UNIVERSITY OF BIRMINGHAM

DEVELOPMENT OF *IN VITRO* MODELS TO INVESTIGATE THE ROLE OF DECIDUALISATION, PDGF, AND HO-1 ON STROMAL – TROPHOBLAST INTERACTION

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Abstract

Decidualisation of human endometrial stromal cells (hESC) is essential for embryo implantation. As ethical barriers prevent investigation of human implantation *in vivo*, this project aimed to develop convenient/reliable co-culture models to evaluate the activity of platelet-derived growth factors (PDGF) and the anti-inflammatory/anti-oxidant haem-oxygenase-1 (HO-1) in hESC–trophoblast interactions. The St-T1b cell line and primary hESC were decidualised *in vitro* and HTR-8 trophoblast line grown as spheroids and used to develop trophoblast-spheroid expansion and invasion/sprouting assays. Fluorescence cell-labelling coupled with ImageJ/trainable weka plugin facilitated monitoring/analysis of assays over 72 h. Decidualisation of hESC reduced their migration in scratch-wound assays, inhibited trophoblast sprouting and promoted trophoblast-spheroid spreading. PDGF-AA was down-regulated whilst PDGF-α/-β receptor expression increased with hESC decidualisation. PDGF-BB increased hESC migration and HTR-8 trophoblast spheroid expansion and invasion in the presence of decidual hESC. HO-1 was upregulated during hESC decidualisation in a forkhead box-O1-dependent manner. Induction of HO-1 inhibited hESC migration and increased HTR-8-spheroid invasion. VEGF secretion increased, whereas sFlt-1 levels reduced with hESC decidualisation. Surprisingly, HO-1 induction enhanced sFlt-1 and VEGF expression in hESC and HTR-8 cells. Collectively, the results support use of these *in vitro* models as a platform to evaluate hESC decidualisation and other factors on trophoblast expansion/invasion and identify decidual regulation of HO-1 and PDGF activity in hESC. As defective hESC decidualisation causes infertility and pregnancy disorders, further investigation of HO-1 up-regulation and PDGF responsiveness in decidual hESC may identify new pathways underpinning these conditions.

Dedication

First of all, I dedicate my work for the sake of the merciful Allah, who brings me everything good.

To my beloved country “Iraq” who granted me the opportunity to make the dream coming to truth through the study in the great United Kingdom.

To my mum “Um Bashir” the irony mother of 14 highly educated siblings. Um Bashir, you inspire me for never giving up in accomplishing the mission successfully.

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List of abbreviations

Abbreviation	Meaning
Ab	Antibody
AKT	Protein kinase B
APS	Ammonium persulphate
ART	Assisted reproductive technology
α-SMA	Alpha-smooth muscle actin
BCA	Bicinchoninic acid
BMP2	Bone morphogenic protein 2
8-Br-cAMP	8-bromoadenosine- 3',5'cyclic monophosphate
BSA	Bovine serum albumin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
CFDA-SE	Carboxy Fluorescein Di Acetate-Succinimidyl Ester
ChIP	Chromatin immunoprecipitation
cMYC	Cellular myelocytomatosis
CREB	cAMP Response Element-Binding Protein
CREM	cAMP Response Element Modulating Protein
DEPC	Diethylpyrocarbonate
DES	Diethylstilbestrol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphide
dPRL	Decidual prolactin
E2	17ß-Oestradiol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked immunosorbent assay
ESC	Endometrial Stromal Cells

EVT	Extra Villous Trophoblast
FBS	Fetal Bovine Serum
Flt-1	Fms-like Tyrosine Kinase-1/VEGFR-1
FOXO	Forkhead Box O transcription factor
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
GPCR	G-protein coupled receptor
HB-EGF	Heparin-Binding Epidermal Growth Factor
hCG	Human Chorionic Gonadotrophin
hESC	Human Endometrial Stromal Cell
HMEC-1	Human Microvessels Endothelial Cell line 1-
HO-1	Heme Oxygenase-1
HOXA10	Homeobox A 10
hTERT	Human Telomerase Reverse Transcriptase
HUVEC	Human Umbilical Vein Endothelial Cell
IGF-1	Insulin-like Growth Factor-1
IGFBP-1	Insulin Growth Factor Binding Protein-1
IL	Interleukin
IRF4	Interferon Regulatory Factor 4
IUGR	Intrauterine Growth Restriction
IVF	*In vitro* fertilisation
LH	Luteinizing hormone
LIF	Leukaemia inhibitory factor
MARK	Mitogen-activated protein kinase
MET	Mesenchymal-epithelial transition
MMP	Matrix metalloproteinase
MPA	Medroxy-Progesterone Acetate
NOX1	Nicotinamide adenine dinucleotide phosphate oxidase
PBS	Phosphate-buffered saline
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor

PDGFR	Platelet-derived growth factor receptor
Pen/Strep	Penicillin/Streptomycin
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIGF	Placenta Growth factor
PKA	Protein kinase A
PLZF	Promyelocytic Leukaemia Zinc Finger
PPARγ	Peroxisome Proliferator-activated receptor gamma
PPH	Protoporphyrin
PGR	Progesterone Receptor
PRE	Progesterone Response Element
PRL	Prolactin
PVDF	Polyvinylidene Fluoride
qPCR	Real-Time Quantitative Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation Assay Buffer
ROS	Reactive Oxygen Species
RPL	Recurrent Pregnancy Loss
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
sFlt-1	Soluble fms-like tyrosine kinase 1
SnPP	Tin protoporphyrin IX dichloride
SOD2	Superoxide dismutase-2
STAT3	Signal transducer and activator of transcription-3
TAE	Tris-Acetate-EDTA
TEMED	N.N.N’N’ Tetramethylethylenediamin-1,2-Diamin
TGFβ	Transforming growth factor-β
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF-α	Tumour Necrosis Factor-α
uNK	Uterine natural killer cell
VEGF	Vascular Endothelial Growth Factor-A
VEGFR	Vascular Endothelial Growth Receptor

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

The use of assisted reproductive techniques (ART) is still a promising and hopeful solution for both clinicians and infertile patients. However, it is a costly procedure that adds an extra burden to NHS in the UK and health services in most countries. Furthermore, the chance of a live birth following ART is only 29% in women less than 35 years (2018), compared to 84% of couples conceiving naturally within a year if they have regular unprotected sex (NHS, 18). One of the main limiting steps in ART is embryo implantation (Spandorfer and Rosenwaks, 1999, Kreyenfeld and Konietzka, 2017). Accordingly, research to increase the rate of success, particularly improving embryo implantation rates, is demanded.

Successful implantation requires the presence of a correctly developed blastocyst stage embryo and a synchronous receptive endometrium leading to a maternal-fetal interaction which comprises maternal decidua and invading trophoblast (Simón *et al.*, 2000). Perturbations of this dialogue can lead to infertility, pregnancy loss (miscarriage), growth restriction of the fetus or maternal pregnancy disorders, including pre-eclampsia (Gellersen *et al.*, 2007a).

1.2. The human endometrium

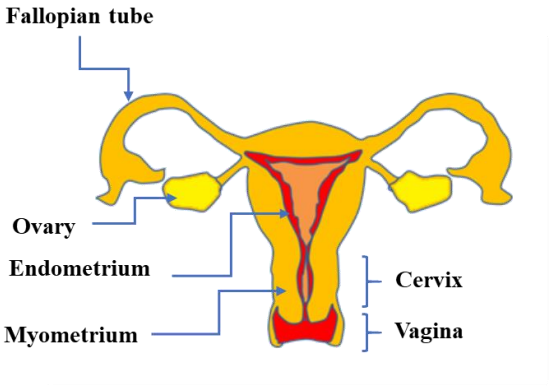
The human uterus composed of perimetrium, myometrium and endometrium (Hermes *et al.*, 2000) (

Figure **1.1** and Figure 1.2). The endometrium is the mucosal lining of the uterine endometrial cavity. Its basic function is to offer a proper place for embryo implantation and growth support. If no implantation occurs, most of the endometrium will be sloughed off. The result will be menstrual flow, followed by endometrial regeneration next cycle (Lessey, 2000). Cyclical endometrial shedding and regeneration are regulated by ovarian oestrogen (E2) and progesterone (P) hormones with a typical 28 day menstrual cycle that occurs approximately 400 times during the

human female reproductive lifespan (Teklenburg *et al*., 2010a, Somasundaram, 2016). The endometrium is subdivided into two layers. The outer thinner layer lies next to the myometrium and is called the stratum basalis. This layer is relatively fixed and does not change during the menstrual cycles (Ham and Cormack, 1987, Ayachit and Kulkarni, 2017). The inner layer is the stratum functionalis which lines the endometrial cavity. It is thicker, extremely sensitive to ovarian hormonal changes, completely shed during menstruation, and regenerated again from progenitors cells in the stratum basalis (Lindenberg, 1994, Ayachit and Kulkarni, 2017). In the presence of conceptus, the stratum functionalis is maintained and forms the decidua.

