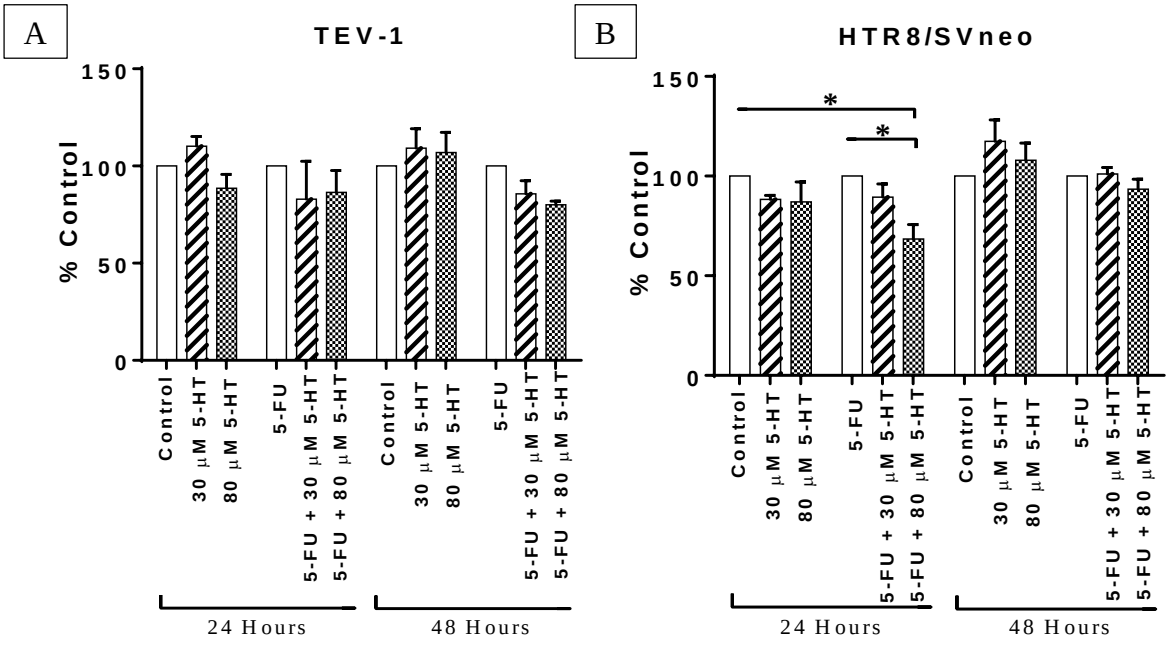


Figure 5.13: ^{3}H incorporation in transformed trophoblast cell lines treated with 5-HT. Statistical analysis was carried out using two-way ANOVA (Tukey's multiple comparisons test). The results are a representation of the mean values for data from a single experiment performed in triplicates ± SEM; *, P<0.05.

- **Effect of 5-HT on placental choriocarcinoma cell proliferation**

5-HT treatment showed a remarkably different pattern in placental choriocarcinoma cell proliferation. As seen from Figure 5.17 (Panel A) the 5-HT treatment had no significant effect on BeWo proliferation at 24 hours incubation. On the contrary, BeWo cells previously treated with 5-FU, when incubated again with 5-HT for 24 hours, showed an increase in proliferation. The 30μM 5-HT showed 25% increase in BeWo proliferation while a significant increase of 80% was observed with 80μM 5-HT for 24 hours ($p<0.05$).

The second choriocarcinoma cell line, JEG3 also showed similar results to BeWo for 5-HT treatment in proliferation (See Figure 5.17, Panel B). Interestingly, a significant increase in cell proliferation (112%) was observed with 80μM 5-HT after 5-FU inhibition. At 48 hours, again there was no significant effect of 5-HT on cell proliferation.

Therefore, for placental choriocarcinoma cell lines 5-HT treatment for 24 hours significantly improved the proliferation of the cells inhibited with 5-FU. These results were contrary to

transformed trophoblast cell lines which showed further decrease in proliferation with 5-HT treatment on 5-FU inhibited cells.

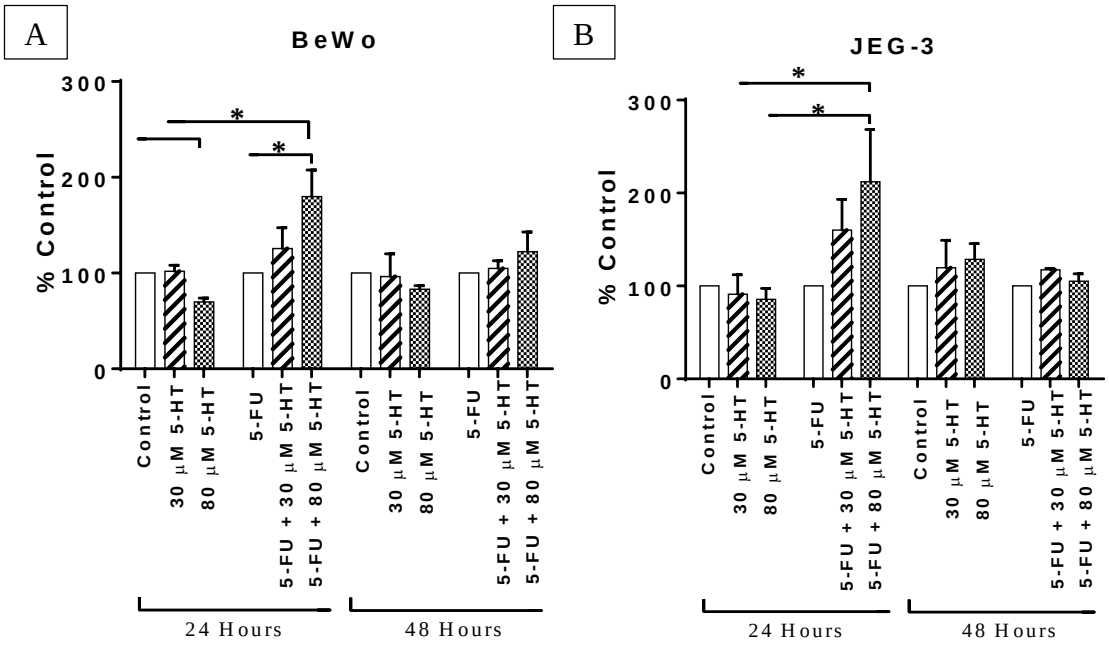


Figure 5.14: ^{3}H incorporation in placental choriocarcinoma cell lines treated with 5-HT.

Statistical analysis was carried out using two-way ANOVA (Tukey's multiple comparisons test). The results are a representation of the mean values for data from a single experiment performed in triplicates ± SEM; *, $P<0.05$.

- **Effect of 5-HT on fibrosarcoma proliferation**

In cell line HT1080, 5-HT showed no significant effect on cell proliferation at 24 as well as 48 hours treatment (See Figure 5.15).

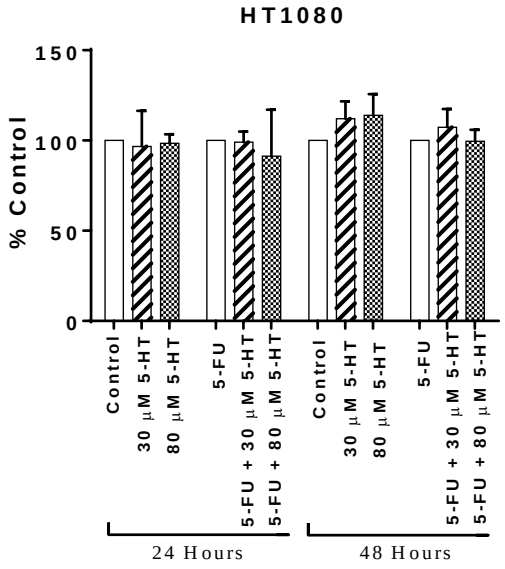


Figure 5.15: ^{3}H incorporation in fibrosarcoma cell line treated with 5-HT.
Statistical analysis was carried out using two-way ANOVA (Tukey's multiple comparisons test). The results are a representation of the mean values for data from a single experiment performed in triplicate ± SEM. Untreated controls are given as unshaded bars in respective time point.

In general, the effect of 5-HT on cell proliferation was cell line dependent. 5-HT increased proliferation of transformed trophoblast cell lines, however it was unable to recover the cells from 5-FU inhibited proliferation. On the other hand, 5-HT reduced the proliferation of placental choriocarcinoma cells, but improved the proliferation of 5-FU treated cells.

5.2.2.4 Effect of 5-HT on cell migration

As mentioned in Section 1.1.1 trophoblast migration and invasion are the key steps in regulating the formation of placenta. Similarly tumour cell migration and its regulation are crucial to control tumour metastasis. This raised interests in investigating the effect of 5-HT on trophoblast and choriocarcinoma cell migration. Therefore, a wound healing assay (often known as scratch assay) was performed due to simplicity and reliability of the method. The scratch assay was performed as described in Section 2.8.9. The WimScratch® image analyser by Wimasis® was used for all the confocal images to calculate the wound closure (%). Due to the differences in scratch area (at time 0) the results are not conclusive with un-aided visual analysis therefore Wimasis® was used. Examples of the summary of cell migration image analysis data (to show how the calculations were made) are given in Figure 5.16. The experiment was performed twice in triplicates each and two-way ANOVA was performed on the data sets. The wound closure (percentage) was used to plot the graphs taking time 0

as 0% closure, thus no migrating cells. Post-hoc Dunnett's test was performed to compare the control to treated cells.

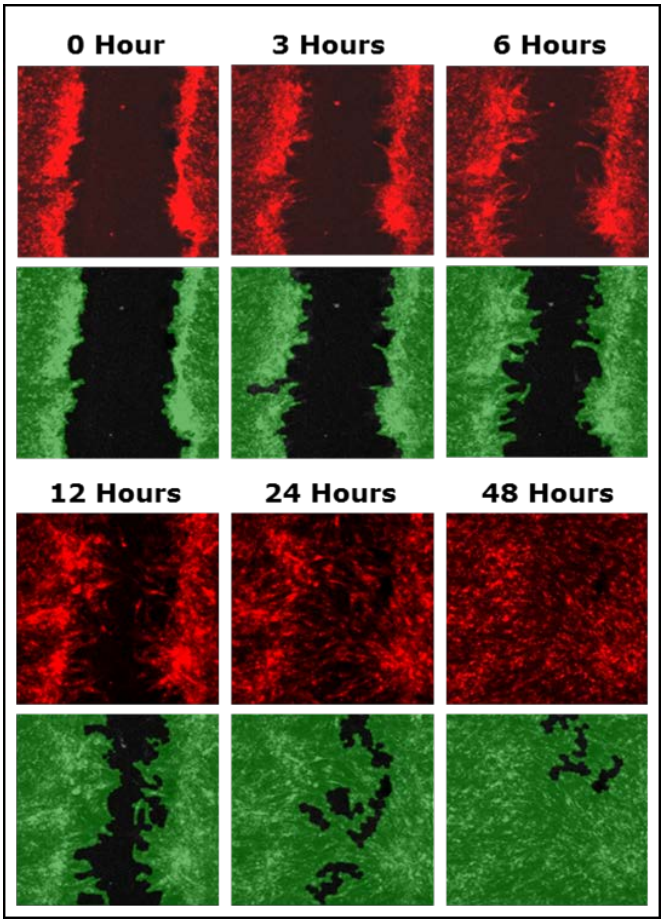


Figure 5.16: Example of Wimasis® quantitation image.

Accurate quantitation of wound healing speed can be achieved using the Wimasis® analyser. **Red**-Represents the cells stained with FM143-FX (Images submitted for analysis). **Green**-Represents the area covered by the cells in Wimasis® program. **Black**- Represents the area of the scratch. *Images taken at 200X magnification.*

- **Effect of 5-HT on transformed trophoblast cell migration**

The percentage of wound closure (along with the wound healing images of time 24 and 48 hours) for transformed trophoblast cells are represented in Figure 5.17. 5-HT treated cells showed significant differences in cell migration compared to control.

In TEV-1 cells, 20µM and 30µM 5-HT treated cells showed higher wound coverage starting from 3 hours (Figure 5.17, Panel A). However, higher concentrations of 5-HT (40µM and 80µM) reduced the wound healing compared to control. At 10µM 5-HT, TEV-1 cells showed similar wound healing rate as control. At 24 hours of incubation it was evident that 20µM and 30µM 5-HT significantly improved the wound closure, whereas 40µM and 80µM significantly reduced it ($p<0.05$). Almost 100% of wound healing was observed at 48 hours with 30µM treatment. This can be clearly seen from the scratch image with total wound

coverage with green compared with other treated and control cells. Thus, the TEV-1 wound coverage progressively increased with time with 20 and 30µM treatment.

HTR8/SVneo cells showed wound healing trend to as TEV-1 (Figure 5.17, Panel B). However in this cell line even 40µM 5-HT improved the wound coverage along with 20µM and 30µM. In fact 40µM 5-HT showed higher rate of wound healing compared to other two doses. Significant enhancement of wound healing was observed with these three doses from 12 hours onwards compared to control. Conversely again 80µM seemed to reduce the wound healing, though it was not statistically significant. Unlike TEV-1, in HTR8/SVneo control cells the healed area at the end of 48 hours was low (indicated by area in the graphs).

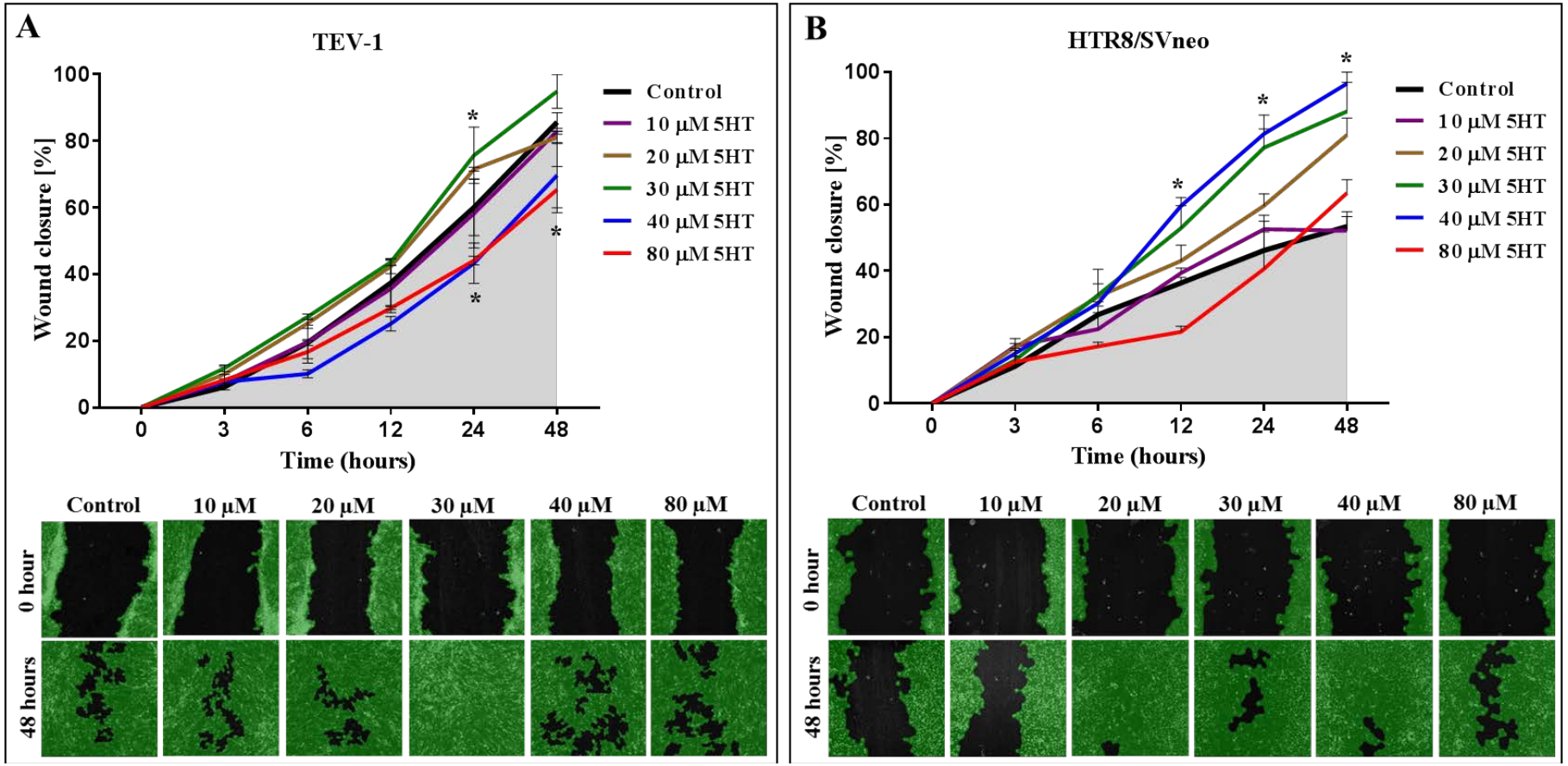


Figure 5.17: Effect of 5-HT on transformed trophoblast cell migration.

Panel A: Represents the wound closure (%) and scratch images at 0 and 48 hours for **TEV-1** cells. Significant differences in wound covered area were observed with 5-HT treated cells when compared to control at 24 and 48 hours (n=2 ±SEM; *p<0.05). Maximum wound coverage observed from the scratch with 30µM 5-HT at 48 hours.

Panel B: Represents the wound closure (%) and scratch images at 0 and 48 hours for **HTR8/SVneo** cells. Significant differences in wound covered area were observed again with 20, 30 and 40µM 5-HT treated cells when compared to control at 24 and 48 hours (n=2 ±SEM; *p<0.05).

The grey shaded area in the graphs represents the wound covered area by control cells. Magnification of images 200X.

- **Effect of 5-HT on placental choriocarcinoma cell migration**

It was challenging to perform scratch assays on choriocarcinoma cell lines due to the syncytial morphology of the cells. The 40µM 5-HT treated BeWo cells showed more wound coverage when compared to control from the very beginning of the assay (Figure 5.18, Panel A). At the end both 30µM and 40µM 5-HT showed significant improvement of wound coverage when compared to control and other 5-HT treated cells ($p<0.05$). While 20µM treatment showed negligible difference to control, both 10µM and 80µM showed reduced wound coverage.

In JEG3 only 20µM 5-HT treatment showed significantly ($p<0.05$) higher wound coverage all throughout the assay when compared to control (Figure 5.18, Panel B). The remaining doses of 5-HT actually reduced the wound coverage with minimum wound coverage observed for 80µM treatment.

The images for the scratch for both the cell lines shows only few cells migrating as clumps into the scratch area. Due to this, only 25% wound healing was observed in these cells even after 48 hours. This was lower when compared to transformed trophoblast cell lines (TEV-1 and HTR8/SVneo). These results for 5-HT effect on placental choriocarcinoma cell lines were not as expected. Since these cell lines originate from cancerous sources it was expected to show higher speed of migration.

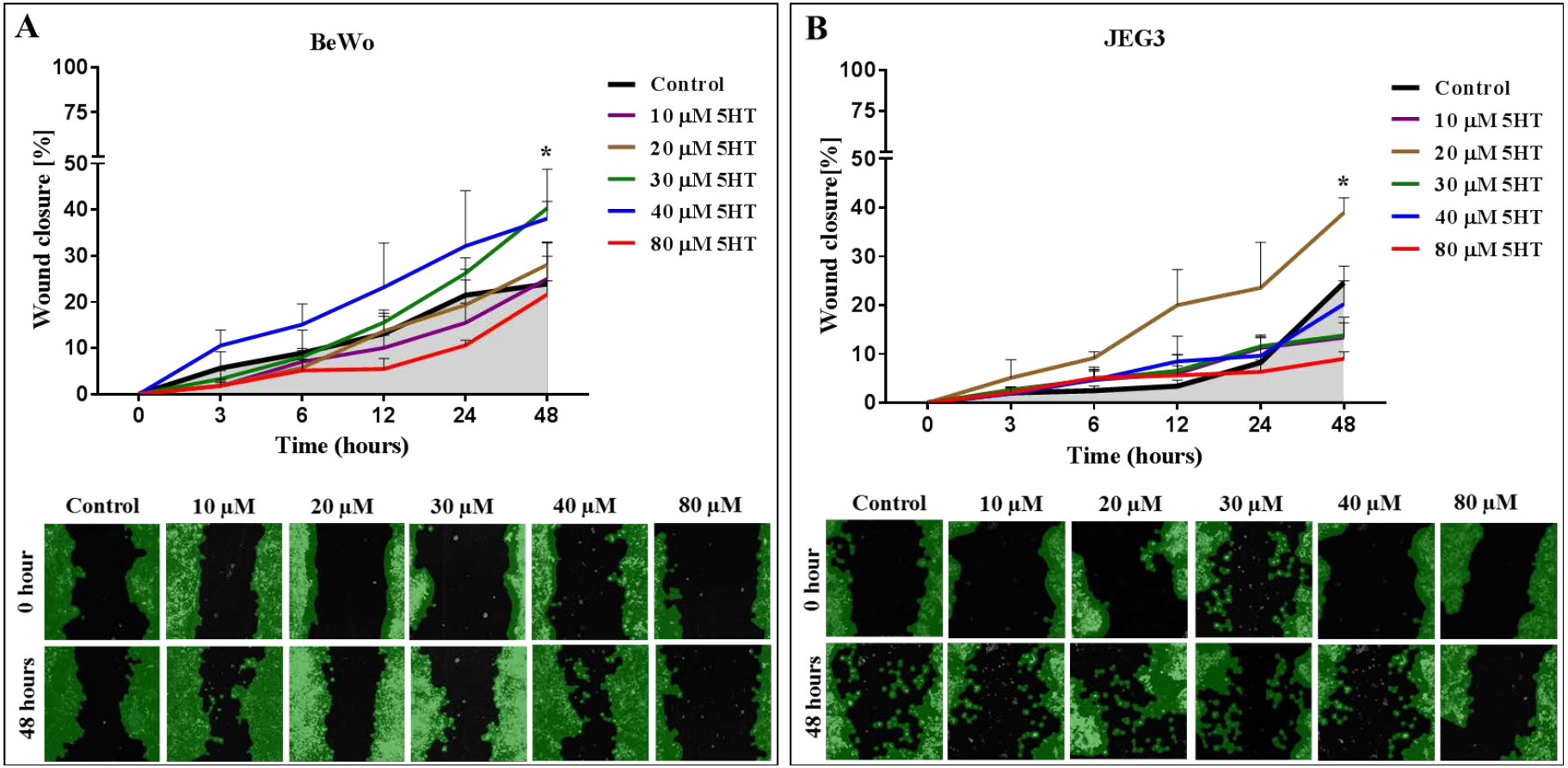


Figure 5.18: Effect of 5-HT on placental choriocarcinoma cell migration.

Panel A: Represents the wound covered area and scratch images at 0 and 48 hours for **BeWo** cells. Significant differences in wound covered area were observed with 30µM and 40µM 5-HT treated cells when compared to control at 48 hours (n=2 ±SEM; *p<0.05).
Panel B: Represents the wound covered area and scratch images at 0 and 48 hours for **JEG3** cells. Significant differences in wound covered area were observed only with 20µM 5-HT treated cells when compared to control at 48 hours (n=2 ±SEM; *p<0.05).
The grey shaded area in the graphs represents the wound *covered area by control cells. Magnification of images 200X.*

➢ Effect of 5-HT on fibrosarcoma cell migration

Since HT1080 is a fibrosarcoma derived cell line, it has been used as a positive control for migration in this study. HT1080 cells show the fastest rate of wound healing compared to the two types of placental cell lines. The scratch area is almost completely covered by these cells before 12 hours of the assay, as a consequence of which, images for 12 hours are shown instead of 48 hours (See Figure 5.19). Interestingly, all the doses of 5-HT improved the rate of wound healing compared to control. Although 80µM 5-HT treatment showed reduced wound coverage until 6 hours, at the end of the assay the wound closure was still higher compared to control. Highly significant differences in wound coverage were observed with 30µM at 12 hours ($p<0.01$). Notice from the graph 10, 20 and 30µM show 100% of wound coverage at 24 hours.

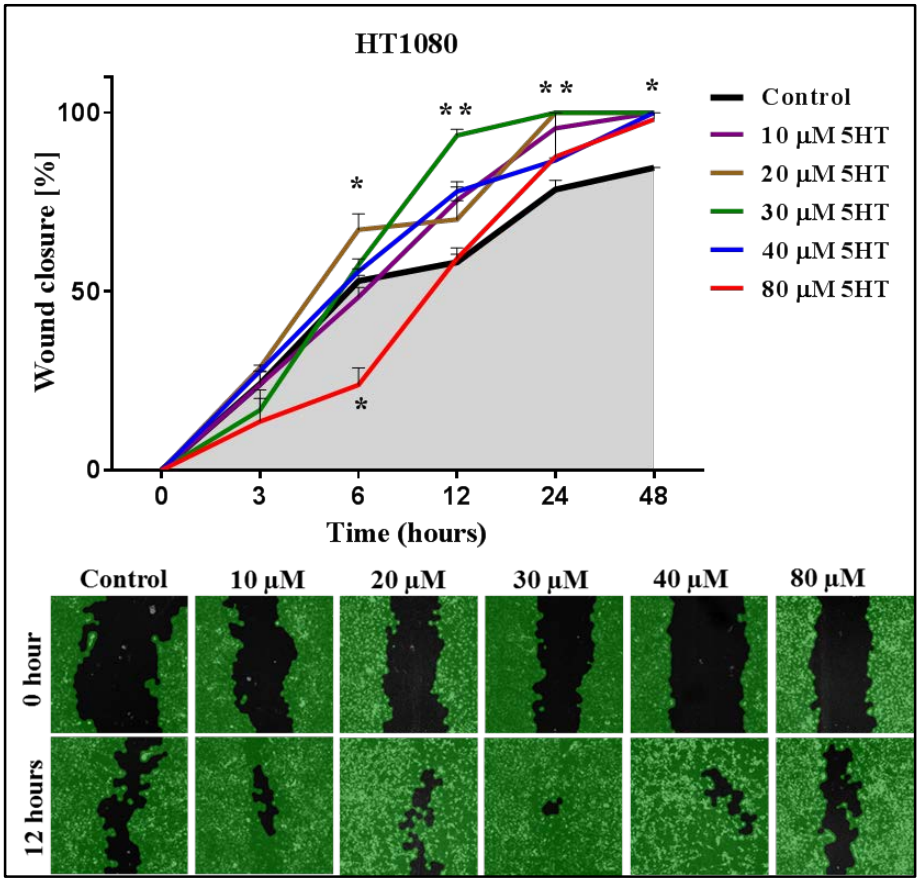


Figure 5.19: Speed of Migration and Cell-covered area in HT1080.

Significant differences in wound coverage were seen from 6 hours onwards (n=2±SEM; *p<0.05, **p<0.01). The wound coverage was approximately 100% even at 12 hours also visible from the scratch images. At 48 hours all 5-HT treated cells show higher wound coverage compared to control. *The grey shaded area in the graphs represents the wound covered area by control cells. Magnification of images 200X.*

5.2.2.5 Effect of 5-HT on cell invasion

The cell migration assay was followed by invasion assay using BD Falcon™ BioCoat tumour invasion plates mentioned in Section 2.8.10**.** Two methods, CTL and Image J, were performed for analysis in order to produce consistent results and to confirm uniform cell seeding at the start of the assay. CTL imager scanned images from the top of the insert wells at beginning of the assay (t=0) and after 48 hours (t=48) of incubation. At t=0 there were equal number of cells seeded into all the wells thus giving similar cell counts. After 48 hours, there were still a few cells seen at the upper chamber of the inserts, but these numbers were maximum in negative control [as there was no chemo-attractant (FBS) at the bottom chambers]. However, the cell numbers decreased in positive (5% v/v FBS in bottom chamber) and 5-HT treated wells (5% v/v FBS plus 5-HT in bottom chambers). Therefore, the negative control was assumed to have maximum number of non-invaded cells (100%).

Image J was also used for the analysis of migrated cells after 48 hours of incubation. It was observed there were high numbers of cells on top of the membrane in negative controls, indicating there were very few cells invaded in these wells. Conversely, in positive and 5-HT treated wells, cells were mostly observed at the lower side of the membrane. The number of invaded cells were compared to positive control (100%) which is assumed to have maximum number of invaded cells.

Therefore, both non-invaded and invaded cell percentages were compared. Variances were analysed using one-way ANOVA and compared against positive control using Dunnett's test.

- **Effect of 5-HT on transformed trophoblast cell invasion**

From Figure 5.20 (Panel A) it can be seen that the TEV-1 cells treated with 20 and 30µM 5-HT showed fewer number of non-invaded cells in the CTL scan, indicating that more cells had invaded through the matrigel. However, this reduction of non-invaded cells was not significant to control (positive). The percentage of non-invaded cells analysed from CTL correlated to the percentage of invaded cells counted by Image J. The positive control showed the least number of non-invaded cells in CTL and 100% of invaded cells in Image J. The percentage of invaded cells in 5-HT treated wells were lower than positive control. In fact, the cells treated with 10, 30 and 40µM 5-HT showed significantly reduced number of invaded cells when compared to positive control. Only 20µM 5-HT treated cells showed similar number of invaded cells as to positive control.

The results from HTR8/SVneo were similar to TEV-1. There was a significant reduction in non-invaded cells in 5-HT treated cells as well as positive control when compared to negative control (See Figure 5.20, Panel B). Furthermore, the reduction was lower in positive controls compared to 5-HT treated cells, indicating that 5-HT treatment did not induce invasion. These results correlated to Image J, where 5-HT treated cells showed reduced number of invaded cells when compared to positive control with significant reductions in 20 and 40µM 5-HT treatment. In summary, the results from invasion assay suggested that 5-HT treatment reduced the invasion of both, TEV-1 and HTR8/SVneo, transformed trophoblast cell lines.

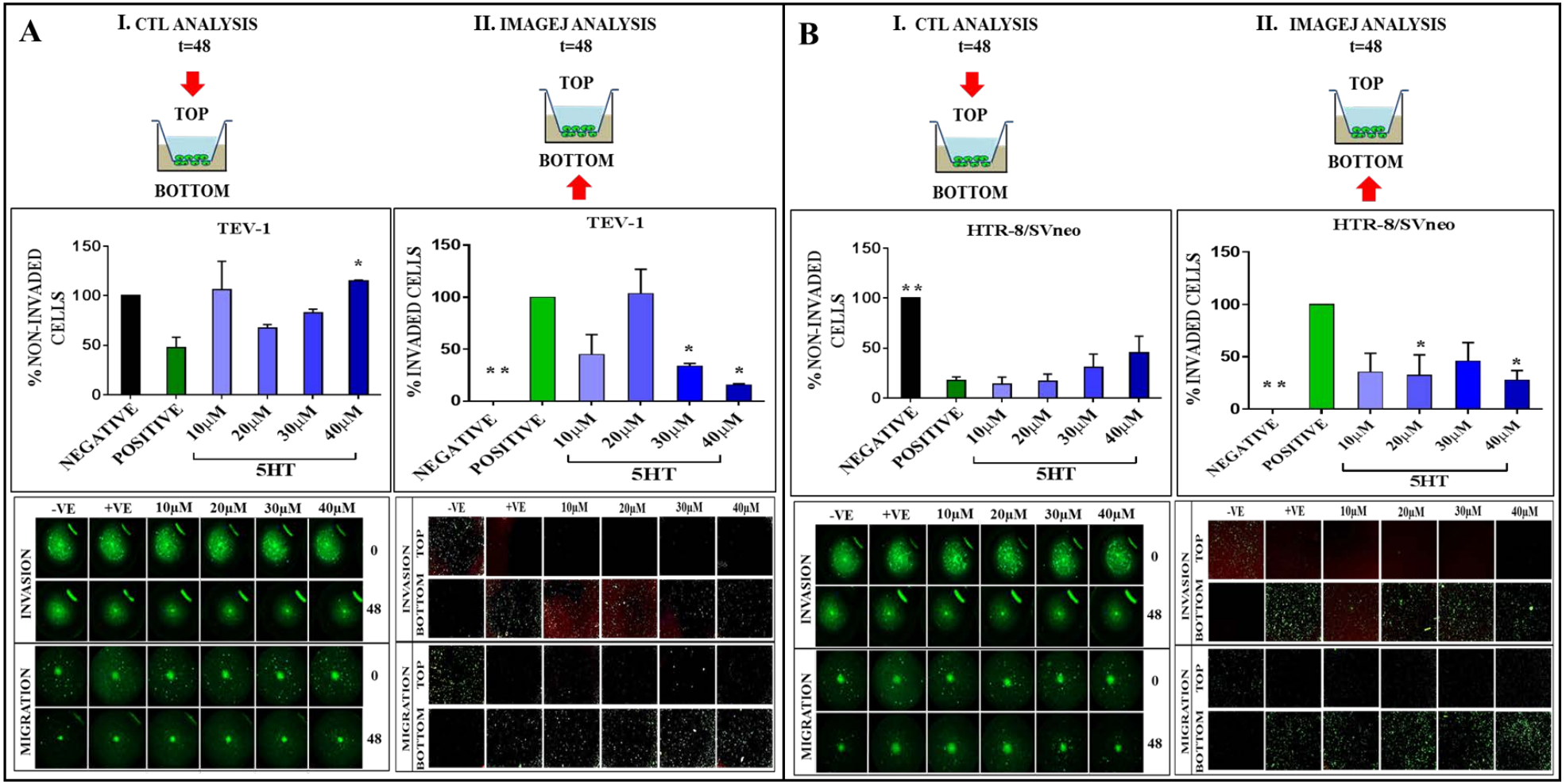


Figure 5.20: Effect of 5-HT on transformed trophoblast cell invasion.

There was a correlation between CTL (**I**) and Image J (**II**) analysis.

Panel A: Represents the invasion of **TEV-1** cells with 5-HT treatment. **I**. There was high percentage of non-invaded cells seen in 10 and 30 µM 5-HT. More number of non-invaded cells were seen in CTL scan for negative at 48 hours. **II**. The 30µM and 40µM 5-HT treatment significantly reduced the % invaded cells (n=2±SEM; **p<0.01; *p<0.05). More invaded cells were seen at bottom of membrane in positive.

Panel B: Represents the invasion of **HTR8/SVneo** cells with 5-HT treatment. **I**. The 40µM 5-HT showed the highest % non-invaded cells when compared to positive and other 5-HT treated cells. **II**. Significant reduction in % invaded cells were observed in 20 and 40µM 5-HT when compared to positive (n=2±SEM; **p<0.01; *p<0.05). More invaded cells were seen on bottom of membrane in positive and 30µM.

- **Effect of 5-HT on placental choriocarcinoma cell invasion**

The effect of 5-HT on cell invasion was also investigated in choriocarcinoma cell lines, JEG3 and BeWo. As seen in Figure 5.21 (Panel A), there was a significant reduction in non-invaded BeWo cells in 30µM 5-HT treatment when compared to control (positive), thereby suggesting that 5-HT induced BeWo cell invasion. Remarkably this was also confirmed by Image J analysis which showed maximum number of cells invaded in 30µM 5-HT treated cells when compared to positive (although not significant).

In JEG3, there was significant reduction of non-invaded cells treated with 5-HT and in positive when compared to negative in CTL scans (See Figure 5.21, Panel B). The CTL results were interestingly correlated by Image J, showing a significant increase in JEG3 invasion treated with 20µM 5-HT, and decrease at 40µM 5-HT, when compared to positive.

In contrast to transformed trophoblast cell lines (TEV-1 and HTR8/SVneo), the placental choriocarcinoma cells (BeWo and JEG3) showed increased cell invasion under the influence of 5-HT.

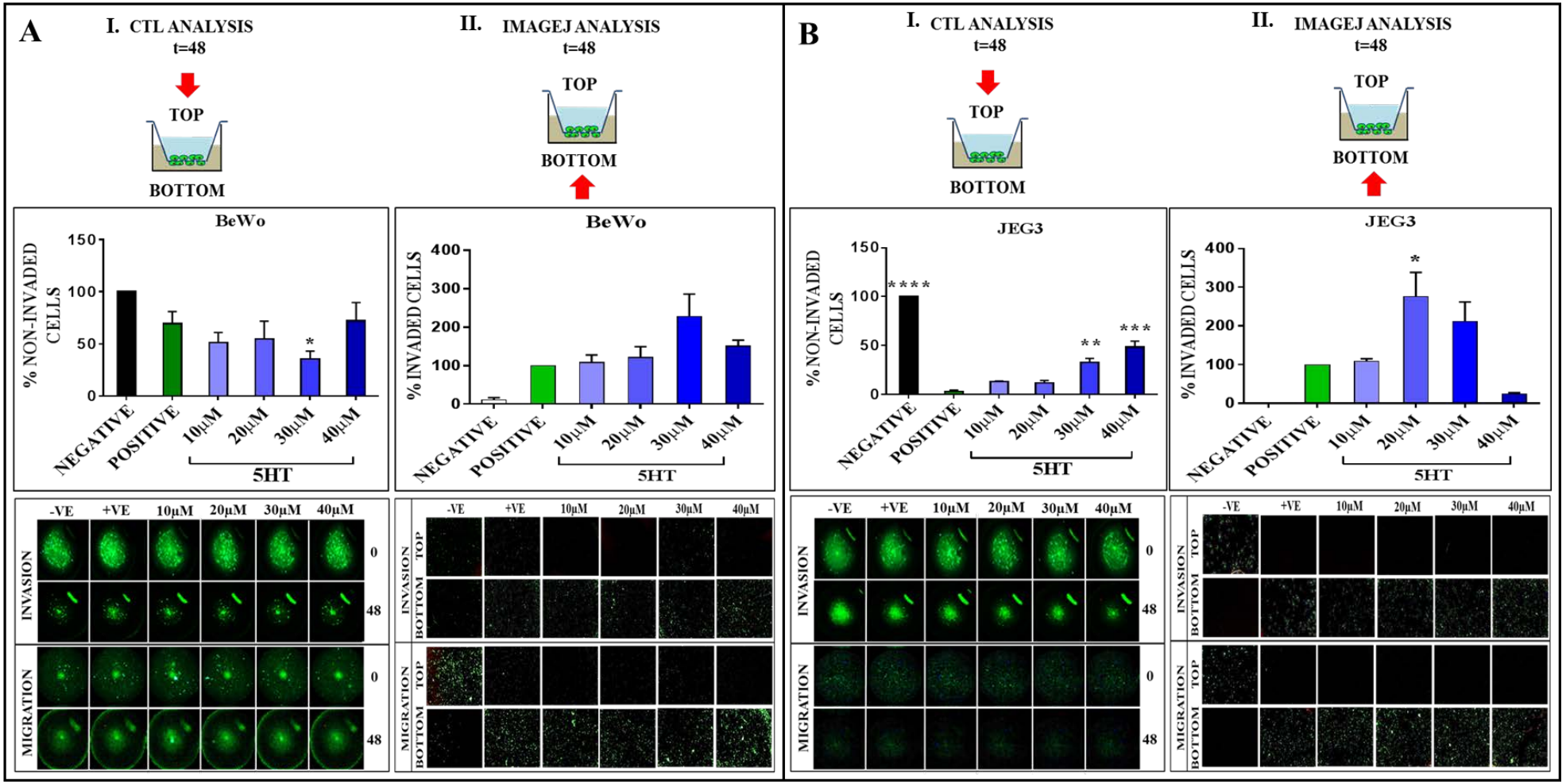


Figure 5.21: Effect of 5-HT on placental choriocarcinoma cell invasion.

Panel A: Represents the invasion of **BeWo** cells with 5-HT treatment. **I**. There was significant reduction of % non-invaded cells seen in 30 µM 5-HT (n=2±SEM; *p<0.05). Fewer cells were seen in CTL scan indicating cell have invaded. **II**. The 30µM 5-HT treatment increased the % invaded cells. More invaded cells were seen at bottom of membrane in 30 and 40µM.

Panel B: Represents the invasion of **JEG3** cells with 5-HT treatment. **I**. The 30µM and 40µM 5-HT showed the highest % non-invaded cells when compared to positive and other 5-HT treated cells. **II**. Interestingly 20 and 30µM 5-HT showed increased % of invaded cells compared to control (positive) (n=2±SEM; **p<0.01; *p<0.05).