Histologically, the endometrium is composed of glandular and luminal epithelia, stromal fibroblasts, endothelial cells, vascular smooth muscle cells and leukocytes (Chan *et al*., 2004). The uterine blood supply is mainly through uterine arteries which pass through the myometrium and divide to give the basal arteries (Ferris *et al*., 1972, Moore *et al*., 2013). The basal arteries are short straight arteries, which enter the endometrium and supply the stratum basalis. Spiral arterioles arise from basal arteries and supply stratum functionalis (Kurup, 2008). They form thick and long tortuous vessels, which are very sensitive to ovarian hormone fluctuation and become more prominent with endometrial secretory changes (Smith, 2018). These arterioles anastomose with each other forming an extensive vascular network invading the stroma (Rogers, 1996) as shown in

Figure **1.1**. In the first trimester of pregnancy, remodelling of spiral arterioles occurs through the loss of maternal vascular smooth muscle and endothelial cells and replacement by extra villus trophoblasts embedded into a fibrinous matrix (Smith *et al*., 2009). Thus, vessels will be changed from narrow elastic to relaxed channel-like structures. Thereby, they can permit a high volume and low resistance blood flow to the fetus. This blood flow maintains healthy fetal growth and successful pregnancy (Burton *et al*., 2009).



Uterus and ovaries

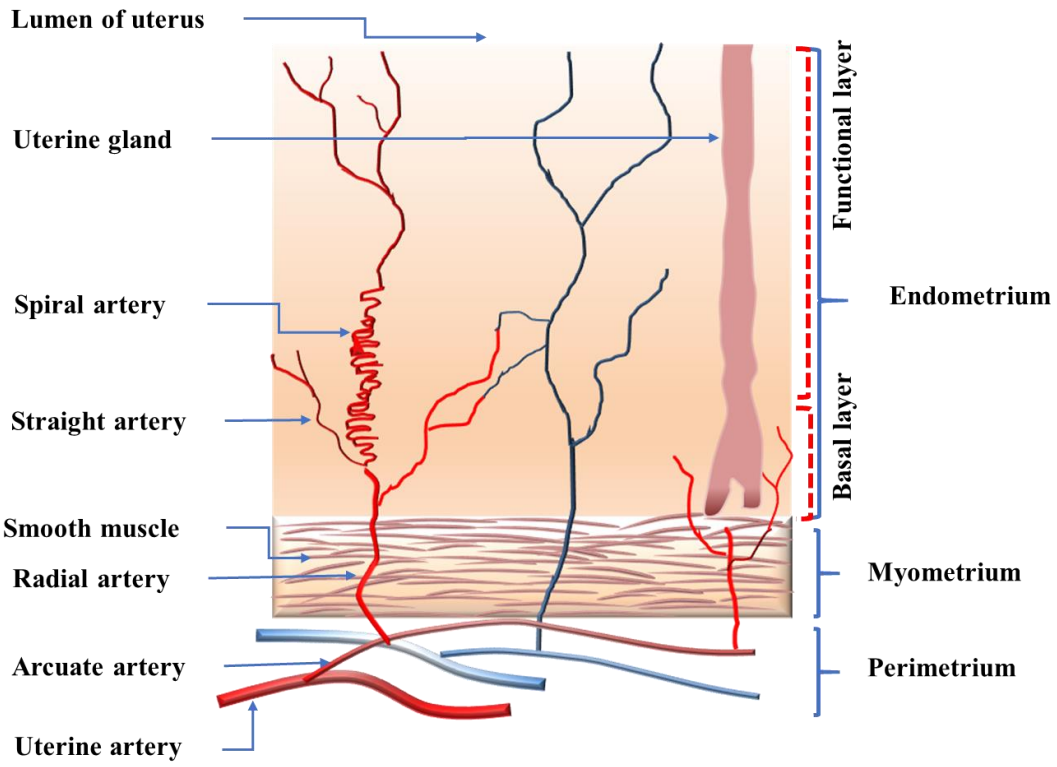


Figure 1.1: **The human female reproductive and endometrium structures**. The upper figure showing the ovary and uterus. The lower figure shows the uterine layers. In the endometrium, the functional layer is the innermost one that contains the epithelial layer, glands, and stroma. This layer receives blood supply from the spiral arteries. The second layer is the basal layer which is the progenitor of the functional layer. Adapted from Pijnenborg *et al* (2006)

1.3. Human endometrial cyclical changes

The cyclical endometrial changes are regulated by E2 and P (Salker, 2013). Ovarian steroids are regulated by hypothalamic-pituitary hormones. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) which stimulates the pituitary to secrete the glycoproteins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Figure 1.2).

FSH and LH hormones induce the ovary to secrete P, E2, activins, and inhibin. Prominent feedback control is operated throughout the axis (Silberstein and Merriam, 2000, Mikhael *et al.*, 2019). FSH and LH release is depending on positive and negative feedback of ovarian hormone. During the luteal phase, E2 regulates LH while P and inhibin B regulate FSH. Both E2 and P regulate FSH and LH during the luteal phase while inhibin A affects FSH. E2 is the main positive feedback sensitiser of the pituitary to the gonadotrophin releasing hormone (Messinis, 2006)

Morphologically, the cyclical endometrial changes can be divided into three phases: follicular, secretory, and menstruation (Ferenczy and Guralnick, 1983, Murray and Orr, 2020). Menstruation spans days 1-5 of the cycle, early to a mid-proliferative phase spans from 6-11 days; late proliferative phase, which extends from days 12-14, early secretory phase, which spans days 15-19. Finally, the mid-late secretory phase extends beyond 20th day until the initiation of menses (Sakamoto, 1985, Reed and Carr, 2018).

1.4. The proliferative phase of the endometrial cycle

This phase is under the influence of E2 secreted from growing follicles in the ovary (Reed and Carr, 2018). Regeneration of the endometrium starts from the basal layer in the early proliferative phase, the endometrium is thin with sparse narrow short glands (Figure 1.1 and Figure 1.2).

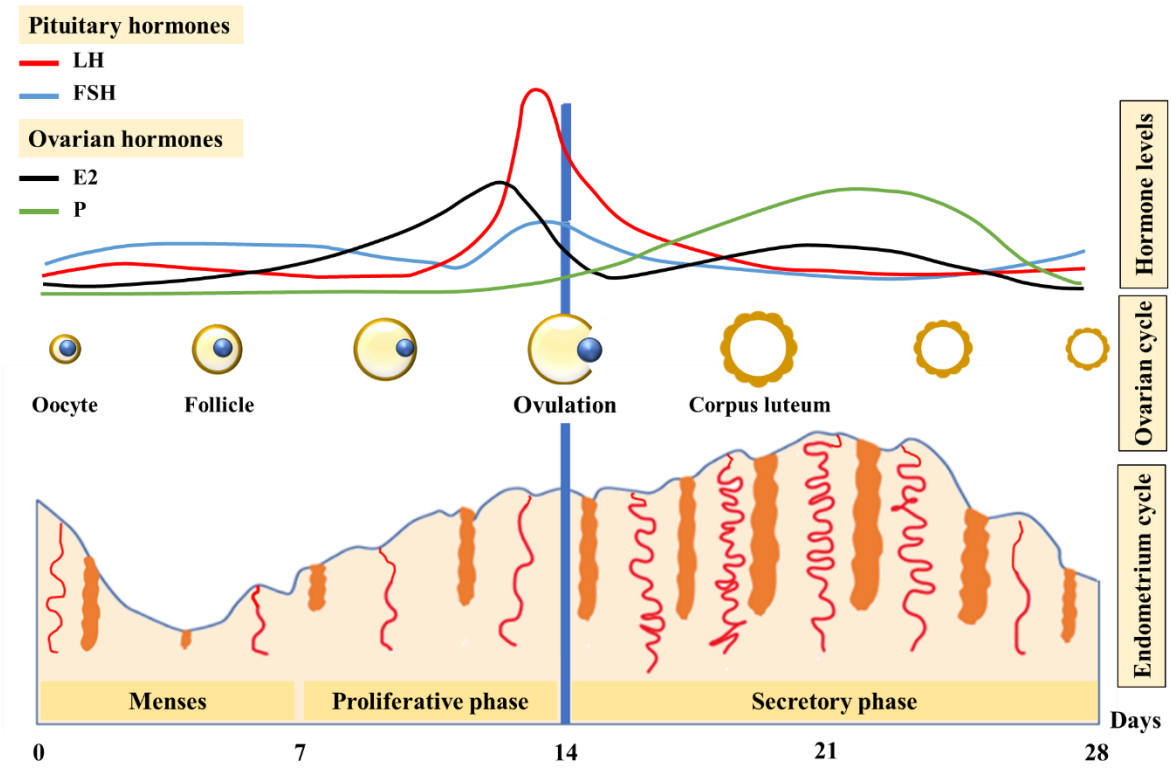


Figure 1.2: Schematic representation of events in the endometrium regulated by pituitary gonadotropins and ovarian steroids during the menstrual cycle. FSH hormone from the anterior pituitary gland stimulates ovarian follicle growth. The growing follicles release E2, which induces proliferation of the endometrium. Increased release of LH from the anterior pituitary (LH surge) stimulates ovulation of the dominant follicle. Post ovulatory P release induces secretory changes and decidualisation in the endometrium to prepare for embryo implantation. The failure of pregnancy causes endometrial sloughing and menstruation begins. Adapted from Hatcher and Namnoum (2004).

From day three of the cycle, proliferation increases both in the glands and stroma and active mitosis is evident. The epithelial cells proliferate to replace the sloughed surface of the endometrium (Mihm *et al*., 2011). The glands grow in size and become elongated. Finally, the spiral arterioles extend in the regenerating endometrium due to the proliferation of their vascular endothelial cells (Gellersen *et al*., 2007b). The progressive production of E2 by the dominant follicle in the ovary induces a rise in LH production – the LH surge (Clarke, 1995). Ovulation occurs approximately on day 14, 24-36 h following this surge, (Miller and Soules, 1996). The remnant of the ovulated follicle in the ovary becomes the corpus luteum, which secretes post-ovulatory P (Niswender *et al*., 2000)

1.5. The secretory phase

The secretory phase occurs as a result of P secretion from the corpus luteum, which inhibits proliferation and induces differentiation of E2-primed endometrial cells (Bentley, 1998, Binder *et al*., 2015). During this phase, the glands become coiled and filled with glycogen-rich secretory product. The spiral arterioles become coiled. The stromal cell enlarges in size with dramatic changes in the extracellular matrix and are transformed into decidual cells in a process called decidualisation (Pan-Castillo *et al*., 2018). In the absence of pregnancy, E2 and P secretion fall as the corpus lutetium ages and undergoes fibrosis (Alam, 2006).

1.6. Menstruation

Following the decline in P secretion (Figure **1.2**), an influx of leukocytes, VEGF release, and spiral vessel constriction occur in the endometrium (Irwin *et al*., 1996, Reavey *et al*., 2019). This constriction is triggered by the production of prostaglandin from stromal cell lysosome and cause ischemia of stratum functionalis (Reavey *et al*., 2019). Later, the process becomes progesterone-independent, due to the secretion of matrix metalloprotease from the leukocytes,

which causes cell membrane lysis and degradation of the extracellular matrix (ECM) (Irwin *et al*., 1996). This leads to the shedding of the stratum functionalis of the endometrium and bleeding. Menstrual bleeding occurs from day 1-5 of the menstrual cycle and aids with the fibrinolytic activity. However, thrombin production from the basal layer secures homeostasis (Mihm *et al.*, 2011). Synchronised regeneration under the influence of E2 occurs from the stratum basalis layer (Mihm *et al*., 2011).

1.7. Decidualisation and implantation window

Decidualisation can be defined broadly as postovulatory uterine remodelling in preparing for embryo implantation and pregnancy that involves all the endometrium and the inner third of the myometrium, which is called the junctional zone (Gellersen *et al*., 2007b). Thus, it leads to the coiling of the spiral arterioles, the entrance of uterine natural killer (uNK) leukocytes, maturation of secretory glands, and stromal fibroblast decidual transformation (Gellersen and Brosens, 2003a). Consequently, decidualising stromal cells attain the unique capability to control trophoblast invasion, to cope with oxidative and inflammatory injuries, and to prevent rejecting maternal immune reactions (Gellersen *et al*., 2007a). A more restricted definition is the morphological and biochemical transformation of ESC to secretory cells, which support immune-privileged and invigorating environments crucial for implantation and success of pregnancy (Gellersen *et al*., 2007b). Typically, menstruating animals undergo spontaneous decidualisation after ovulation, while in non-menstruating species, decidualisation require signals from an implanting embryo (Emera *et al*., 2012). Decidualisation occurs mainly in haemochorial placentation where the fetal chorion achieves direct contact with maternal blood for nutrient and gas exchange in species such as the human, mouse, rabbit, and guinea pig (Brosens *et al*., 2002). In humans, decidualisation is initially visible in the area surrounding the spiral arterioles around the 23rd day of the menstrual cycle (Figure **1.2**). Decidualisation

continues until the 28th day of the cycle (Brosens and Gellersen, 2006). It later migrates evenly throughout the endometrium during pregnancy.

The programmed remodelling of uterine endometrium during decidualisation occurs via rising postovulatory P acting on E2-primed endometrial stromal cells leading to the stimulation of the cyclic adenosine monophosphate (cAMP) system (Telgmann *et al.*, 1997). Various paracrine or autocrine regulators operate beside hormonal control (Carson *et al*., 2000). During early pregnancy, decidualisation is maintained by blastocyst-derived human chorionic gonadotropin (hCG), which preserves the corpus luteum to maintain P production (Berndt *et al*., 2006) leading to the formation of pregnancy decidua.

Decidualisation indicates the end of a restricted time of endometrial receptivity known as the implantation window (IW) (Brosens and Gellersen, 2006). The IW refers to the time during which a competent blastocyst can attach to a receptive endometrium and usually spans the 20th to 24th day of a regular menstrual cycle (Quinn and Casper, 2008, Wilcox *et al.*, 1999). Embryo implantation out of this period is likely to be unsuccessful (Brosens and Gellersen, 2006). A restricted time for implantation receptivity allows for the recruitment of a competent embryo more than abnormally developed but highly invasive embryos as in the study of Wilcox *et al* (1999), who found that implantation out of the IW is associated with early pregnancy loss. Approximately 50% of embryo implantation failure in ART is due to defective endometrial receptivity (Salamonsen, 2009). Some authors consider the establishment of a receptive phenotype is synchronised with decidualisation of the endometrial stroma. This notion is based on the idea that the receptivity of the endometrial epithelia, which is the primary barrier of implantation, is achieved by a substance secreted from the endometrial stromal cell under P control. E.g. morphogen Indian hedgehog in murine uterine epithelium which is essential for normal P effect on the uterus depends on the P receptor in the stromal cells (Simon *et al*., 2009,

Salker *et al.*, 2010). During the IW, the endometrium expresses proteins that enable critical modulations to occur in a sequence starting with a fundal localisation of the embryo and ending with trophoblast invasion into the uterine stroma (Kimber, 2008, Wang and Dey, 2006).

1.7.1. Morphological changes in stromal cells

During decidualisation, endometrial stromal cells (ESC) undergo differentiation into active secretory cells associated with characteristic morphological changes. They transform from elongated stellate fibroblasts to large rounded polygonal epithelioid secretory cells with an enlarged rounded active nucleus, well developed Golgi cisternae and rough endoplasmic reticulum, and cytoplasmic deposition of lipid and glycogen droplets (Fazleabas *et al*., 2004). Decidualised ESC are structurally close to myofibroblasts expressing desmin, α-smooth muscle actin (α-SMA), and vimentin (Oliver *et al*., 1999). During decidualisation, re-arrangement of the cyto-skeleton occurs regulated by the activation of myosin light chain and α-SMA dynamics. There is down-regulation of α-SMA (Fazleabas *et al*., 2004) and polymerisation of filamentous actin combined by stress fibre formation (Teklenburg *et al.*, 2010a).

1.7.2. Biochemical changes in stromal cells

The biochemical transformation of the decidual cells is manifested through the expression of products such as insulin-like growth factor binding protein-1 (IGFBP-1), decidual prolactin (dPRL), Forkhead box O-1 (FOXO1), tetraspanin CD82 (KAI1), (Gonzalez *et al*., 2011), interferon regulatory factor 4 (IRF4), Promyelocytic leukaemia zinc finger (PLZF), and superoxide dismutase-2 (SOD2) (Sugino *et al.*, 2000). Additionally, the decidualised endometrial cells express chemoattractant factors such as cytokines and growth factors, which attract an influx into the decidua of specialised uterine natural killers leukocytes (uNK), which represent 90% of resident lymphocytes in the early conception (Brosens and Gellersen, 2006).

The uNKs have a primary action in the formation of both angiogenic factors and cytokines (Demir *et al.*, 2010, Li *et al.*, 2008), the regulation of trophoblast invasion, uterine vascular remodelling (Cartwright *et al.*, 2010), and additional cytotoxic effects (Dosiou and Giudice, 2005). An important role of decidual cells is their regulatory role in the trophoblastic invasion through a not fully understood involvement of the cytokine/chemokine environment (Loke and King, 1995, Menkhorst *et al*., 2019) with various expressions of regulatory factors such as metalloproteinase, cell adhesion molecules, and immune complex (Lunghi et al., 2007).