- **Effect of 5-HT on fibrosarcoma cell invasion**

The differences in invasion of HT1080 cells treated with 5-HT and the control were not statistically significant (See Figure 5.22). However, all 5-HT treated cells showed lower cell numbers in CTL (non-invaded) and higher numbers in Image J (invaded) compared to control (positive). The 40µM 5-HT exhibited an almost 100 fold increase to control.

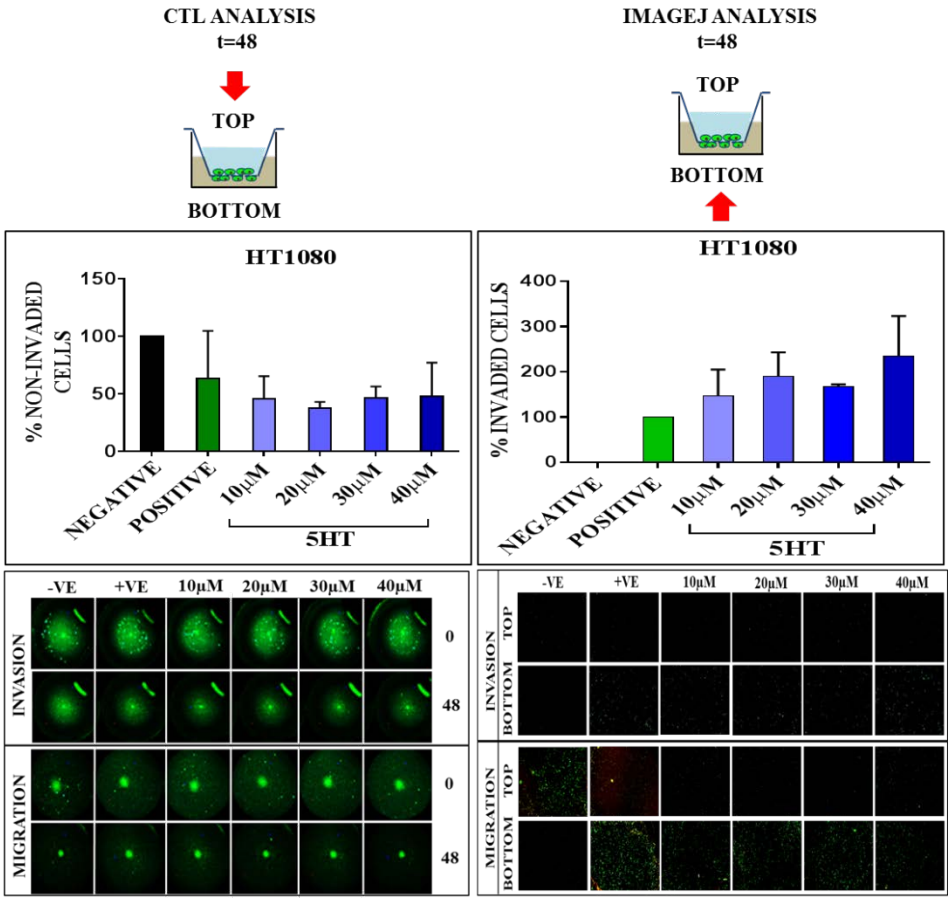


Figure 5.22: HT1080 invasion under the influence of 5-HT.
All the 5-HT treated cells showed a non-significant increase cell invasion in HT1080 (n=2±SEM).

Furthermore, HT1080 was used as a positive control to compare the invasion in other cell lines, as recommended by the manufacturer. The invasion in transformed trophoblast cell lines (TEV-1 and HTR8/SVneo) was reduced by 5-HT and was lower when the invasion by HT1080. On the other hand, the 5-HT treatment significantly increased the invasion in placental choriocarcinoma cells (BeWo and JEG3) and the percentage of cell invasion was similar (BeWo) or higher (JEG3) to that of HT080. 5-HT treatment induced the invasion of BeWo and JEG3 cells but reduced the invasion of TEV-1 and HTR8/SVneo cells. Overall the effect of 5-HT on cell invasion was cell line dependent.

5.2.3 Effect of 5-HT in *in vitro* under hypoxic conditions

The 5-HT acts as a regulator to maintain the homeostasis of the epithelial layer of many organs e.g. breast, liver, prostate. Therefore, the effects of 5-HT on the cells were analysed under hypoxic conditions in order to determine if the effects were still prevalent under hypoxic conditions. Furthermore, the first trimester trophoblast cells are more resistant to hypoxic conditions, as has been reported previously (Esterman *et al*, 1997). Additionally, due to increased level of 5-HT found in PE, higher 5-HT doses (20-100μM) were used for hypoxic investigations compared to normoxia (10-80μM) in order to better mimic the PE conditions. In order to investigate this, the cells were first grown in normoxic conditions (20% v/v O_2) and trypsinised when confluent (See Section 2.8.1). The trypsinised cells were then transferred to hypoxic conditions with 2% v/v O_2 and maintained for further investigations.

5.2.3.1 Morphological changes due to 5-HT treatment under hypoxia

The cells were grown under hypoxic conditions for 24 hours and then treated with 5-HT. The H&E staining was then performed to observe the morphological changes in these cells. The 5-HT treated cells showed more elongated extensions when compared to the control cells. However, the cell number per field have reduced at very high doses of 80μM and 100μM. Moreover, at these concentrations higher numbers of apoptotic cells with blebs and necrotic cells were observed. It is known that 5-HT induces the migration of these cell lines in normoxic condition, as seen from Section 5.2.2.4. Therefore, the extended features of the cells under hypoxic condition could be due to effect of 5-HT on migration. However, features like membrane ruffling and filopodium could not be distinctly identified. Please refer to Figures 5.23 to 5.25 for further details on individual cell lines.

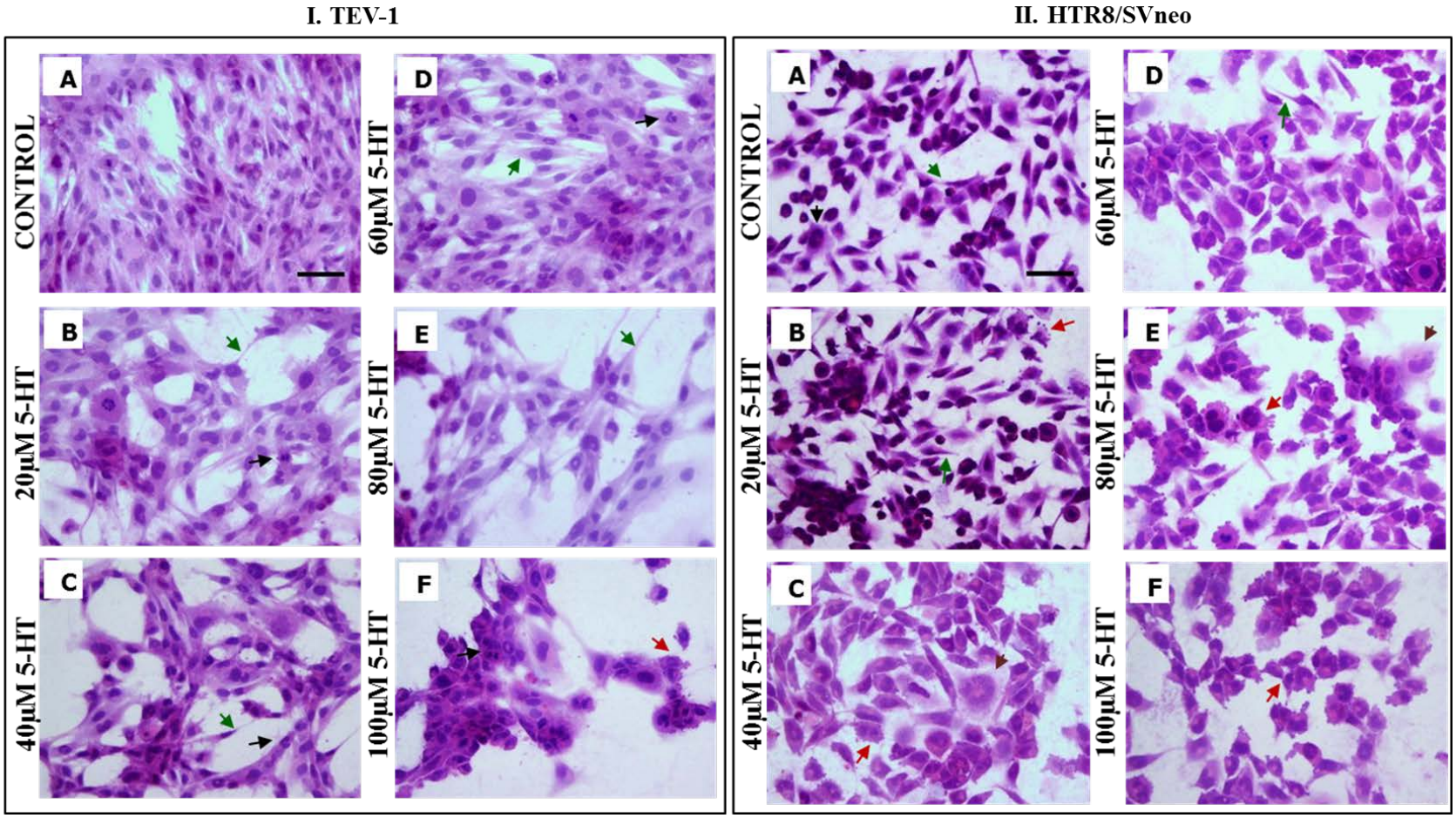


Figure 5.23: H & E staining for 24 hours effect of 5-HT on transformed trophoblast cells under hypoxia.

Section I. and II. Shows images for **TEV-1** and **HTR8/SVneo** cells respectively. **Panel A** represents control cells and **Panels B-F** represents 5-HT treated cells (20-100µM 5-HT respectively).

Long extensions from the cells were seen (green arrows in Panels B to E). The mitotic cells undergoing division are indicated by black arrows. Apoptotic cells with clear blebbing were observed in most of the 5-HT treated cells (red arrows); also visible at lower doses of 20 and 40µM 5-HT in HTR8/SVneo (II. Panels B and C). The apoptotic cell numbers increased in 80µM and 100µM 5-HT treatment. Necrotic cells with faint structures were seen only in HTR8/SVneo (II. Panels C and E; brown arrows). *Scale bar= 100µm. Images taken at 200X magnification.*

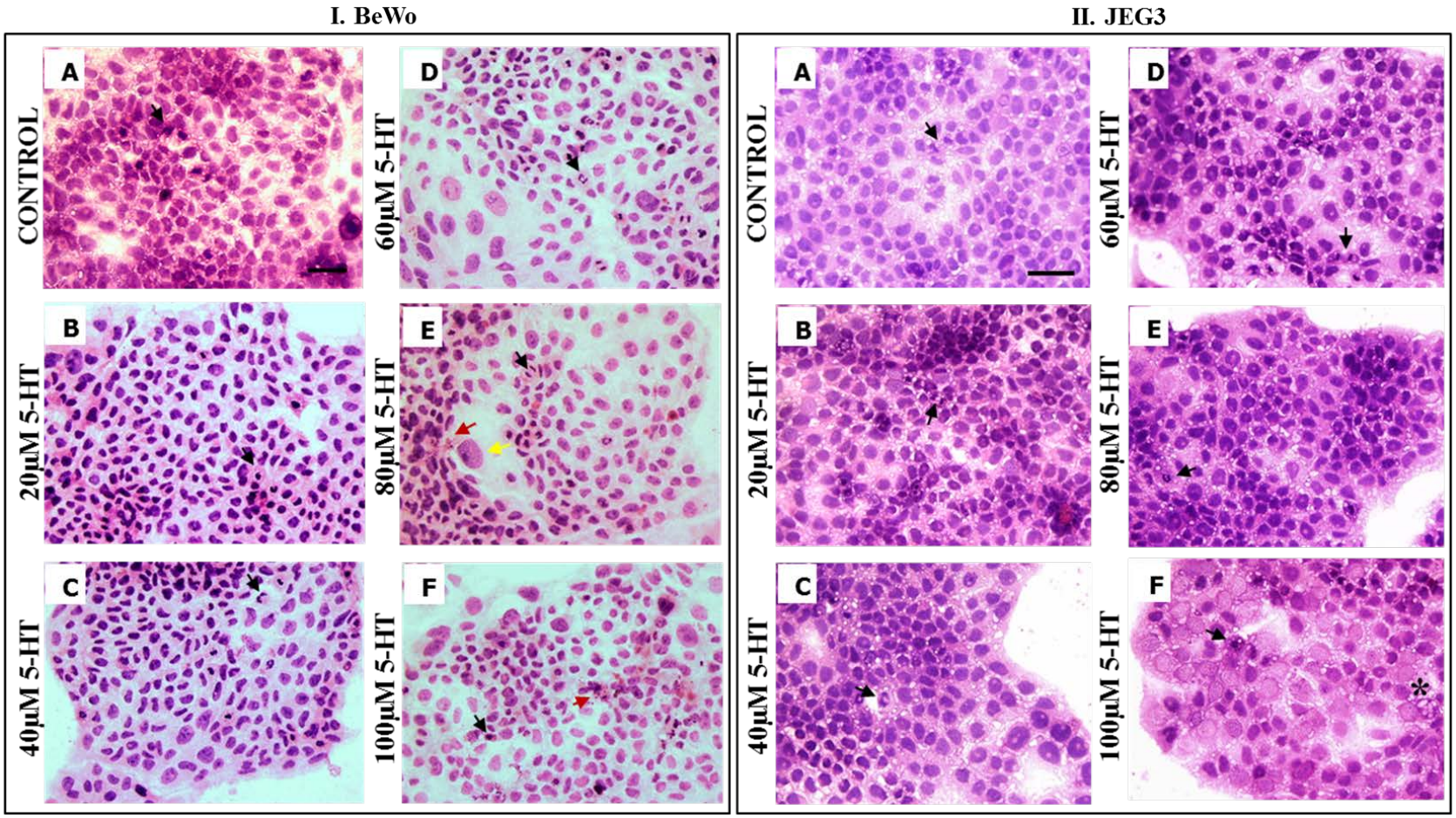


Figure 5.24: H & E staining for 24 hours effect of 5-HT on placental choriocarcinoma cells under hypoxia.

Section I. and II. Shows images for **BeWo** and **JEG3** cells respectively. **Panel A** represents control cells and **Panels B-F** represents 5-HT treated cells (20-100µM 5-HT respectively).

Cells undergoing mitotic divisions during anaphase with chromosomal division were observed **(**black arrows). Cell debris after apoptosis of the cells were frequently observed in BeWo cells treated with 80µM and 100µM 5-HT (represented with red arrows in Panel E and F). Also there were large giant anaplastic cells observed at higher concentrations of 5-HT represented with yellow arrows in Panel E, Section I. However, these morphological features were difficult to be identified in JEG3 cell line. Nonetheless, in 100µM 5-HT treated JEG3 peculiar distortion in the cell chromosome was seen (✻; Panel F). This may be due to the combined effect of hypoxia and high dose of 100µM treatment. *Scale bar= 100µm. Images taken at 200X magnification.*

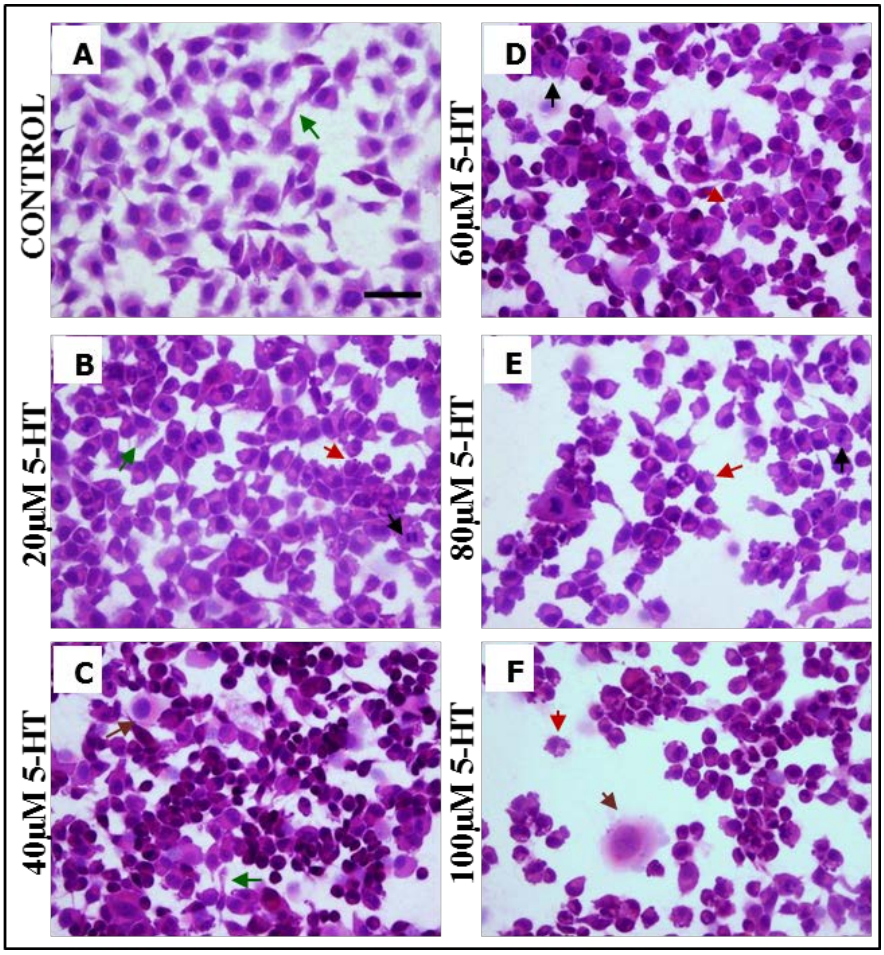


Figure 5.25: H & E staining for 24 hours effect of 5-HT on HT1080 under hypoxia.

Panel A represents control cells and **Panels B-F** represents 5-HT treated cells (20-100µM 5-HT respectively). Extended features like lamellipodium indicated with green arrows were observed in viable cells in control, 20 and 40µM 5-HT treatment (Panel A, B and C respectively). Mitotic cells with condensed chromatin division are indicated with black arrows. These features were reduced in higher doses like 60, 80 and 100µM and replaced with increased number of apoptotic cells with blebs (red arrows) and necrotic cells (brown arrow) (Panel D, E and F respectively). *Scale bar= 100µm. Images taken at 200X magnification.*

5.2.3.2 Effect of 5-HT on cell viability and toxicity under hypoxia

The cell-cycle can be affected by environmental changes, particularly changes in the level of oxygen supply (Alarcon *et al*, 2004). It has been proven that the cell cycle progression is hindered during hypoxic conditions. Hence, the MTT and LDH assay were performed to investigate the effects of hypoxic in cell viability and toxicity, and to understand the role of 5-HT during these changes.

- **Plating cells along with 5-HT**

The cells were seeded into plates with different doses of 5-HT and incubated in hypoxic chambers for 24 or 48 hours.

- **Plating transformed trophoblast cells along with 5-HT**

When TEV-1 cells were treated with 5-HT under hypoxic conditions it was observed that the cell viability increased significantly by approximately 25% with 60µM 5-HT treatment upon 24 and 48 hours incubation when compared to control (no treatment) (Refer to Figure 5.26.A). There was no significant effect of higher doses of 5-HT (80 and 100µM) on TEV-1. Moreover, no conclusive results were obtained from LDH assay on 5-HT toxicity in this cell line.

There was no significant difference in viability of 5-HT treated HTR8/SVneo cells compared to control (Refer to Figure 5.26.C). The LDH results confirmed the MTT findings concluding the 40 and 60µM 5-HT was least toxic to the cells when compared to other doses and control (See Figure 5.26.D).

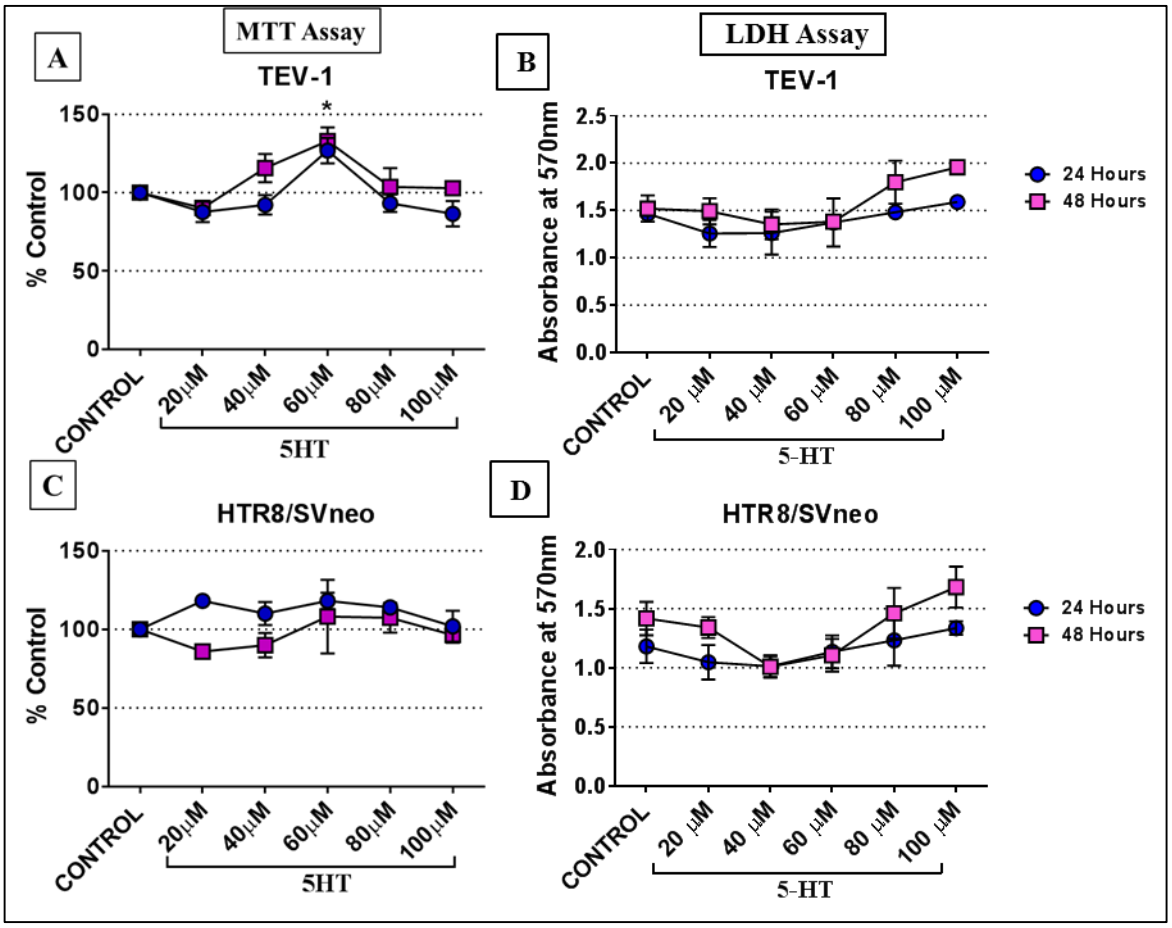


Figure 5.26: Dose-response curve for transformed trophoblast cell lines seeded with 5-HT under hypoxic conditions.

Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *, $p<0.05$.

- **Plating placental choriocarcinoma cells along with 5-HT**

Incubation of BeWo cells with 5-HT for 24 hours showed a significant decrease of approximately 16% in cell viability with 80µM 5-HT when compared to control (Refer to Figure 5.27.A). The same cell line revealed significant decrease in viability with 20µM (15% decrease) and 100µM (approximately 20% decrease) 5-HT treatment for 48 hours. There was no significant differences observed in cell toxicity at 24 and 48 hours for BeWo cells treated with 5-HT (See Figure 5.27.B). Once again 5-HT had no significant effect on JEG3 metabolic activity and toxicity (See Figure 5.30.C and D).

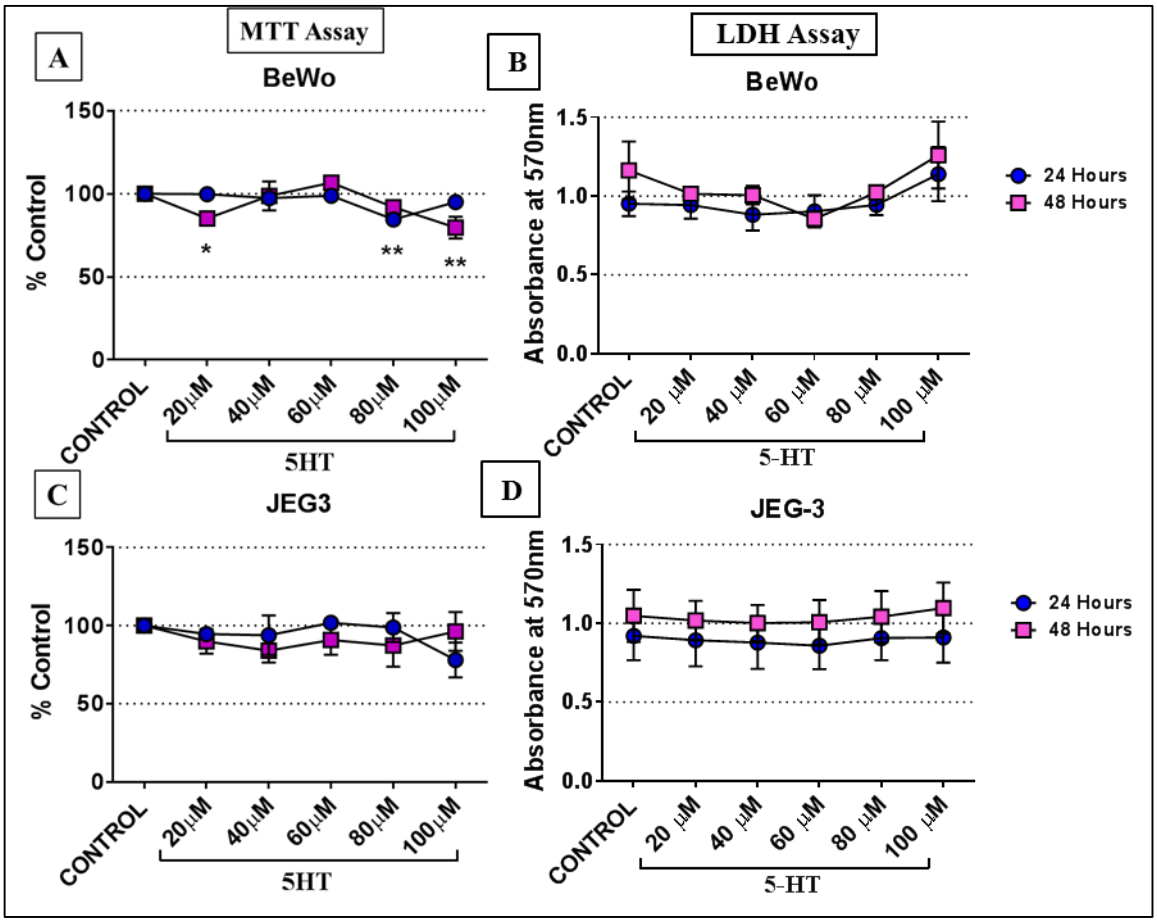


Figure 5.27: Dose-response curve for placental choriocarcinoma cell lines seeded with 5-HT under hypoxic condition.

Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *, $p<0.05$; **, $p<0.01$.

- **Plating fibrosarcoma cells along with 5-HT**

In 5-HT treated HT1080, a peak at 40-60µM was observed for metabolic activity (Refer to Figure 5.28.A). However, all the 5-HT treated cells showed increase in viability when compared to control. The 60µM 5-HT treated cells showed the maximum viability of approximately two fold (183%) during 24 hours treatment. The viability, however, decreases with the increase in 5-HT dose with the minimum at 100µM 5-HT. When the cells were treated for 48 hours, 40µM 5-HT treatment increased the viability of the cells by 88% when compared to control. However, no conclusive results could be drawn from the LDH results (See Figure 5.28.B). Therefore, the toxicity of the 5-HT on the HT1080 cells could not be estimated.

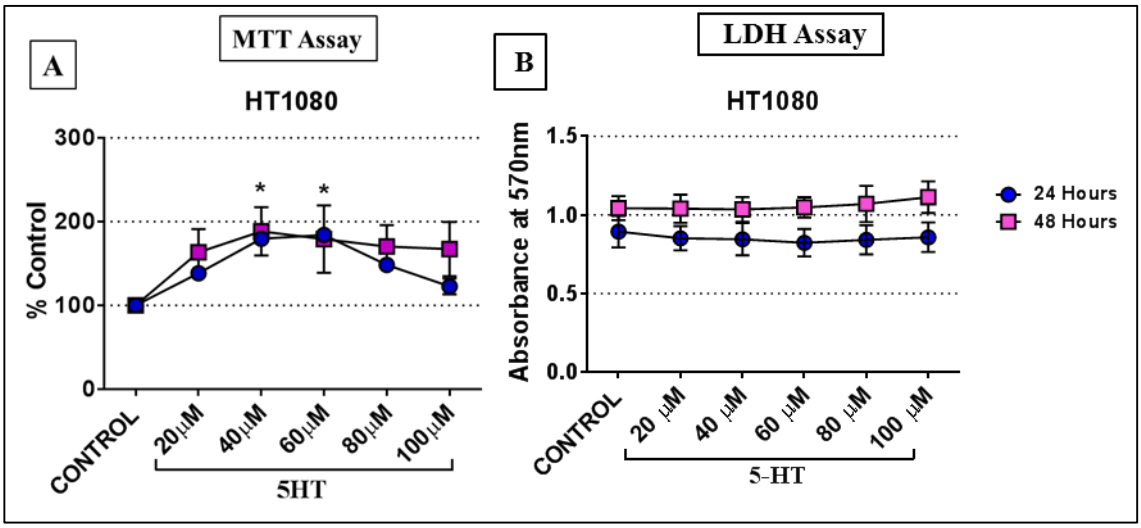


Figure 5.28: Dose-response curve for fibrosarcoma cell line HT1080 seeded with 5-HT. Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *, $p<0.05$.

Overall, it could be concluded that 40-60µM 5-HT treatment was ideal for all the cell lines under hypoxic conditions.

- **5-HT treatment after 24 hours of recovery**

The cells that were grown under normoxic conditions (20% v/v O_2) were plated and then incubated in hypoxic chambers (2% v/v O_2) for 24 hours before treatment with 5-HT. Thus, the cells would be pre-acclimatised to hypoxic conditions.

- **Effect of 5-HT on transformed trophoblast cells**

TEV-1 cells treated with 5-HT showed an increase of 20% cell viability at 60µM treatment, however at higher concentrations the viability reduced when compared to control (Refer to Figure 5.29.A). The viability of TEV-1 cells was further increased when treated with 60µM 5-HT for 48 hours under hypoxic incubation (approximately 60% more viable cells than the control). The LDH results confirmed the MTT results, but were not statistically significant (See Figure 5.29.B).

Although the second transformed trophoblast cell line, HTR8/SVneo, exhibited an increase in cell percentage viability with 5-HT treatment, this effect was not significant (See Figure 5.29.C). Nonetheless, the LDH results was analysed for the effects of 5-HT as seen in Figure 5.29.D and not significant changes were observed.

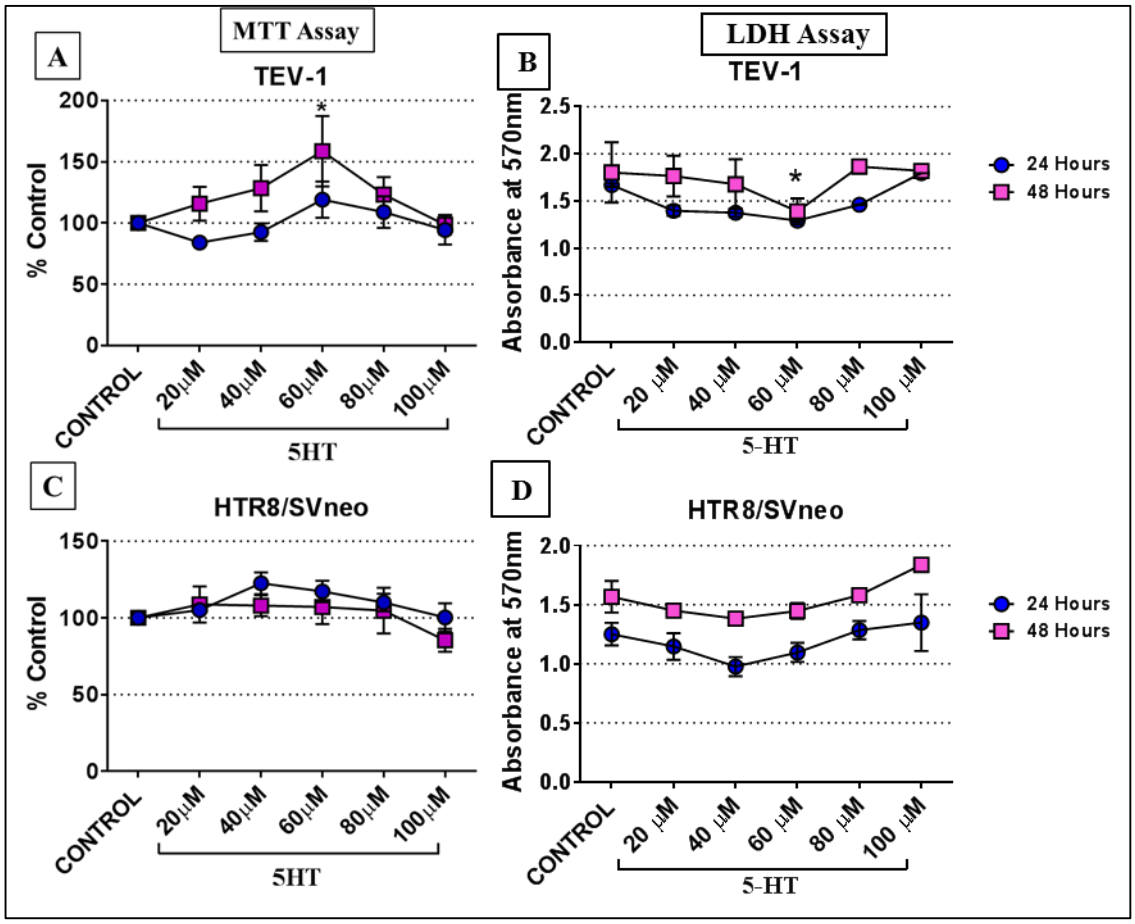


Figure 5.29: Dose-response curve for transformed trophoblast cell lines treated with 5-HT after 24 hours under hypoxic condition.

Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *, $p<0.05$.

- **Effect of 5-HT on placental choriocarcinoma cells**

The effect of 5-HT on BeWo cells is given in Figure 5.30.A. There were no significant change in cell viability with 5-HT. The LDH results indicated there was increase in toxicity when the cells were treated with 5-HT for 48 hours when compared to control (See Figure 5.30.B). The 100µM 5-HT treatment showed significantly high release of LDH when compared to control showing high toxicity to the cells.

In the second choriocarcinoma cell line, JEG3, there was an increase of approximately 25% in cell viability with 60µM 5-HT treatment for 24 and 48 hours when compared to control (See Figure 5.30.C). There were very little differences in results for 24 hours and 48 hours of incubation. Increased release of LDH was indicated in 80-100µM 5-HT treated cells for 24 hours (See Figure 5.30.D). The LDH results are consistent with the MTT results.

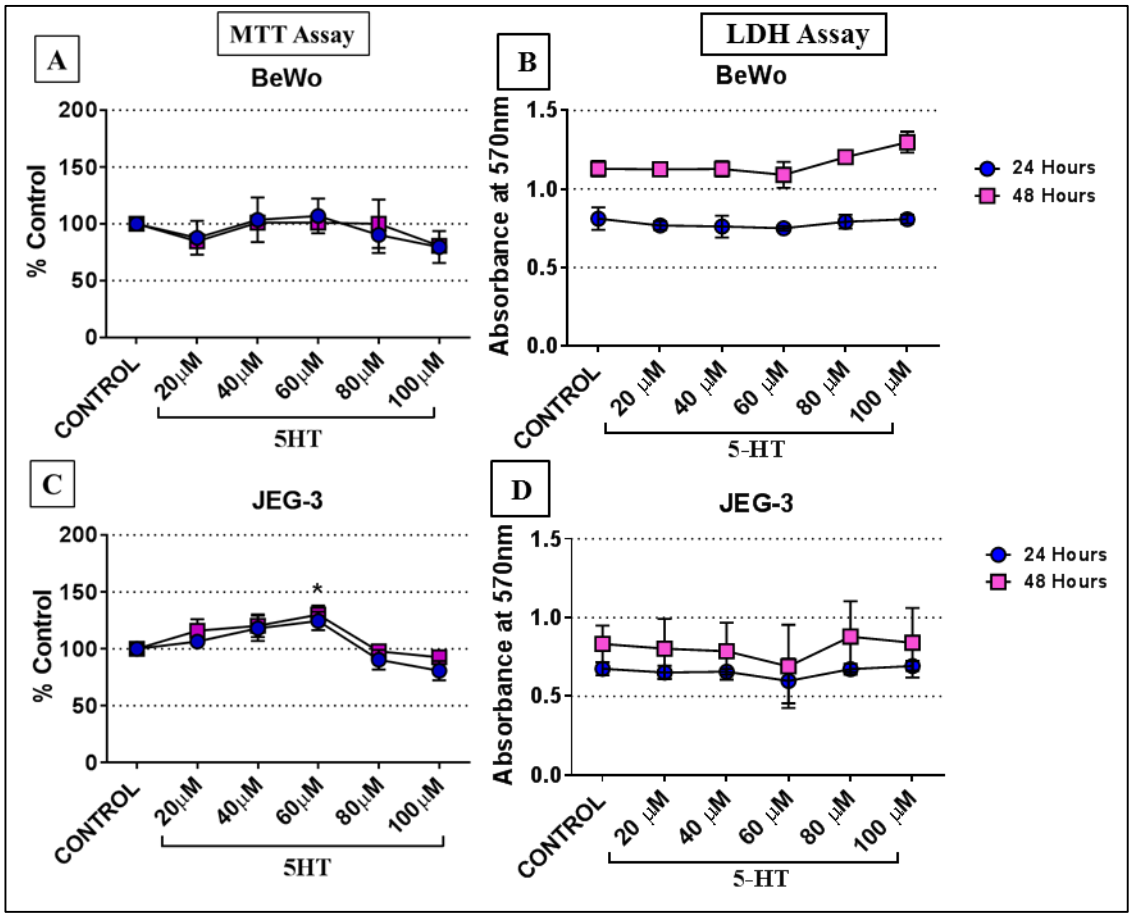


Figure 5.30: Dose-response curve for placental choriocarcinoma cell lines treated with 5-HT after 24 hours of hypoxia.

Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *, $p<0.05$.

- **Effect of 5-HT on fibrosarcoma cells**

HT1080 cells treated after 24 hours of recovery showed an increase in cell viability as shown in Figure 5.31.A. The cells treated with 60µM 5-HT for 24 hours and 48 hours showed an increase of approximately 33% and 21% respectively when compared to control. The viability reduced with increasing concentration of 5-HT. The LDH released from the cells reciprocally corresponded to the MTT assay, but were not statistically significant (See Figure 5.31.B).

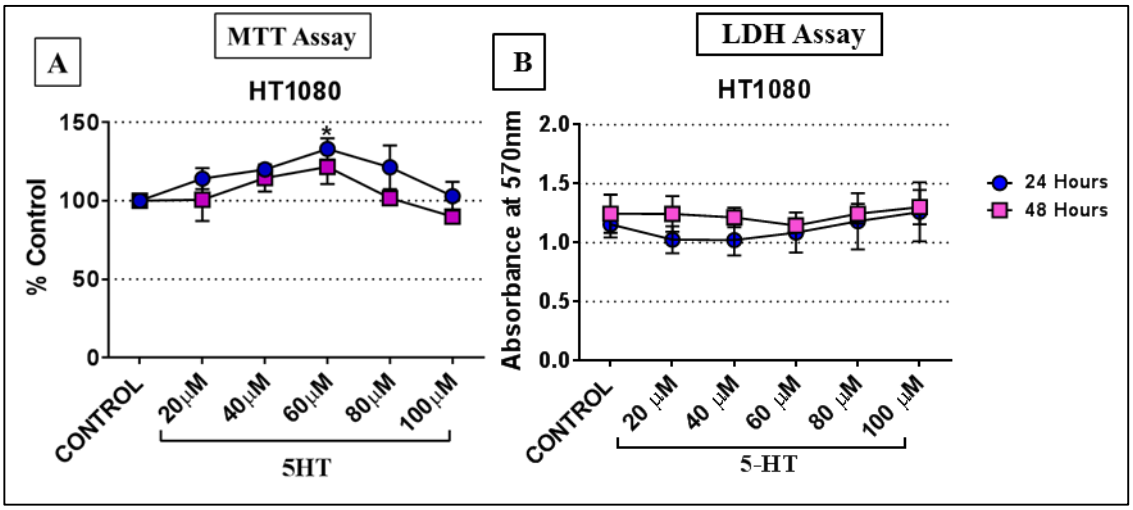


Figure 5.31: Dose-response curve for fibrosarcoma cell line HT1080 treated with 5-HT after 24 hours of under hypoxic conditions.

Statistical analysis was carried out using two-way ANOVA (Dunnett's multiple comparison test against control). The results are a representation of the mean values for data from 3 independent experiments performed in triplicates ± SEM; *, $p<0.05$.

5.2.3.3 Effect of 5-HT on cell proliferation under hypoxic conditions

Considering the almost inconclusive MTT/LDH data, the effect of 5-HT on cell proliferation was examined after 5-HT treatment by 3H incorporation into the cells (see Sections 2.8.7 and 5.2.2.3) after 5-HT treatment. Only two doses of 5-HT were examined for proliferation, 60µM (that indicated maximum percentage increase in viability of the cells from MTT/LDH assays) and 100µM (which reduced viability of the cells). The role of 5-HT in the recovery of cell proliferation after 5-FU inhibition was also investigated. The cells were plated and allowed to recover for 24 hours before 5-FU and 5-HT treatment.

- **Effect of 5-HT on transformed trophoblast cell proliferation**

5-HT had no significant effect on the proliferation of TEV-1 and HTR8/SVneo cells even after 24 and 48 hours treatment (Refer to Figure 5.32). Furthermore, 5-HT did not induce any recovery of cells that were previously inhibited by 5-FU. The proliferation further reduced with 24 hours 5-HT treatment and a significant reduction of 56% was observed for 100µM when compared to control in TEV-1 cells. This reduction was not observed in HTR8/SVneo cells.

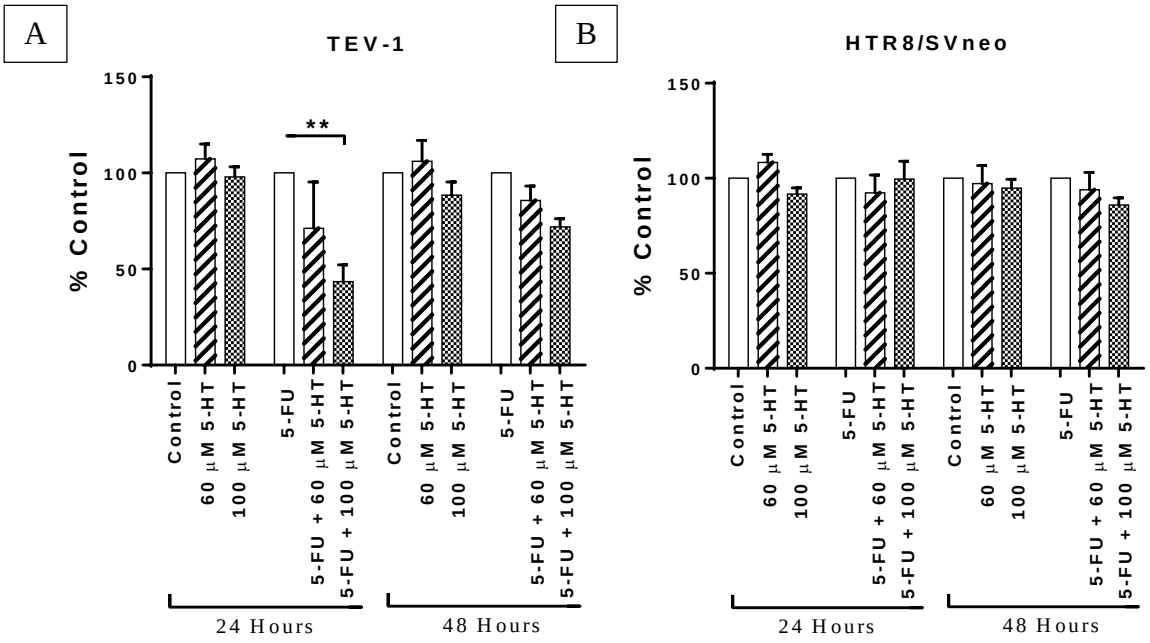


Figure 5.32: ^{3}H incorporation into transformed trophoblast cell lines treated with 5-HT.

Statistical analysis was carried out using two-way ANOVA (Tukey's multiple comparisons test). The results are a representation of the mean values for data from an experiment performed in triplicates ± SEM; **, $p<0.01$.

- **Effect of 5-HT on placental choriocarcinoma cell proliferation**

A significant increase of 85% of proliferation was observed in BeWo treated with 100μM for 24 hours when compared to control (See Figure 5.33.A). Apart from this, there were no other significant changes in proliferation due to 5-HT in BeWo cells, and 5-HT had no capacity to reverse the influence of 5-FU on proliferation.

In JEG3, the 60μM 5-HT treatment for 48 hours significantly increased the proliferation, whereas 24 hours recovered the proliferation inhibited with 5-FU by 16% (See Figure 5.33.B).

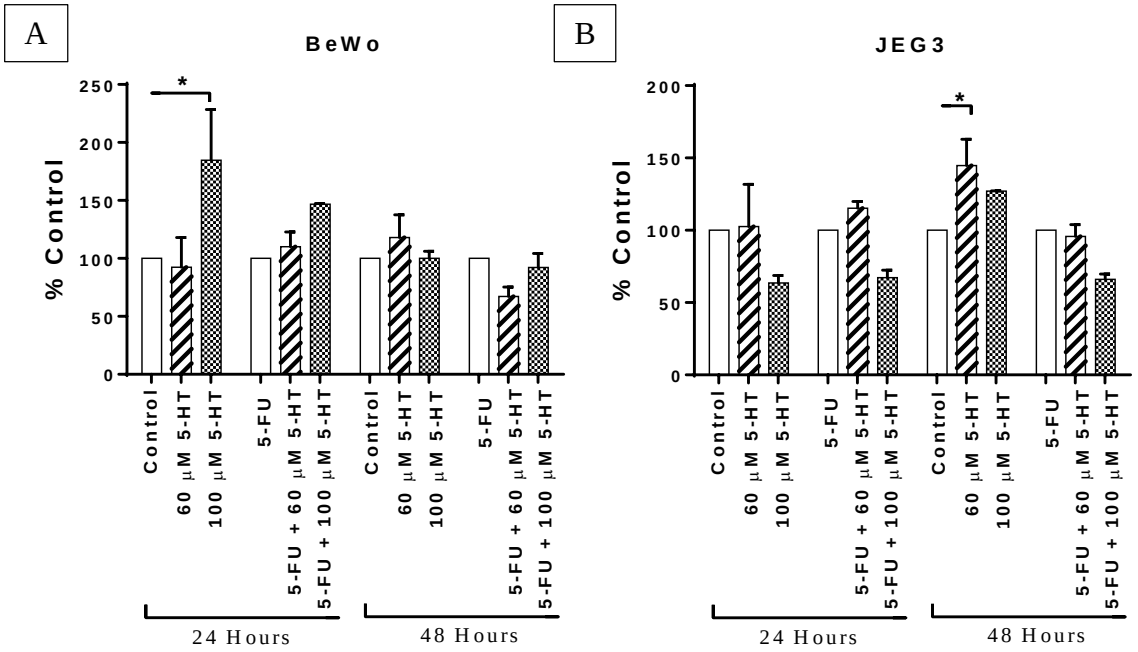


Figure 5.33: ^{3}H incorporation in placental choriocarcinoma cell lines treated with 5-HT.

Statistical analysis was carried out using two-way ANOVA (Tukey's multiple comparisons test). The results are a representation of the mean values for data from an experiment performed in triplicates ± SEM; *, $p<0.05$.

In conclusion 5-HT treatment improved the proliferation of BeWo cell line at 24 hours incubation. The recovery effect of 5-HT on cell proliferation was observed only in JEG3 cell line.

- **Effect of 5-HT on fibrosarcoma proliferation**

5-HT had no significant effect on the proliferation of fibrosarcoma cell line, HT1080 (See Figure 5.34).

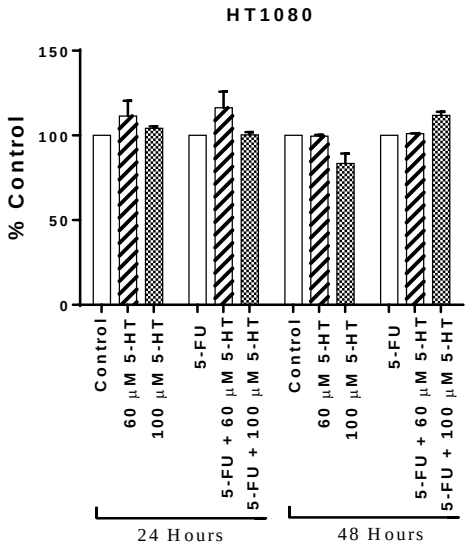


Figure 5.34: ^{3}H incorporation in fibrosarcoma cell line treated with 5-HT.
Statistical analysis was carried out using two-way ANOVA (Tukey's multiple comparisons test). The results are a representation of the mean values for data from an experiment performed in triplicates ± SEM.

5.2.3.4 Effect of 5-HT on cell migration under hypoxic conditions

In Section 5.2.2.4 it is clear that 5-HT induced the migration of transformed trophoblast cell lines at optimal doses of 20µM-40µM under normoxia (20% v/v O_2). However, due to the disparity in cell migration (and/or invasion) under hypoxic conditions in literature it was imperative to investigate the effects of 5-HT under hypoxic conditions too. Therefore, the scratch/wound healing assay was performed under 2% v/v O_2 treated with different doses of 5-HT. The oxygen concentration was recorded to confirm/maintain 2% v/v O_2 throughout the experiment period. Additionally the cells were not stained with fluorescent dye in order to minimise any stress to the cells under hypoxic conditions. Therefore, the images using confocal microscopy were taken with bright field and analysed again using Wimasis®. Images were only analysed for 12 hours as to avoid any changes in oxygen levels due to experimental set up. The variances were analysed as mentioned earlier in Section 5.2.2.4.

- **Effect of 5-HT on transformed trophoblast cell migration**

Unusually, 5-HT at lower doses (20, 40 and 60µM) seemed to have little or no effect on the migration of transformed trophoblast cells (See Figure 5.35). In TEV-1 cells at high concentrations of 80µM and 100µM showed a significant reduction in wound healing ($p<0.05$). However, in HTR8/SVneo cells, only 100µM showed significant reduction in wound covered area beginning from 6 hours. Although not significant, 60µM 5-HT treated HTR8/SVneo cells showed slight improvement in wound coverage when compared to control.

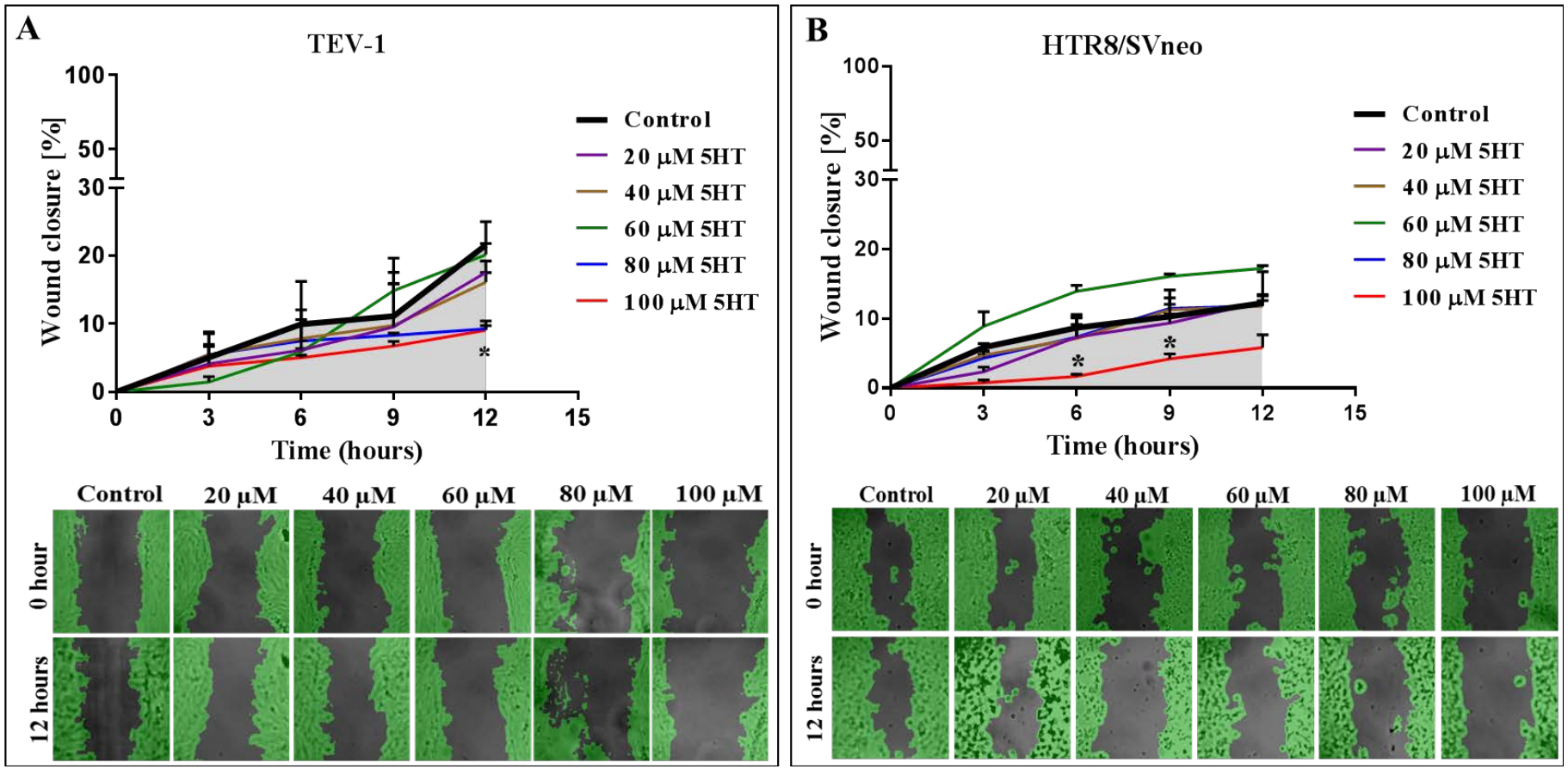


Figure 5.35: Effect of 5-HT on transformed trophoblast cell migration under hypoxic conditions.

Panel A: Represents the wound covered area (%) and scratch images at 0 and 12 hours for **TEV-1** cells. No improvement in wound coverage was observed with 5-HT treatment. There was a significant reduction in wound covered area with higher doses of 80 and 100µM 5-HT (n=2 ±SEM; *p<0.05).

Panel B: Represents the wound covered area (%) and scratch images at 0 and 12 hours for **HTR8/SVneo** cells. The 60µM treated cells showed higher wound coverage compared to control. However 100µM significantly reduced wound coverage (n=2 ±SEM; *p<0.05).

The grey shaded area in the graphs represents the wound covered area by control cells. Magnification of images 200X.

- **Effect of 5-HT on placental choriocarcinoma cell migration**

The effect of 5-HT on placental choriocarcinoma cells under hypoxic conditions were also comparable to transformed trophoblast cells (See Figure 5.36). Both BeWo and JEG3 treated with 5-HT showed lower wound coverage when compared to the controls. Only 5-HT concentrations of 60µM in BeWo and 40µM in JEG3 showed similar wound coverage to control cells. However even the wound coverage by control cells within 12 hours was only approximately 5%. This wound coverage area at 12 hours for BeWo under hypoxic conditions was lower by approximately 15% in control cells under normoxia at the same time point (See Section 5.2.2.4). Conversely the wound coverage area was almost similar under normoxia and hypoxia for JEG3 at the same time point.

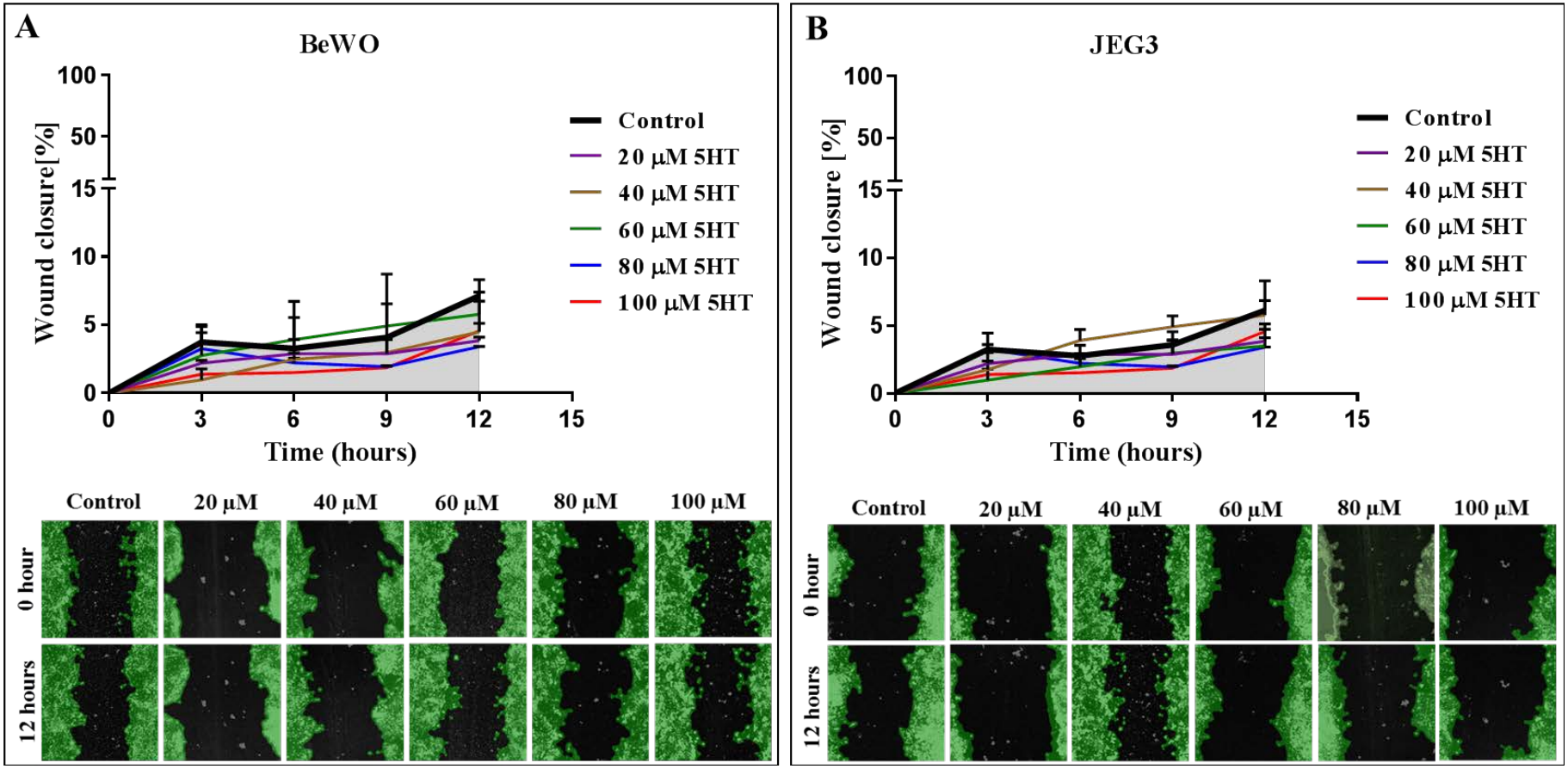


Figure 5.36: Effect of 5-HT on placental choriocarcinoma cell migration under hypoxic conditions.

Panel A and B: Represents the wound covered area (%) and scratch images at 0 and 12 hours for **BeWo** and **JEG3** respectively. No improvement in wound coverage was observed with 5-HT treatment compared to control. Furthermore the wound covered area by control was very low (n=2 ±SEM). *The grey shaded area in the graphs represents the* wound *covered area by control cells. Magnification of images 200X.*

- **Effect of 5-HT on fibrosarcoma cell migration**

Comparable to the transformed trophoblast cell line HTR8/SVneo, there was a significant improvement in wound coverage of HT1080 treated with 60µM 5-HT when compared to control (seen in Figure 5.37). However, 5-HT concentrations below 60µM showed no significant improvement, whilst higher dose (100µM) reduced the wound healing. Overall, hypoxia reduced the migration of all the cells treated with 5-HT and only 60µM showed improvement when compared to control.

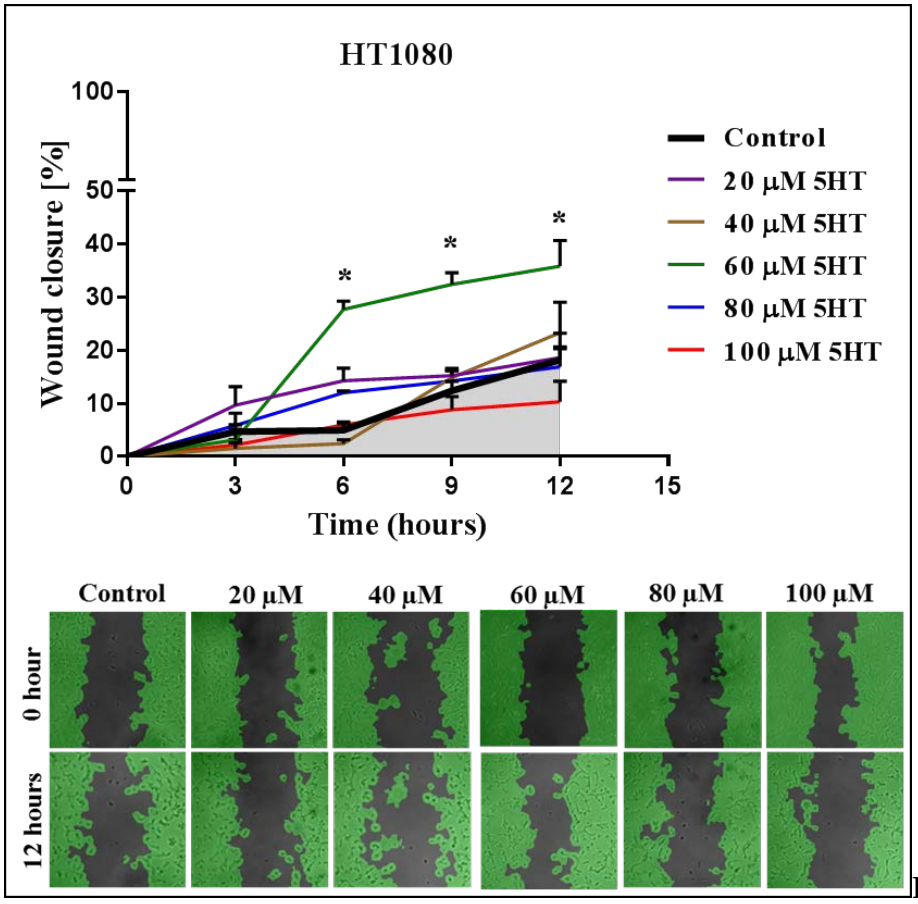


Figure 5.37: Effect of 5-HT on HT1080 migration under hypoxia.

Significant increase in wound covered area was seen with 60µM 5-HT treated cells compared to control (n=2±SEM; *p<0.05). Unlike normoxia, there were scratch areas still visible after 12 hours.

Overall, hypoxia reduced the cell migration of all the cells investigated except for JEG3 which showed similar wound healing under both conditions. In transformed trophoblast and fibrosarcoma cells, 60µM 5-HT showed an increase in cell migration. However, there was no improvement of migration of placental choriocarcinoma cells with 5-HT under hypoxia. Furthermore, higher doses of 5-HT (80-100µM) were detrimental to the cells.

5.2.3.5 Effect of 5-HT on cell invasion under hypoxic conditions

Interestingly, since hypoxia had reduced the migration of the cells, even in fibrosarcoma cells, the effects of 5-HT on cell invasion under hypoxia were investigated in order to determine the effects of 5-HT on cell invasion under hypoxic conditions. Variances were analysed using one-way ANOVA and compared against positive control using Dunnett's test.

- **Effect of 5-HT on transformed trophoblast cell invasion**

In TEV-1, a significantly higher number of non-invaded cells in CTL scans were observed in cells treated with 20µM and 80µM 5-HT when compared to control (positive) ($p<0.05$) (See Figure 5.38.A). Although 60µM showed reduction in non-invaded cells, it was not statistically significant. There was correlation between CTL scans and the Image J results. The same doses of 5-HT (20 and 80µM) showed less invaded cells in the membrane.

Similarly, in HTR8/SVneo cells, the 60µM treated cells showed a significant increase in cell invasion compared to control, as confirmed by both CTL scan (lowest number of non-invaded cells) and Image J counts (highest number of invaded cells; $p<0.05$). The increase was almost 100%, more than the control, as seen from Figure 5.38.B. The 80µM 5-HT treatment once again significantly reduced the cell invasion in HTR8/SVneo cells. Therefore 60µM 5-HT was considered as the optimal concentration for improvement of transformed trophoblast cell invasion under hypoxic conditions. Concentrations above and below 60µM 5-HT reduced the cell invasion.

- **Effect of 5-HT on placental choriocarcinoma cell invasion**

When the effects of 5-HT on placental choriocarcinoma cell invasion were investigated, there was no significant changes reported within treated and control groups (See Figure 5.39). The 60µM 5-HT showed very little or slight increase in invaded cell numbers when compared to control. Image J analysis demonstrated that 80µM 5-HT treated cells showed significantly lower number of JEG3 cells on bottom of the membrane.

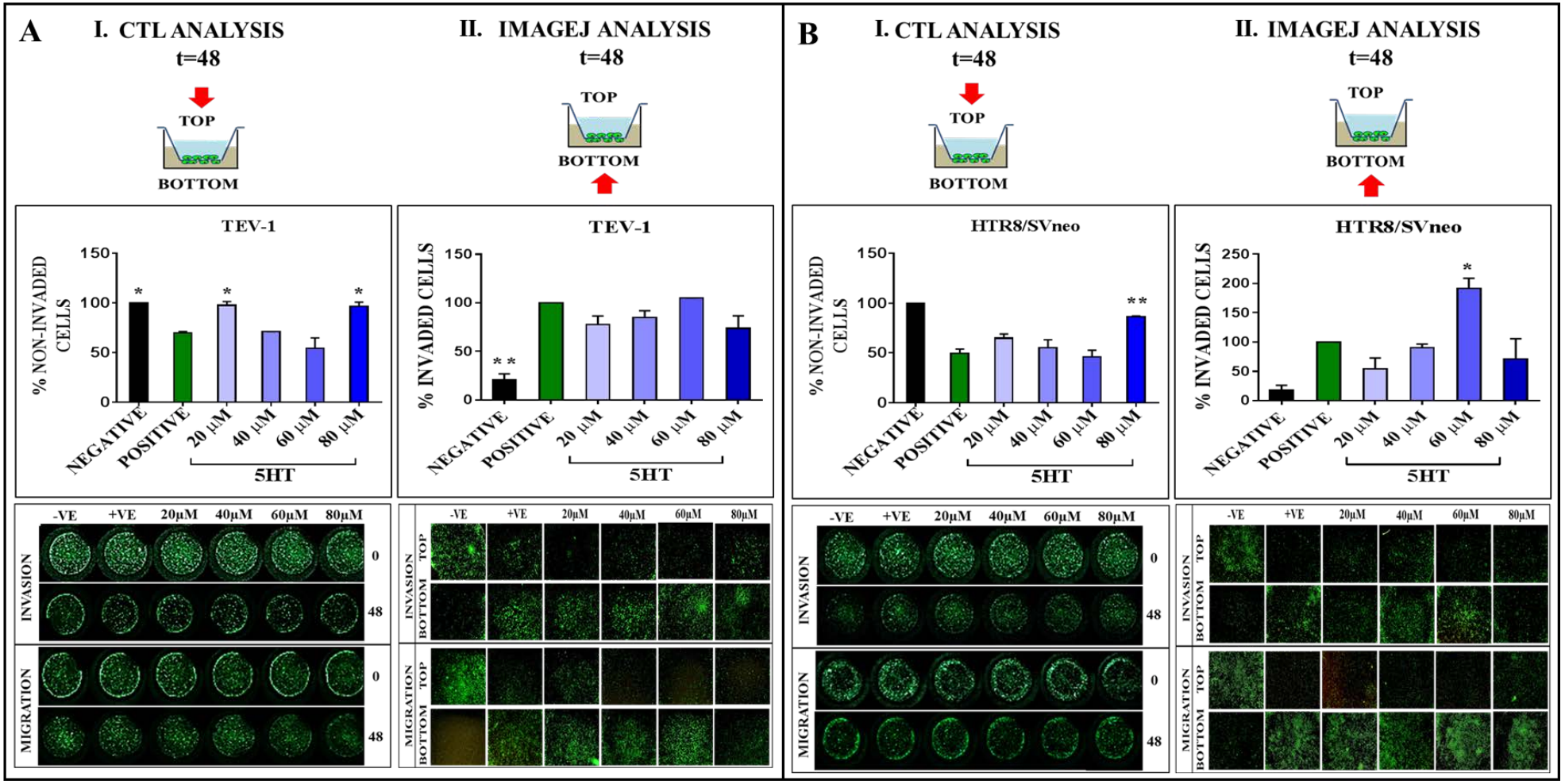


Figure 5.38: Effect of 5-HT on transformed trophoblast cell invasion under hypoxic conditions.

A correlation was observed between CTL (**I**) and Image J (**II**) analysis.

Panel A: Represents the invasion in **TEV-1** cells. I. There was significantly high percentage of non-invaded cells seen in negative, 20 and 80µM 5-HT when compared to control (positive) (n=2±SEM;**p<0.01; *p<0.05). The same doses showed reduced number of invaded cells (II).

Panel B: Represents the invasion of **HTR8/SVneo**. I. The 80µM 5-HT showed the highest % non-invaded cells when compared to positive and other 5-HT treated cells. II. Approximately 100% significant increase in invaded cells were observed with 60µM when compared to control (n=2±SEM; **p<0.01; *p<0.05). More invaded cells were seen on bottom of membrane in positive and 60µM.

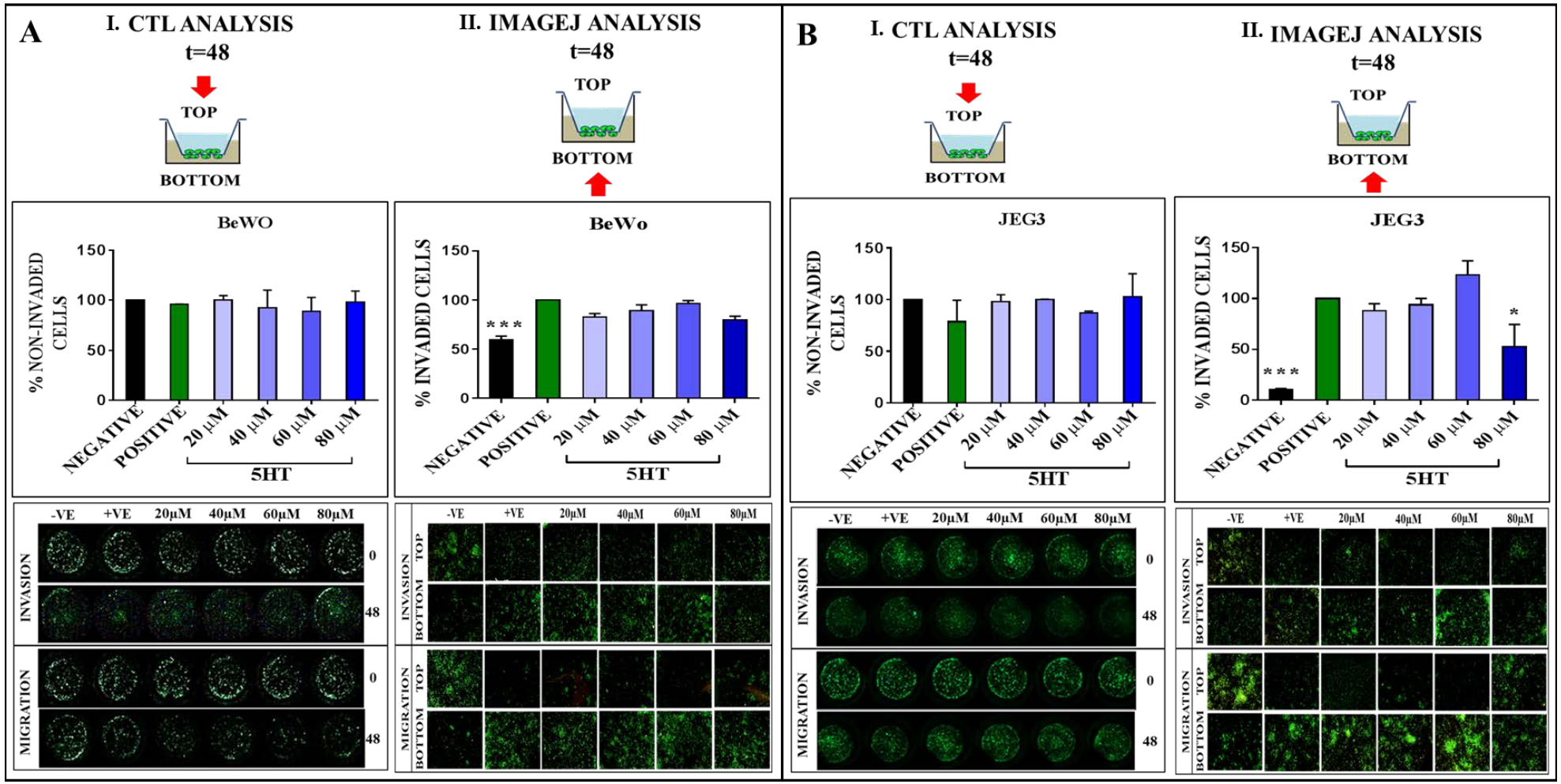


Figure 5.39: Effect of 5-HT on placental choriocarcinoma cell invasion under hypoxic conditions.

A correlation was observed between CTL (**I**) and Image J (**II**) analysis.

Panel A: Represents the invasion in **BeWo**. I. There was no significant changes in cell invasion with 5-HT treatment when compared to control (positive) (n=2±SEM). II. Only negative control showed a significantly lower number of invaded cells (***p<0.001).

Panel B: Represents the invasion of **JEG3**. I. 5-HT treated JEG3 cells showed very little or slight variation in cell invasion when compared to control (positive). II. The 80µM 5-HT however shows a significant reduction in invaded cells by Image J analysis (n=2±SEM; *p<0.05).

- **Effect of 5-HT on fibrosarcoma cell invasion**

Although a significantly lower number of non-invaded cells ($p<0.05$) were observed in 60µM 5-HT treated HT1080 cells, it failed to show similar significance in increase of invaded cells when compared to control (See Figure 5.40). The cell invasion reduced with 80µM 5-HT. Therefore there was no improvement of fibrosarcoma invasion with 5-HT under hypoxic conditions.

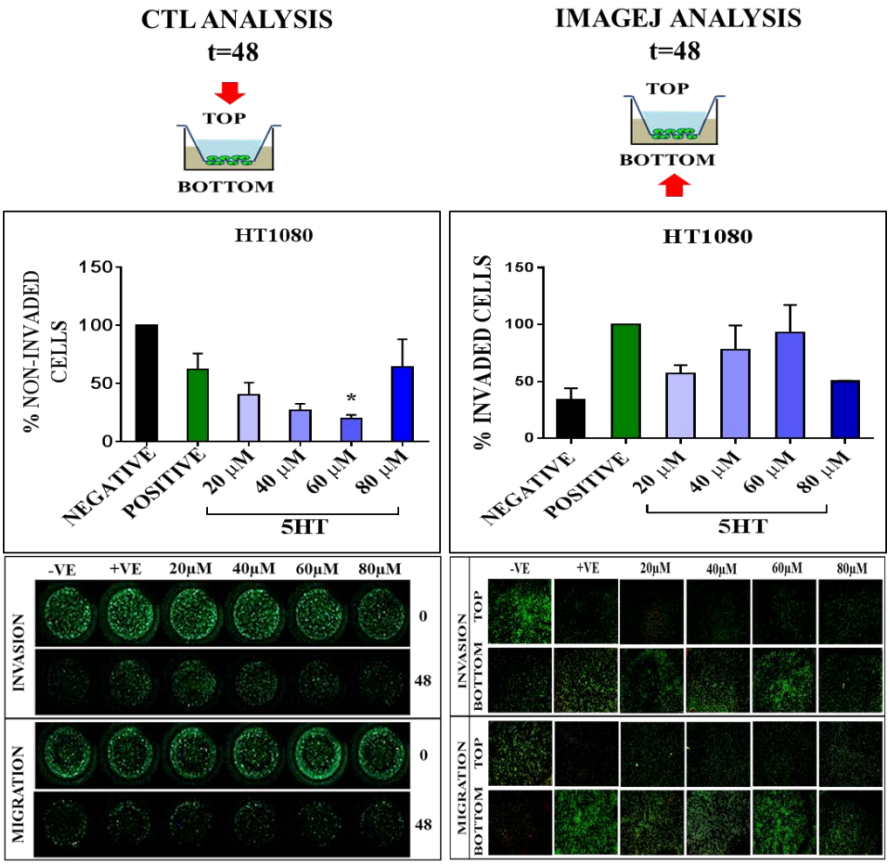


Figure 5.40: Effect of 5-HT on fibrosarcoma cell invasion under hypoxic conditions.
A significantly low number of non-invaded cells (%) were observed with 60µM 5-HT treatment from the CTL analysis (n=2±SEM; *$p<0.05$). Although CTL and Image J analysis showed same patterns of invasion for 5-HT treated cells, the 60µM increase was not higher than control.

Overall, among all the 5-HT doses investigated, only 60µM 5-HT improved the invasion of all the five cell lines under hypoxic conditions, whereas higher or lower doses reduced the cell invasion. When compared to the invasion in normal conditions, the invasion under hypoxic conditions with 5-HT were reduced.

5.3 Discussion

Systematic investigation on the status of any proteins/biomarkers in placenta *in situ* has always been challenging due to ethical constraints. In addition, the morphology of placenta is distinctly different from one another, even in NT pregnancies. To circumvent these problems, placental explants and many different types of placental cells have been isolated and immortalised to be used as *in vitro* models (Orendi *et al*, 2011). These cell lines have been convenient *in vitro* models for expression analysis with/without interventions. In this study, four placental cell lines; TEV-1, HTR8/SVneo (representing early first trimester trophoblast), BeWo and JEG3 (choriocarcinoma) were used along with a positive fibrosarcoma cell line HT1080.

It is a proven fact that in PE pregnancies, the circulating 5-HT is increased (Cruz *et al*, 1998). Furthermore, it has been postulated that 5-HT and its receptors may have a fundamental role in placental development and pregnancy maintenance (Oufkir *et al*, 2010). However, due to ethical constraints, it is impossible to compare the effects of 5-HT on the expression of its receptors between early stages of human pregnancy and term placentae. A few previous studies have looked at the expression profiles of 5-HT receptors *in vitro* using term choriocarcinoma cell lines such as JEG3 and BeWo (Sonier *et al*, 2005). However, there was not enough information on the effects of 5-HT on early first trimester trophoblast (EVT) cell lines. This current study gives an outline of the events occurring in these cell lines due to 5-HT treatment. The results from this chapter are summarised in Table 5.2.