1.7.2.1 dPRL

Prolactin (PRL) is a hormone produced by lactotroph cells of the anterior pituitary, dermal fibroblasts (Richards and Hartman, 1996), hematopoietic cells (De Bellis *et al*., 2005), and decidual endometrial cells when it is known as decidual prolactin (dPRL). The secretion of dPRL needs cAMP stimulation (Telgmann *et al*., 1997). Progesterone has no noticeable effect on dPRL secretion (Telgmann and Gellersen, 1998). dPRL secretion is increased by insulin and relaxin (Li *et al*., 1993). In contrast, dPRL secretion by hESC is reduced by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), tumour necrosis factor- α (TNF-α), and interleukin (IL) (Ben-Jonathan *et al*., 1996).

The initiation of dPRL secretion by the decidual cell is synchronised with the histological decidualisation and increases with decidualisation. dPRL can act locally or on the corpus luteum (Maslar and Riddick, 1979, Marano and Ben-Jonathan, 2014). The PRL function in the endometrium depends on the Janus Kinase (JAK) 2/ Signal transducer and activation of transcription protein (STAT) pathway. Upon stimulation with dPRL, the phosphorylation of the pathway occurs, and functions are initiated (Jabbour *et al*., 1998). dPRL plays a crucial role during embryo implantation by affecting the growth and differentiation of trophoblast cells,

enhancing the endometrial gland's secretory function that promotes the trophoblastic invasion, regulation of angiogenesis, immunological role through antiapoptotic effect, and stimulation of immune cells proliferation (Jabbour and Critchley, 2001).

1.7.2.2. IGFBP-1

IGFBP-1 belongs to the insulin-like growth factor (IGF) family. IGF-I and II are hormone modulators as well as major binding proteins at the time of the secretory changes of the endometrium. IGF binding to IGFBP-1 increases its half-life and modulating its interaction with the cell surface receptor. The IGF family plays a crucial role in cell metabolism and proliferation. The IGFBP-1 expression is stimulated by glucocorticoids and cAMP in the presence of low insulin concentrations (Suwanichkul *et al*., 1993), relaxin, and P via glucocorticoid response element (Gao *et al*., 1994). Both IGF and insulin both have an inhibitory effect on IGFBP-1 production (Lewitt *et al*., 1991).

IGFBP-1 is not only a marker of decidualisation, but it also promotes trophoblast invasion and migration (Chakraborty *et al*., 2002). Excess production of IGFBP-1 is associated with intrauterine growth restriction (Crossey *et al*., 2002), while low levels are found in polycystic ovarian disease (Apter *et al*., 1995).

1.7.2.3. FOXO1

The FOXO subfamily includes FOXO1, FOXO3a, FOXO4, and FOXO6 (Hannenhalli and Kaestner, 2009). FOXO1 is not constitutively expressed by the uterine stromal cells and is induced during decidualisation while FOXO3a is reported to be suppressed (Christian *et al*., 2002, Labied *et al*., 2006). FOXO4 is not expressed by endometrium (Labied *et al*., 2006). The FOXO1 protein is encoded in humans by the *FOXO1* gene (Galili *et al*., 1993), which is induced

on the first day of decidualisation (Gellersen *et al*., 2007b) by the increase in the cAMP activity. FOXO1 is a master transcriptional regulator involved in the diverse cellular functions such as apoptosis, cell death, immune cells haemostasis, DNA repair, and coping with oxidative stress. All of these are critical features of decidualisation in hESC (Kajihara *et al*., 2006). These functions are regulated by post-translation modifications of FOXO1 protein by phosphorylation, acetylation, deacetylation, and proteolytic degradation. These modifications guide the localisation of FOXO1 either to the nucleus leading to transcriptional activity or the cytoplasm and exposure to proteasomal degradation (Vogt *et al*., 2005) (Figure 1.3). Growth factors such as EGF activate the phosphatidylinositol/AKT signalling pathway leading to transcriptional inhibition of FOXO1 through its phosphorylation and transport out of the nucleus to the cytoplasm (Kops *et al*., 1999, Brunet *et al*., 1999). Nuclear localisation of FOXO1 upon phosphorylation of alternative residues by the mitogen-activated protein kinase (MAPK) family member, JUN N-terminal kinase (JNK) stimulates FOXO1 transcriptional activity as the production of SOD2 and oxidative stress resistance (Essers *et al*., 2004, Wang *et al*., 2005). FOXO1 controls a number of genes involved in decidualisation like IGFPB-1, dPRL, and tissue inhibitors of matrix metalloproteinases (TIMP) (Buzzio *et al*., 2006). Using a luciferase reporter assay, FOXO1 was shown to be a transcriptional coregulator of homeobox-A (HOXA) 11 and CEBP-β leading to the activation of PRL (Lynch *et al*., 2009, Christian *et al*., 2002) and IGFBP-1 promotors (Kim *et al*., 2005).

The promotors of dPRL and IGFBP-1 are stimulated by a FOXO1 transcription complex regulated by the interaction of P and cAMP signals (Gellersen and Brosens, 2003b) in addition to FOXO1 regulation by the PKA pathway and the nuclear P receptor (PGR).

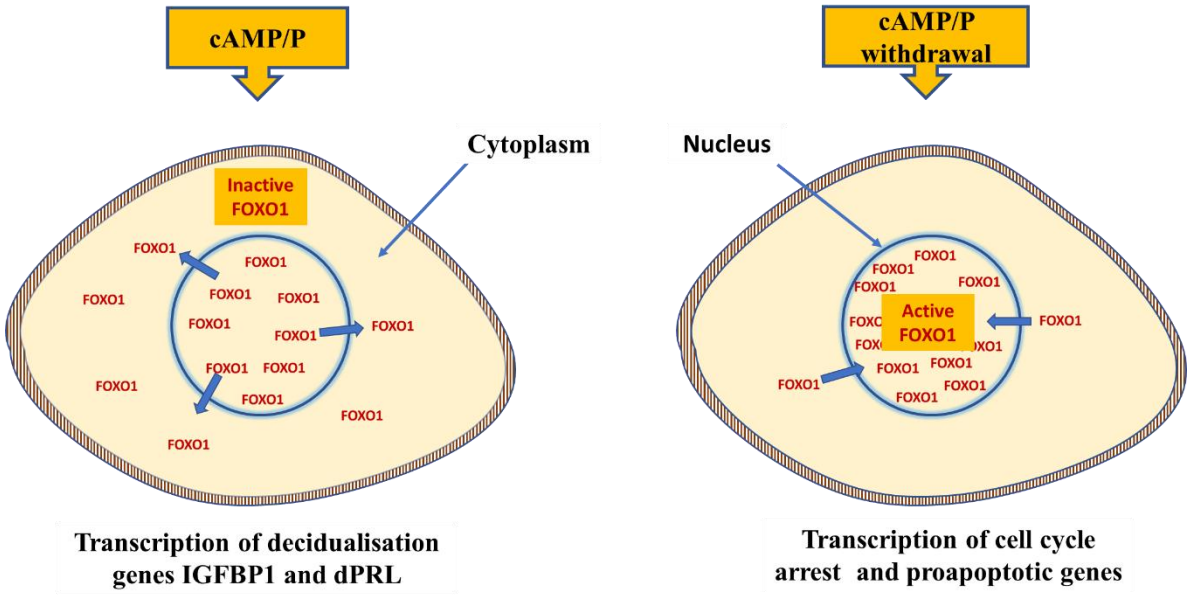


Figure 1.3: Schematic representation of FOXO1 transcriptional activity regulation by P/cAMP in ESCs. Left panel: In the late luteal phase, elevated intracellular cAMP is synergized with P to enhance FOXO1 transcription. However, it causes release of a small nuclear fraction into the cytoplasm to serve as a latent reservoir. The remaining large fraction of nuclear FOXO1 induces the expression of decidualisation genes like IGFBP-1 and dPRL. Right panel: P4 withdrawal before menstruation. Re-entrance of cytoplasmic FOXO1 into the nucleus. The plentiful amount of active nuclear FOXO1 binds to its direct receptor and activates cycle arrest and apoptotic genes. Adapted from Brosens and Gellersen (2006).

A few days following treatment with cAMP, cultured endometrial stromal cells accumulate FOXO1 and this further increases in the presence of P. However, treatment with P alone does not induce FOXO1 expression (Labied *et al.*, 2006). FOXO1 knockdown in hESC resulted in a more than 50% inhibition of IGFBP-1, dPRL, TIMP3, and somatostatin mRNAs (however, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin were unaffected). Collectively, these results robustly suggest a vital role for FOXO1 in inducing the process of decidualisation (Grinius *et al.*, 2006).