- **Effect of 5-HT on cell viability:**

The morphological changes monitored by H&E staining in the cells treated with 5-HT showed that the cell density increased at 20-40µM of 5-HT concentrations. However, at higher concentrations there was an increase in the amount of apoptosis and/or necrosis. In addition, giant anaplastic cells with distorted features such as multiple nuclei were also observed at these concentrations (especially at 80µM 5-HT treatment). Therefore, to confirm the behaviour of these cells upon 5-HT treatment, two assays were performed for cell viability (MTT) and toxicity (LDH). The MTT results for the effect of 5-HT confirmed that the mitochondrial activity of the cells also increased at 20-40µM concentrations. The LDH assay on the other hand showed minimum LDH release in cells treated with 20-40µM 5-HT concentrations, but maximum LDH releases was observed with higher concentrations (80µM). This shows that, at lower concentrations, 5-HT enhances mitochondrial activity; whereas at higher concentrations, it reduces it. This variation in cell behaviour with dose confirms the biphasic effects of 5-HT that have been reported earlier in mammary cells (Pai and Horseman, 2008). This increased mitochondrial activity at 20-40µM could also be due

to the inhibition of apoptosis. Previous studies using similar doses have shown inhibition of apoptosis in monocytes (Soga *et al*, 2007). There were very limited variations in the cells plated along with 5-HT and the cells treated with 5-HT after attachment (24 hours of cell recovery). This shows 5-HT does not have any immediate effects on the cell viability or cell attachment. However, prolonged treatment with optimum doses may increase viability (mitochondrial activity) of the cells. This phenomenon has also been reported in hepatic tumour cells (Ahlund *et al*, 1989).

Table 5.2: Summary of the effects of 5-HT on cell behaviour.

Effect of -5HT on	Transformed trophoblast		Placental choriocarcinoma		Fibrosarcoma
	TEV-1	HTR8/SVneo	BeWo	JEG3	HT1080
	Normal				
Mitochondrial activity	(++) 30µM	(++) 30µM	(+++) 20-30µM	(+) 20µM	(++) 20-30µM
Toxicity	(-) 30µM	(-) 30µM	(-) 20-40µM	(-) 20µM	(-) 20-40µM
Proliferation	nc	(-) 30-80µM	nc	nc	nc
Recovery of Proliferation	nc	(-) 30-80µM	(++) 30-80µM	(++) 30-80µM	nc
Migration	(++) 30µM	(++) 20-40µM	(+) 30-40µM	(++) 20µM	(+++) 10-80µM
Invasion	(-) 10-40µM	(-) 10-40µM	(+++) 10-40µM	(+++) 10-30µM	(++) 10-40µM
	Hypoxia				
Mitochondrial activity	(++) 60µM	(+) 60µM	(+) 60µM	(+) 60µM	(+++) 40-60µM
Toxicity	(- -) 60µM	(-) 40-60µM	(+) 100µM	(+) 80-100µM	(-) 40-60µM
Proliferation	nc	nc	(+++) 100µM	(++) 60µM	nc
Recovery of Proliferation	(- -) 60-100µM	nc	(+) 100µM	nc	nc
Migration	(- -) 80-100µM	(- -) 100µM	(-) 20-100µM	(-) 20-100µM	(++) 60µM
Invasion	nc	(++) 60µM	nc	(+) 60µM	(-) 20-80µM

The significant changes in the cell behaviour due to 5-HT treatment are shown compared to control (non-treated) cells. The 5-HT concentration that induces these changes are given below the changes. +++ = High increase; ++ = Moderate increase; + = Low increase; - - = Moderate decrease; - = Low decrease; nc= no change.

- **Effect of 5-HT on cell proliferation:**

It should be noted that MTT and LDH assays can only show the viability (or the mitochondrial activity) and toxicity of the 5-HT respectively. In other words, it is impossible to predict whether the increase in mitochondrial activity (and therefore cell viability) is a direct results of increased cell proliferation or due to the enhanced cellular metabolic activity.

In order to prove the increased mitochondrial activity by 5-HT was mainly due to cell proliferation, tritium labelled (^{3}H) thymidine incorporation assay was employed. In the method, the methyl group of thymidine is labelled with tritium to give radioactive ^{3}H-thymidine. During cell proliferation the cells undergo DNA duplication and cell division at S phase of the cell cycle (Cavanagh *et al*, 2011). At this stage, treated cells will incorporate ^{3}H-thymidine into the duplicating DNA instead of thymidine thus labelling all the daughter cells. Thus, the detection of radioactivity of ^{3}H-thymidine at the end of the assay would indicate cell proliferation.

Furthermore, the effect of 5-HT on cell proliferation recovery were also examined. The cells were treated with 5-Fluoro-Uracil (5-FU) for 24 hours before 5-HT treatment. 5-FU is a pyrimidine analogue with a fluoride atom attached to the base of pyrimidine ring. 5-FU has extensively been used as an anti-metabolite to treat solid tumours such as colorectal, breast and liver carcinomas (Ghoshal and Jacob, 1997). 5-FU has also been used as anti-mitotic agents in cell culture to differentiate mitotic cells from migrating cells (Gordon *et al*, 2005). Therefore, by pre-treating the cells with 5-FU, it was possible to study the effects of 5-HT on cell recovery. However, no studies could be found on this in the literature.

The results for ^{3}H thymidine incorporation indicated that 5-HT did not improve the cell proliferation of these cell lines significantly. This shows that 5-HT at lower concentrations may increase both the viability and proliferation of placental trophoblast, choriocarcinoma and fibrosarcoma (the positive control) cell lines. However, 5-HT was also able to recover placental choriocarcinoma cell lines (BeWo and JEG3) proliferation which were inhibited with 5-FU treatment previously. Thus, 5-HT had a recovery effect on inhibited proliferation of only cancerous and not transformed trophoblast cell lines. This effect could be mediated through the interaction of 5-HT with several 5-HT receptors studied in previous chapters which trigger several signalling pathways. However, there are no previous reports on this and need to be critically reviewed.

- **Effect of 5-HT on cell migration and invasion:**

The study of 5-HT inducing human aortic endothelial cell migration by Matsusaka and Wakabayashi in 2005 instigated similar interests in placental cells. The scratch/wound healing assay on placental cell lines indicated that 5-HT could improve the ‘wound healing’ response compared to untreated cells (control). When the wound closure of transformed trophoblast cells, TEV-1 and HTR8/SVneo were compared, it was observed that lower doses of 5-HT (20-40µM) improved the migration.

The wound closure of TEV-1 cells was higher at the end of the assay than HTR8/SVneo. Similar concentrations of 5-HT (30µM and 40µM) in placental choriocarcinoma cells (BeWo and JEG3), showed higher rates of wound healing compared to controls. However, when the wound healing of the two placental cell types were compared, transformed trophoblast cells showed higher wound coverage than placental choriocarcinoma. This unexpectedly reduced rate of wound healing in BeWo and JEG3 may be due to the fact that these cell lines, despite having originated from carcinoma, have a syncytial-like morphology. That is, they both grow in clusters of cells and migrate as sheets or clumps. Therefore, individual cell motility cannot be observed as in transformed trophoblast cells. As expected, the fibrosarcoma cell line (HT1080) which was used as positive control showed the fastest wound coverage within 12 hours of the assay. Also, all the doses of 5-HT seemed to increase the wound coverage when compared to untreated cells.

It should be noted during the migration assay, cells were treated with 5-FU. This, together with the fact that all migration assays were carried out using serum free media (SFM) rules out any effects of 5-HT induced cell proliferation. Therefore it can be safely argued, under the present experimental conditions, 5-HT activated cell migration; may be via a different intra-cellular pathway. Previously, it was reported that lower doses of 5-HT increased the migration of neural crest cells during early development (Reviewed by Moiseiwitsch, 2000). However, treatment with higher doses of 5-HT resulted in the reduction of cell migration and promoted differentiation. 5-HT is also known to play an important role in epithelial homeostasis of the lung, breast, liver and prostate (Pai *et al*, 2009). The studies on tumour cell lines such as breast and hepatocellular cancer have confirmed the migratory effects of 5-HT through its interactions with various 5-HT receptors, especially via 5-HT_{2B} receptor subtype (Pai and Horseman, 2008; Soll *et al*, 2010). These studies have also suggested the role of 5-HT on the epithelial-to-mesenchymal cell transition (EMT) which is one of the important features for cell migration and invasion. Although the signalling pathways for the 5-HT receptors have been investigated in placental choriocarcinoma cell lines (BeWo and JEG3) (Sonier *et al*, 2002), no previous studies could be found on the migratory effects on transformed trophoblast cell lines (TEV-1 and HTR8/SVneo).

The data from the invasion assay did not correlate with data from above migration studies. There was no significant increase in cell invasion with 5-HT treated for transformed trophoblast cell lines. However, 20-30µM 5-HT increased the invasive capacity of placental cancer cell lines (BeWo, JEG3 and HT1080). This was expected, as cancerous cells show higher cell invasion than normal cells. Overall, this study has confirmed 5-HT increases the migration of all the four placental cell lines (TEV-1, HTR8/SVneo, BeWo and JEG3), but

on the other hand, increases cell invasion only in placental choriocarcinoma cells. A study by Oufkir *et al* (2010), have proposed the involvement of 5-HT2_A receptor in the invasion of BeWo and JEG3 cell lines. However, the pathways involving transformed trophoblast cell invasion are yet to be revealed.

- **Effects of 5-HT under hypoxic conditions:**

In this study, the cells under hypoxic conditions were treated with higher concentrations of 5-HT to recreate a PE placental environment for the cells. As discussed earlier, during PE placenta the oxygen levels are low (less than 2% v/v) and the serotonin levels are higher (0.5-0.7µg/mg) than in normal placenta (0.4µg/mg) (Ramadan *et al*, 1973).

The H&E staining revealed long extended cell morphology in transformed trophoblast cells with 20-80µM 5-HT treatment. At much higher dose of 100µM, frequent appearance of apoptotic cells with blebbing were observed. However, due to the syncytial-like appearance of the placental choriocarcinoma cells, these features were not distinguishable in BeWo and JEG3. There was frequent appearance of anaplastic cells with some showing chromosomal distortions at higher concentrations. The fibrosarcoma cells also showed similar features. The MTT and LDH data suggest that 5-HT increased the viability of all the cell lines except for BeWo under hypoxic conditions. This proves the earlier finding within this chapter that the effects of 5-HT on cell viability may be cell line dependant. The optimum 5-HT dose which induced the highest cell viability in all cell lines was found to be 60µM. Cells treated with 100µM 5-HT showed the least viability with maximum release of LDH, indicating this dose was toxic to the cells under hypoxic conditions.

The ^{3}H assay revealed 5-HT did not have any significant effect on the proliferation of transformed trophoblast cells and also did not show recovery of inhibited proliferation. However, in placental choriocarcinoma cells, BeWo and JEG3, 5-HT induced significant increase in proliferation and also recovery. Although a slight increase in fibrosarcoma cell proliferation was observed, this was not significant. These studies have confirmed that placental cells are tolerant to hypoxic conditions and the mitogenic effects of 5-HT under hypoxic conditions in cancerous cell lines.

On the other hand, under hypoxic conditions, no improvement in cell migration was observed with 5-HT except for 60µM in HT1080. In contrast, in invasion studies, HTR8/SVneo and JEG3 showed an increase in cell invasion with 60µM 5-HT. There was no significant changes with 5-HT in the remaining cell lines. This was paradoxical, as increased invasion was expected during hypoxic conditions, especially in cancerous cell lines (such as BeWo and HT1080).

Although the cells were more tolerant to high doses of 5-HT under hypoxic conditions, the migratory and invasive properties induced by 5-HT were not significant. It is also a very well-known fact that hypoxia alters the expressions of genes involved in migration and invasion *(*Graham *et al*, 2000). The data from this chapter confirms that high levels of 5-HT may not enhance placental cell migration or invasion under hypoxic conditions. This agrees with the findings that high levels of circulating 5-HT in PE conditions may result in shallow placental invasion. This may be due to the alterations in the 5-HT receptors. To confirm these effects of 5-HT on the cell behaviour, receptor expression studies need to be carried out (See Chapter 6).

Conclusion:

The data from this chapter suggests that under normoxic conditions 5-HT at low concentrations induces migration and invasion of the placental cells. However under hypoxic conditions the cells are more "tolerant" to higher doses of 5-HT and therefore do not show any changes either in migration or invasion.

Chapter 6
Characterising the effects of 5-HT on its receptors and C/T antigens

6.1 Introduction

Although 5-HT is one of the most extensively studied molecules in human physiology/pharmacology, previous studies mainly concentrated on the brain and neuronal development (Burnet *et al*, 1995). The effect of 5-HT on cell migration and invasion via its interaction with 5-HT receptors have been reported in glioma and hepatocellular cancer cells (Merzak *et al* 1996; Soll *et al*, 2010). The few studies that have investigated the involvement of 5-HT on regulation of placental choriocarcinoma cell lines (JEG3 and BeWo) migration have only highlighted the importance of 5-HT_{2A}R subtype (Klempan *et al*, 2011; Sonier *et al*, 2005). Interestingly, these studies have concluded that the differential expression patterns of several intracellular mitogenic proteins are dependent on the concentration of 5-HT. However, both BeWo and JEG3 are tumour originated cell lines. Therefore, it is essential to repeat this study using cell lines that are derived from first trimester trophoblast (TEV-1 and HTR8/SVneo) which are involved in early placental invasion. Moreover, 5-HT has been reported to be involved in early trophoblast invasions (Ugun-Klusek *et al*, 2011; Oufkir *et al*, 2010**;** Bolte *et al*, 2001). Therefore, it is essential to study the effects of 5-HT on its main receptors using cell lines derived from early first trimester trophoblast.

On the other hand, neither the expression of C/T antigens in placental cell lines, nor the effects of 5-HT on C/T antigen expressions, have ever been studied before. In fact, C/T antigens are expressed mainly in tumour and germ cells (such as testis). Therefore, very little is known about the functions of these antigens. From the few studies that have been carried out, these antigens are known to be involved in invasion, tumorigenesis, and apoptosis (Olesen *et al*, 2011; Sang *et al*, 2011; Gjerstorff *et al*, 2006). Interestingly, from Chapter 3, it was clear that C/T antigens were expressed in human placenta, both NT and PE. The immunohistochemistry has revealed that these antigens are mostly expressed in the syncytiotrophoblast (STB) and cytotrophoblast (CTB) layers. Given the fact that the expression of many C/T antigens are generally altered in PE where the circulating 5-HT is increased (Sivasubramaniam, 2002), it would be interesting to see if there are changes in C/T antigen expression with 5-HT. In addition, the data from Chapter 5 have confirmed that the migration and invasion of transformed trophoblast and choriocarcinoma cell lines are affected by different doses of 5-HT. This, together with the fact that 5-HT receptors and C/T antigens have shown differential expression patterns in NT and PE placentae (See Chapter 3), have warranted the need to study whether 5-HT (a) brings about its mitogenic actions via 5-HT_{1B}R and 5-HT_{2B}R; and (b) affects the expression of C/T antigen.

6.2 Results

The expression of the 5-HT receptors and C/T antigens in NT and PE placentae were compared previously in Chapter 3. This chapter deals with the effects of 5-HT on the

functional expression of the above receptors/antigens, using transformed trophoblast (TEV-1 and HTR8/SVneo) and placental choriocarcinoma (BeWo and JEG3) cell lines. Firstly, the basal expressions of 5-HT receptors and C/T antigens in placental cell lines were studied using qRT-PCR, to confirm that the cell lines used express these receptors and especially the C/T antigens. These cells were treated with different doses of 5-HT for 24 hours to analyse the mRNA (qRT-PCR) and protein (Western blotting) expression (see Chapter 2; Sections 2.6.5 and 2.7.4). Commercially available total RNA and proteins (Clontech, UK) from human brain and testis were used as positive controls for 5-HTR and C/T antigens respectively (data not shown).

A one-way ANOVA statistical analysis was performed on the $2^{-\Delta\Delta Ct}$ values from qRT-PCR (two individual experiments each for two different passage numbers; n=4) and densitometry data from Western blots (one blot each for two different passages; n=2). Where appropriate, the cellular localisation of the receptors and antigens were also investigated by immunofluorescent labelling with FITC (green) and nuclear counter staining with DAPI (Section 2.9.4).

6.2.1 5-HT receptors

The two 5-HT receptor subtypes, 5-HT_{1B}R and 5-HT_{2B}R were investigated in transformed trophoblast and placental choriocarcinoma cell lines.

6.2.1.1 Basal 5-HT_{1B} receptor expression in cell lines

The mRNA expression of 5-HT_{1B}R were investigated in the cell lines relative to expression in human brain (positive; arbitrarily set to 1) (See Figure 6.1). The mRNA expression of 5-HT_{1B}R in brain was significantly higher than the expressions in the cell lines (****$p<0.0001$). When the expressions were compared amongst cell lines, the level of 5-HT_{1B}R in transformed trophoblast group was significantly higher than other groups. On the other hand, the placental choriocarcinoma group expressed the lowest level of 5-HT_{1B}R which was significantly lower to HT1080 (*$p< 0.05$)

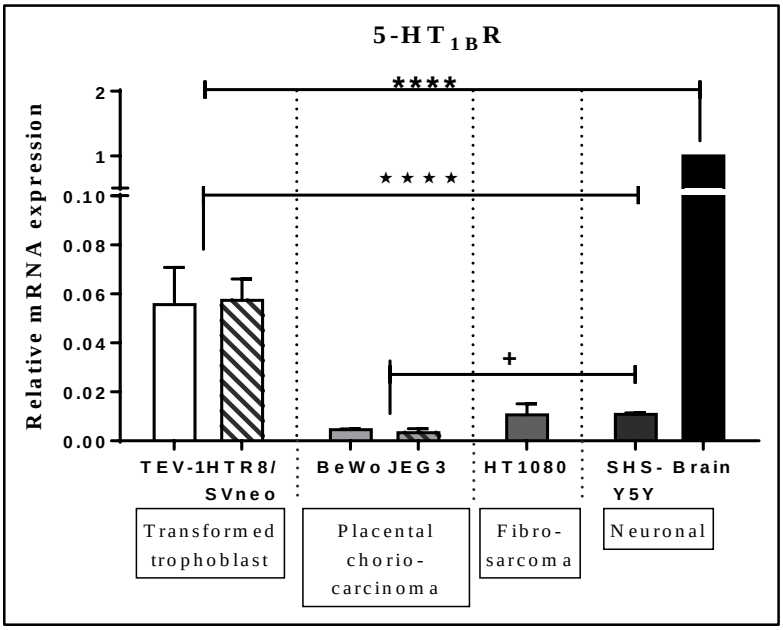


Figure 6.1: Basal mRNA expression of 5-HT$_{1B}$R in cell lines.

The data are means of 2 experiments (each performed in tripliactes; ±SEM; ****, ★★★★p<0.0001; +p<0.05). The mRNA expression is relative to expression in brain (arbitrarily set to 1).

- ***The effects of 5-HT on 5-HT$_{1B}$R expression***

Although the expression of 5-HT$_{1B}$R was detected at mRNA level for 5-HT treated placental choriocarcinoma cells (BeWo and JEG3), these differences in expression were not confirmed by Western blotting and immunofluorescence. Thus, only 5-HT$_{1B}$R expression patterns in transformed trophoblast cells are discussed below.

As can be observed from Figure 6.2 (Panels A and B), a significant increase in expression of 5-HT$_{1B}$R was observed in TEV-1 cells treated with 5-HT relative to the control (not treated). The mRNA and protein expressions showed similar trends with 10µM 5-HT treated cells showing the maximum up-regulation. The highest concentration of 80µM 5-HT showed a down-regulation in mRNA expression relative to control. On the other hand, Western blots have shown a significant increase in the 5-HT$_{1B}$R with all the different concentrations of 5-HT. The immunofluorescent staining for 5-HT$_{1B}$R showed a cytoplasmic localisation and the fluorescence intensity correlated with the protein expression data (See Figure 6.2.C).

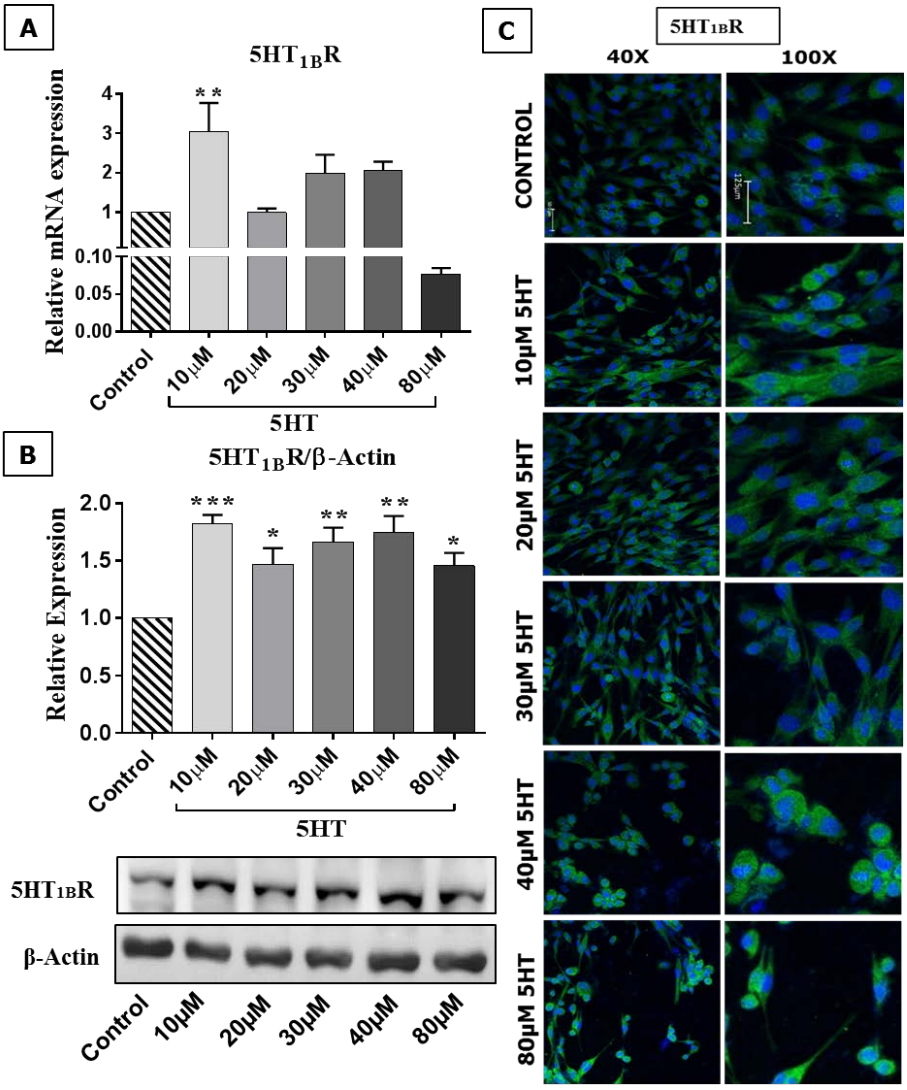


Figure 6.2: Effect of 5-HT on 5-HT$_{1B}$R expression in TEV-1 cells.

Panel A represents the relative mRNA expression of 5-HT$_{1B}$R to control (n=4; ±SEM) which shows a significant up-regulation with 10µM 5-HT treatment (**p<0.01). **Panel B** represents the densitometry analysis of the western blots (n=2 ±SEM). Bands for 5-HT$_{1B}$R were visible at 33kDa. Equal loading of protein concentration was confirmed by β-actin expression. There was a significant increase of 5-HT$_{1B}$R proteins with 5-HT treatment (average *p<0.05). **Panel C** reveals the cytoplasmic localisation of 5-HT$_{1B}$R (FITC-Green). Nucleus was counter stained with DAPI (blue). [Objective magnification: 40X (Scale bar= 50µm) and 100X (Scale bar= 125µm)]

The 5-HT treatment in the second transformed trophoblast cell line, HTR8/SVneo, produced an overall increase of 5-HT$_{1B}$R (See Figure 6.3). There was a gradual up-regulation in mRNA expressions with increasing concentrations of 5-HT (except the cells treated with 10µM). Significant increase in 5-HT$_{1B}$R was seen in 30µM and 40µM 5-HT treated cells (p<0.01). The densitometry analysis of the Western blots correlated to mRNA expression. However, the expression patterns were not significant at protein levels. The immunostaining also revealed cytoplasmic localisation. There were no differences in fluorescent intensities observed between the 5-HT treated and control cells.

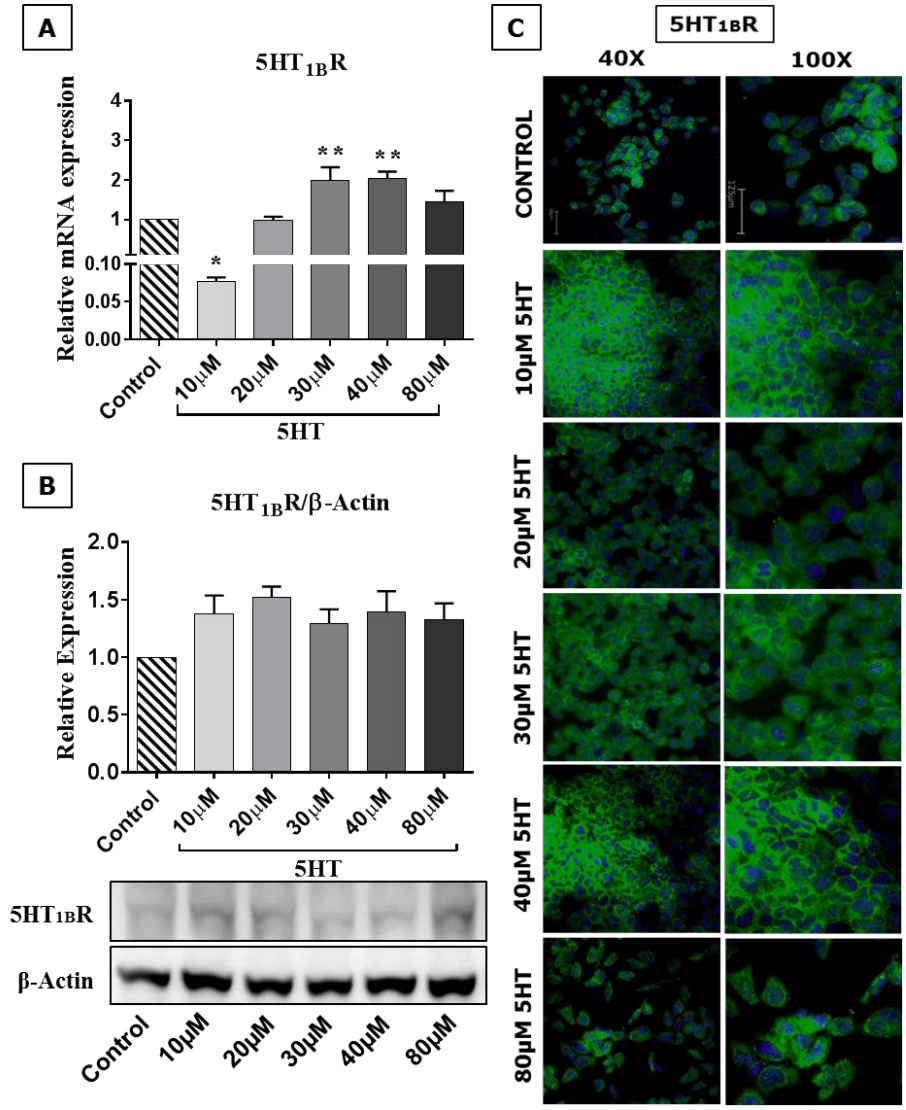


Figure 6.3: Effect of 5-HT on 5-HT$_{1B}$R expression in HTR8/SVneo cells.

Panel A and B represent the relative mRNA (n=4) and protein (n=2) expression of 5-HT$_{1B}$R in HTR/SVneo. The mRNA expression was upregulated with 30 and 40 μM (±SEM; **p<0.01). The cytoplasmic localisation of 5-HT$_{1B}$R (FITC) is confirmed by immunofluorescent staining in **Panel C** and nucleus counter stained (DAPI). [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

6.2.1.2 Basal 5-HT$_{2B}$ receptor expression in cell lines

The placental choriocarcinoma cell lines (BeWo and JEG3) showed a significantly higher level of 5-HT$_{2B}$R mRNA expression when compared to all other cell lines (see Figure 6.4). When the expressions were compared within the groups, there was a significant difference observed between the two transformed trophoblast cell lines, TEV-1 and HTR8/SVneo (*p<0.05). It was also observed that the mRNA expression was significantly higher in human brain (positive control).

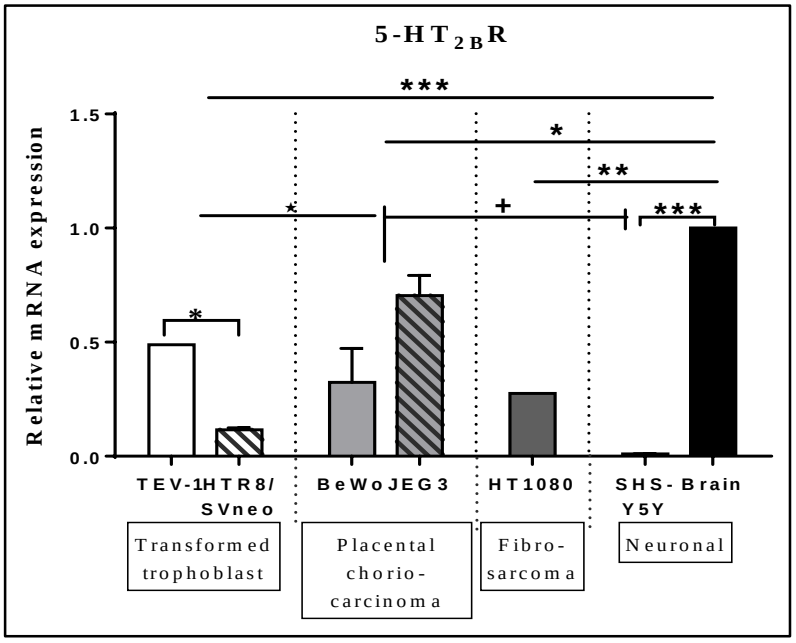


Figure 6.4: Basal mRNA expression of 5-HT$_{2B}$R in cell lines.

The data are means of 2 experiments (each performed in triplicates; ±SEM; ***p<0.001; **p<0.01; ⋆,*, *p<0.05). The mRNA expression is relative to expression in brain (arbitrarily set to 1).

- ***The effects of 5-HT on 5-HT$_{2B}$R expression***

Both the transformed trophoblast cells, TEV-1 and HTR8/SVneo were investigated for 5-HT$_{2B}$R expressions. However, only JEG3 from placental choriocarcinoma group contributed to expression data for 5-HT$_{2B}$R.

- **The effects of 5-HT on 5-HT$_{2B}$R expression in transformed trophoblast cell lines**

The TEV-1 cells treated with 5-HT showed a significant reduction in 5-HT$_{2B}$R expression when compared to control (See Figure 6.5.A and B). This difference in expression was observed at both mRNA and protein level. A single protein band of approximately 53kDa was detected on the Western blots (molecular weight as specified by the primary antibody supplier, biorbyt, UK). However, a greater staining of the cell membrane rather than a diffuse cytoplasmic stain was observed by immunofluorescent staining (FITC) (Figure 6.5.C).

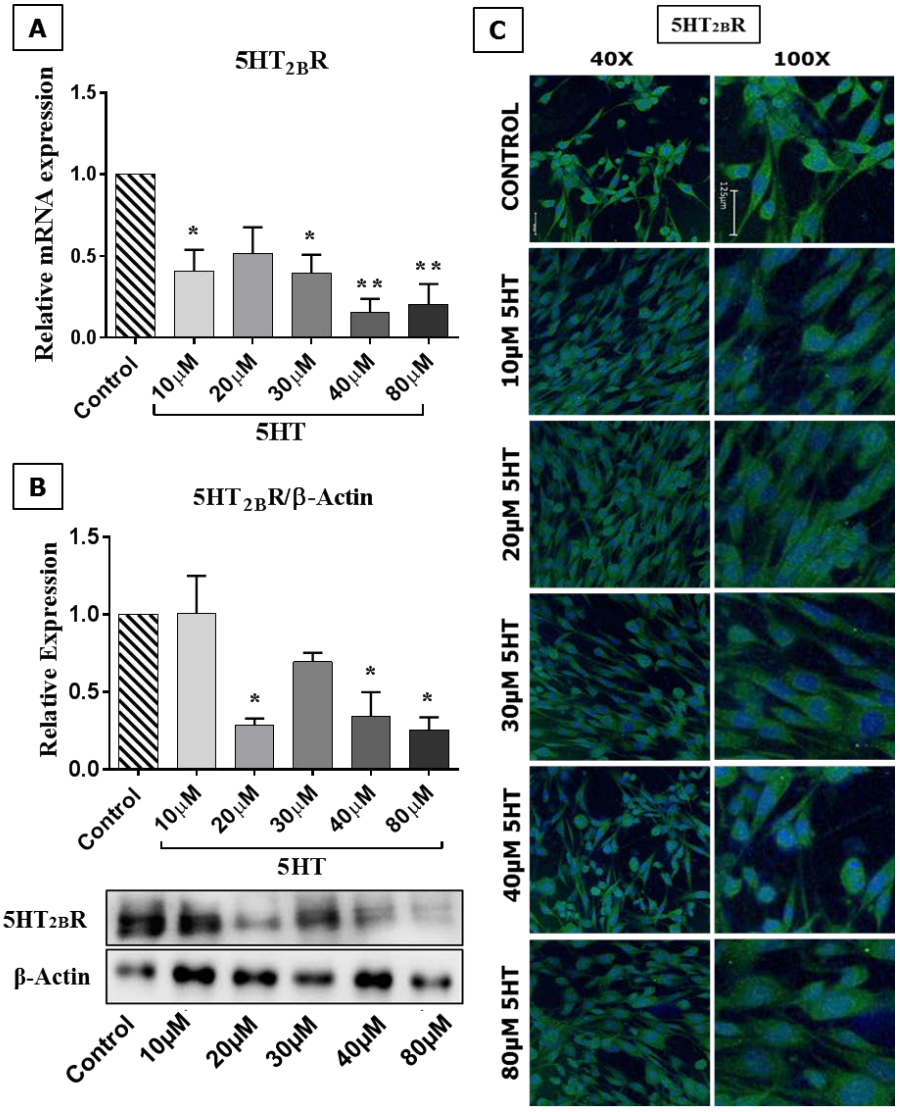


Figure 6.5: Effect of 5-HT on 5-HT$_{2B}$R expression in TEV-1 cells.

Panel A represents the relative mRNA expression of 5-HT$_{2B}$R [(n=4) ±SEM] which shows significant down-regulation in 5-HT treated cells (except 20μM). The significance is generally *p<0.05). **Panel B** represents the immunoblot analysis [(n=2) ±SEM]. Protein bands were visible at 50kDa. Significant reduction in protein concentrations were also observed with 5-HT treatment (*p<0.05). **Panel C** displays the cytoplasmic localization of 5-HT$_{2B}$R (FITC) with nuclear staining (DAPI). [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

In HTR8/SVneo cells, there was a significant up-regulation in expression of 5-HT$_{2B}$R mRNA in 5-HT treated cells. This change was not demonstrated on the basis of protein expression. Instead, the protein expression was significantly only increased following 10μM 5-HT treatment, with a gradual decrease with increasing 5-HT concentrations. Like TEV-1 cells, a cytoplasmic localisation of 5-HT$_{2B}$R is evident in immunofluorescent cytochemistry (Figure 6.6).

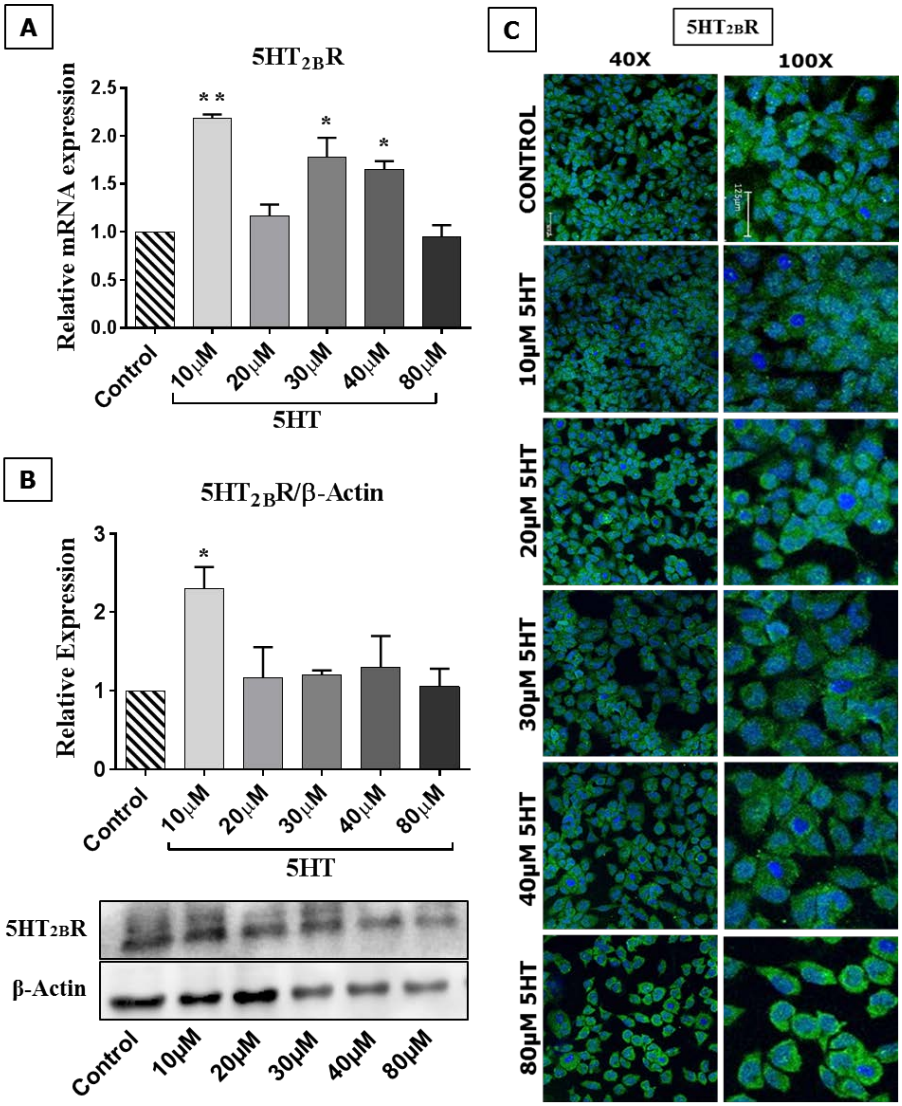


Figure 6.6: Effect of 5-HT on 5-HT$_{2B}$R expression in HTR8/SVneo cells.

Panels A and B represent the relative mRNA (n=4) and protein (n=2) expression of 5-HT$_{2B}$R. The mRNA expression was up-regulated with 10μM (**p<0.01), 30 μM (*p<0.05) and 40 μM (*p<0.05) 5-HT treatments. 10μM also shows a significant increase in protein expression. **Panel C** displays the cytoplasmic localisation of 5-HT$_{2B}$R (FITC) with nuclear staining (DAPI). [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

- **The effect of 5-HT on 5-HT$_{2B}$R expression in placental choriocarcinoma cell lines**

Although the mRNA expression of 5-HT$_{2B}$R in both BeWo and JEG3 were analysed by qRT-PCR, the expression at protein levels was confirmed only for JEG3 by western blotting. The immunofluorescent cytochemistry was unsuccessful for both cell lines. The expression patterns in JEG3 were similar to HTR8/SVneo (See Figure 6.7). A significant increase was seen with 10μM 5-HT treatment in both mRNA and protein expressions (p<0.01). However the higher concentrations of 5-HT reduced the expression of 5-HT$_{2B}$R.

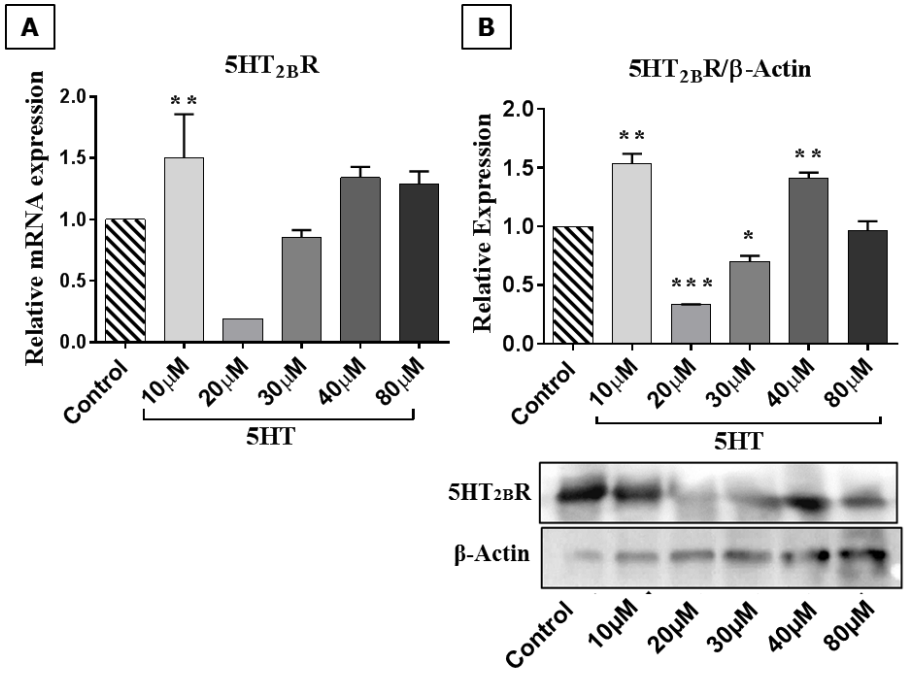


Figure 6.7: Effect of 5-HT on 5-HT$_{2B}$R expression in JEG3 cells.

Panel A and B, represent the relative mRNA (n=4) and protein (n=2) expressions of 5-HT$_{2B}$R in JEG3 respectively. The mRNA and protein expressions were significantly up-regulated with 10μM 5-HT treatment (**p<0.001). On the other hand, higher concentrations up to 30 μM of 5-HT has significantly reduced the protein expressions (***p<0.001; *p<0.05).

6.2.2 C/T antigens

The six C/T antigens; CAGE1, FATE1, GAGE1, GAGEE1, MAGEA1 and MAGEA4 were investigated at mRNA and protein levels in *in vitro*. Since the status of these antigens in any of these cell lines have not been studied before, the basal (mainly mRNA) expressions of these antigens in different placental cell lines were checked in comparison with fibrosarcoma (cancer) cell line and testis (positive; arbitrarily set to 1).

6.2.2.1 Basal expression CAGE1 in cell lines

There were no differences in the basal mRNA expression observed between the three groups of cells namely transformed trophoblast, placental choriocarcinoma and fibrosarcoma (See Figure 6.8). However, there was significant differences reported within the members of the placental choriocarcinoma group, BeWo and JEG3 (***p<0.001). The fibrosarcoma HT1080 cell line unexpectedly showed the lowest level of expression for CAGE1 when compared to placental originated cells. As expected, the mRNA expression of CAGE1 in testis sample was significantly higher when compared to all the cell lines (********p<0.0001).

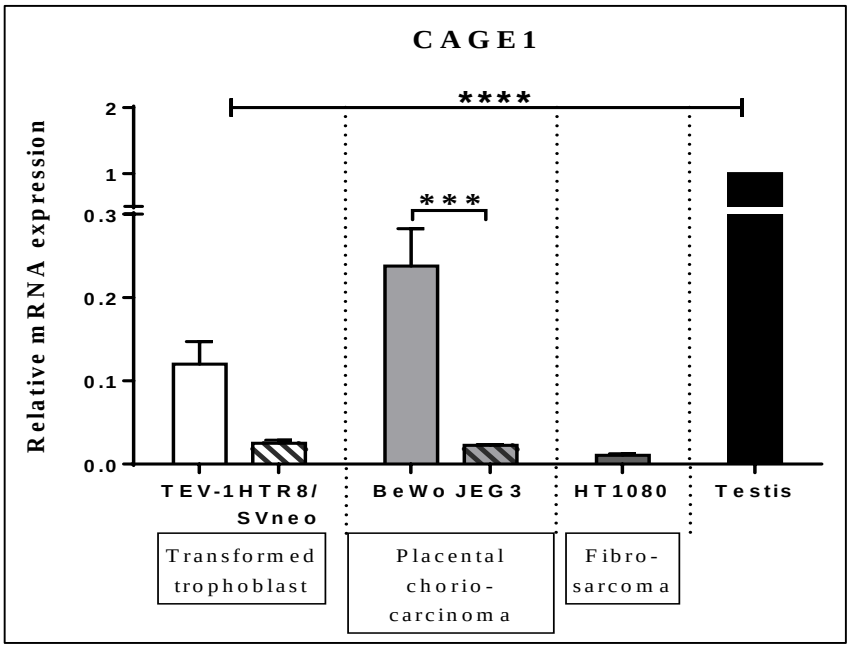


Figure 6.8: Basal mRNA expression of CAGE1 in cell lines.

The data are means of 2 experiments (each performed in triplicates; ±SEM; ****$p<0.0001$; ***$p<0.001$). The mRNA expression is relative to expression in testis (positive set as 1).

- ***The effect of 5-HT on CAGE1 expression***

Although the effect of 5-HT on CAGE1 mRNA expression were investigated in all the four cell lines, confirmation of expression at the protein level was only possible for TEV-1 and BeWo, discussed as follows.

- **The effect of 5-HT on CAGE1 expression in transformed trophoblast cell lines**

The mRNA expression of CAGE1 in TEV-1 did not correlate to the protein expression, as determined using the Western blot (See Figure 6.9.A and B). A significant down-regulation of the antigen at mRNA was seen in 5-HT treated TEV-1 cells when compared to control ($p<0.001$). However, only higher concentrations (40µM and 80µM) of 5-HT have resulted in significant reduction of CAGE1 at protein level ($p<0.05$). In contrast, there was increased expressions of the protein in 10µM ($p<0.05$) and 30µM ($p<0.01$) 5-HT treated cells. Granular cytoplasmic staining of CAGE1 can be seen in immunofluorescence cytochemistry (See Figure 6.9.C). However, there was no significant difference in staining intensities between 5-HT treated and control cells.

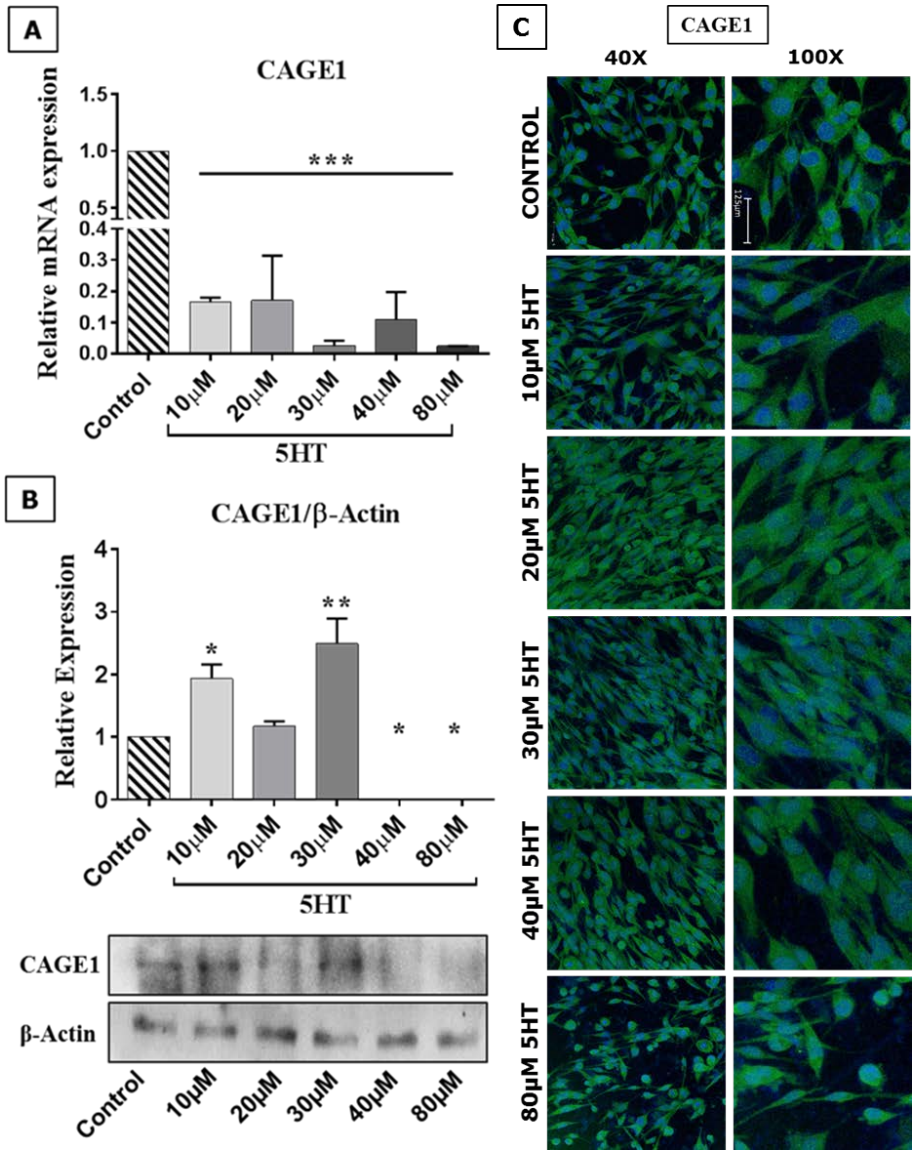


Figure 6.9: Effect of 5-HT on CAGE1 expression in TEV-1 cells.

Panel A and B represent relative mRNA (n=4) and protein (n=2) expressions of CAGE1 (±SEM; ***p<0.001; **p<0.01; *p<0.05). Single band for CAGE1 in Western blot was observed at approximately 100kDa. Granular cytoplasmic localisation of CAGE1 (FITC) was confirmed by immunofluorescent staining in **Panel C**. [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

- **The effect of 5-HT on CAGE1 expression in placental choriocarcinoma cell lines**

The expression of CAGE1 was significantly up-regulated in BeWo cells treated with 10-80μM 5-HT when compared to control. The mRNA and protein expression patterns were comparable (See Figure 6.10). A significant peak of protein expression was observed at 40μM 5-HT treatment (p<0.0001). However, at highest concentration (of 80μM) the expression levels declined.

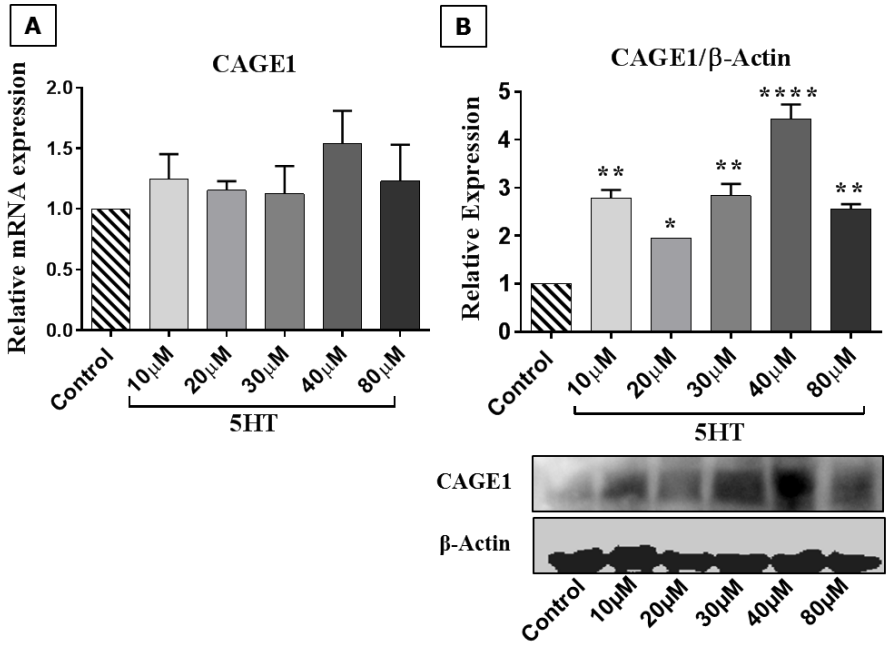


Figure 6.10: Effect of 5-HT on CAGE1 expression in BeWo cell line.

Panel A represents mRNA expression of CAGE1 in 5-HT treated cells relative to control (n=4±SEM). **Panel B** confirms the significant increase in protein expression in 5-HT treated cells (n=2 ±SEM; ****p<0.0001;**p<0.01;*p<0.05).

6.2.2.2 Basal expression of FATE1 in cell lines

The placental choriocarcinoma cell lines (BeWo and JEG3) showed a significantly higher FATE1 mRNA expression than the transformed trophoblast (⋆p<0.05) and fibrosarcoma (*p<0.05) cell lines (shown in Figure 6.11). Testis (positive control) showed a significantly higher level of expression to all the cell lines (********p<0.0001).

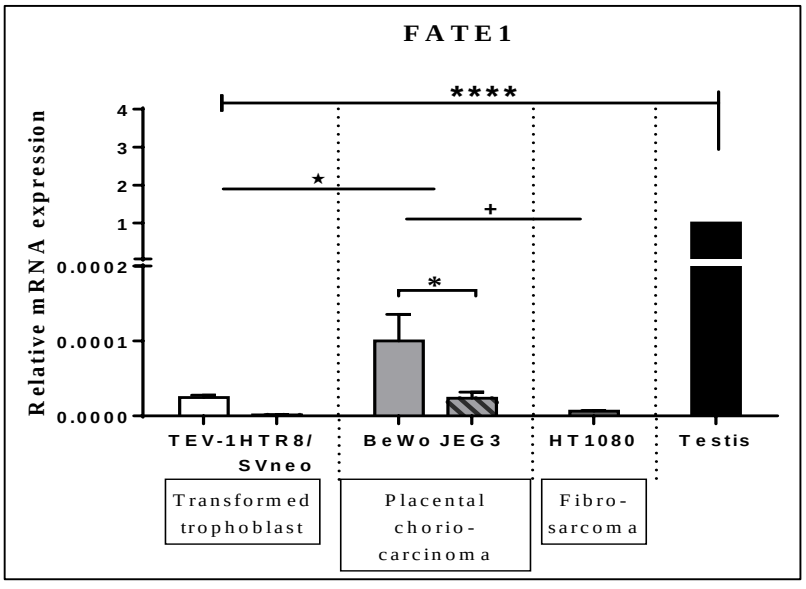


Figure 6.11: Basal mRNA expression of FATE1 in cell lines.

The data are means of 2 experiments (each performed in triplicates; ±SEM; ********p<0.0001; ⋆, +,*p<0.05). The mRNA expression is relative to expression in testis (positive set as 1)

➢ ***The effects of 5-HT on FATE1 expression***

The mRNA and protein expression of FATE1 in 5-HT treated cells were investigated. However, as stated earlier the localisation of the protein by immunofluorescence was not possible.

- **The effect of 5-HT on FATE1 expression in transformed trophoblast cell lines**

There was no correlation between the mRNA and protein expression of FATE1 in the two transformed trophoblast cell lines, TEV-1 and HTR8/SVneo. Nevertheless, both cells showed down-regulation of the FATE1 mRNA as well as protein expressions with high concentrations of 80µM 5-HT (See Figure 6.12). The protein bands on the Western blots were detected at approximately 20kDa molecular weight.

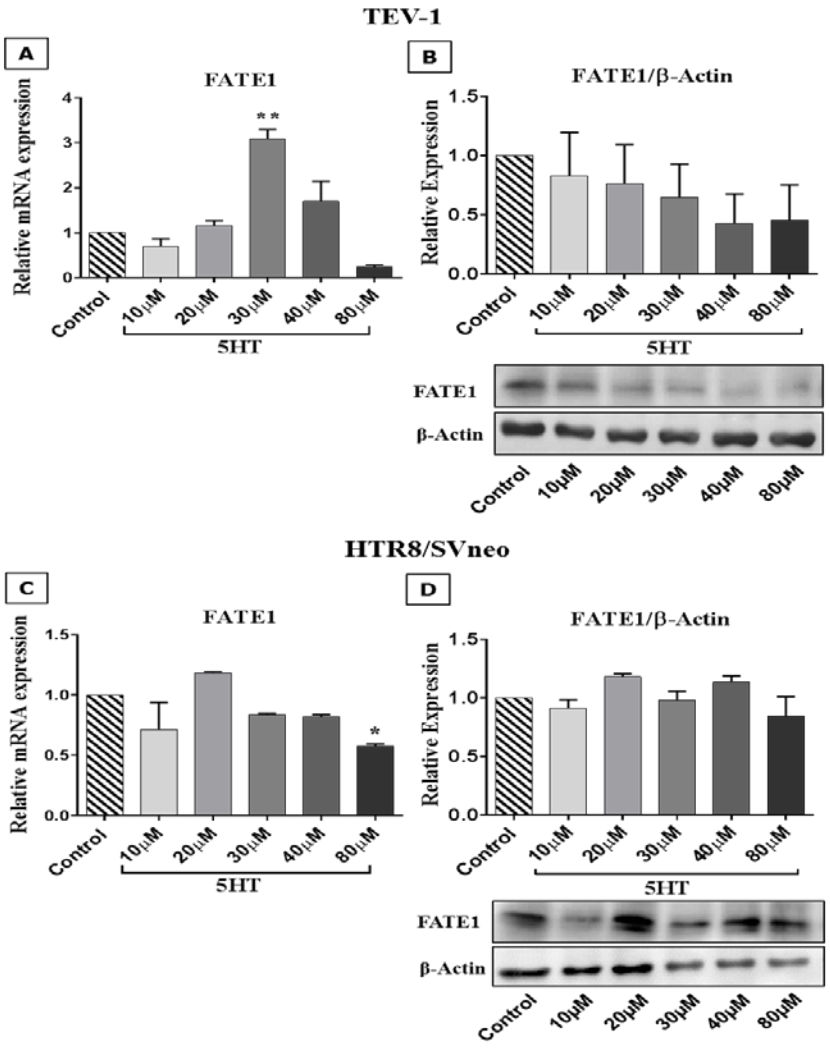


Figure 6.12: Effect of 5-HT on FATE1 expression in transformed trophoblast cells.

Panels A and B represent relative mRNA and protein expression of FATE1 in TEV-1 cells respectively (n=2; ±SEM; **p<0.01). **Panels C and D** represents relative mRNA and protein expression in HTR8/SVneo cells respectively (n=2; ±SEM; *p<0.05). No significant differences in protein expression were detected.

- **The effect of 5-HT on FATE1 expression in placental choriocarcinoma cell lines**

There was significant down-regulation of FATE1 mRNA in 10µM and 80µM 5-HT treated BeWo and JEG3 cells (See Figure 6.13.A and C). However, the mRNA expression in both the cells treated with concentrations between 20-40µM 5-HT showed minimum variations to control. As for protein expression, high concentrations of 5-HT (40µM and 80µM) have shown a non-significant reduction of FATE1. Remarkably, there was a significant up-regulation in protein expression in 20µM 5-HT treated BeWo cells. Unlike FATE1 protein expression in transformed trophoblast cells, the immunoblots of FATE1 in placental choriocarcinoma cells showed two distinct bands at 20kDa and 25kDa.

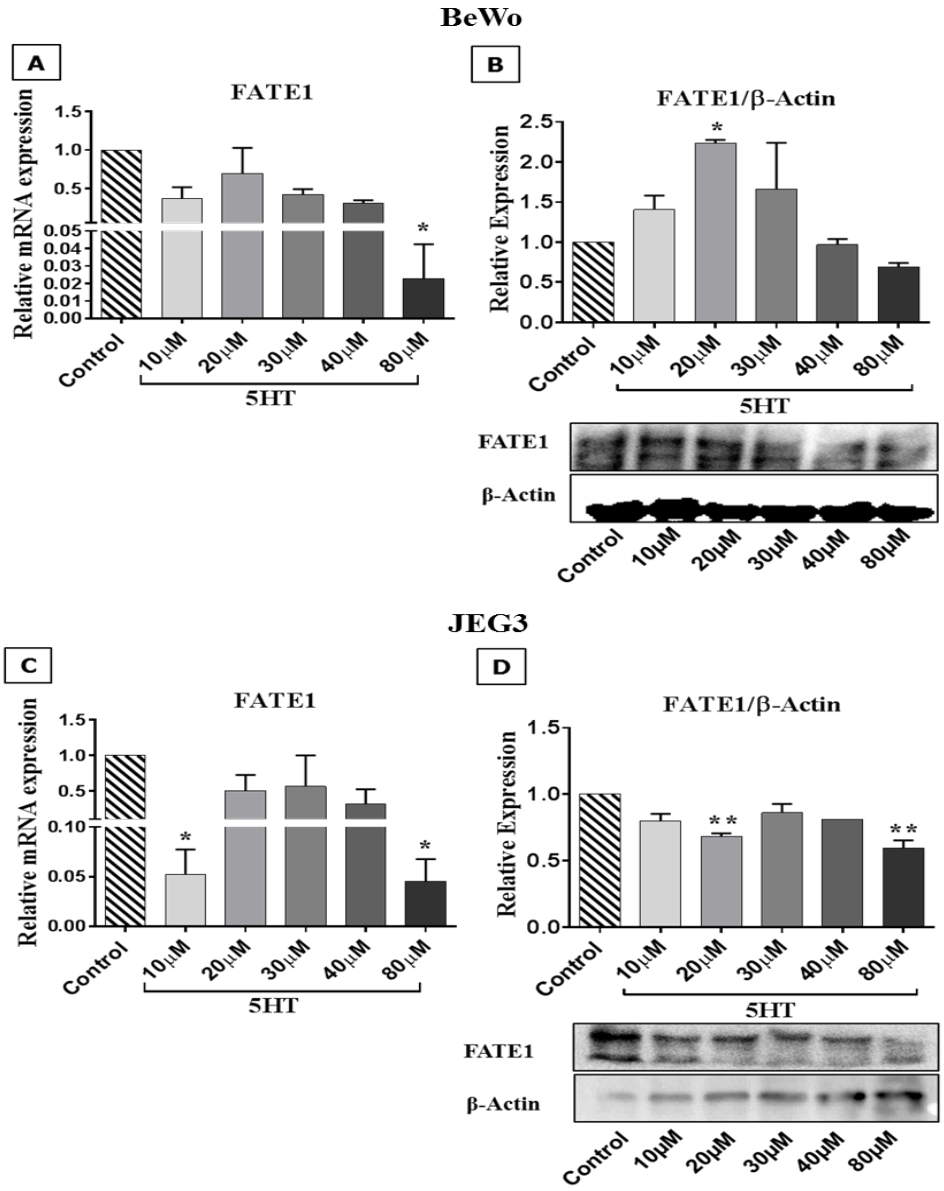


Figure 6.13: Effect of 5-HT on FATE1 expression in BeWo and JEG3 cells.

Panels A and B represent relative mRNA and protein expression of FATE1 in BeWo cells (n=2; ±SEM; *p<0.05). **Panel C and D** represents relative mRNA expression in JEG3 cells (n=2; ±SEM; *p<0.05, **p<0.01). Two distinct bands are seen in the western blot images.

6.2.2.3 GAGE family

The expression of two members of the GAGE family, GAGE1 and GAGEE1, were investigated in the cell lines.

6.2.2.3.1 Basal expression of GAGE1 in cell lines

As can be seen in Figure 6.14, there was no variation in GAGE1 mRNA expression between the transformed trophoblast and placental choriocarcinoma cell lines. The expression in human testis and HT1080 cells was significantly higher when compared to the cells originated from placenta.

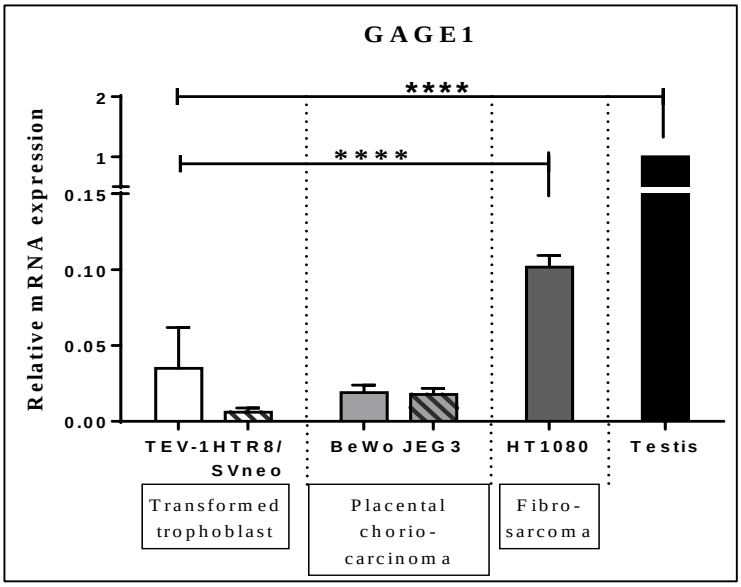


Figure 6.14: Basal mRNA expression of GAGE1 in cell lines.

The data are means of 2 experiments (each performed in triplicates; ±SEM; ****, ****$p<0.0001$). The mRNA expression is relative to expression in testis (positive set as 1).

- ***The effects of 5-HT on GAGE1 expression***

The mRNA and protein expression of GAGE1 was investigated in all the four cell lines.

- **The effect of 5-HT on GAGE1 expression in transformed trophoblast cell lines**

The 5-HT treatment altered the expression of GAGE1 in TEV-1 and HTR8/SVneo cells. In TEV-1, with 10µM 5-HT treatment, the mRNA and protein expression was reduced when compared to control (See Figure 6.15.A and B). However, with increasing concentrations of 5-HT the expression of GAGE1 was slightly increased. A significant increase was observed with 40µM 5-HT, then expression level decreased at 80µM 5-HT. Two protein bands were detected on the western blots with molecular weights between 30-40kDa, which was within the predicted range specified by the antibody supplier (Novus). Granular cytoplasmic localisation of GAGE1 was also detected with immunofluorescent staining with FITC as seen in Figure 6.15, Panel C.

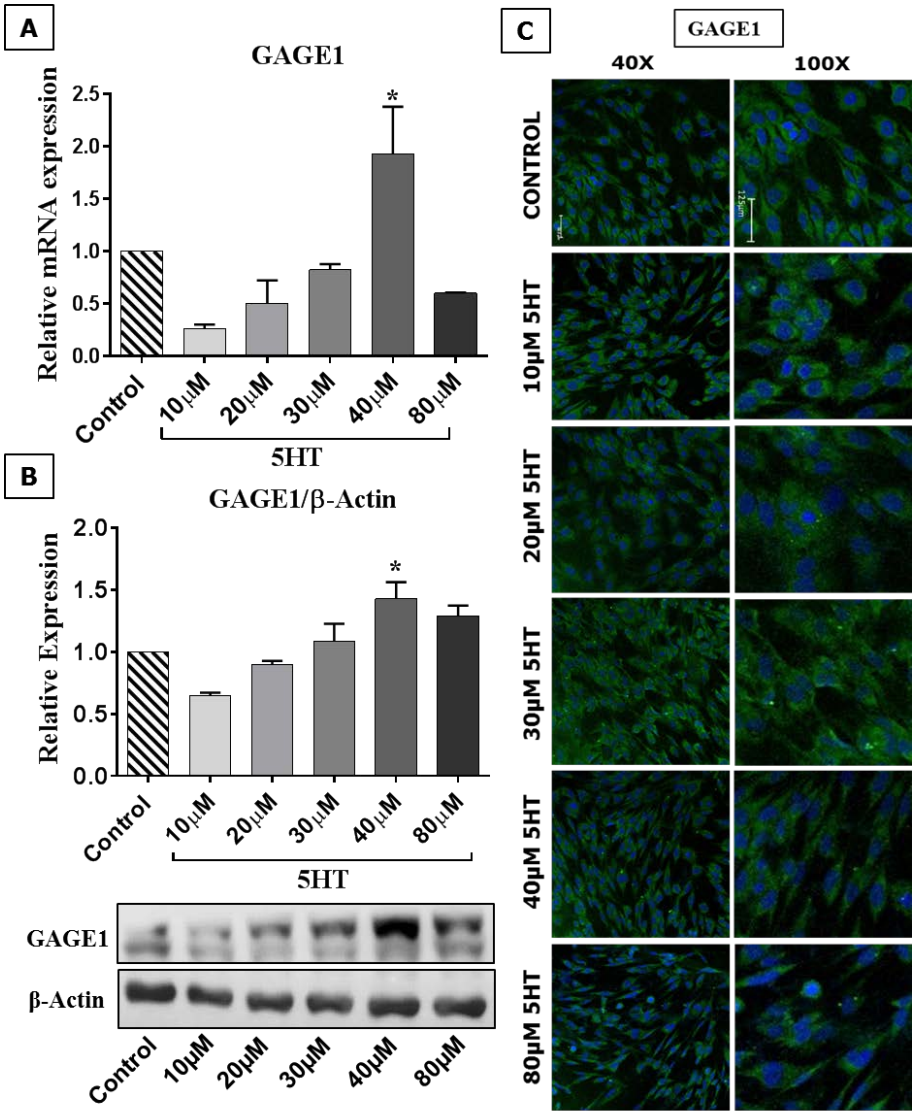


Figure 6.15: Effect of 5-HT on GAGE1 expression in TEV-1 cells.

Panels A and B represent relative mRNA (n=4) and protein (n=2) expression of GAGE1 in TEV-1 cells (±SEM; *$p<0.05$). Two bands within 30-40kDa were observed in Western blots. Granular cytoplasmic localisation of GAGE1 (FITC) was confirmed by immunofluorescent staining in **Panel C**. [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

The expression of GAGE1 in 5-HT treated HTR8/SVneo cells followed comparable expression patterns to TEV-1 (See Figure 6.16). However these differences in mRNA and protein expressions were not statistically significant except for 80μM treated. Furthermore the immunofluorescent labelling of GAGE1 revealed very faint marginal staining of the cytoplasm in HTR8/SVneo.

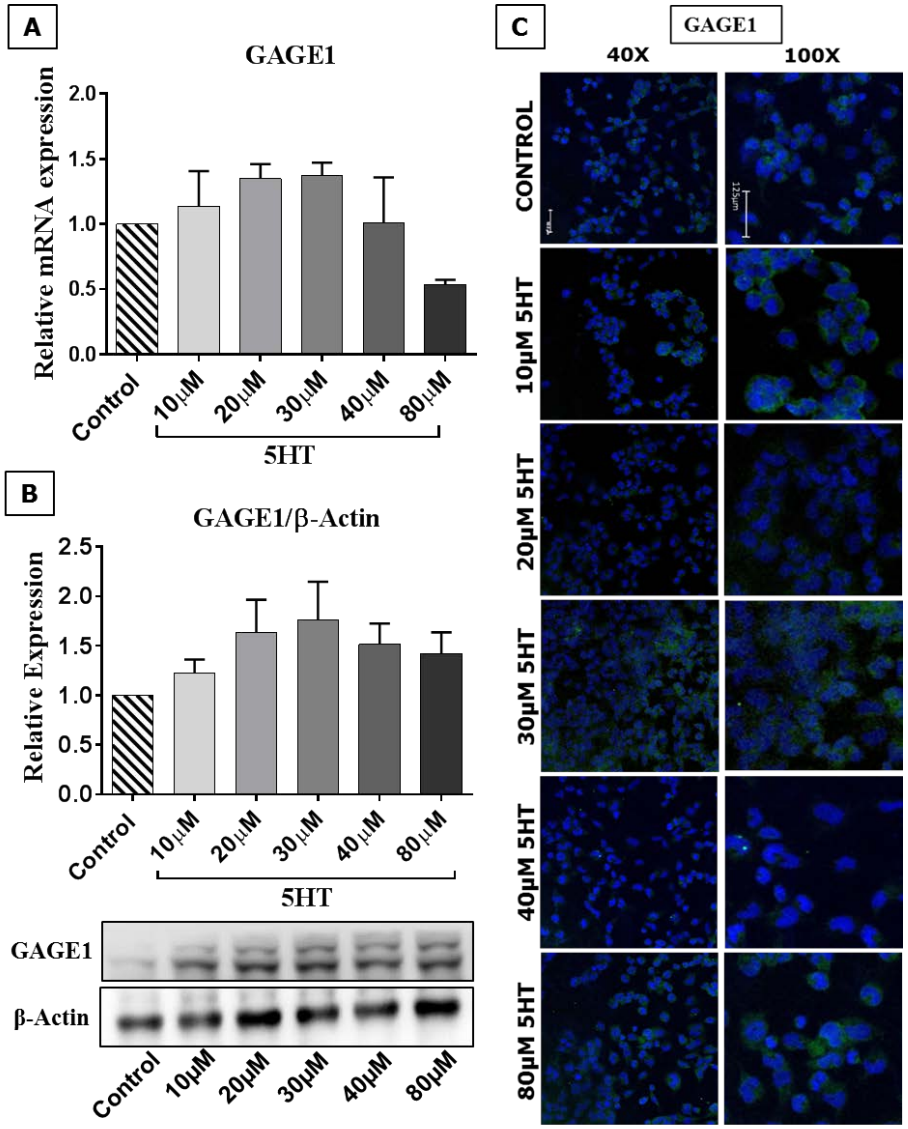


Figure 6.16: Effect of 5-HT on GAGE1 expression in HTR8/SVneo cells.

Panels A and B represents relative mRNA (n=4) and protein (n=2) expression of GAGE1 in HTR8/SVneo cells (±SEM; *p<0.05). The immunofluorescent labelling of GAGE1 (FITC) reported a marginal staining of the cytoplasm (**Panel C**). [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

- **The effect of 5-HT on GAGE1 expression in placental choriocarcinoma cell lines**

In BeWo cells, the mRNA and protein expressions of GAGE1 was observed to have increased with increasing concentration of 5-HT (See Figure 6.17.A and B). Compared to control, the level of mRNA expression was significantly up-regulated in 80μM 5-HT treatment. For protein expression, both 20μM as well as 80 μM treated BeWo cells showed significant increase relative to control. On the contrary, the expression of GAGE1 was significantly down-regulated in JEG3 cells treated with higher concentrations of 5-HT; especially with 80μM (See Figure 6.17.C and D). Although the commercial antibody was able to detect the proteins in Western blot, it was unable to detect the localisation of GAGE1 by immunofluorescence.

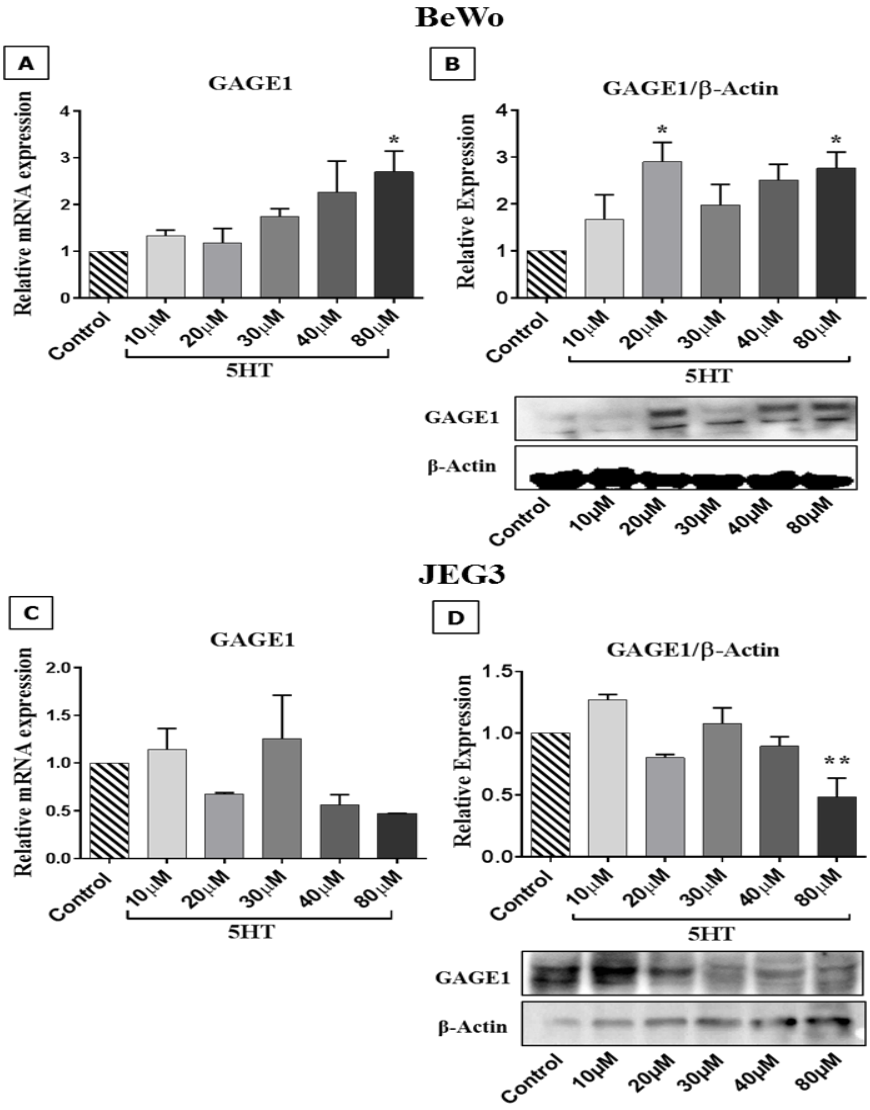


Figure 6.17: Effect of 5-HT on GAGE1 expression in BeWo and JEG3 cells.

Panels A and B represent the relative mRNA and protein expression of GAGE1 in BeWo cell line respectively (*p<0.05). **Panels C and D** represent the relative mRNA and protein expression in JEG3 cells (**p<0.01).

6.2.2.3.2 Basal expression of GAGEE1 in cell lines

The mRNA expression of the second member of GAGE family, GAGEE1 (also known as PAGE5), was lower in transformed trophoblast cells (TEV-1 and HTR8/SVneo) when compared to placental choriocarcinoma cell lines (BeWo and JEG3). Furthermore, the expression was significantly lower when compared to fibrosarcoma (HT1080; ★★p<0.01) and testis (★★★★p<0.0001) (See Figure 6.18). Furthermore, the mRNA expression of GAGEE1 in placental choriocarcinoma group (★★★p<0.001) and HT1080 (★p<0.05) was significantly lower when compared to testis.

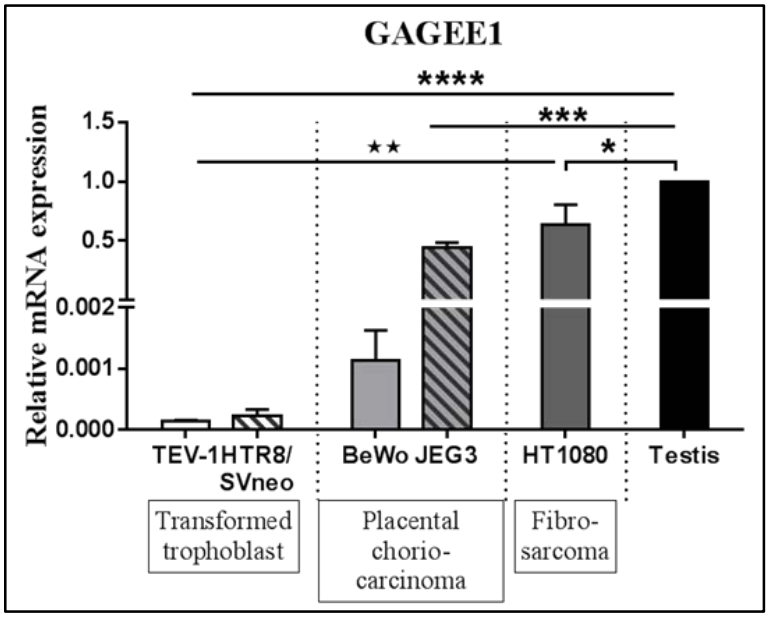


Figure 6.18: Basal mRNA expression of GAGEE1/PAGE5 in cell lines.

The data are means of 2 experiments (each performed in triplicates; ±SEM; ********p<0.0001; *******p<0.001; ******p<0.01; *****p<0.05]. The mRNA expression is relative to expression in testis (positive set as 1)

- ***The effects of 5-HT on GAGEE1 expression***

The mRNA and protein expression of GAGEE1/PAGE5 was verified in all the four cell lines. Although JEG3 showed higher mRNA expression to other cell lines in basal expression as discussed above, it was not detected by immunofluorescent labelling.