1.7.2.4. KAI1

KAI1 is a cell surface protein; it is also called cluster differentiation 82 (CD82). It is classified according to function as a tumour suppressor while it is categorised structurally within the tetraspanin family (Vogt *et al.*, 2002) with two extracellular domains and four transmembrane domains (Stipp *et al.*, 2003). Tetraspanins regulate diverse functions such as cell motility, proliferation (Tohami *et al.*, 2007), and immunity (Toyo-oka *et al.*, 1999). By immunohistochemistry, Gellersen *et al* (2007a) reported the expression of KAI1 by decidual endometrial cells and absence of its expression by the trophoblast cells. Upon KAI1 knockdown, there was a loss of the decidual product decorin, which is involved in limiting trophoblast invasion (Gellersen *et al.*, 2007a).

1.7.2.5. PLZF

PLZF was first recognised in promyelocytic leukaemia (Xue *et al.*, 2000). It is a member of the Krüppel-like zinc finger family that is essential for limb development, myeloid cell differentiation, and nervous system formation (Fahnenstich *et al.*, 2003). It inhibits transcription by recruiting suppressor proteins like histone deacetylase I, nuclear receptor corepressor, silencing mediators for retinoid and thyroid hormone receptors, and Sin3 (Costoya

and Pandolfi, 2001, Melnick *et al.*, 2000). PLZF can offer resistance to apoptosis by inhibiting the transcription of the pro-apoptotic protein (Parrado *et al.*, 2004). PLZF is anti-proliferative through the transcriptional repression of genes regulating the cell cycle such as cyclin A (Yeyati *et al.*, 1999). However, during the early phase of decidualisation before the transformation of ESC into decidualised cells, PLZF may promote proliferation (Gellersen and Brosens, 2014). PLZF is directly induced by P stimulation of endometrial cells, and it is crucial in their progesterone-mediated decidualisation (Kommagani *et al.*, 2016). *In vivo*, PLZF is found in the nuclei of ESC at the mid to late secretory phase (Fahnenstich *et al.*, 2003). Brosens and Gellersen (2006) reported that PLZF mRNA is induced rapidly upon P stimulation of hESCs while cAMP cannot induce it. It is reported that PLZF knockdown in hESC abolishes morphological changes associated with decidualisation, attenuation of the expression of the PRL and IGFBP1 decidual markers, and the reduction of hESC motility (Szwarc *et al.*, 2017).

1.7.2.6. IRF4

IRF4 is an important decidualisation regulator in hESC and a genomic target of both P and FOXO1 (Vasquez *et al.*, 2015). It was found that the knockdown of FOXO1 led to the removal of P from its DNA binding site in IRF4 and decreased the transcription of IRF4 with the reduction of decidualisation. IRF4 regulates the production of interleukin-10 (IL-10) (Lee *et al.*, 2011a), which is predominantly detected in the decidual site (Viganò *et al.*, 2002).

1.7.2.7. SOD2

SOD2 (a.k.a Mn-SOD) belongs to the ubiquitous enzyme family that catalyses the dismutation of superoxide ions. Other family members are SOD1 (CuZn-SOD), which is mostly intracellular, while SOD3 has a particular extracellular localisation (Zelko *et al.*, 2002). In the female reproductive system, embryo implantation and placentation can be considered as

inflammatory processes and changes in oxygen tension can produce an oxidative stress environment (Burton, 2009); decidual cells express SOD2 which contribute to their oxidative stress resistance (Sugino *et al*., 1996, Storz, 2011). FOXO1 is the transcription factor regulating SOD2 expression during decidualisation (Brosens and Gellersen, 2006).

1.8. Decidualisation signalling

1.8.1. Progesterone

Progesterone binds to acts via two dominant PGRs produced by alternative mRNA splicing. PGR-A is a truncated version of PGR-B lacking 164 amino acids (Kastner *et al*., 1990, Gellersen and Brosens, 2014). PGRs display cyclical and cell type-dependent expression in the human endometrium. PGR-A is the predominant receptors in hESC (Mote *et al*., 1999) and is a more powerful transactivator role of dPRL and IGFBP-1promotor (Gao *et al*., 2000). PGR-A is abundantly expressed by hESC along the menstrual cycle and gestation, whereas its epithelial cell expression subsides following ovulation. In contrast, PGR-B's stromal and epithelial cell expression peaks in the mid proliferative phase, followed by a gradual decline afterwards (Jabbour and Critchley, 2001). Selective ablation of PGR receptors in mice revealed that PGR-A is essential for ovarian and endometrial function, while PGR-B is indispensable for mammary development (Mulac-Jericevic and Conneely, 2004). Progesterone is a lipophilic hormone and can pass freely through the cell membrane (Georget *et al*., 1997) to exert its effects via PGR-A which is located mainly in the nucleus, whilst PGR-B shuttles between the nucleus and cytoplasm (Boonyaratanakornkit and Edwards, 2007). Progesterone binding induces structural changes in PGRs leading to dissociation from their chaperone proteins and dimerization (Rekawiecki *et al*., 2017). Subsequently, the PGR binds to specific response elements in target genes and interacts with steroid receptor co-activators to enable transcription. Post-translation

modifications such as sumoylation modulate PGR activity. Reduced PGR sumoylation is partly responsible for increased hESC progesterone sensitivity (Jones *et al*., 2006a).

1.8.2. cAMP

cAMP is a ubiquitous second messenger generated by stimulation of G protein-coupled receptors (GPCR) from ATP upon activation of adenylate cyclase (Hur and Kim, 2002). Adenylate cyclase and cAMP levels are higher during the secretory phase of the cycle (Tanaka *et al*., 1993). The main target of cAMP is the protein kinase A pathway (PKA). In its inactive state PKA is a dimer of two catalytic submits linked to two regulatory units. Upon binding of cAMP the catalytic units dissociates, facilitating diffusion into the nucleus and phosphorylation of the cAMP response binding protein (CREB) and cAMP modulating protein (CREM) leading to transcription of target genes such as dPRL and IGFBP-1 (Gellersen and Brosens, 2014). hCG signals via this cAMP pathway to maintain decidualisation in early pregnancy (Weedon-Fekjær and Taskén, 2012). Cyclic AMP levels are regulated by the rate of production as well as degradation by cyclic nucleotide phosphodiesterase (PDEs), and the inhibition of PDE can induce the expression of decidual markers (Bartsch *et al*., 2004). Another mechanism controlling cAMP activity is catabolism of PKA catalytic subunits and upregulation of regulatory subunits decreasing cAMP levels (Kopperud *et al*., 2003).

1.9. The convergence of cAMP and P activity during decidualisation

Historically, P was considered as the only inducer of hESC decidualisation. Now, other vital factors have been identified, including cAMP. Progesterone induces modest decidual changes over ten days in hESC *in vitro* which can proceed. In contrast, treatment with 8-Br-cAMP can induce strong decidualisation, which is not maintained in long-term culture (Brosens *et al*., 1999).

Cyclic AMP sensitises hESC to P primarily through stimulation of the PKA pathway alters the interaction of PGRs with the nuclear receptor corepressor and silencing mediator of retinoid thyroid receptors (Wagner *et al*., 1998). This promotes the selection of the steroid receptor co-activators (Rowan *et al*., 2000). The further mechanism by stimulation of different subsidiary transcription factors like FOXO1, SV40 promoter binding protein 1 (SP1), C/EBP-ß, and signal transducer and activators of transcription 3 and 5 (STAT3 and STAT5) (Christian *et al*., 2002, Mak *et al*., 2002). All these transcription factors share the ability to interact with PGRs. Thereby, the generation of multimeric transcription complexes gives PGR regulatory activity over gene transcription without the involvement of the PGR element (PGRE) binding (Salker, 2013).

1.10. Functions of decidualisation

1.10.1. Trophoblast invasion and embryo encapsulation and selection

The process of embryo implantation is analogous to tumour invasion with the decidua controlling embryo invasion. The decidua should limit extensive pathological invasion and prevent placenta accreta by modulating the local cytokine environment. It should also promote the growth of an embryo and prevent growth retardation. This means that the decidua both accommodates and promotes trophoblast invasion to an equal extent which has been called "cooperation and conflict in the placental bed" (Haig, 2010). Trophoblast invasion requires the remodelling and degradation of the extracellular matrix by matrix metalloproteinases (MMP) produced by trophoblasts. MMPs proteolytic action is counteracted by TIMPs, which are secreted from both decidual cells and trophoblasts (Lambert *et al*., 2004). Transforming growth factor β (TGFβ) is another inhibitor of MMPs produced by decidual cells as well as its storage proteoglycan, which is called decorin (Jones *et al*., 2006c). The decidua also produces plasmin

which is activated by the membrane-anchored receptor (uPAR) binding with urokinase-type plasminogen activator (uPA). This pathway is inhibited by plasminogen activator inhibitor type-1 (Wu *et al*., 1995, Jones *et al*., 2006c).