- **The effects of 5-HT on GAGEE1 expression in transformed trophoblast cell lines**

A bell shaped (or Gaussian distribution) GAGEE1 expression pattern was observed in both TEV-1 and HTR8/SVneo cells; with 10µM and 80µM 5-HT treated cells showing the lowest levels of expression. Although both the mRNA and protein expressions showed bell shaped pattern with increasing concentrations of 5-HT in TEV-1, the mRNA expression was significantly down-regulated in 5-HT treated cells when compared to control. However, the protein expressions in the same cells were increased when compared to control (See Figure 6.19.A and B). Nevertheless, the significance was only observed in 30µM 5-HT treated cells for both mRNA and protein expressions ($p<0.05$). A single protein band of approximately 20kDa in molecular weight was detected by Western blotting. Immunofluorescent labelling of GAGEE1 with FITC revealed cytoplasmic localisation of the protein in TEV-1 (See Figure 6.19.C). There was no significant reduction in staining intensities detected in 5-HT treated cells and control.

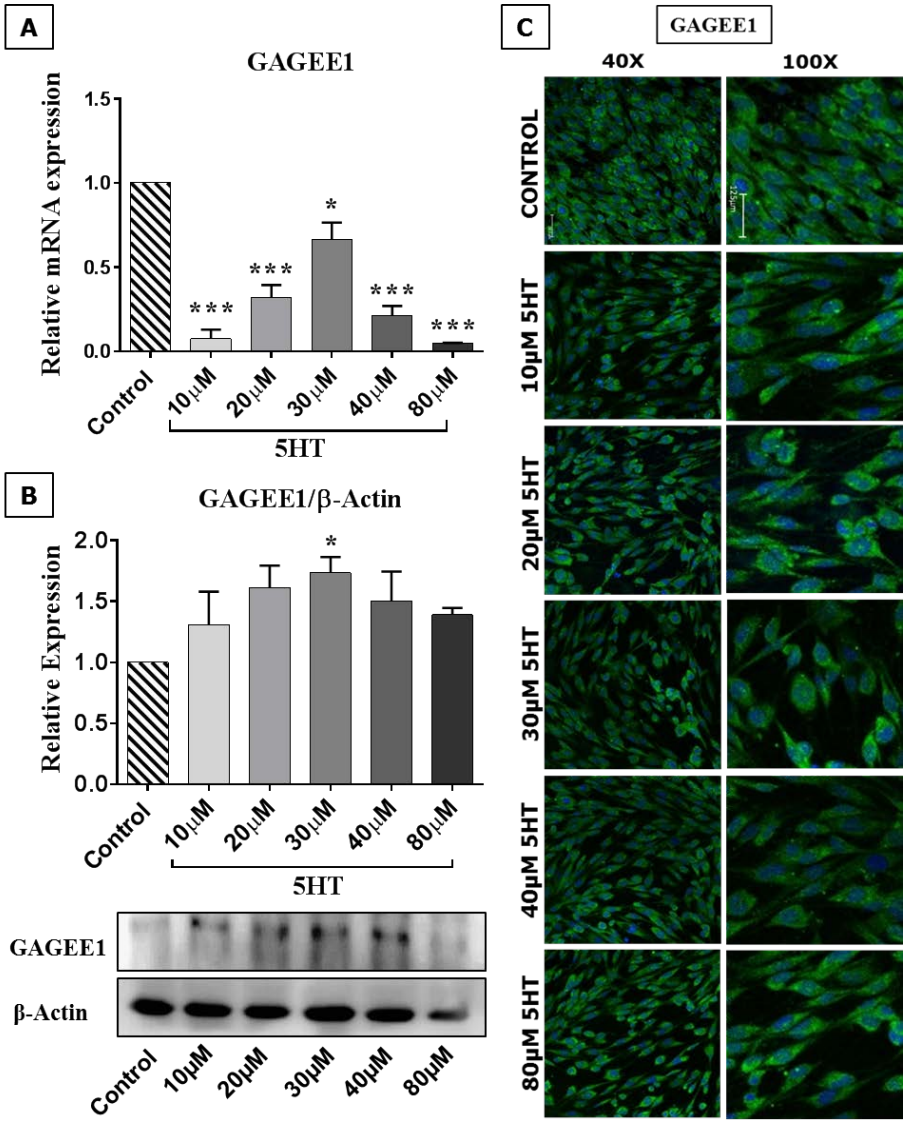


Figure 6.19: Effect of 5-HT on GAGEE1 expression in TEV-1 cells.

Panels A and B represent relative mRNA (n=4) and protein (n=2) expression of GAGEE1 in TEV-1 cells (±SEM; ***p<0.001; *p<0.05). A single protein band of ~20kDa was observed in Western blots. Granular cytoplasmic localisation of GAGEE1 (FITC) can be seen in the immunofluorescent staining in **Panel C** counter stained with DAPI for nucleus. [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

In HTR8/SVneo cells, the expression levels of both GAGEE1 mRNA and protein were up-regulated by 5-HT treatment when compared to control (See Figure 6.20). A significance was observable with 30μM 5-HT treatment ($p<0.05$). The staining was observed on overall cytoplasmic regions of the cells.

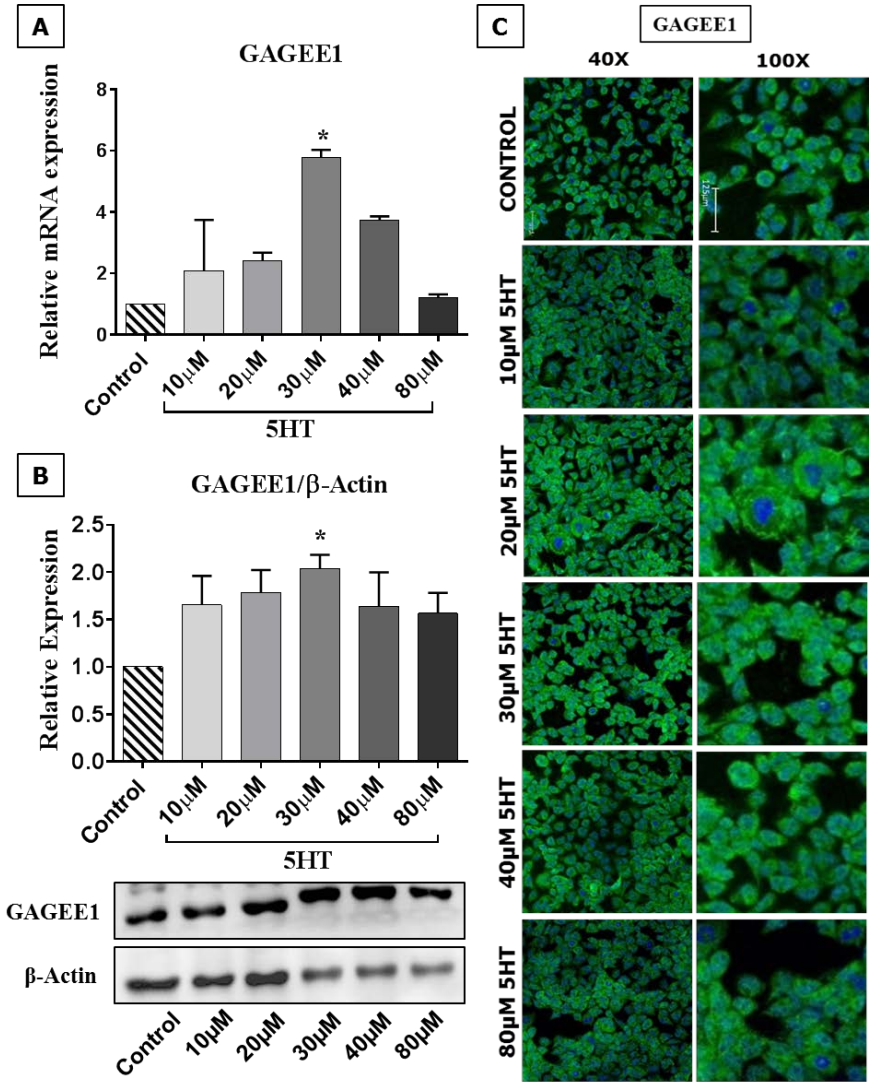


Figure 6.20: Effect of 5-HT on GAGEE1 expression in HTR cells.

Panels A and B represent relative mRNA (n=4) and protein (n=2) expression of GAGEE1 in HTR/SVneo cells, with significant up-regulation at 30μM 5-HT treatment (±SEM; *p<0.05). Intense staining for GAGEE1 (FITC) was observed in the cytoplasmic region of the cells (**Panel C**). [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

- **The effect of 5-HT on GAGEE1 expression in placental choriocarcinoma cell lines**

There was a variation in expression of GAGEE1 observed in the two placental choriocarcinoma cell lines (BeWo and JEG3). The 5-HT treated BeWo cells expressed a similar bell shaped (Gaussian distribution) pattern with increasing concentrations of 5-HT as in transformed trophoblast cells (See Figure 6.21.A and B). At lower (10μM) and higher concentrations (40μM and 80μM) of 5-HT, the expression of GAGEE1 was down-regulated when compared to control. However, the mRNA as well as protein expression of GAGEE1 were significantly increased with 30μM 5-HT treatment relative to control. Remarkably, of all the proteins investigated, only GAGEE1 protein was detected by immunofluorescent staining in BeWo cell lines and, interestingly, the staining were observed to be localised at certain regions of the cytoplasm, as illustrated from Figure 6.21.C.

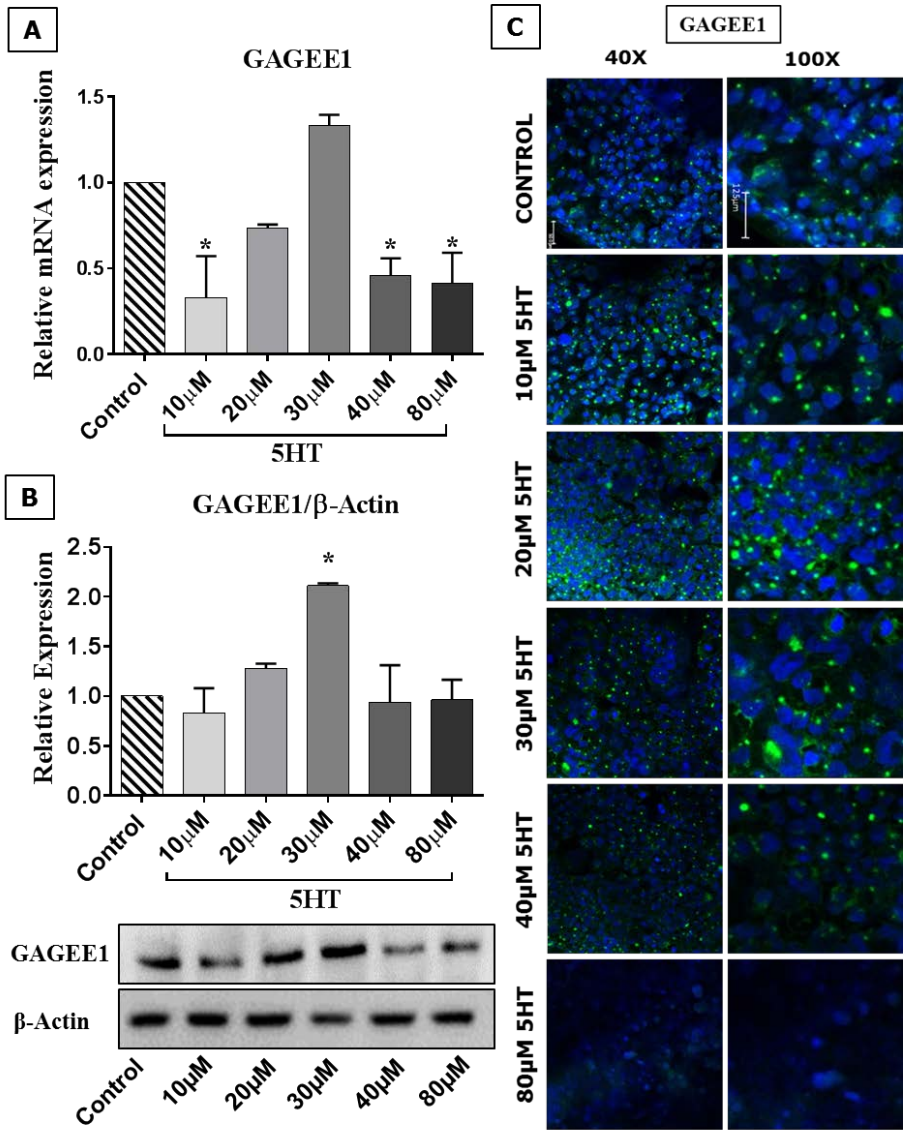


Figure 6.21: Effect of 5-HT on GAGEE1 expression in BeWo cells.

Panels A and B represent relative mRNA (n=4) and protein (n=2) expression of GAGEE1 in BeWo, with significant up-regulation at 30μM 5-HT treatment (±SEM; *$p<0.05$). Localised staining of GAGEE1 (FITC) of the cytoplasm can be seen in **Panel C**. [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

Although the GAGEE1 mRNA expression in JEG3 showed similar bell shaped (Gaussian distribution) expression pattern as BeWo, the protein expression did not reveal any comparable results. The two expression patterns were almost contrasting, as is illustrated in Figure 6.22. The 10μM and 80μM 5-HT treatments resulted in down-regulation of mRNA expression, but significantly increased the protein expressions when compared to control ($p<0.001$; $p<0.01$).

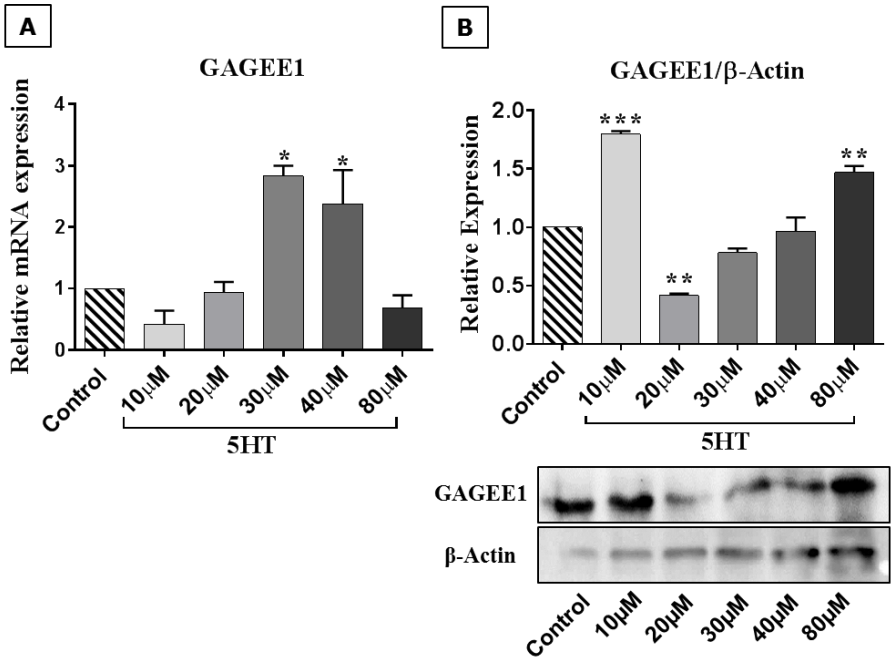


Figure 6.22: Effect of 5-HT on GAGEE1 expression in JEG3 cells.

Panel A shows a significant up-regulation of GAGEE1 mRNA expression in 30μM and 40μM 5-HT treated JEG3 cells when compared to control [(n=4) ±SEM; *p<0.05]. As for protein expression, 10μM and 80μM 5-HT treated cells showed a significant up-regulation **Panel B** [(n=2) ±SEM; ***p<0.001; **p<0.01].

6.2.2.4 MAGE family

The expression of two members of MAGE family, MAGEA1 and MAGEA4, were investigated in the cell lines. The protein expression of these antigens were not detected in placental choriocarcinoma cell lines, hence only expression analysis in transformed cells (TEV-1 and HTR8/SVneo) is discussed as follows.

6.2.2.4.1 Basal expression of MAGEA1 in cell lines

When the overall mRNA expression of MAGEA1 in the cell lines were compared, a significant difference in expression was observed between the transformed trophoblast and placental choriocarcinoma groups (★★p<0.01; See Figure 6.23). It was also worth noting that the mRNA expression in the fibrosarcoma cell line, HT1080, was remarkably higher when compared to the other cell lines (****p<0.0001), as well as to the positive control (testis; *****p<0.05).

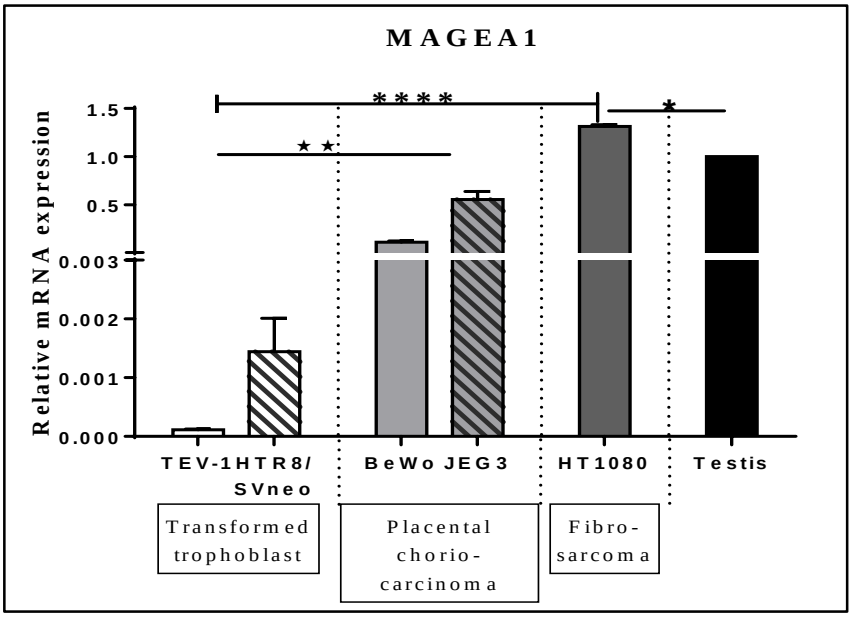


Figure 6.23: Basal mRNA expressions of MAGEA1 in cell lines.

The data are means of 2 experiments (each performed in triplicates; ±SEM; ********p<0.0001; ★★p<0.01; *****p<0.05]. The mRNA expression is relative to expression in testis (positive set as 1)

- ***The effects of 5-HT on MAGEA1 expression analysis***

The mRNA and protein expression of MAGEA1 was observed to be down-regulated in 5-HT treated TEV-1 cells when compared to control. There was a significant decline in the expression with increasing concentrations of 5-HT (See Figure 6.24). Two protein bands were observed on the Western blots, with molecular weights approximately 34kDa and 36kDa. The predicted molecular weight supplied by the antibody provider was 34kDa. Granular cytoplasmic presence of the protein was indicated by fluorescent labelling of the protein by FITC.

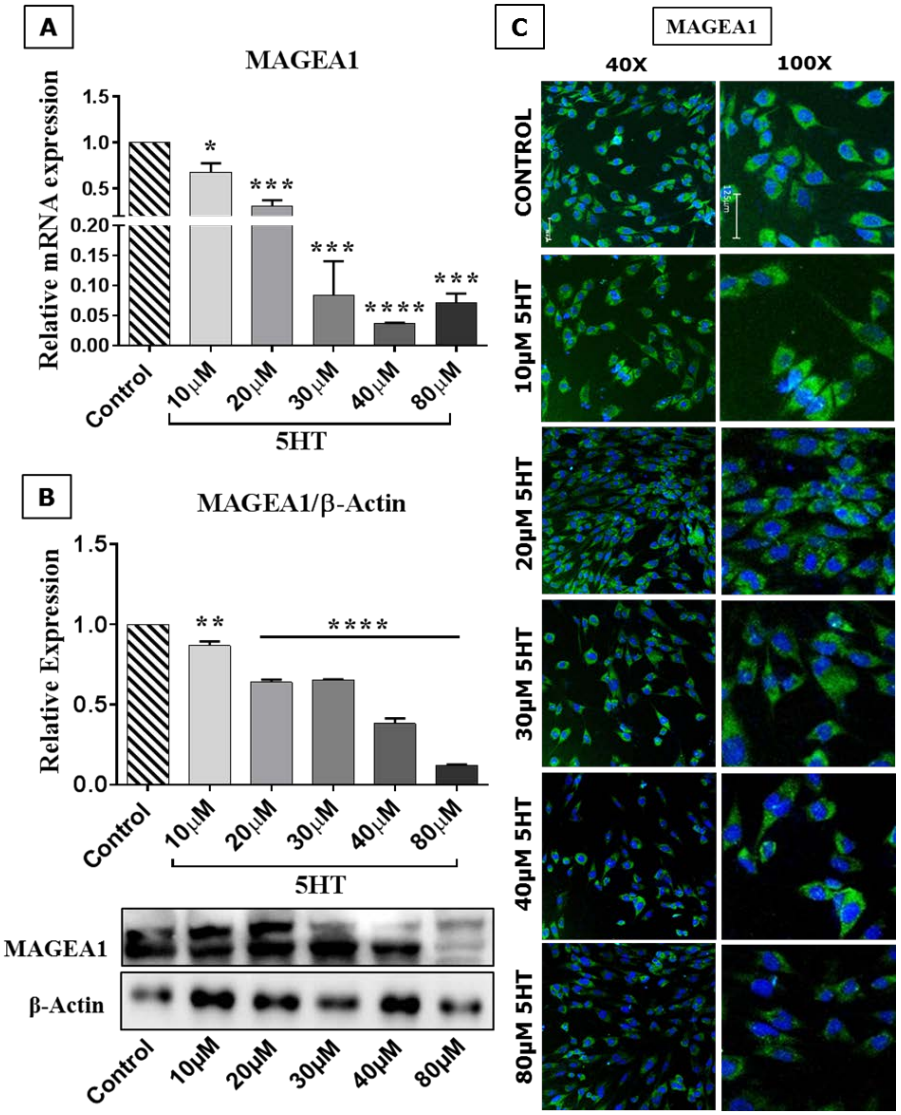


Figure 6.24: Effect of 5-HT on MAGEA1 expression in TEV-1 cells.

Panels A and B show a significant decline in mRNA and protein expression of MAGEA1 in 5-HT treated TEV-1 cells respectively (****p<0.0001; ***p<0.001; **p<0.01; *p<0.05). **Panel C** shows the cytoplasmic localisation of the protein in the cells. [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

The mRNA and protein expressions of MAGEA1 in 10μM 5-HT treated HTR8/SVneo were significantly increased when compared to control ($p<0.05$; See Figure 6.25.A and B). However, the expression declined at higher 5-HT concentrations. A statistically significant down-regulation was observed only for 80μM 5-HT treated HTR8/SVneo cells ($p<0.05$). The two protein bands at 34kDa and 36kDa were also detected in HTR8/SVneo cells. Granular staining in the cytoplasm was observed for MAGEA1 protein.

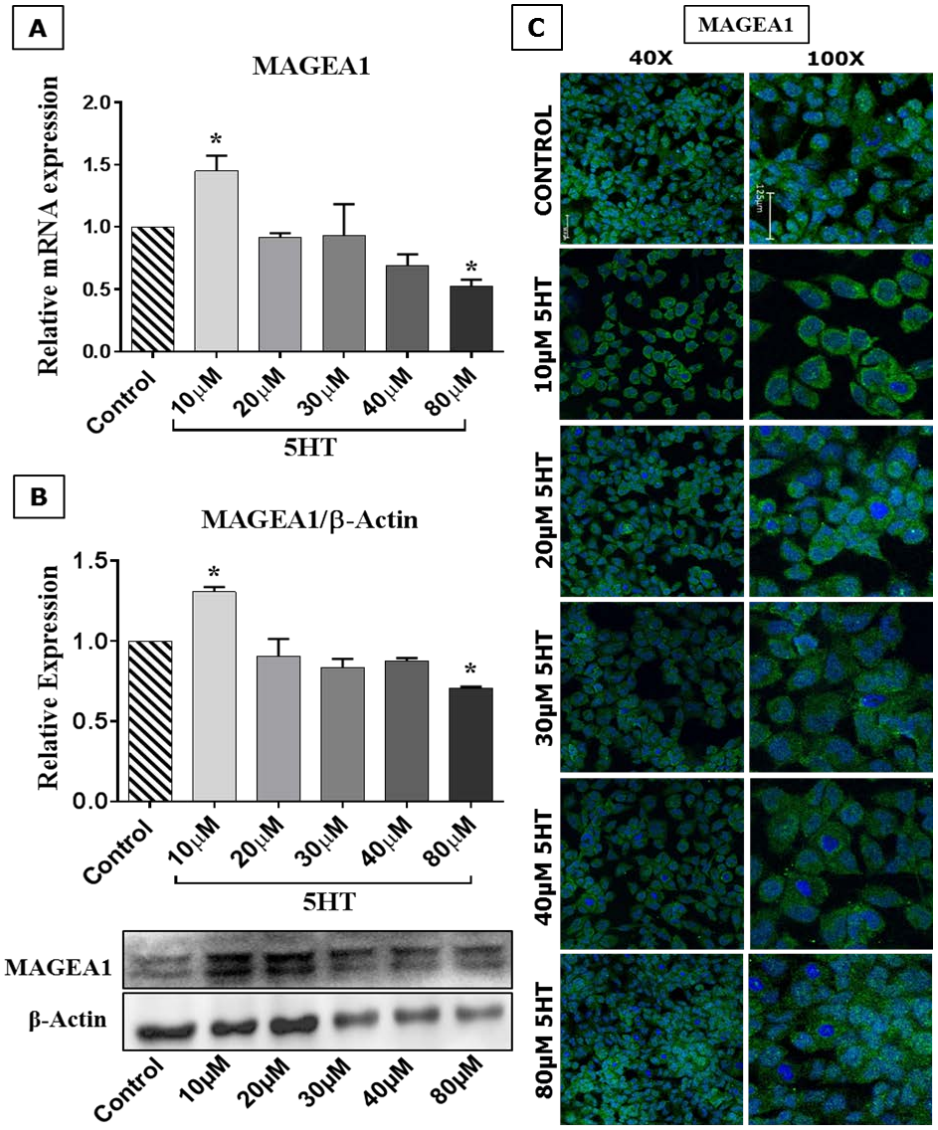


Figure 6.25: Effect of 5-HT on MAGEA1 expression in HTR8/SVneo cells.

Panels A and B show significant changes in mRNA and protein expression of MAGEA1 in 10μM and 80μM 5-HT treated HTR8/SVneo cells relative to control (*p<0.05). **Panel C** shows the cytoplasmic localisation of the protein labelled with FITC in the cells. [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

6.2.2.4.2 Basal expression of MAGEA4 in cell lines

There were no variations in the mRNA expressions of MAGEA4 between the three cell groups (See Figure 6.26). However, there were variations in expressions within the group members, with significant differences in expression between BeWo and JEG3 (****p<0.0001). The expression was also significantly higher in testis when compared to all the cell lines.

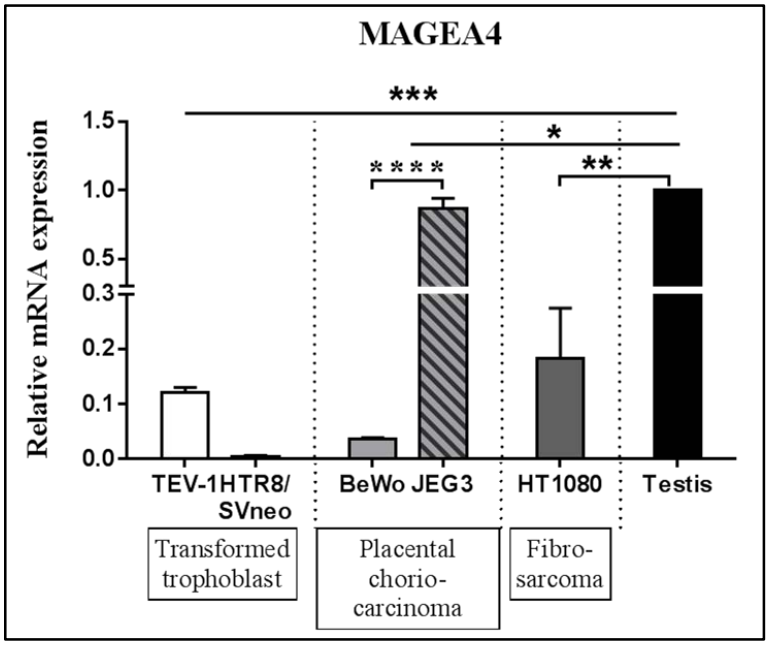


Figure 6.26: Basal mRNA expression of MAGEA4 expression in cell lines.

The data are means of 2 experiments (each performed in triplicates; ±SEM**;******p<0.0001; *******p<0.001; ******p<0.01; *****p<0.05]. The mRNA expression is relative to expression in testis (positive set as 1)

- ***The effects of 5-HT on MAGEA4 expression analysis***

Although MAGEA1 and MAGEA4 belong to the same family of antigens, the expressions patterns for the two were different in the transformed trophoblast cell lines. Like MAGEA1, the expression of MAGEA4 was down-regulated in 5-HT treated TEV-1 cells compared to control (See Figure 6.27.A). When the expressions between the different doses of 5-HT were compared, the 10µM 5-HT showed the lowest expression of MAGEA4 and the levels increased with increasing concentrations of 5-HT. A single protein band was observed at approximately 35kDa by western blot investigation and at 80µM 5-HT treatment a significant up-regulation of MAGEA4 protein was detected (p<0.05). Overall, weak granular staining of the protein was shown by immunofluorescent labelling with few intense focalised regions.

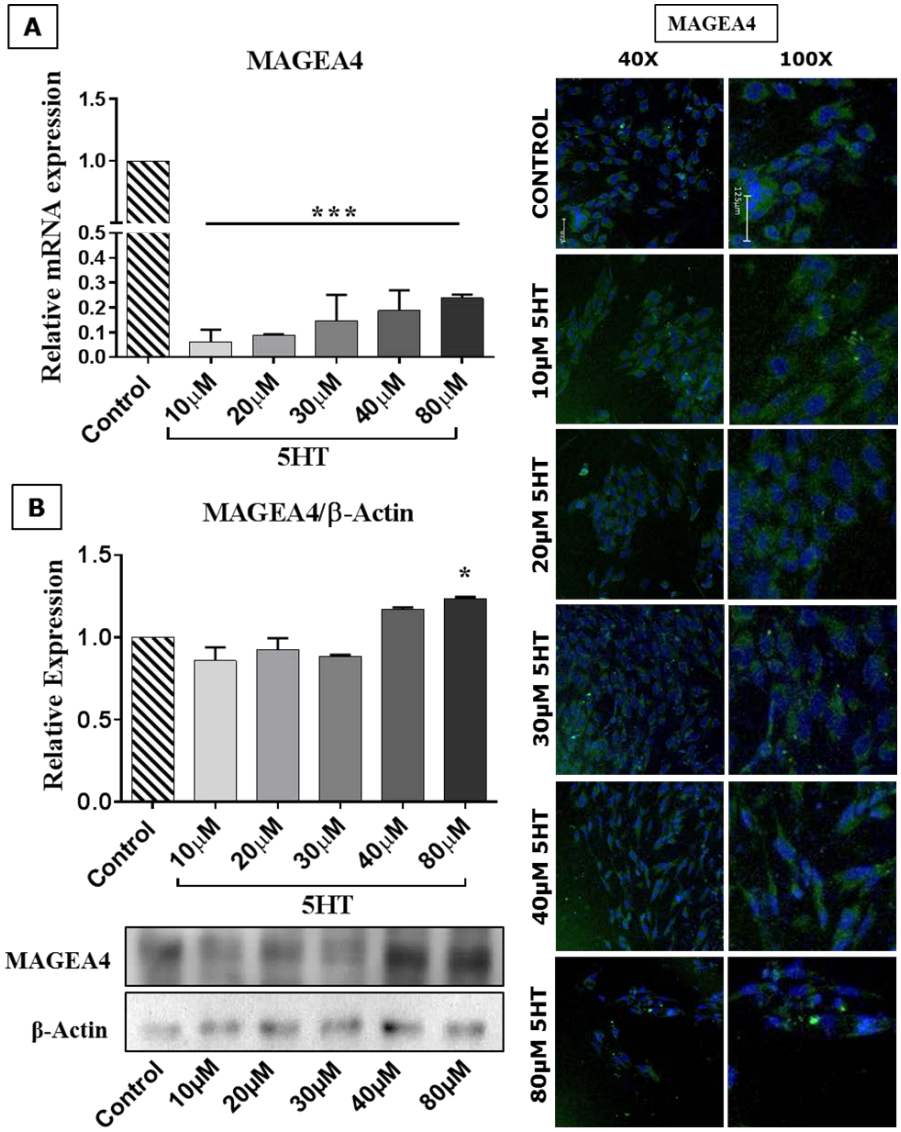


Figure 6.27: Effect of 5-HT on MAGEA4 expression in TEV-1 cell line.

Panel A represents the gradual increase in mRNA expressions with increasing concentration of 5-HT treatment in TEV-1 cells (n=4±SEM). **Panel B** represents the significant increase in the protein expression with higher concentrations of 5-HT (n=2±SEM; *$p<0.05$). Bands were observed at ~35kDa. Granular cytoplasmic staining of the MAGEA4 protein can be seen in **Panel C**. [Objective magnification: 40X (Scale bar= 50μm) and 100X (Scale bar= 125μm)]

In HTR8/SVneo cells, except for 30μM, the remaining 5-HT concentrations showed no significant variations in the mRNA and protein expression of MAGEA4 when compared to control (See Figure 6.28.A and B). The 30μM 5-HT treated cells showed a down-regulation in the mRNA ($p<0.05$) and protein expressions (not significant). The MAGEA4 protein was located in the cytoplasm of the cells.

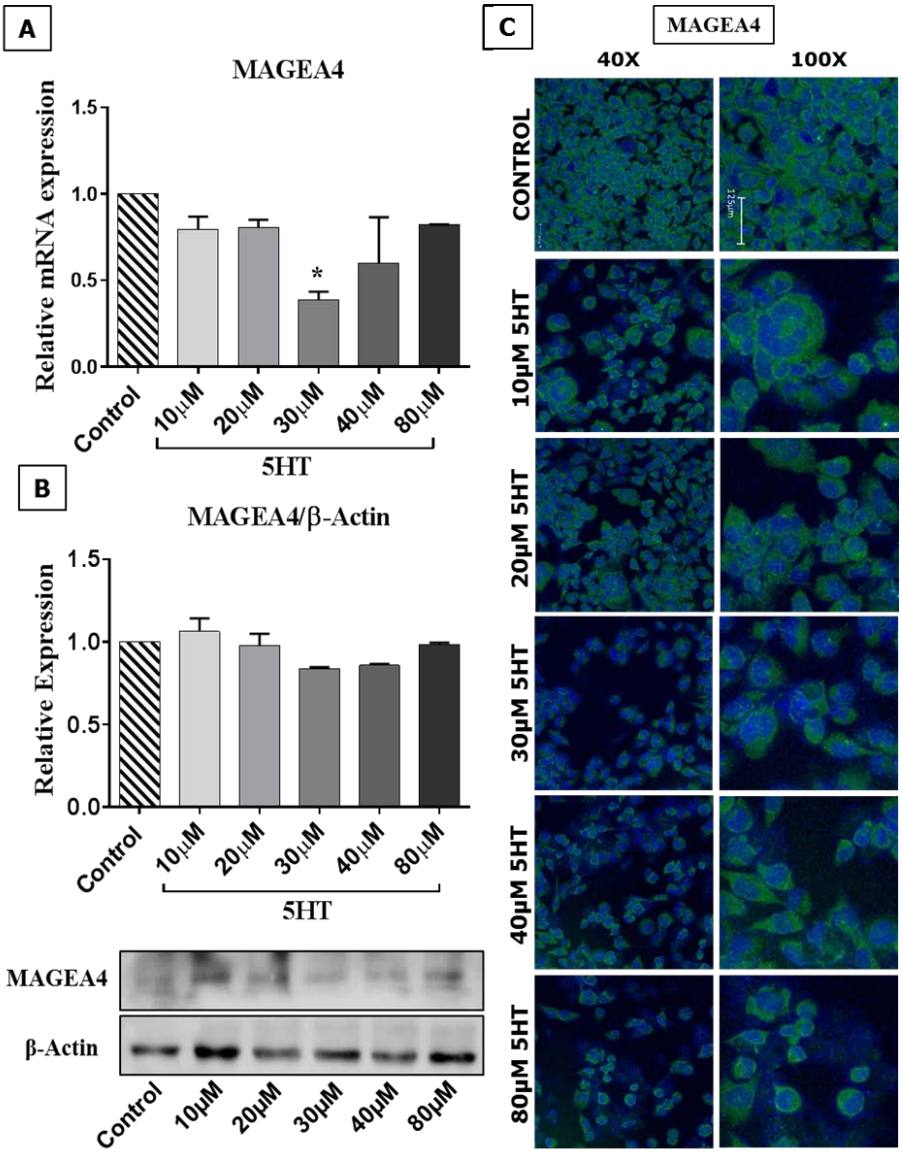


Figure 6.28: Effect of 5-HT on MAGEA4 expression in HTR8/SVneo cell line.

Panel A shows a significant down-regulation of mRNA expression in 30µM 5-HT treated HTR8/SVneo cells when compared to control (n=4±SEM; *p<0.05). Down-regulation in the protein expression (but not significant) was also observed at the same 30µM 5-HT treated cells, as shown in **Panel B** (n=2). **Panel C** shows the granular cytoplasmic staining of the protein MAGEA4 (FITC). [Objective magnification: 40X (Scale bar= 50µm) and 100X (Scale bar= 125µm)]

6.2.2.5 Basal mRNA expression of PASD1 in cell lines

Overall, there was no significant variation in mRNA expression among the three cell groups, transformed trophoblast, placental choriocarcinoma and fibrosarcoma (See Figure 6.29). However, there were variations among the group members like placental choriocarcinoma, where the mRNA expressions were significantly higher in BeWo cells when compared to JEG3 cells (p<0.0001). Interestingly, the expressions in BeWo was the highest in the investigation. PASD1 was investigated only at the mRNA level, as the proteins were not detectable by Western blotting with the commercial antibody which was available for study.

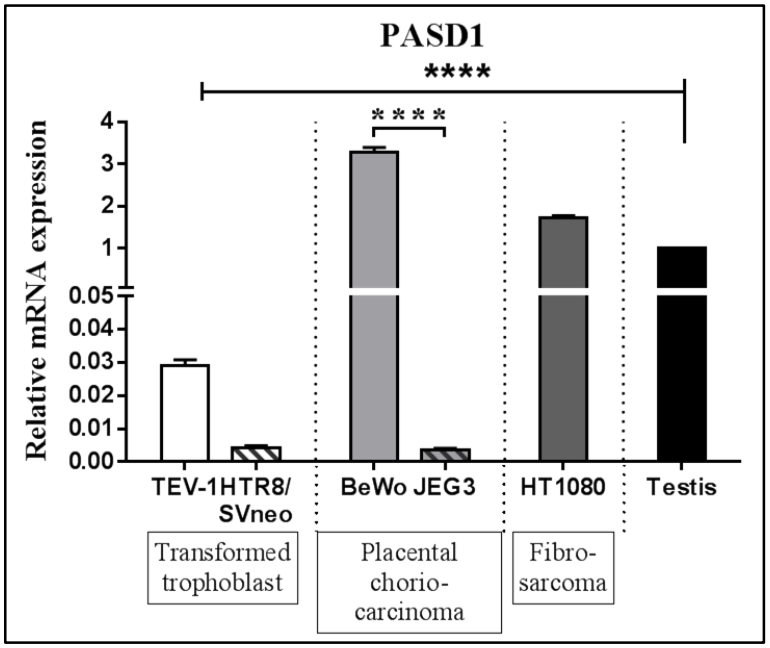


Figure 6.29: Basal mRNA expression of PASD1 in cell lines.

The data are means of 2 experiments (each performed in triplicates; ±SEM; ********p<0.0001; ********p<0.0001). The mRNA expression is relative to expression in testis (positive set as 1).

6.2.3 Relative mRNA expressions under hypoxic condition

As seen in Section 5.2.3, hypoxic conditions (2% v/v O_2) altered the metabolic, proliferative, migrative and invasive effects induced by 5-HT on the cell lines. After confirmation of the effect of 5-HT on gene expression above, the basal mRNA expressions of these genes under hypoxic conditions were also investigated (See Figure 6.30). The mRNA expression in the cell lines were evaluated relative to expression in positive controls (brain for 5-HT receptors and testis for C/T antigens). The mRNA expression under normoxia and hypoxia were then compared. Variance in mRNA expression was analysed using two-way ANOVA followed by Sidak's test for comparison under normoxia and hypoxia. Significant up-regulation was observed in mRNA expression of most of the receptors and antigens under hypoxic conditions, except for PASD1 which was down-regulated in BeWo cells under hypoxia. Thus, hypoxia altered the mRNA expression of these important receptors and antigens in the placental cell lines. These preliminary results need to be confirmed at the protein level (See Section 7.4).

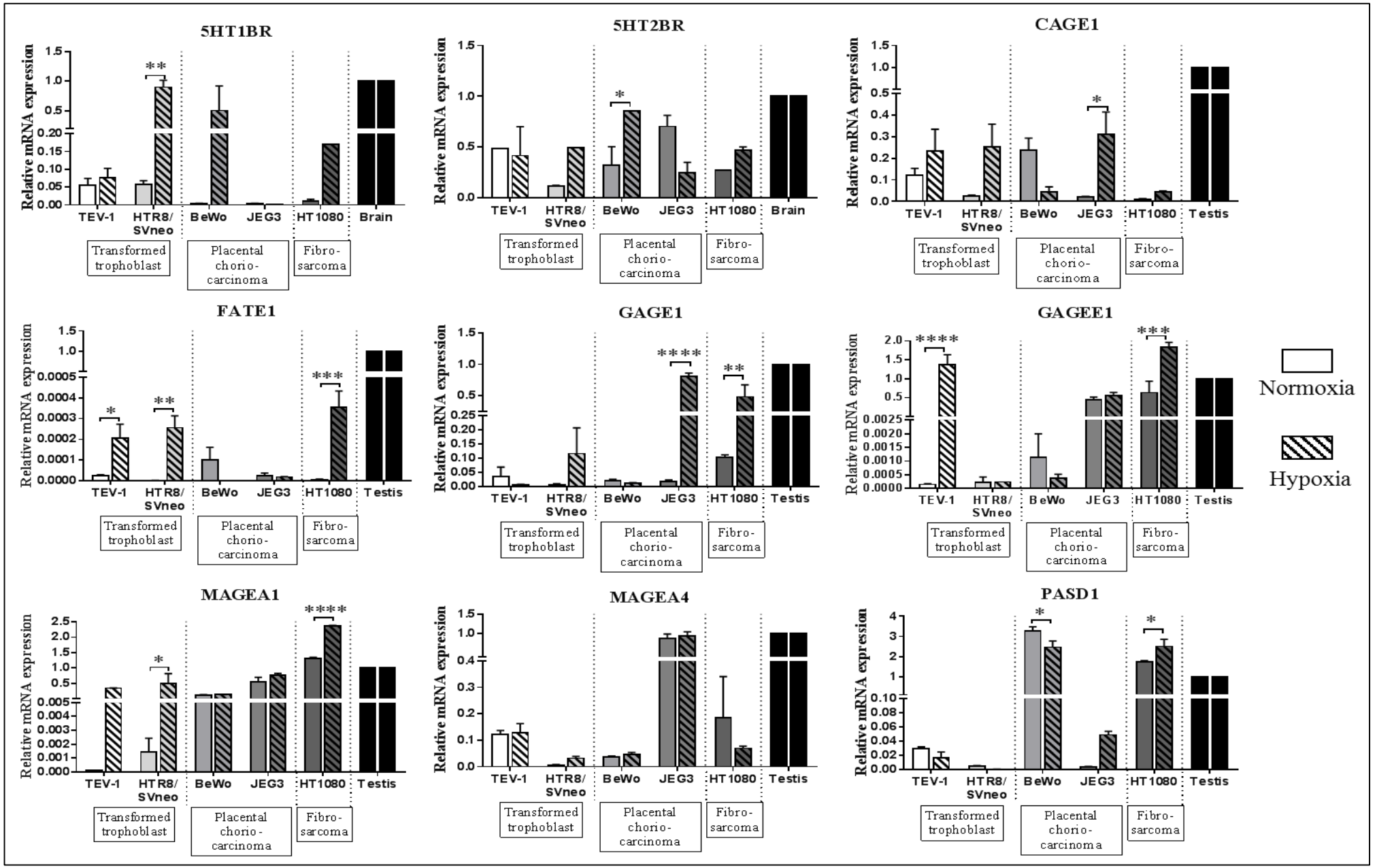


Figure 6.30: Relative mRNA expression under hypoxic condition.

The data are means of 2 experiments (each performed in triplicates; ±SEM; ********p<0.0001; *******p<0.001; ******p<0.01; *****p<0.05]. The mRNA expression is relative to expressions in positive controls (brain and testis; arbitrarily set as 1).

6.3 Discussion

The aim of this chapter was to investigate the effect of 5-HT on expressions of 5-HT receptors and C/T antigens in transformed trophoblast (TEV-1 and HTR8/SVneo) and placental choriocarcinoma (BeWo and JEG3) cell lines. Two different cells in each group were investigated, as there were variations in gene expression, even within the same group. The fibrosarcoma (HT1080) cell line was used as representative tumour cell line (as explained in Chapter 5). From the results, it can be seen that the effect of 5-HT on protein expression was dependant of the protein type, cell line and concentration of 5-HT. It was initially intended to compare the mRNA (by real-time PCR) and protein (quantitatively by immunoblotting and qualitatively by immunofluorescence). However, the commercially available antibodies were unable to detect the protein expression on some of the cell lines within the study period, despite repeated attempts. To the author's knowledge, this is the first study to attempt to comparatively analyse protein and mRNA expressions of C/T antigens in placental derived cells.

- **Comparison of Basal mRNA expressions**

Since the status of C/T antigens in trophoblast and choriocarcinoma cell lines are not known, their basal mRNA expression, together with the expression of 5-HT_{1B}R and 5-HT_{2B}R were compared with that which is present in fibrosarcoma (HT1080) cells. Also, it was important to compare the expression levels in all of the cell lines in order to select the best model for placental trophoblast study. The obtained data suggest there is difference in basal mRNA expression patterns in different cell lines. The mRNA expression of the majority of C/T antigens in HT1080 (the control cancer cell line), were high and comparable with the positive control (human testis). The exceptions were FATE1 and CAGE1 mRNAs which were found to be the lowest in HT1080 amongst the cell lines used in this study. As for the main 5-HT receptors, the choriocarcinoma cells showed a very low expression pattern of 5-HT_{1B}R. On the other hand, the transformed trophoblast cell lines have shown a relatively high basal expressions of 5-HT_{1B}R. Nevertheless, compared to choriocarcinoma cells, the expression of 5-HT_{2B}R was found to be lower in transformed trophoblast cells.

The results also show the basal mRNA expressions of C/T antigens were generally lower in transformed trophoblast cell lines. This is consistent with the data from term and first trimester placenta (Chapters 3 and 4 respectively). In contrast, both choriocarcinoma cells exhibit higher mRNA expressions of many C/T antigens compared to HT1080 cells. This was expected as the C/T antigens are tumour-specific, and the choriocarcinoma cell lines are derived from placental tumours.

Between the choriocarcinoma cell lines, BeWo has shown higher mRNA expression of CAGE1, and PASD1 than JEG3 cells. In contrast, the mRNA expression for MAGEA1, MAGEA4 and GAGEE1 were found to be higher in JEG3 cells. The above results show the data from the *in vitro* studies are cell line specific, as has been highlighted by other authors in cancer research (Holliday and Speirs, 2011; Domcke *et al*, 2013).

- **Effect of 5-HT on expression of 5-HT receptors**

There was an increase in 5-HT_{1B}R expression observed in both TEV-1 and HTR8/SVneo cells treated with 5-HT when compared to control. However, the expression of 5-HT_{2B}R in TEV-1 and HTR8/SVneo cells did not correlate. Furthermore, the expression of only 5-HT_{2B}R was detected in JEG3 placental choriocarcinoma cell line, whereas the expression of this two receptors were not detected at protein levels in BeWo. The HTR8/SVneo and JEG3 cells treated with 10µM 5-HT showed a significant increase in 5-HT_{2B}R expression relative to controls. Thus it was noticed, at low 5-HT concentrations, that the expression of these two receptors were increased, but at higher concentration (80µM) the expression levels decreased. This suggests that 5-HT being a mitogen would enhance the expression of 5-HT_{1B}R and 5-HT_{2B}R at low concentrations, yet down-regulate expression at higher concentration.

The cellular localisation of the proteins in the cytoplasm was confirmed by immunofluorescent staining. However, these were not detected in placental choriocarcinoma cells. One reason for this could be that the syncytial nature of choriocarcinoma cells with minimal cytoplasmic mass hinders the epitope binding of the antibodies. Both 5-HT_{1B} and 5-HT_{2B} receptors have been reported to show increased expression during hepatocellular cancers and the mitogenic effects of 5-HT in liver cells have been confirmed (Soll *et al* 2010; 2012). In a previous placental study, the presence of 5-HT as well as its receptors on trophoblast cells suggested that the physiological effects of 5-HT are mediated via its autocrine and paracrine interactions to receptors (Huang *et al*, 1998).

As can be seen in Chapter 5, there is a direct correlation between effects of 5-HT on cell proliferation, migration and invasion of TEV-1, HTR8/SVneo and JEG3, and the observed effects of 5-HT on 5-HT_{1B} and 5-HT_{2B}R receptor expression. This suggests that the physiological actions of 5-HT on these cell lines may be via 5-HT_{1B} and 5-HT_{2B}R receptors. Since 5-HT has been implicated in the implantation (Cikos, 2011), the finding that the expression of 5-HT_{1B}R and 5-HT_{2B}R in both HTR8/SVneo and JEG3 cells are enhanced would confirm the role of 5-HT in trophoblast invasion. Also, the finding that 5-HT at high concentrations reduces both receptors in these cell lines, confirms the hypothesis (explained

in Chapter 1) that the drastic increase in the circulating 5-HT may be the cause for the reduced trophoblast invasion in PE (Bolte *et al*, 2001; 2001a, Sivasubramaniam *et al*, 2002).

- **Effect of 5-HT on expression of C/T antigens**

The C/T antigens have not been investigated earlier in transformed trophoblast (TEV-1 and HTR8/SVneo) and placental choriocarcinoma (BeWo and JEG3) cell lines. In fact, there is no literature on the effects of 5-HT on C/T antigen expressions overall. Therefore, this element of the research was focussed on establishing whether 5-HT may have an effect on C/T antigens. The 5-HT treatment either increased or decreased the expression of the C/T antigens compared to control. The effects were dependant on specific antigens, the concentration of 5-HT and the origin of cell line. Most of the C/T antigens like CAGE1, FATE1 and GAGE family (GAGE1 and GAGEE1) showed a bell shaped (or Gaussian distribution) expression patterns with range of 5-HT concentrations in nearly all the cell lines. At the lowest (10 µM) and highest (80 µM) concentrations, the levels of C/T antigen expression were relatively low. However, between 20-40µM, the expression levels increased, with the highest being shown at 30-40µM 5-HT. This suggests that 5-HT at concentrations between 20 to 40µM may be the most probable optimal range to produce effective changes in the expressions of these C/T antigens. In contrast, MAGEA1 expression levels decreased with increasing concentration of 5-HT, whilst MAGEA4 expression was directly proportional to 5-HT concentrations. In general most of the C/T antigens expressions were reduced at 80µM 5-HT.

On the protein expression analysis, the Western blots of some of the C/T antigens such as FATE1, GAGE1 and MAGEA1 showed two distinct bands. Similar band patterns were also observed in placental tissue samples (See Section 3.2.3.5). The appearance of two distinct protein bands could be either due to transcript variants of the antigens or post-translational modifications. FATE1 expression showed two bands in the immunoblots of placental choriocarcinoma cell lines (BeWo and JEG3) similar to placental tissues. However, both TEV-1 and HTR8/SVneo cells showed only a single band in the Western blots. It is not clear whether the appearance of the two bands in choriocarcinoma cells is a result of splice variants specific to these cells. Although functions of most of the C/T antigens have not been confirmed, some of them are implicated in cell differentiation (MAGEA1), tumour suppression (MAGEA4), apoptosis (GAGE1; GAGEE1) and tumorigenesis (FATE1) (Monte *et al*, 2006; Sang *et al*, 2011; Gjerstorff *et al*, 2006; Olesen *et al*, 2011).

Interestingly, it was noticed in TEV-1 cells the increasing concentration of 5-HT has decreased the MAGEA1 antigen expression, whilst in the same cell line the MAGEA4

expression increased with increasing concentrations of 5-HT. Thus, there appears to be an inverse correlation between cell differentiation (suggested function of MAGEA1) and tumour suppression (function of MAGEA4) in this cell line. Furthermore, the expression of other antigens involved with apoptosis (such as GAGE1 and GAGEE1) was decreased with high 5-HT concentrations, thereby inhibiting the apoptotic effects of 5-HT at these concentrations. These findings suggest that high concentrations of 5-HT decrease the cell proliferation through different mechanism. Previous hepatocellular cancer studies have suggested that high concentrations of 5-HT may activate an autophagy mechanism (Soll *et al*, 2010). Similar mechanism may have been involved in these cell lines. The immunofluorescent cytochemistry confirmed the cytoplasmic localisations of many of these antigens in transformed trophoblast cells especially in TEV-1. However, it was not possible to show the cellular localisation of all the antigens in some cell lines. Although a complete comparison of mRNA, protein and localisation of all C/T antigen was not achieved due to time constraints, to the best of author's knowledge this is the first study which reports basal expressions of these C/T antigens in placental cell lines and its regulation by different doses of 5-HT.

- **Conclusion:**

Since this was a preliminary investigation and no previous studies were available for comparison, concrete conclusions could not be drawn from the data available. However, the study has shown that all the placental cell lines express most of the C/T antigens similar to the expression patterns of placenta. Therefore, these cell lines, especially TEV-1 and HTR8SV/neo cells can be used as *in vitro* models in the functional studies involving C/T antigens.

Also, it could be suggested that 5-HT affects the expressions of the C/T antigens and their functions. An in depth analysis of the intracellular pathways of C/T antigens and the influence of 5-HT on these pathways is needed to establish link between them (see Chapter 7).

Chapter 7
General Discussion

7.0 Discussion

The aims of this thesis were firstly to establish the status and the regional expression of (a) all 5-HT receptor subtypes and (b) C/T antigens in pre-eclamptic (PE) placentae in comparison with normotensive (NT). Following this, an analysis of the expression of 5-HT receptors and C/T antigens in the placentae from first trimester (FT) pregnancies was carried out. This was followed by an investigation on the effects of 5-HT on the invasive potential of transformed early first trimester placental cells in relation to choriocarcinoma (placental cancer) cell lines under normoxic/hypoxic environment and an elucidation on the effects of 5-HT on the expressions of C/T antigens in placental cell lines under normal and hypoxic conditions.

To satisfy the initial aim, the expression profiles of 5-HT receptors and C/T antigens were investigated in three different human placental samples (NT, PE and FT) with different trophoblast invasive potentials. A three way status analysis of these receptors and antigens was conducted, firstly between NT and PE placentae, followed by FT and term. After the confirmation of differences in expression between these placental groups, the study focussed on the effects of 5-HT on trophoblast invasion and the expression of the above receptors/antigens using placental cell line models. The expression of C/T antigens was investigated with 5-HT insult- firstly to identify the antigens that are differently expressed with 5-HT and, secondly, to elucidate any probable functions of these antigens in placental invasion and development.

7.1 Expression analysis on placental samples

7.1.1 Normotensive *versus* pre-eclamptic placentae

In the past, the effects of 5-HT and the status of 5-HT_1 and 5-HT_2 receptors on placental vessels have extensively been studied (Cruz *et al*, 1998; Ugun-Klusek *et al*, 2011; Cordeaux *et al*, 2008). Apart from its vascular effects, 5-HT is involved in proliferation and migration of cells. These effects were found to be brought about by 5-HT_{1B} (Sibella-Argüelles, 2001; Banasr *et al*, 2004; Gurbuz *et al*, 2014; Matsusaka and Wakabayashi, 2005) and 5-HT_{2B} receptors (Pai and Horseman, 2008; Soll *et al*, 2012). To begin with, the mRNA expression of the seven 5-HT receptor families and their sub-types was compared in NT and PE placentae. The expression was compared with that in brain (positive) control after normalisation with house-keeping genes. Significant down-regulation of mRNA expressions of 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{2B} receptors in PE was observed.

Due to the close homology of the members of 5-HT_1 receptor subtype, the protein expression of only 5-HT_{1B} and 5-HT_{2B} receptor was examined. Although the difference in protein expression of 5-HT_{1B}R in NT and PE was not significant, it agreed with the mRNA expression patterns. Both 5-HT_{1B} and 5-HT_{2B} receptors showed cytoplasmic staining in the STB and CTB layers of villi by IHC. The staining in the decidua of PE samples was more intense than in the villous section.

The findings from this study suggest that the proliferative and migratory effects of 5-HT cannot be exploited due to the low expression levels of 5-HT_{1B} and 5-HT_{2B} receptors in PE, although high levels of 5-HT have been reported during PE (Cruz *et al*, 1998). Hence, lack of proliferation and migration of the placental cells leads to poor placental development.

The present study represents the first time that the expression profiles for C/T antigens between NT and PE have been compared. Earlier studies have only reported localisation of some antigens by IHC (Jungbluth *et al*, 2007) and expression of one C/T antigen (CAGE1) by Western blotting in FT (Khan *et al*, 2014). In the present study, it was observed that seven C/T antigens displayed significant differences in mRNA expression between NT and PE, thus the hypothesis was proved to be true atleast at the mRNA level. This was followed by protein validation, which correlated to mRNA expression with the exception of FATE1. The protein levels of FATE1 showed an increase in PE when compared to NT, whereas the mRNA expressions were the opposite. It may be due to the half-life of mRNA, as protein cannot be translated from mRNA with very short or long half-life (Wang *et al*, 2002; Yang *et al*, 2003).

As explained by one of the manufacturers (Sigma-Aldrich – **www**[13]), Western blots can give four different types of result:

- a supportive result which shows the band of predicted size (e.g. MAGEA4 and PAGE5);
- a supportive result of predictive size band with additional bands present (as in FATE, CAGE1 and GAGE1);
- an uncertain result, with no/very faint bands (e.g. MAGEA1); or with a single band differing more than +/- 20% from the predicted size;
- None-supportive results with weak bands of predicted sizes but with additional bands of higher intensity also present.

Interestingly, none of the Western blot data presented herein showed bands differing more than +/- 20% from the predicted size; nor were there bands beyond predicted sizes. Additionally, two bands of different molecular weights (20kDa and 25kDa) were observed for FATE1 in placental samples as well as the positive control (human testis). In testis, the intensity of the 20kDa band was higher than that of the 25kDa, whereas for placental samples the opposite was observed. Although no splice variants have been reported for FATE1, Olesen *et al*, (2003) reported two mutations within FATE1 gene which were linked to infertility in males. Therefore, it would be interesting to explore the nature of these two bands further to identify any possible variants. In addition, this C/T antigen is predicted to be involved in tumourigenesis, due to the presence of binding sites for steroidogenic factor-1 (SF-1) and Wilms' tumor gene 1 (WT1) (Yang *et al*, 2005). Both SF-1 and WT1 have been reported to be expressed in placental trophoblast linking to placental growth and invasion (Bamberger *et al*, 1993; Feingold *et al*, 1998). Hence, a possible function for FATE1 in placenta could also be trophoblast differentiation and invasion.

Similar observations were made for CAGE1 and GAGE1 proteins with additional molecular weight bands. Since these bands were also consistently shown in positive controls, the total sum of the intensities of these bands was comparatively analysed. Although this may be a limitation of this study, it was not feasible, within the study period, to extensively analyse whether these extra bands actually represent the original protein or if they are the splice variants. The current study is one of the very few that has used C/T antigen specific antibodies in Western blots. Since all the antibodies used were polyclonal in nature, these additional, but faint, bands may also be due to non-specific binding. A comparable band pattern for FATE1 and GAGE1 was also observed in *in vitro* expression analysis (see Section 7.2).

The CAGE1 antigen was down-regulated in PE placentae. Although the function of CAGE1 in placenta is unknown, differential expression patterns with gestational age (weeks) have been described sooner in FT placentae (Khan *et al*, 2014). Unusually, antigens involved in tumour suppression and apoptosis (MAGEA4, GAGE1 and GAGEE1/PAGE5) were significantly down-regulated in PE placentae. As higher apoptotic rates were shown in STB during PE complications (Ishihara *et al*, 2002), an up-regulation of these antigens was expected. This paradox may suggest alternative functions for these antigens in placenta. The fact that the CTB cells in PE are not as physiologically active as in NT to undergo constant differentiation, proliferation and apoptosis cycle may also be a likelihood.

Although the MAGEA1 mRNA expression was up-regulated in PE, protein bands on Western blots were found to be too faint for analysis. Similarly, the PASD1 down-regulation

in PE was investigated only at mRNA level. Due to this, the cellular localisation of these antigens in NT and PE placentae was also investigated using immunohistochemistry (IHC). The immunoreactivity (IRS) for MAGEA1 staining intensity confirmed the unfaithful translation of MAGEA1 mRNA to protein in PE. As MAGEA1 is linked with cell differentiation in spermatogenesis and cell signalling (Monte *et al*, 2006), the low levels of this antigen in PE can thus be linked to poor differentiation of the CTB stem cells into STB during early placentation . However, it can also be argued that, as the presence of CTB stem cells is low even in NT term placentae, their prevalence is lower in PE, possibly accounting for the low expression of the antigen.

Finally, PASD1 mRNA expression was significantly down-regulated in PE, but not confirmed by IRS. Very little is known about this antigen, but its function is predicted to be stimuli-dependent (Campbell *et al*, 2011).

The C/T antigens, like 5-HT receptors, were mostly localised on the STB and CTB layers of the villi in NT. In PE the staining was concentrated around the decidua, embedded with giant cells and iCTB. The staining of these antigens appeared in the cytoplasm of these cells; even though FATE1 and PASD1 were reported to be present in the nucleus in the Uniprot database (Q969F0; Q8IV76). Previous studies have also reported both cytoplasmic and nuclear localisation of FATE1 and PASD1 (Yang *et al*, 2005; Ait-Tahar *et al*, 2009). In general, these C/T antigens which are involved in tumour development are more prevalent in the trophoblast cells which share invasive characteristics with cancer cells.

7.1.2 First trimester *versus* term placentae

The reliability and practicality of microarray studies have led to several expression analyses on first trimester (FT) placentae (Sitras *et al*, 2012; Chen *et al*, 2002; Khan *et al*, 2010, 2004). In the present study the first obvious difference in expression pattern was observed between FT and term placentae due to the placental structure and thus the second hypothesis was proved to be true (Chapter 1: Introduction). The C/T antigen mRNA expression in FT were significantly up-regulated in FT when compared to term placentae, except for GAGEE1/PAGE5. The up-regulation of these antigens that are predicted to be involved in placental development agrees with the high metabolic activity of trophoblast cells during FT than at term (Huppertz, 2008; Jones and Fox, 1991). However, the down-regulation of GAGEE1 and 5-HT_{2B} receptor in FT could not be explained (as up-regulation was expected).

On a closer examination, a heterogeneity in the mRNA expression was observed for FT placentae for these receptors and antigens, which was comparable to previous findings with gestational ages (weeks 6-8) (Khan *et al*, 2010, 2004). No changes in mRNA expression were observed for 5-HT_{1B} and 5-HT_{2B} receptors between weeks 7 to 9 of FT placentae. Although the number of samples was limited, the data from the current study agreed with previous expression analyses carried out by Huang *et al* (1998). Using IHC, these reported that the 5-HT staining intensity in STB reduced with gestational age, but that 5-HT receptors staining showed no variations with age.

Some of the C/T antigens (GAGE family and FATE1) have also been reported to show differences in expression with maturity of the germ cells (spermatocytes) (Gjerstorff *et al*, 2008; Yang *et al*, 2005). Therefore, it was of primary importance to check if the expression of C/T antigens also differed with pregnancy progression.

The current study shows CAGE1 mRNA expression was up-regulated in weeks 7 and 9, but was down-regulated in week 8. This agreed with previous findings by Khan *et al* (2014) of CAGE1 expression in FT samples of weeks 6 to 8. Significant differences were also observed for FATE1, GAGE1 and MAGEA1 with down-regulation at week 8, but up-regulation at week 9. This might be a result of the dramatic changes in trophoblast invasion and placental development during these weeks. It would be interesting to explore this further using a larger number of samples (See Section 7.4).

Protein localisation of 5-HT receptors and C/T antigens was investigated by IHC in villous and decidua of FT. The two receptors, 5-HT_{1B} and 5-HT_{2B} were localised on the cytoplasm of the STB and CTB layers of the villi. The mesenchymal cells were also positive for only 5-HT_{1B} receptors. Staining of the receptors was focused on the stromal cells, epithelial cells (uterine and luminal) and spiral arteries of the endometrium. The expression of 5-HT receptors on the decidua may indicate the effects of 5-HT on decidua. This finding could be linked to observation of the effects of 5-HT on the endometrium development before implantation (reviewed by Cikos *et al*, 2011), and suggests that the decidualisation of the endometrium controls the invasion of the trophoblast cells (Norwitz *et al*, 2001; Arnold *et al*, 2001). The presence of C/T antigens on the endometrial cells also indicates these antigens play parallel roles during early implantation.

7.2 *In vitro* expression analysis

Studies involving human tissue samples are always challenging due to various constraints such as ethics, sample availability and delay in collection. During the course of this study, 13 normal (NT) and 12 PE samples were studied. NT and PE samples were comparable in their gestational ages. In addition, it was possible to collect only 11 fresh FT samples from the EU collaborator and it was not feasible to collect considerable amounts of RNA and protein due to the minute structure of FT placentae from aborted pregnancies. However, previous small scale placental studies have reported identification of new proteins involved in placental development (Apps *et al*, 2011; Khan *et al*, 2010, 2004). Hence, to overcome the shortage of samples, placental cell lines were used for the rest of the study.

As mentioned earlier, the 5-HT levels in PE are higher than in normal placentae and placental trophoblast invasion is also shallow in PE. Hence, the next phase was to investigate the effects of 5-HT on trophoblast cell lines. Four cell lines originating from placenta, TEV-1 and HTR8/SVneo (transformed first trimester trophoblast cell lines) and BeWo and JEG3 (choriocarcinoma) were used for this study.

Increased apoptotic and anaplastic features were observed in cells treated with high doses of 5-HT (80-100µM) visualised with H&E staining under normoxia (20% v/v O_2) and hypoxia (2% v/v O_2). There were differences in cell density at various 5-HT concentrations. Thus, an in depth analysis using three different assays was implemented to study the effects of 5-HT on the behaviour of cells of placental origin.

It was deduced that the effect of 5-HT was bi-phasic i.e. at very low (10µM) and high concentrations (80-100µM) the mitogenic effects of 5-HT on all the cell lines were minimal. However, at certain concentrations (20-40µM for normoxia and 60µM for hypoxia) cells showed the highest mitochondrial activity with minimum LDH release. The proliferation rates were also maximal at these concentrations. Former studies have also reported such bi-phasic and mitogenic effects of 5-HT on other cell lines (Pai and Horseman, 2008). Remarkably, 5-HT also showed recovery of cancerous cell proliferation (BeWo, JEG3 and HT1080) inhibited with 5-FU treatment under both normoxia and hypoxia. Under hypoxia the cells were more tolerant to higher doses of 5-HT (60µM). The fibrosarcoma HT1080 showed the highest proliferation rates and activity, thus proving to be the ideal positive control for the migration and invasion assays conducted. The cell lines in each group were identical in their behaviour under 5-HT insult.

5-HT is a multifunctional monoamine which triggers various activities in the cells. Migration of other cells from normal as well as a malignant origin prompted by 5-HT have been described in the past (Matsusaka and Wakabayashi, 2005; Pai *et al,* 2009). No reference to the migratory effects of 5-HT on trophoblast could be found in the literature. Therefore, the effects of different doses of 5-HT on migration of four different placental cell lines were investigated by performing a simple wound-healing (scratch) assay. The bi-phasic effect of 5-HT was also observed on the migratory pattern of the cells. 5-HT in moderate doses of 20-40µM (TEV-1 and HTR8/SVneo) and 30-40µM (BeWo and JEG3) showed increased wound coverage when compared to controls (without 5-HT treatment).

The findings described in this study confirm the bi-phasic effects of 5-HT on cell migration as reviewed by Moiseiwitsch (2000) who reported that high doses of 5-HT reduced the migration of neural crests and induced differentiation of the cells instead. Unexpectedly the wound covered area was greater for transformed trophoblast cell lines than for choriocarcinoma cell lines. This may be due to the syncytial nature of these choriocarcinoma cell lines which would hinder cell migration. HT1080 being the positive and cancerous cell lines showed the fastest wound coverage within 12 hours. Since the assay was performed after 24 hours treatment with 5-FU and with serum free media (SFM) the possibility of movement by proliferating cells was ruled out.

These assays were repeated under hypoxia with constant monitoring of percentage O_2 with an oxygen sensor for 12 hours. The effects of 5-HT on the migration of these cells under hypoxia and normoxia were compared for 12 hours. Hypoxia, overall, increased the wound covered area of all the cells, except JEG3. Only 60µM 5-HT treated transformed trophoblast and fibrosarcoma cells showed improved wound coverage (in comparison) to the control. Hence, it was confirmed that optimal doses of 5-HT induced the migration of all cell lines under normoxia, but induced only migration of transformed trophoblast and fibrosarcoma cells under hypoxia. Higher wound coverage was expected for choriocarcinoma due to their cancerous nature in comparison to transformed trophoblast cells. It can thus be proposed that the trophoblast cells are more tolerant under hypoxic conditions (hypoxia during placental development) than choriocarcinoma (hypoxia during tumour formation).

Since this was the first study to report 5-HT induced migration of transformed first trimester trophoblast, it was reasonable to investigate if the 5-HT also affected cell invasion. BD Falcon™ BioCoat tumour invasion systems coated with BD matrigel Matrix were used to investigate the invaded cells and compared to migrated cells through uncoated plates.

Two methods of analysis were utilised to eliminate any drawbacks from either of the two methods and a reciprocal agreement was derived. It was confirmed that 5-HT only improved the invasion of cancerous cell lines (BeWo, JEG3 and HT1080), but not transformed cell lines. This could be due to the highly invasive nature of the cancerous cells when compared to the trophoblast cells. Under hypoxic conditions, only HTR8/SVneo and JEG3 cells showed increased invasion with 60µM 5-HT when compared to controls. Thus, a cell line dependency was observed for the effect of 5-HT on cell migration and invasion. This proves that it is critical to choose the right cell models for placental study, and HTR8/SVneo and JEG3 cells can be considered to be the best models to investigate invasion during the developmental stages of placenta.

The basal mRNA expressions of the two 5-HT receptors were analysed in all the cell lines. The mRNA expression of 5-HT_{1B} receptor was higher in transformed trophoblast cell lines (TEV-1 and HTR8/SVneo), whereas 5-HT_{2B} receptor expression was higher in placental choriocarcinoma cell lines (BeWo and JEG3). Thus, the expression of these receptors is cell line dependent. The 5-HT treatment increased the expression of the 5-HT_{1B} receptor in transformed trophoblast cell lines and showed a similar Gaussian distribution, as seen for MTT, with lower doses (10-30µM) showing maximum expression. A significant increase in expression was observed for 5-HT_{2B} receptor with 10µM 5-HT in TEV-1, HTR8/SVneo and JEG3 cell lines. It can be suggested that the effects of 5-HT are mediated through these two receptors, but further studies are needed for confirmation (See Section 7.4). The immuno-fluorescent staining for these receptors verified the expression of these receptors in the cytoplasm of the cells, confirming the previous result obtained using IHC staining.

Owing to the lack of literature on the expression of C/T antigens on placental cell lines, the four placental cell lines along with HT1080 were next analysed for C/T antigen mRNA expressions.

As expected, the cancerous cell lines (BeWo, JEG3 and HT1080) showed higher levels of C/T antigens (as the name suggests). Though expression was higher in BeWo and JEG3, variations were observed between the two cell lines. Thus, the expression of C/T antigens was also cell line dependent. The expression patterns for some of the C/T antigens with 5-HT doses followed a Gaussian distribution, similar to that of 5-HT receptors. These C/T antigens were CAGE1 (structural protein), FATE1 (tumourigenesis) and GAGE family (GAGE1 and GAGEE1; apoptosis). This hints that at optimal 5-HT concentrations (normal pregnancy), the receptors and antigens involved in proliferation, migration and tumourigenesis are maintained. The increase in the expressions of antigens related to

apoptosis at these concentrations maybe due to high proliferation, differentiation and apoptosis cycle of the trophoblast cells similar to cytotrophoblast during FT.

Fascinatingly, MAGEA4 which has tumour suppressing properties increased with increasing 5-HT concentrations. Thus, high 5-HT concentrations during PE can cause up-regulation of MAGEA4 antigen, which suppresses the proliferation of trophoblast cells. Conversely, MAGEA1 was the only gene which did not support the above hypothesis of 5-HT inducing expression of proteins involved in placental development. MAGEA1 is predicted to be involved in cell differentiation as confirmed earlier and its expression decreased with increasing 5-HT concentration. This substantiates the previous findings that 5-HT induces cell differentiation at higher concentrations (Reviewed by Moiseiwitsch, 2000).

The effect of 5-HT also showed alteration under hypoxic conditions, therefore basal mRNA expressions of the receptors and antigens were investigated to examine any changes. Significant differences in basal mRNA expressions were observed under normal and hypoxic conditions in all the cell lines, a finding that needs to be confirmed by protein study.

7.3 Conclusion

The data from this study suggest that

- The expression patterns of 5-HT receptors and C/T antigens are altered in PE placentae, demonstrating their importance in trophoblast functions. The expression of C/T antigens were also different between FT and term placentae, indicating their functional importance in the early trophoblast invasion.
- 5-HT acts as a mitogen for placental cell lines and induces migration as well as invasion, signifying the importance of optimum 5-HT levels especially during FT. The levels of 5-HT in FT, may alter the expression of 5-HT receptors and C/T antigens and therefore regulate their functions during trophoblast invasion and placental development. The effects of 5-HT on these cell lines is altered during hypoxia which may be linked to expression of these receptors and C/T antigens.
- In cell culture models it is important to use more than two different cell lines for effective analysis of the proteins/factors and their functions. Compared to other cell lines, HTR8/SVneo and JEG3 cells can be considered to be the best models to investigate invasion during the developmental stages of placenta.

Refer to Table 7.1 and Table 7.2 for overall summary of results obtained for human placental and *in vitro* study.

Table 7.1: Summary of human placental study.

Genes Investigated	Significant	NT (Term)		PE				FT				
		IHC		mRNA (compared to NT)	Protein (compared to NT)	IHC		Villous			Decidua	
								mRNA (compared to Term)	IHC		IHC	
		Cell type	Cellular Compartment			Cell type	Cellular Compartment		Cell type	Cellular Compartment	Cell type	Cellular Compartment
12 5-HT receptors	5-HT$_{1B}$R	CTB; STB; EVT; SA;	Cytoplasm	(-)	(-)	CTB; STB; EVT; SA	Cytoplasm	(+)	CTB; STB; MC	Cytoplasm	UE; DS;SA	Cytoplasm
	5-HT$_{2B}$R	CTB; STB; SA; DS	Cytoplasm	(-)	ND	CTB; STB; SA; D	Cytoplasm	(-)	CTB; STB	Cytoplasm	UE; DS;SA	Cytoplasm
14 C/T antigens	CAGE1	CTB; STB; EVT; SA; C&F	Cytoplasm	(-)	(-)	CTB; STB; EVT; SA; C&F; D	Cytoplasm	(+)	CTB; STB	Cytoplasm	UE; SA	Cytoplasm
	FATE1	CTB; STB; EVT; C&F; VS	Cytoplasm	(-)	(+)	EVT; DS	Cytoplasm	(+)	CTB; STB; MC	Cytoplasm & nucleus (STB)	UE; DS;SA	Cytoplasm
	GAGE1	CTB; STB; SA; EVT	Cytoplasm & nucleus (EVT)	(-)	(-)	CTB; STB; EVT; C&F	Cytoplasm	(+)	CTB; STB	Cytoplasm	UE; DS;SA	Cytoplasm
	GAGEE1	Overall	Cytoplasm	(-)	(-)	EVT; DS	Cytoplasm	(-)	CTB; STB	Cytoplasm	UE; DS;SA	Cytoplasm
	MAGEA1	CTB; STB; VS;	Cytoplasm	(+)	ND	EVT	Cytoplasm	(+)	CTB; STB	Cytoplasm	UE	Cytoplasm
	MAGEA4	CTB; STB; EVT; C&F	Cytoplasm	(-)	(-)	CTB; STB; EVT; C&F	Cytoplasm	(+)	CTB; STB	Cytoplasm	UE; DS;SA	Cytoplasm
	PASD1	CTB; STB; EVT; VS	Cytoplasm	(-)	ND	CTB; STB; EVT; C&F	Cytoplasm	(+)	CTB; STB	Cytoplasm & nucleus (CTB)	UE; DS;SA	Cytoplasm

C&F- Collagen & fibrin; CTB- Cytotrophoblast; D-Decidua; DS-Decidual stroma; EVT-Extravillous trophoblast; MC- Mesenchymal cells; SA-Spiral arteries; STB-Syncytiotrophoblast; UE-Uterine epithelium; VS- Villous stroma;

(-) down regulated; (+) upregulated; ND- Not detected

Table 7.2: Summary of *in vitro* study.

Effect of -5HT on	Transformed trophoblast		Placental choriocarcinoma		Fibrosarcoma	Transformed trophoblast		Placental choriocarcinoma		Fibrosarcoma
	TEV-1	HTR8/SVneo	BeWo	JEG3	HT1080	TEV-1	HTR8/SVneo	BeWo	JEG3	HT1080
	Normal					Hypoxia				
Mitochondrial activity	(++) 30µM	(++) 30µM	(+++) 20-30µM	(+) 20µM	(++) 20-30µM	(++) 60µM	(+) 60µM	(+) 60µM	(+) 60µM	(+++) 40-60µM
Toxicity	(-) 30µM	(-) 30µM	(-) 20-40µM	(-) 20µM	(-) 20-40µM	(- -) 60µM	(-) 40-60µM	(+) 100µM	(+) 80-100µM	(-) 40-60µM
Proliferation	nc	(-) 30-80µM	nc	nc	nc	nc	nc	(+++) 100µM	(++) 60µM	nc
Recovery of Proliferation	nc	(-) 30-80µM	(++) 30-80µM	(++) 30-80µM	nc	(- -) 60-100µM	nc	(+) 100µM	nc	nc
Migration	(++) 30µM	(++) 20-40µM	(+) 30-40µM	(++) 20µM	(+++) 10-80µM	(- -) 80-100µM	(- -) 100µM	(-) 20-100µM	(-) 20-100µM	(++) 60µM
Invasion	(-) 10-40µM	(-) 10-40µM	(+++) 10-40µM	(+++) 10-30µM	(++) 10-40µM	nc	(++) 60µM	nc	(+) 60µM	(-) 20-80µM

Effect of -5HT on gene expression	Transformed trophoblast						Placental choriocarcinoma					
	TEV-1			HTR8/SVneo			BeWo			JEG3		
	mRNA	Protein	IF	mRNA	Protein	IF	mRNA	Protein	IF	mRNA	Protein	IF
5-HT receptors												
5-HT1BR	(+) 10µM	(+) 10-80µM	cytoplasm	(+) 30, 40µM	ns	cytoplasm	ND	ND	ND	ND	ND	ND
5-HT2BR	(-) 10-80µM	(-) 20, 40, 80µM	cytoplasm	(+) 10,30,40µM	(+) 10µM	cytoplasm	ND	ND	ND	(+) 10µM	(+) 10, 40µM	ND
C/T antigens												
CAGE1	(-) 10-80µM	(+) 10, 30µM	cytoplasm	ND	ND	ND	ns	(+) 10-80µM	ND	ND	ND	ND
FATE1	(+) 30µM	ns	ND	(-) 80µM	ns	ND	(-) 80µM	(+) 20µM	ND	(-) 10, 80µM	(-) 20, 80µM	ND
GAGE1	(+) 40µM	(+) 40µM	cytoplasm	ns	ns	cytoplasm	(+) 80µM	(+) 20, 80µM	ND	ns	(-) 80µM	ND
GAGEE1	(-) 10-80µM	(+) 30µM	cytoplasm	(+) 30µM	(+) 30µM	cytoplasm	(-) 10, 40, 80µM	(+) 30µM	Focalised areas in cytoplasm	(+) 30, 40µM	(+) 10, 80µM	ND
MAGEA1	(-) 10-80µM	(-) 10-80µM	cytoplasm	(+) 10µM	(+) 10µM	cytoplasm	ND	ND	ND	ND	ND	ND
MAGEA4	(-) 10-80µM	(+) 80µM	cytoplasm	(-) 30µM	ns	cytoplasm	ND	ND	ND	ND	ND	ND

The significant changes in the cell behaviour due to 5-HT treatment are shown compared to control (non-treated) cells. The 5-HT concentration that induces these changes are given below the changes. (+++) = High increase; (++) = Moderate increase; (+) = Low increase; (- -) = Moderate decrease; (-) = Low decrease; nc= no change; ND= Not detected; ns= Not significant.