Recently, it has been shown that the decidual endometrial cells are motile and invasive and can encapsulate the embryo (Weimar *et al*., 2013). Grewal *et al* (2008, 2010) demonstrated that decidualised ESC motility is crucial for trophoblast invasion during implantation. Continuous *in vitro* observation using time-lapse microscopy demonstrates that the decidualised ESC moves around the implanting embryo to enhance its expansion. Additionally, decidualised cells moved towards the excellent embryo that gives them embryo selection ability. Failure of this endometrial discrimination can result in recurrent miscarriage (Weimar *et al*., 2012). Dramatic upregulation of important implantation genes was observed in murine uteri treated with conditioned media from competent blastocysts that were compared with incompetent blastocysts (Brosens *et al*., 2014).

Massive desmosomal cell-cell contact is seen between trophoblasts and decidual cells. These cells do not display signs of degeneration which ensures embryonic invasion is controlled to avoid disturbance of the feto-maternal interface (Babawale *et al*., 2002). Collectively, it is evident that the decidua not only controls trophoblast invasion but also actively participates in embryo encapsulation and selection.

1.11. Oxidative stress responses

The occlusion of spiral arterioles by trophoblasts limits the vulnerability of the fetus and placenta to blood flow and oxygen from the maternal side and consequently, oxidative stress. Later on, dislodging of this trophoblastic plug permits blood flow to the inter-villus space and accordingly, massive fluctuations in oxygen concentrations ensues at the feto-maternal area.

Thereby, partial oxygen pressure rises from 20 mmHg at 7-10 week to 50 mmHg at 14 weeks of gestation. This fluctuation induces a flare-up of reactive oxygen species (ROS) (Wu *et al.*, 1995, Burton *et al.*, 2009). In the lack of potent defence mechanism, ROS such as hydrogen peroxide, superoxide anion, and hydroxyl radicals result in non-selective destruction of protein, nucleic acids, and lipids. The cells constitutively produce neutralising enzymes to fight oxidative stress and reverse the resulting damage by ROS (Sies, 1997, Lee *et al*., 2019). Furthermore, an adaptive response manifested by upregulation of antioxidant enzymes as peroxidase, superoxide dismutase, glutathione S-transferase, and activation of repair genes such as growth arrest proteins and heat shock proteins (Barnouin *et al*., 2002, Tran *et al.*, 2002).

It is still envisaged that oxidative damage at the maternal-fetal interface can result in a spectrum of pathological pregnancy ranging from miscarriage to pre-eclampsia and growth restriction. Even though the primary pathology of miscarriage or pre-eclampsia such as focal bleeding, necrosis, or spiral artery pathology is located on the maternal side of decidua, however, the decidual contribution in oxidative stress defence has been primarily skipped (Gellersen *et al.*, 2007a). Decidualisation is associated with the extraordinary ability of OS coping revealed by the induction of ROS scavenger such as Monoamine oxidase A and B, SOD2, glutarerdoxin, thioredoxin, and peroxiredoxin 2 (Maruyama *et al.*, 1997, Riesewijk *et al.*, 2003). Moreover, glutathione peroxidase 3 (GPx3), which considered a powerful extracellular neutraliser of hydrogen peroxide and fatty acid hydroperoxide (Gellersen *et al*., 2007a). Expressing GPx3 permits the decidual cell involvement in trophoblasts defence against ROS. In contrast to undifferentiated cells which on exposure to ROS express the pro-apoptotic FOXO3A, the decidual cell does not exhibit this. Therefore, oxidative-stress induced cell death is selectively disabled in decidualised cells (Kajihara *et al.*, 2006).

1.12. Immune responses

The decidualisation process plays an essential role in securing the immuno-tolerant environment around the allogenic feto-placental unit, whereas keeping the fetus and mother protected from pathogens. This role may be attributed to recruitment of $CD56^{bright}/CD16^{-ve}$ uNK cells which express immune-modulatory particles as glycodelin A (placental protein 14) and galectins (Koopman *et al*., 2003). The latter inhibits T-cell survival, proliferation and pro-inflammatory cytokine expression. Likewise, glycodelin A strongly inhibits T-cell activation. Recurrent miscarriage is associated with reduced serum levels of glycodelin A in the secretory phase of the cycle in fertile couples (Tulppala *et al*., 1995). There is compelling evidence that decidual cells can inhibit the T-cell proliferation-inducing activity of tryptophan through the expression of indoleamine 2 3-dioxygenase (IDO) as IDO inhibitors result in massive inflammation and fetal resorption in mice (Munn *et al*., 1998). Lastly, induction of activated-T cell apoptosis by decidualised cells is promoted through expression of Fas ligands and c-FLIP, which inhibit the auto-activation of cell death pathways (Kajihara *et al*., 2006).

In conclusion, decidualisation of the human endometrium is primarily considered as a critical component in immunological tolerance of pregnancy (Ringden *et al*., 2013). Accordingly, transplant pathologists explored the utilisation of decidual stromal cells in the treatment of steroid-refractory acute transplant rejection (Ringden *et al*., 2013).

1.13. Clinical perspective of aberrant decidualisation

1.13.1. Infertility

The steroid hormone effect on the endometrium is profoundly changing and dependant on a programmed cells regeneration, and because of this, the human endometrium has an endogenous potential for modulating its function to a secure successful conception (Gellersen

and Brosens, 2014). The perturbation of such programmed cell changes during decidualisation can result in reproduction failure as in, polycystic ovarian syndrome (Younas *et al*., 2019), recurrent pregnancy loss (Salker *et al*., 2010), and endometriosis (Klemmt *et al*., 2006).

1.13.2. Endometriosis

Endometriosis is a common gynaecological problem and affects approximately 10% of women of reproductive age (Benaglia *et al.*, 2009). It is diagnosed in up to 30% of women with infertility problems (Bulletti *et al.*, 2010) and is a major cause of low pregnancy success following IVF (Barnhart *et al*., 2002). Endometriosis is characterised by ectopic endometrial tissue outside the uterine cavity and it is frequently accompanied by pelvic pain. One of the oldest theories to explain endometriosis is menstrual blood retrograde deposition which is called Sampson's theory (Sampson, 1927). An alternative theory attributes endometriosis to the metaplasia of Mullerian epithelia into an endometrial tissue (Fujii, 1991). Despite these theories, it is still difficult to explain why some women develop endometriosis while others not. To find effective treatments for endometriosis demands a clear understanding of the underlying cellular and molecular pathology. There is clear evidence of poor endometrial decidualisation of both ectopic and eutopic endometrial tissue which leads to endometrial shedding (Klemmt *et al*., 2006, McCluggage and Kirk, 2000, Aghajanova *et al*., 2010, Yang *et al*., 2012). This appears related to P resistance in eutopic endometrial tissue due to the abnormal expression of PGRs mainly PGR-B. Progesterone resistance leads to reduced 17-hydroxysteroid dehydrogenase type 2 (17-HSD-2) enzyme expression in the epithelium resulting in the accumulation of biologically active E2, which stimulates endometrial tissue growth (Bulun *et al.*, 2006).

1.13.3. Miscarriage

Miscarriage (early pregnancy loss/spontaneous abortion) is defined as loss of pregnancy before the conceptus gains the ability to live independently and usually occurs before the 24th week of gestation. Miscarriage is classified according to the week of gestation at which it occurs. It is categorised as pre-clinical if occurring before five weeks, or clinical from the 6th-20th week of pregnancy (Bienstock *et al.*, 2015). Miscarriage can affect 10-40% of pregnancies (Bienstock *et al*., 2015). Increased the risk of miscarriage is recorded in teenagers and the over 40s. Miscarriage is associated with emotional trauma, anxiety and depression (Klier *et al.*, 2000, Brier, 2004), which predispose to obstetric problems such as prematurity in the future pregnancies (Bhattacharya and Bhattacharya, 2009) and increased risk of maternal cardiovascular problems (Oliver-Williams *et al.*, 2013).

Recurrent pregnancy loss (RPL) or habitual abortion is another significant obstetric complication that may be the result of abnormal decidualisation. RPL is defined as two (American definition) or three or more consecutive miscarriages (European definition) (Stirrat, 1990, Medicine, 2012b). Half of these cases are due to chromosomal abnormality (Bienstock *et al*., 2015). Most cases of RPL are treated empirically using aspirin and heparin based management to reduce immunological rejection of the fetus, which results in improved pregnancy rates (Heuser and Branch, 2019).

A new understanding of the role of decidualised ESC in embryo selection may explain RPL. This selectivity is demonstrated by the ability of the decidualising cells to accept the implantation of a good embryo and reject the implantation of a suboptimal embryo (Brosens *et al.*, 2014, Teklenburg *et al*., 2010b, Salker *et al.*, 2010, Macklon and Brosens, 2014). The response in RPL endometrium is more pro-inflammatory. It exhibits a prolonged receptive phase and expression of the powerful pro-inflammatory factor IL-33 and attenuated expression

of auto-inflammatory decoy receptor, sST2, resulting in bleeding and fetal resorption (Macklon and Brosens, 2014). In contrast, this hESC response is attenuated in endometriosis (Salker *et al*., 2012).