7.4 Future directions

The study needs to be extended with a larger number of samples used to confirm and validate its findings. This would include protein expression studies, especially for FT, possibly using specific monoclonal antibodies. Furthermore, the expression analysis can also be improved by cellular fractionation (cytoplasm, mitochondria and nucleus) of placental tissues. Micro-dissection techniques should also be used to study and quantify the mRNA/protein expressions of these antigens, in particular cell groups such as CTB, STB and decidua.

On the Western blotting side, if additional molecular weight bands persist (as in CAGE1, FATE1 and GAGE1), then it would be interesting to investigate the individual bands for detecting possible variants and/or post-translational modifications such as phosphorylation. The bands can be isolated from the gels and analysed using Mass-spectrometry to identify the peptide sequences, which can be further investigated for their properties and localisation in the cells.

The 5-HT receptor inhibition studies are to be conducted to confirm the pathway followed by 5-HT to induce its effects on placental cell lines. The functions of the C/T antigens can be studied by performing knock-down using small interfering RNA (siRNA) or small hairpin RNA (shRNA). A further literature review is to be carried out on this to confirm the advantages of these techniques.

The trophoblast cells from the PE and NT samples can be isolated and the effects of 5-HT studied on these primary cell cultures to categorise any changes. Preliminary studies on the effect of 5-HT on trophoblast cells isolated from FT villous have been performed under the supervision of Dr. Martin Gauster. The protocol for isolation and purification of the trophoblast has been optimised during this period. Using these established protocols, the primary cell culture can be carried out at least for a limited period of 2 weeks (8 passages). This will confirm the differences in the expressions of C/T antigens between NT and PE detected in this study.

Appendix

A.1 Ethical approval

NHS
Health Research Authority

NRES Committee North East - County Durham & Tees Valley
Room 002
TEDCO Business Centre
Viking Industrial Park
Rolling Mill Road
Jarrow
Tyne & Wear
NE32 3DT

Telephone: 0191 428 3561
Facsimile: 0191 428 3432

12 March 2012

Dr Shiva Das Sivasubramaniam
Senior Lecturer
School of Science and Technology
Nottingham Trent University
Clifton Lane
Nottingham
NG11 8NS

Dear Dr Sivasubramaniam

Study title:	**Analysis of the expression patterns of cancer testis (C/T) and onco-foetal antigens in normotensive and pre-eclamptic placentae**
REC reference:	**12/NE/0112**
Protocol number:	**N/A**

The Proportionate Review Sub-committee of the NRES Committee North East - County Durham & Tees Valley reviewed the above application on 09 March 2012.

Ethical opinion

The Sub Committee raised the following issues and the Chief Investigator, Dr Sivasubramaniam, responded accordingly as follows:

1. The Committee noted that question B-2 of the IRAS form stated that the samples being used are anonymised and linked anonymised, and that the text of the application states that the samples held in the tissue bank have already been anonymised. Members requested that the researcher clarify this, particularly to confirm that the researchers will not be able to identify the original patient from the samples.

   Dr Sivasubramaniam responded that the samples were collected from Nottingham City Hospital and that they were received as anonymised (numbered) samples and the researchers therefore do not hold any patient details, or the possibility to obtain them. Dr Sivasubramaniam confirmed that by "linked anonymised" it was meant that the samples could be linked to a previous study he was involved in (Ugun-Klusek et al, 2011) to see whether there is a link between the changes in the expression of 5HT receptor and C/T antigens. Dr Sivasubramaniam confirmed that the researchers do not have (nor is there any possibility they could obtain) any patient/participant details at all and so would never be able to identify any patients from any sample.

2. It was noted that in question A-50 of the IRAS form the researchers had indicated

that they do not intend to register the study on a public database; the Committee questioned why this would not be done.

Dr Sivasubramaniam replied that he would be happy to register the study on a public database if one was available to non-NHS researchers.

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Confirmation should also be provided to host organisations together with relevant documentation.

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
Evidence of insurance or indemnity		23 February 2012
Investigator CV	1.0	15 February 2012
Letter from Sponsor		23 February 2012
Letter from Statistician		23 February 2011
Other: PIS & Consent Form for "Normal Pregnancy" used in original study	1	04 November 2004
Other: PIS & Consent Form "Pre-eclampsia" used in original study	1	04 November 2004
Other: Invalidation letter from Cambridge Central REC		16 February 2012
Protocol	1.0	10 January 2012
REC application	IRAS 3.4	27 February 2012

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/NE/0112 **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

pp. S. Grimshaw

Dr A MacSween
Chair

Email: sarah.grimshaw@sotw.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

"After ethical review – guidance for researchers"

Copy to: *Roger Eccleston, Nottingham Trent University*

NRES Committee North East - County Durham & Tees Valley

Attendance at PRS Sub-Committee of the REC meeting on 09 March 2012

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mr S Chandler	Retired Consultant Medical Physicist	Yes	
Dr A MacSween	Principal Lecturer in Research Governance	Yes	
Mrs Sophie Welch	Research Governance & Policy Officer	Yes	

A.2 Wimasis results

Table A.1: Summary of the scratch

	Speed [% / Time units]	Start Point: Time [Time units]	Start Point: Cell-covered Area [%]	End Point: Time [Time units]	End Point: Cell-covered Area [%]
Overall Average	0.9229	0	51.6	48	95.9
Image Name	Time [Time units]	Scratch Area [%]	Cell-covered Area [%]	Speed [% / Time units]	Acceleration [% / (Time units)^2]
Pos2TEV1_control-00.0	0	48.4	51.6	0.5333	0.9556
Pos2TEV1_control-03.0	3	46.8	53.2	3.4	-0.4667
Pos2TEV1_control-06.0	6	36.6	63.4	2	-0.1361
Pos2TEV1_control-12.0	12	24.6	75.4	1.1833	-0.0767
Pos2TEV1_control-24.0	24	10.4	89.6	0.2625	
Pos2TEV1_control-48.0	48	4.1	95.9		

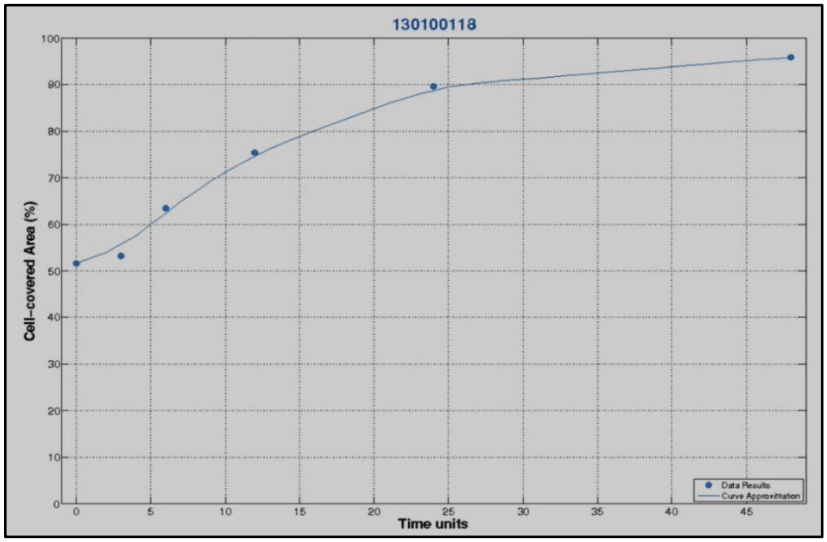


Figure A.7.1: Representation of Cell-covered area [%] for TEV-1 control at different time points.

A.3 Clinical data of patients

Serial No.	Normotensive:	Maternal age (Years)	Gestational age (Weeks)	Systolic BP (mmHg)	Diastolic BP (mmHg)	Placental Weight (Grams)	Mode of Delivery	Gestational weight (Kg)	Gestational Sex	BMI	Protein in urine (g/L)
N1	**V6**	34	38.4	122	60	855	Vaginal	4.24	male	21.6	N/D
N2	**V7**	32	38.9	120	90	700	Vaginal	3.3	female	24.5	N/D
N3	**V8**	29	40.1	140	68	520	Vaginal	3.8	male	23	N/D
N4	**V9**	36	39.2	128	68	579	Vaginal	3.12	male	24.2	N/D
N5	**V10**	39	39.2	130	70	636	Vaginal	3.03	male	23.5	N/D
N6	**V11**	35	38.1	120	70	654	Vaginal	3	male	23	N/D
N7	**V12**	34	38.3	120	72	725	Vaginal	3.33	female	23	N/D
N8	**V13**	40	38.4	123	70	852	Vaginal	4.24	male	24.2	N/D
N9	**V15**	29	39.2	122	66	625	Vaginal	3.21	male	23.5	N/D
N10	**V23**	36	39.1	110	70	579	Vaginal	3.01	male	24	N/D
N11	**V24**	35	39.2	125	71	560	Vaginal	3.01	female	24.7	N/D
N12	**SITE-1**	35	38.5	109	78	698	Vaginal	3.5	female	23.5	N/D
N13	**ALISON**	34	39.3	122	67	524	Vaginal	3.51	male	24.5	N/D
	Average	34.46	38.92	122.38	70.77	654.38		3.41		23.63	
	STD	3.20	0.55	7.94	7.04	109.85		0.44		0.86	

Serial no.	Pre-eclamptic:	Maternal age (Years)	Gestational age (Weeks)	Systolic BP (mmHg)	Diastolic BP (mmHg)	Placental Weight (Grams)	Mode of Delivery	Gestational weight (Kg)	Gestational Sex	BMI	Protein in urine (g/L)
P1	**Kirsty**	28	33	150	92	360	Vaginal	1.66	male	23.5	2.5
P2	**PE3**	27	36	168	95	470	Cesearean	2.3	male	22.8	1.5
P3	**PE5**	29	37	152	110	505	Cesearean	1.87	male	22.5	1.14
P4	**V14**	29	38	150	111	420	Vaginal	1.82	female	24.4	1.01
P5	**V16**	27	36	160	92	545	Cesearean	2.6	male	24.6	1
P6	**V17**	28	39	160	90	480	Cesearean	1.76	male	22.8	0.9
P7	**V18**	32	37	150	90	510	Cesearean	2.98	male	22.5	0.98
P8	**V19**	28	38	150	90	529	Cesearean	3.1	female	21	1
P9	**V22**	28	39.5	155	95	510	Cesearean	2.8	male	23.5	0.91
P10	**V25**	28	38	155	112	517	Cesearean	2.9	male	22.8	0.875
P11	**V26**	29	39	140	108	620	Vaginal	3.59	female	23	0.88
P12	**Angela**	29	39.4	156	98	610	Cesearean	3.12	male	22	1.1
	Average	28.5	37.5	153.3	98.5	506.3		2.54		22.95	1.14
	STD	1.31	1.86	6.99	8.98	71.79		0.64		0.98	0.46

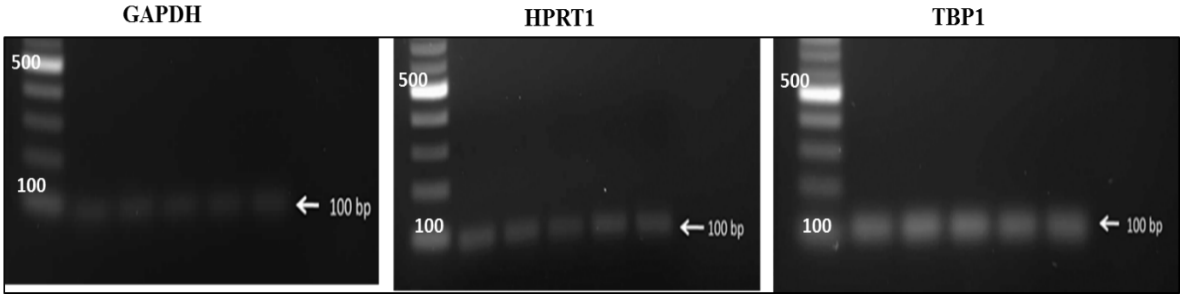
A.4 Accession numbers for target genes used for primer designing

C/T antigen	NCBI accession number
BUC11	Primer provided by the John van Geest Cancer Research Centre
CAGE1	NM_001170692 ; NM_01170693 ; NM_205864
FATE1	NM_033085
GAGE1	NM_001040663.2
GAGE C1/PAGE 4	AK312039
GAGE E1/PAGE 5	NM_001013435
HAGE	Primer provided by the John van Geest Cancer Research Centre
MAGE A1	NM_004988
MAGE A3	NM_005362
MAGE A4	NM_001011548 ; NM_002362 ; NM_001011549 ; NM_001011550
NY-ESO-1	Primer provided by the John van Geest Cancer Research Centre
PASD1	NM_173493
T128	Primer provided by the John van Geest Cancer Research Centre
T21	Primer provided by the John van Geest Cancer Research Centre

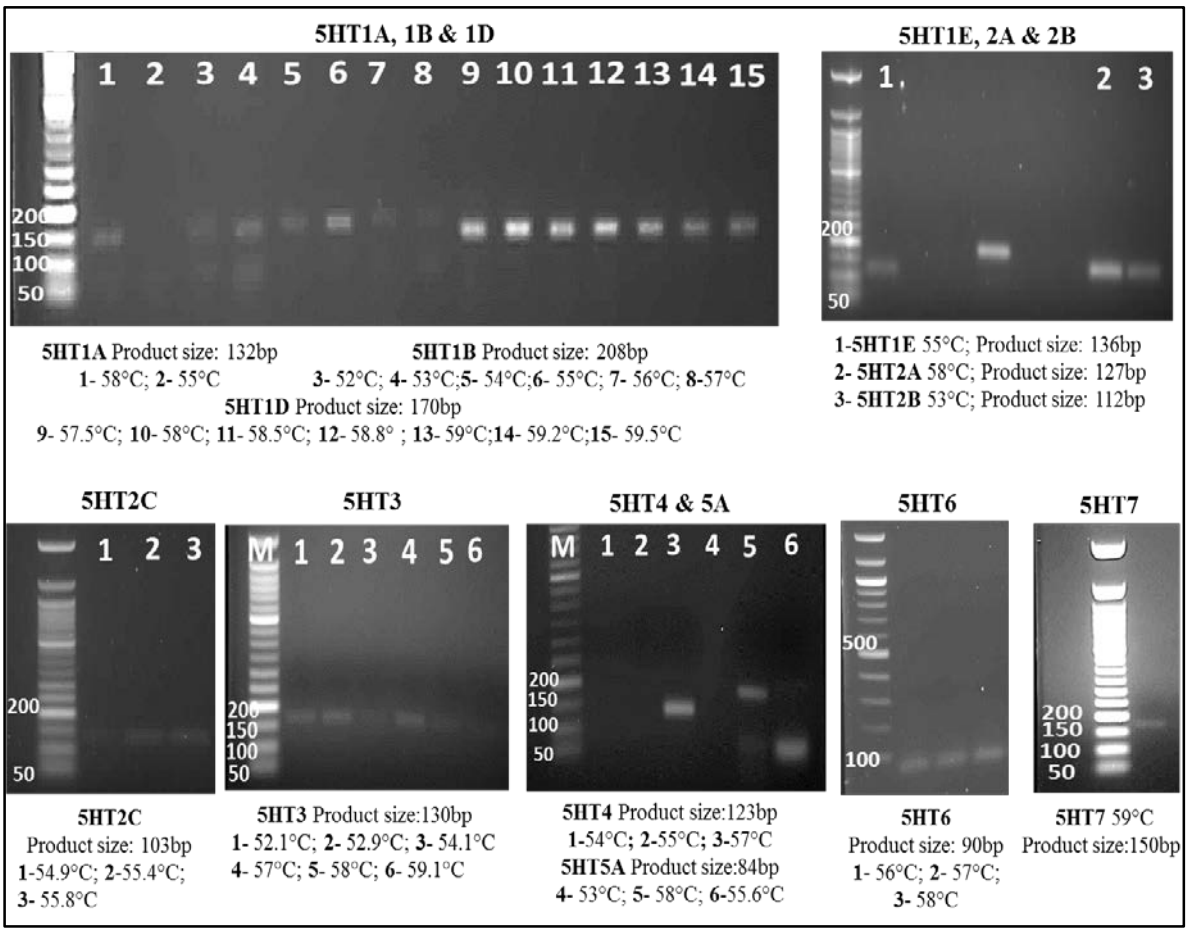
5HT receptors	NCBI accession number
5HT 1A	NM_000524
5HT 1B	NM_000863
5HT 1D	NM_000864
5HT 1E	NM_000865
5HT 2A	NM_000621; NM_001165947
5HT 2B	NM_000867
5HT 2C	NM_000868
5HT 3	NM_213621; NM_000869; NM_001161772
5HT 4	NM_001040173; NM_001040172 NM_001040171; NM_001040169; NM_199453; NM_000870
5HT 5A	NM_024012
5HT 6	NM_000871
5HT 7	NM_019860; NM_019859; NM_000872

A.5 DNA confirmation by gels and sequencing

A.5.1 Housekeeping genes



A.5.2 Temperature optimisation for 5-HT receptors



5HT1A Product size: 132bp
1- 58°C; **2**- 55°C

5HT1B Product size: 208bp
3- 52°C; **4**- 53°C;**5**- 54°C;**6**- 55°C; **7**- 56°C; **8**-57°C

5HT1D Product size: 170bp
9- 57.5°C; **10**- 58°C; **11**- 58.5°C; **12**- 58.8° ; **13**- 59°C;**14**- 59.2°C;**15**- 59.5°C

1-5HT1E 55°C; Product size: 136bp
2- 5HT2A 58°C; Product size: 127bp
3- 5HT2B 53°C; Product size: 112bp

5HT2C
Product size: 103bp
1-54.9°C; **2**-55.4°C;
3- 55.8°C

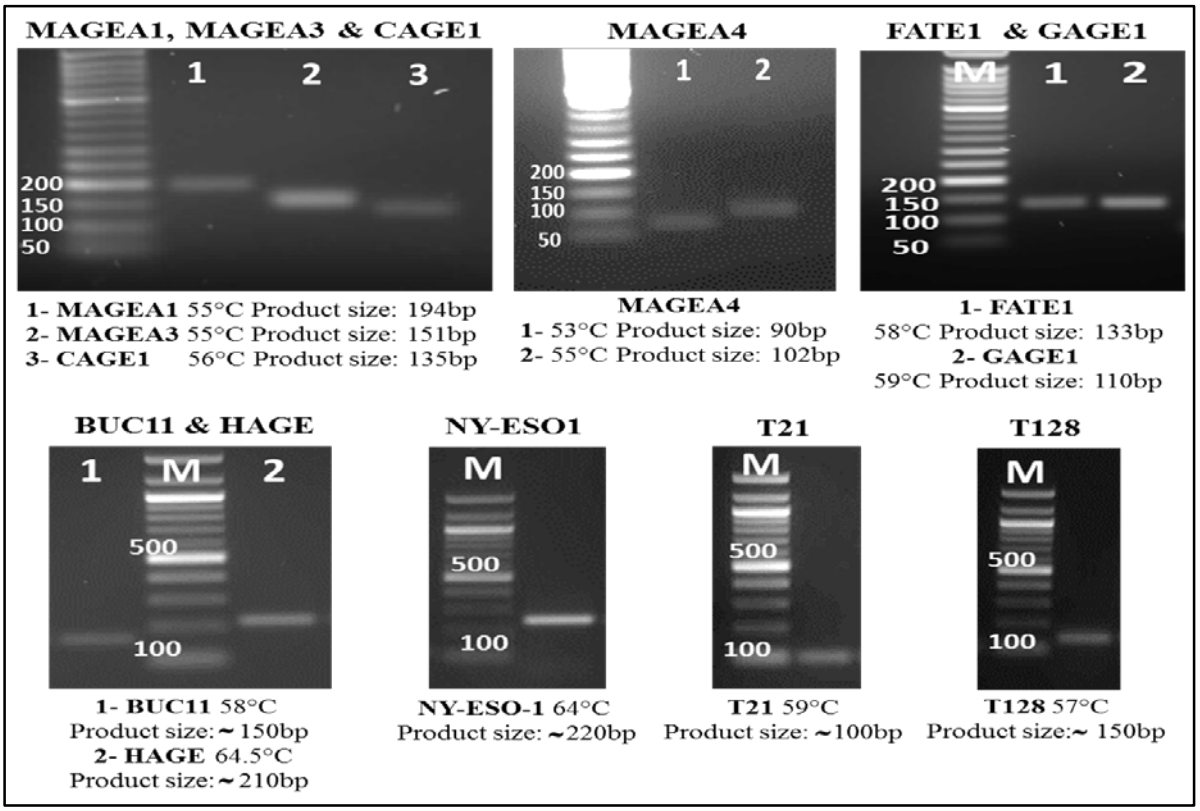
5HT3 Product size:130bp
1- 52.1°C; **2**- 52.9°C; **3**- 54.1°C
4- 57°C; **5**- 58°C; **6**- 59.1°C

5HT4 Product size:123bp
1-54°C; **2**-55°C; **3**-57°C
5HT5A Product size:84bp
4- 53°C; **5**- 58°C; **6**-55.6°C

5HT6
Product size: 90bp
1- 56°C; **2**- 57°C;
3- 58°C

5HT7 59°C
Product size:150bp

A.5.3 Temperature optimisation for C/T antigens



1- MAGEA1 55°C Product size: 194bp
2- MAGEA3 55°C Product size: 151bp
3- CAGE1 56°C Product size: 135bp

MAGEA4
1- 53°C Product size: 90bp
2- 55°C Product size: 102bp

1- FATE1
58°C Product size: 133bp
2- GAGE1
59°C Product size: 110bp

1- BUC11 58°C
Product size:~150bp
2- HAGE 64.5°C
Product size:~210bp

NY-ESO-1 64°C
Product size: ~220bp

T21 59°C
Product size: ~100bp

T128 57°C
Product size:~ 150bp

A.5.4 Example of sequencing

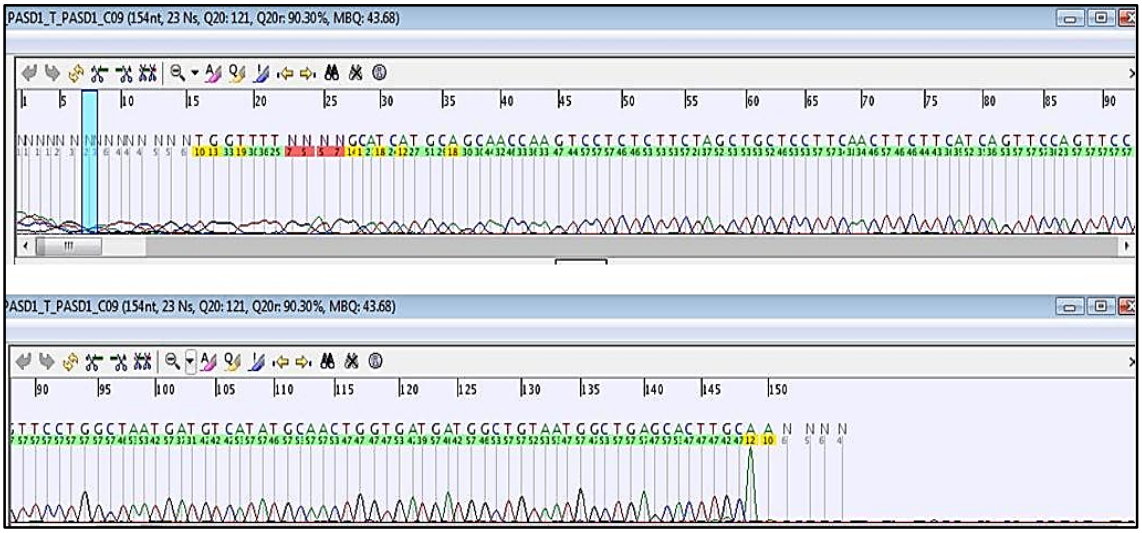


Figure A.7.2: The DNA sequencing data for PASD1 PCR product.

The bases represented by N were replaced by the specific bases identified by their coloured peaks (A- Green, T- Red, G- Black and C- Blue).

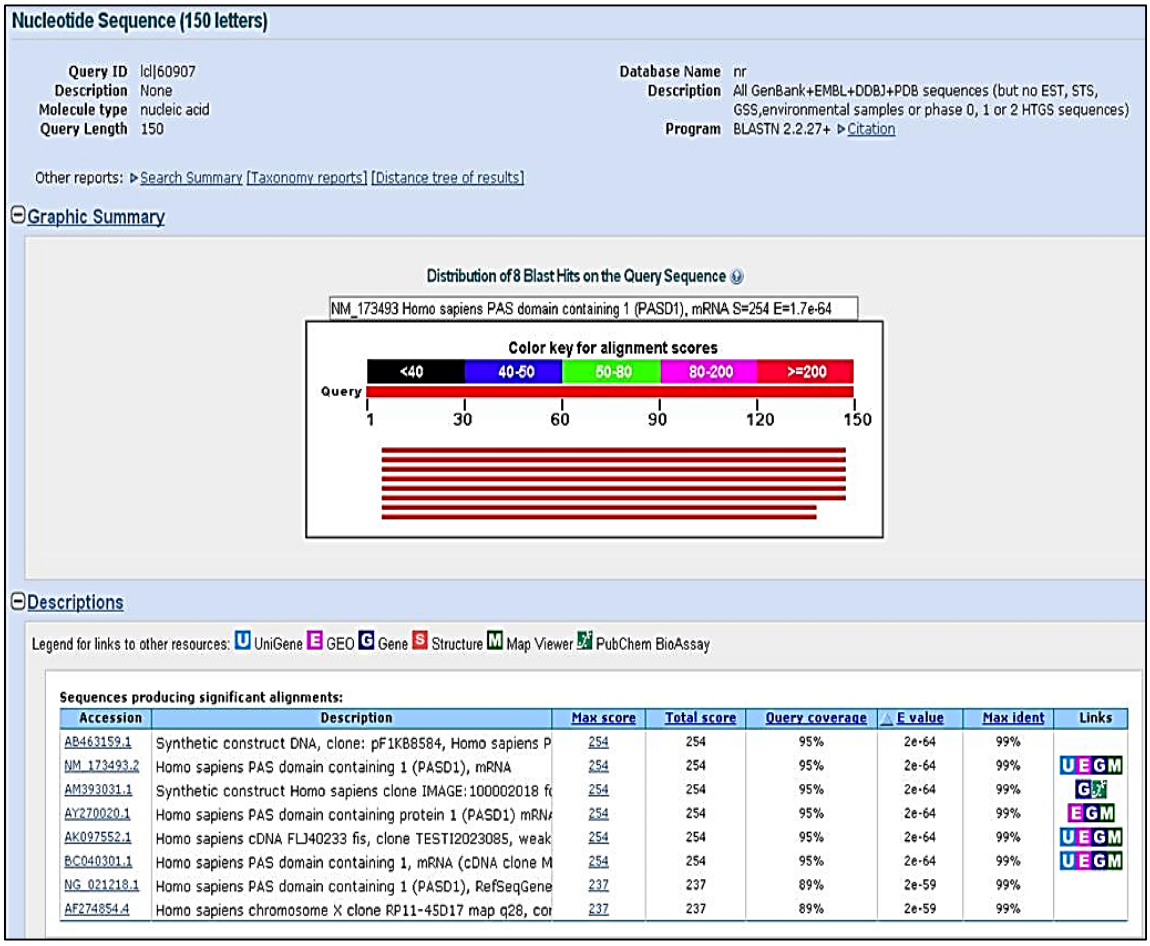


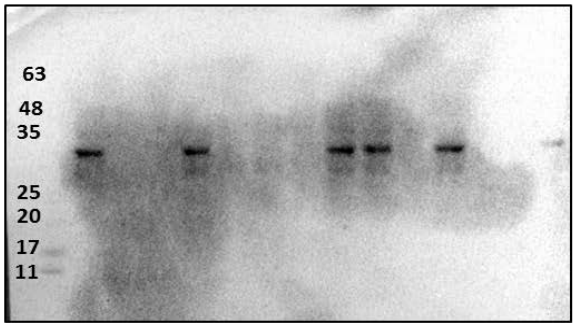
Figure A.7.3: Example of BLAST matching data for PASD1 sequence obtained from sequencing.

The red bands shows >=200 alignment score for the DNA sequence received from the company and the gene of interest PASD1 (with 99% maximum identity).

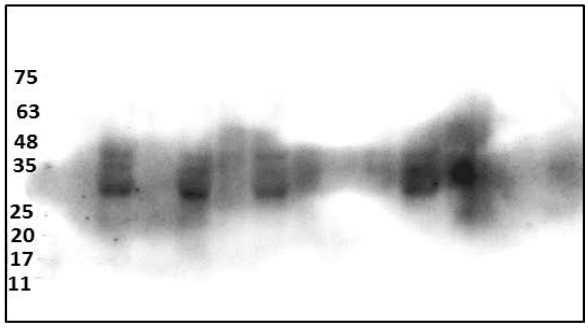
A.6 Western blot whole blots

5HT1BR NT

Predicted	Detected
47kDa	Approx. 33kDa

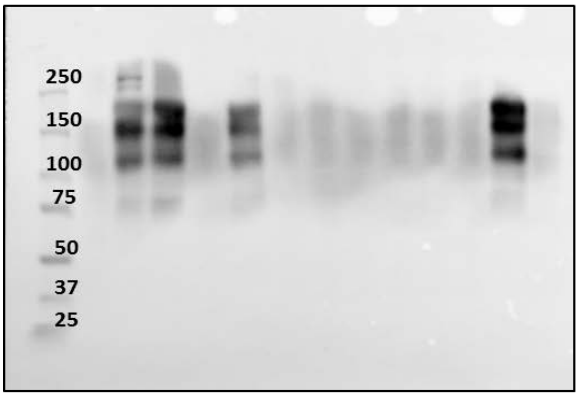


5HT1BR PE

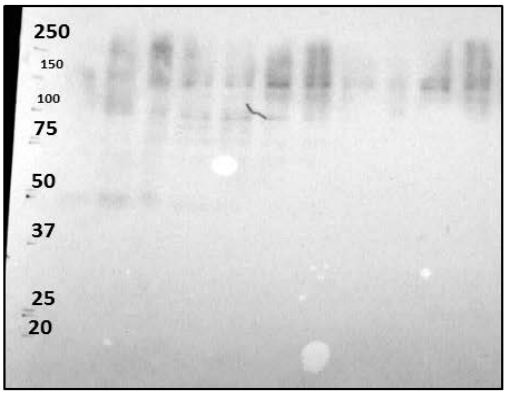


CAGE1 NT

Predicted	Detected
95-230 kDa	100,150,175kDa

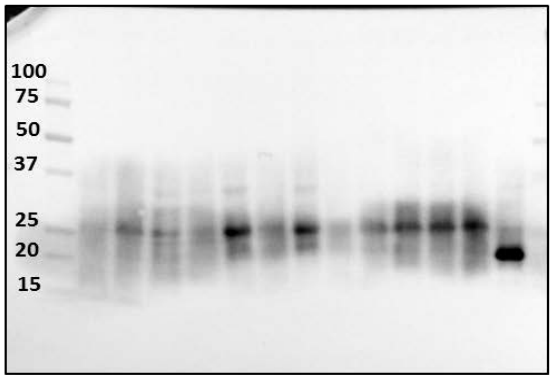


CAGE1 PE

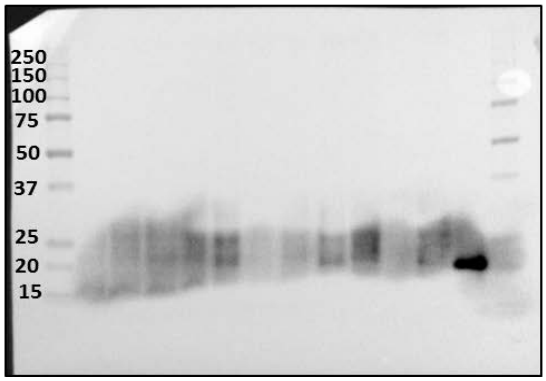


FATE1 NT

Predicted	Detected
17-28 kDa	17-28 kDa

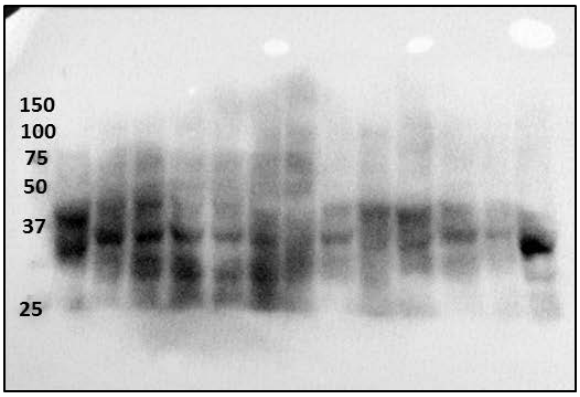


FATE1 PE

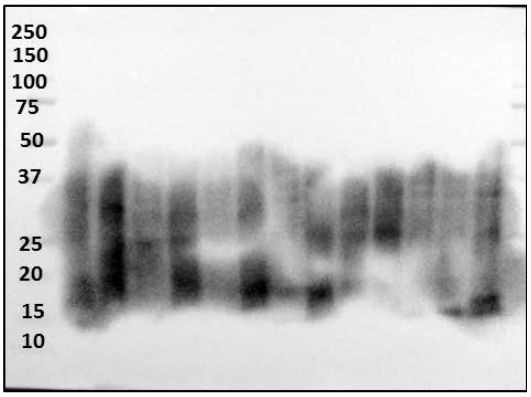


GAGE1 NT

Predicted	Detected
25-37kDa	30-40



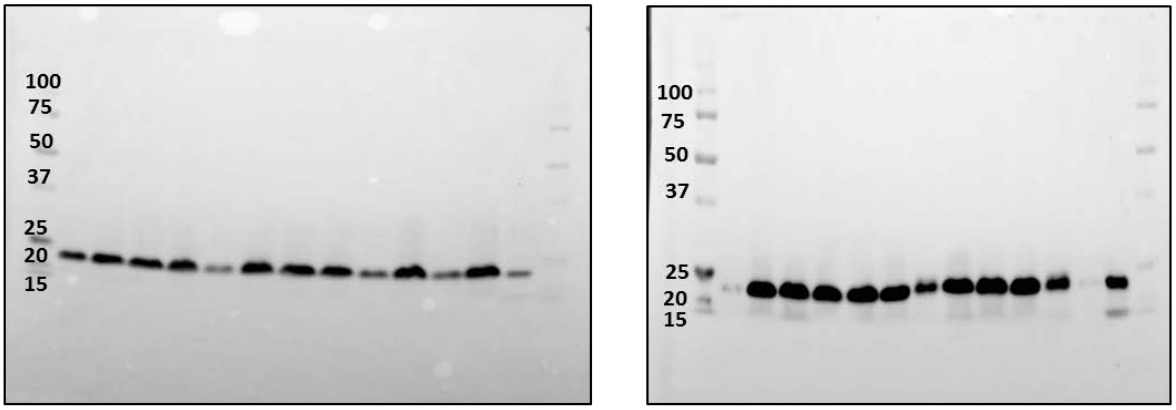
GAGE1 PE



GAGEE1 NT

Predicted	Detected
14 kDa	22kDa

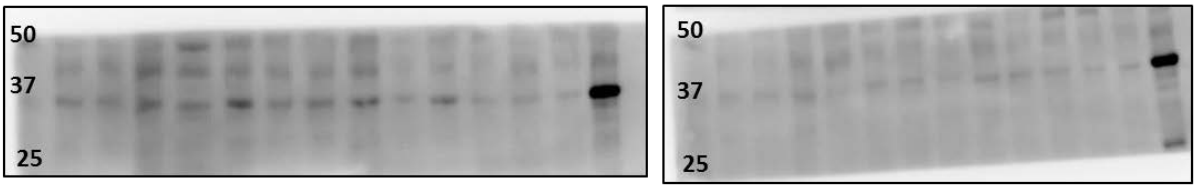
GAGEE1 PE



MAGEA4 NT

Predicted	Detected
25-37 kDa	37kDa

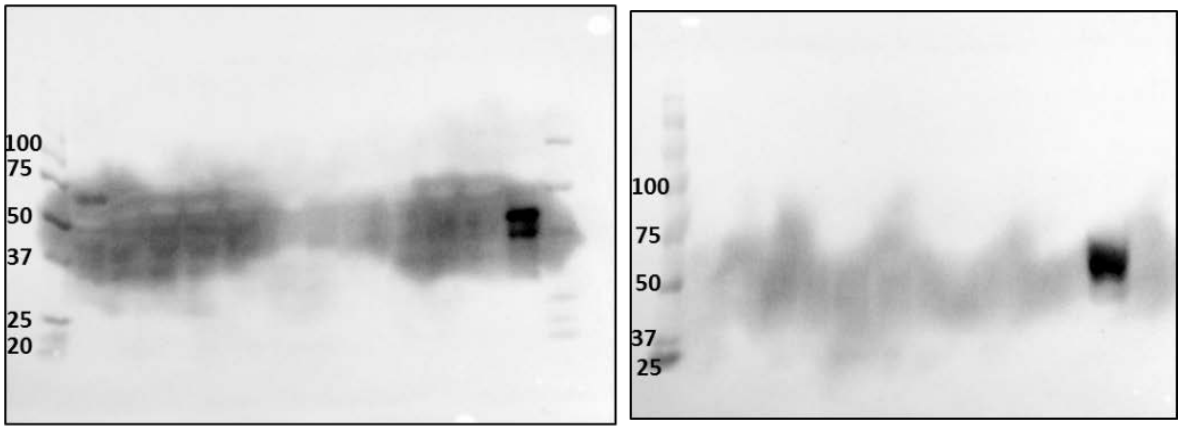
MAGEA4 PE

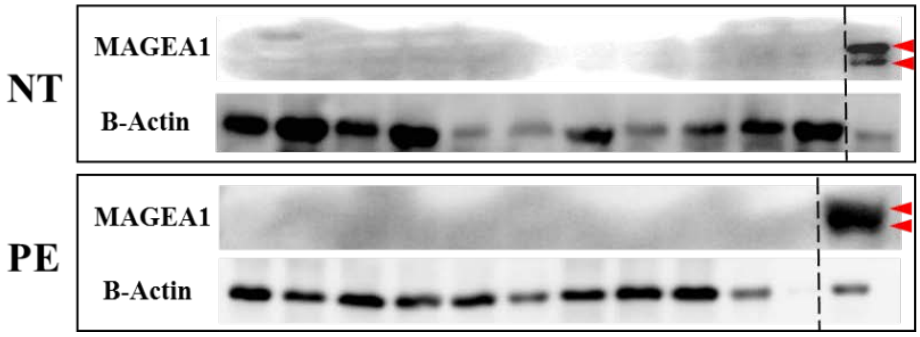


MAGEA1 NT

Predicted	Detected
34kDa	37-50kDa

MAGEA1 PE





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Communications resulting from the study

Research Article:

Ugun-Klusek A, **Tamang A**, Loughna P, Billett E, Buckley G, and Sivasubramaniam S (2011) Reduced placental vascular reactivity to 5-hydroxytryptamine in pre-eclampsia and the status of 5-HT_{2A} receptors . *Vascular Pharmacology*. 55(5-6), pp. 157-162.

Conference Abstracts:

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