Recently it has been found that defective decidualisation leads to a reduction of brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 transcriptional factor expression, which controls trophoblast invasion by regulating TIMP levels increasing an individual's risk of RPL (Lv *et al*., 2019). Additionally, impaired decidualisation in women with RPL may be attributed to premature cellular senescence, which is an essential mechanism for healthy tissue remodelling to prevent uterine fibrosis (Lucas *et al*., 2016). Due to fluctuating P levels, the growth of embryos that lack fitness may also fail due to their low hCG production (Delcour *et al*., 2019) leading to low/fluctuating P levels or through the OS challenge on the transition from the histiotrophic phase to active maternal blood perfusion at the feto-maternal interface at the end of the first trimester (Azizi *et al*., 2019).

1.14. Pre-eclampsia

Pre-eclampsia is a disorder that complicates 2-8% of pregnancies(Ghulmiyyah and Sibai, 2012, Rana and Hameed, 2019), resulting in global maternal morbidity of 29750 deaths/year (Conti-Ramsden *et al*., 2019). It is characterised by the development of hypertension and high proteinuria at or following 20th week of gestation in previously fit women (Magee *et al*., 2014). Symptoms can start with sudden weight gain, oedema, headaches, and visual disturbances. However, some women may be asymptomatic. Pre-eclampsia can result in a serious sequelae as impaired placental blood flow and intrauterine growth restriction (IUGR), eclampsia, placental abruption, acute renal failure, HELLP (haemolysis, elevated liver enzymes and low platelet count) syndrome, disseminated intravascular coagulation, pulmonary oedema, future cardiovascular disease (Boniface *et al*., 2019, Magee *et al*., 2014). Pre-eclampsia can be

classified as early-onset if it occurs before the 34th week of gestation or late-onset (Raymond and Peterson, 2011, Burton *et al.*, 2019). The early-onset form can be described as of fetal origin due to a malfunctioned placenta. This type results in an adverse effect on the fetus, mother, small placenta, IUGR, and multi-organ failure (Burton *et al*., 2019). Late-onset pre-eclampsia is thought to be of maternal origin and has a more favourable prognosis, with larger placenta, and higher average birth weight (Raymond and Peterson, 2011). The leading risk factors of pre-eclampsia are a history of hypertension, diabetes mellitus, obesity, null parity, and immunological factors as antiphospholipid syndrome, renal problems, IVF, and family history. These factors can predispose to placental malfunction, consequently result in the release of inflammatory mediators and antiangiogenic factors as soluble endoglin (sENG) and soluble fms-like tyrosine kinase-1 (sFlt-1), which induce preeclampsia (Rana *et al*., 2019). Furthermore, the chorionic villus transcriptional profile showed poor decidualisation in cases of severe pre-eclampsia (Rabaglino *et al*., 2015). The transcriptional profile of decidual cells of severe pre-eclamptic pregnancies showed genetic abnormalities. These cells were unable to decidualise *in vitro*, and their conditioned medium cannot support trophoblastic invasion (Garrido-Gomez *et al.*, 2017). The histological examination of pre-eclamptic placenta showed shallow placental invasion (Stanek, 2019).

1.15. Implantation overview

Implantation is a highly complicated developmental process, which is pivotal in the progression of embryonic development inside the mother's uterus (Das, 2009). Implantation extends from the time of blastocyst hatching to the generation of primitive placental circulation. It is initiated on the contact of the blastocyst to the endometrium.

1.15.1. Pre-implantation embryo development

Pre-implantation development leads to the generation of an attachment-competent embryo. The pre-implantation period spans 6 days from the time of fertilisation to implantation (Vassena *et al.*, 2011). In humans, fertilisation occurs 24-48 h after ovulation in the ampulla region of the Fallopian tube (Gwatkin, 2012) (Figure 1.4). The fertilised oocyte undergoes cleavage, activation of embryonic genome, compaction, blastocyst formation or cavitation, and finally zona pellucida hatching (Kidder, 1992). The hallmark result of the pre-implantation period is the formation of a fluid-filled structure called the blastocyst at the time of entering the uterus (Krüssel *et al.*, 2003). The blastocyst consists of an outer epithelial layer of extra-embryonic trophectoderm (TE), which will form the placenta. The inner cell mass (ICM), which forms the embryo, and a fluid-filled space called the blastocoel (Larsen, 1998).

The activation of the embryonic genome and gradual degradation of maternal genome begins at the 2-cell stage (Duranthon *et al.*, 2008). At the 8-cell stage compaction occurs with increasing intracellular communication between individual blastomeres due to the formation of adherence junctions (Telford *et al.*, 1990) leading to a 16-cell morula formation with an inner cell mass (ICM) surrounded by the progenitor TE (Johnson and McConnell, 2004). On the 3rd to 4th day after ovulation, the morula enters the uterine cavity (Croxatto, 2002). Cavitation leads to the formation of the blastocyst which achieves polarity by adopting a specific orientation towards the uterus (Diedrich *et al.*, 2007) followed by hatching from the zona pellucida (ZP) ~72 h after entering the uterine cavity (Watson and Barcroft, 2001). The TE cells are the origin of trophoblasts, which are paramount in the process of implantation as they are the first cells achieving contact with and subsequent invasion into the endometrium (Duc-Goiran *et al.*, 1999).

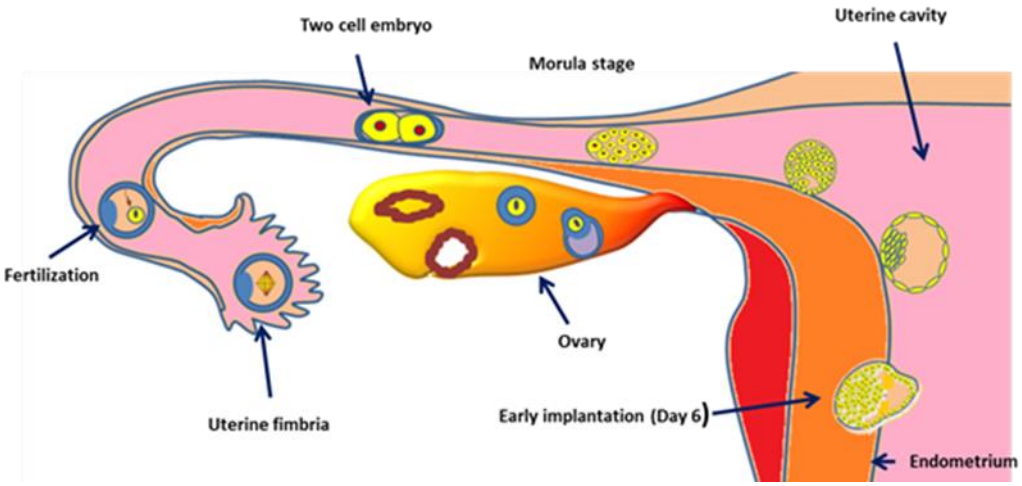


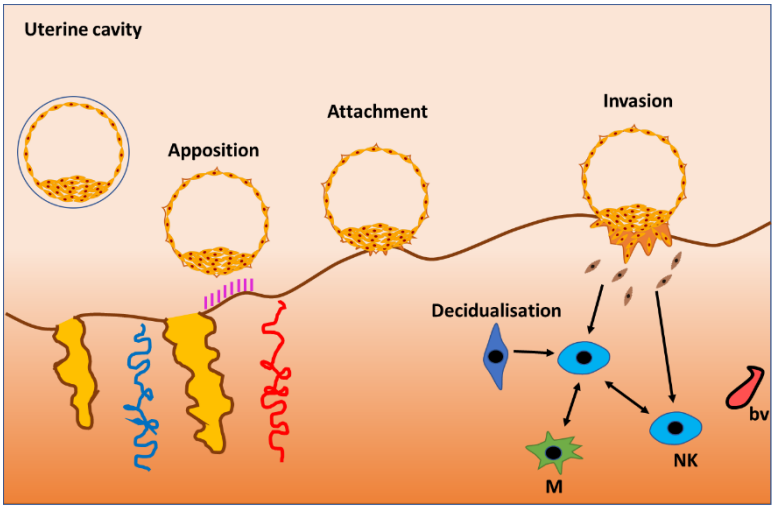
Figure 1.4: The journey of a blastocyst during the post fertilisation period. Schematic illustration of blastocyst differentiation from fertilisation time till implantation in the uterine endometrium. The fertilised oocyte undergoes cleavage, activation of embryonic genome, compaction, blastocyst formation or cavitation, and finally zona pellucida hatching. Adapted from www.drgpbiology.com. 2017.

The trophoblasts secrete a number of growth factors and cytokines such as tumour necrosis factor alpha (TNF-α), interleukins, MMPs, and hCG. These factors work in a paracrine or autocrine manner to communicate with the endometrium and support implantation (Merviel *et al.*, 2001)

1.15.2. Implantation

Implantation usually takes place between six to seven days post fertilisation (Vigano *et al.*, 2003). In humans, implantation occurs in three stages (**Error! Reference source not found.**A): The initial unstable stage is the contact between the blastocyst and the endometrial epithelia and is called apposition (Modi *et al.*, 2012). During this stage, there is interdigitation between microvilli on the trophectoderm and pinopodes on the endometrial epithelia with marked oedema and increased vascular permeability at the attachment point (Cha *et al.*, 2012). Apposition is followed by firm adhesion to the endometrium (Johnson, 2015). In the adhesion stage, an active interaction between blastocyst and endometrium exists.

The trophoblast differentiates into well defined, proliferating cytotrophoblasts in the inner part, and the fused multinucleated syncytiotrophoblast layer on the outer part (Anin *et al.*, 2004). The final step of implantation is the invasion stage when the syncytiotrophoblast invades the endometrium by lytic activity that weakens the adjacent endometrial cells (Denker, 2012). On the tenth-day post fertilisation, the embryo becomes entirely buried in the uterus, and the uterine epithelia rebuild over it (Glasser *et al.*, 2002). The syncytiotrophoblast (**Error! Reference source not found.**B) is responsible for oxygen and nutrient support of the fetus and located at the free surface of the chorionic villi, while the villus cytotrophoblast is located underneath the syncytiotrophoblast (Norwitz *et al.*, 2001). Extra villus trophoblasts (EVT) (Anin *et al.*, 2004) invade the decidua as endovascular or intestinal EVT.



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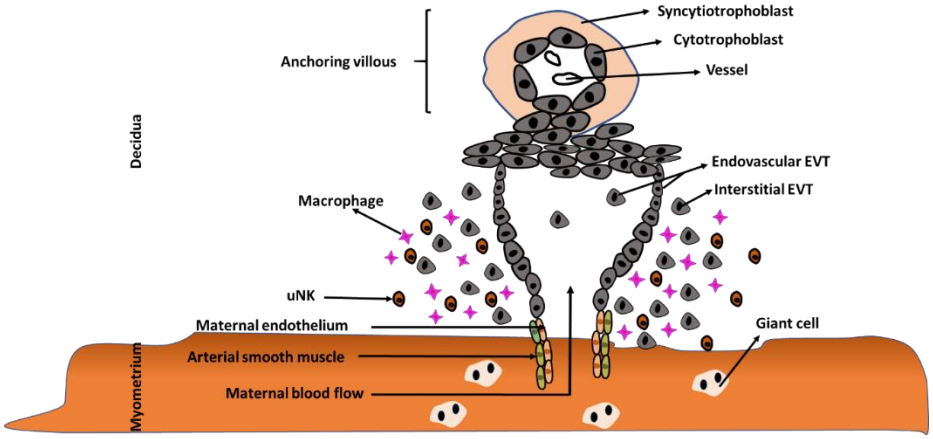


B

Figure 1.5: Stages of embryo implantation. Schematic representation of embryo implantation and spiral artery remodelling. (A) Embryo implantation has three distinct stages as apposition, attachment, and invasion. Implantation requires receptive endometrium represented by decidualisation with the influx of uNK and microphage. (B) Trophoblastic invasion as endovascular invasion and interstitial invasion. uNK = uterine natural killer cells, EVT = extra villous trophoblast. Amended from Lounghi et al (2007a).

The interstitial EVT anchor the placenta to decidua leading to the development of the utero-placental circulation (Pijnenborg, 1988). The endovascular trophoblasts invade into uterine spiral arteries and replace their endothelial and muscle tissue to form a high capacitance and low resistance vessels in the fetal-maternal interface in the process of spiral arteriole remodelling (Goldman-Wohl and Yagel, 2002). By the end of the first trimester, the EVT invade the endometrium and the inner third of myometrium (Kaufmann *et al.*, 1996, Staun-Ram and Shalev, 2005).

1.15.3. Trophoblast cell function during embryo implantation

Implantation and successful pregnancy are dependent on the proliferative, migratory, adhesive, and invasive potential of the trophoblast (Chakraborty *et al*., 2002, Ferretti *et al*., 2007). Despite intensive research, the regulatory mechanisms are still not fully understood. Trophoblast function is regulated by paracrine and autocrine mechanisms involving the trophoblasts and the uterine microenvironment (Lunghi et al., 2007). The importance of embryo-uterine crosstalk is highlighted here. For example, during the uterine invasion, the trophoblast secretes a wide range of protease enzymes, the MMPs for degrading the ECM barrier. In turn, MMP activity is widely influenced by the uterine microenvironment through the opposing action of TIMP. Further contributors could also be involved in this crosstalk such as IGFBP-1, cytokines, LIF, TNF-α, P, and hCG (Staun-Ram and Shalev, 2005). The decidualised endometrial stroma produces an ECM, which forms a cytokine-controlled environment controlling trophoblast invasion (Kliman, 2000)

1.16. Growth factors, interleukins and cytokines of the fetal-maternal interface

Decidualised hESC produced a lot of cytokines and growth factors that further engage in stromal differentiation. Additionally, the surrounding immune cells, epithelial cells, and

endometrial vasculature further secrete signal factors that might influence decidualisation and embryo implantation. These factors include ILs, the TGFβ superfamily, and growth factors such as EGF, PDGF, VEGF, and PlGF. They are involved in the fetal-maternal interface to regulate the proliferative, adhesive and invasive ability of trophoblasts (Bischoff *et al*., 2000, Lala and Chakraborty, 2003).

1.16.1. Interleukins

Differentiating hESC secrete IL-11 in a coincident manner to IGFBP-1 and PRL (Dimitriadis *et al*., 2000). IL-11 is a pleiotropic cytokine which has a heterodimeric receptor complex consisting of the signalling component gp130 and IL-11 receptor α chain (IL-11Rα). IL-11 is a mandatory factor for the process of implantation and mice lacking IL-11, or its receptors are infertile due to inefficient endometrial decidualisation (Bilinski, 2010, Robb *et al*., 1998). A reduction of IGFBP-1 and PRL accompanies the inhibition of IL-11 (Dimitriadis *et al*., 2005, Karpovich *et al*., 2005).

IL-1β is an essential pro-inflammatory cytokine that is produced in the embryo as well as the endometrium, especially in the peri-implantation period. It plays a crucial role in decidualisation and embryo implantation. It is proposed that IL-1β intervenes in the endometrial-blastocyst cross-talk during implantation through the induction of integrin αvβ3, which is critical for implantation (Simón *et al*., 1997). This ligand binds to a number of substances in the ECM, including laminin and fibronectin (Fazleabas *et al*., 1997). IL-1β enhances the cAMP accumulation and the expression of prostaglandin E2 through upregulation of cyclo-oxygenase 2 (Tamura *et al*., 2002). Additionally, IL-1β stimulates the action of MMP-3 in the degradation of ECM during implantation (Fazleabas *et al*., 2004).

1.16.2. LIF

LIF is a glycoprotein of the IL-6 family that is highly expressed in mouse endometrial glands before implantation at day 4 of pregnancy (Bhatt *et al*., 1991). In human, its secretion is limited to endometrial epithelia during the luteal phase (Stewart *et al*., 1992). There is growing evidence that refers LIF as one of the entities that participate in human implantation and uterine receptivity (Munro, 2019). Hence, its inhibition results in implantation failure in mice (Song *et al*., 2000). However, the administration of LIF to women with RPL in IVF did not improve their pregnancy outcomes (Brinsden *et al*., 2009).

1.16.3. EGF

EGF is 53 amino acid protein that is expressed by trophoblastic and decidual cells (Hofmann *et al*., 1991). It binds the epidermal growth factor receptor (EGFR) in humans ErbB-1and HER1. EGF and its receptor expression increase with endometrial stromal decidualisation. It is released at the uterine wound site and enhances epithelial, but not stromal cell proliferation at the edge of the wound (Alsat *et al*., 1993). In pregnancy, EGF improves embryonic development and trophoblastic differentiation through phosphatidylinositol 3-kinase (PI3K) and MAPK pathways activation, trophoblast invasion (Bass *et al*., 1994) and proliferation (Li and Zhuang, 1997, Qiu *et al*., 2004). It promotes the function of PAI-1, MMP-2 and 9, and uPA in trophoblast cells (Anteby *et al*., 2004) and this stimulates trophoblast invasion. Therefore, EGF is considered as a chief regulator of embryo implantation. The placenta of IUGR pregnancies showed a low expression of EGFR (Evain-Brion and Alsat, 1994). EGFR increases the expression of IL-8, VEGF, and MMP-1 in decidual cells as well as increases wound healing (Goto *et al*., 2019). EGF is involved in regulating cell growth, laminin, PRL, and fibronectin expression in hESC (Irwin *et al*., 1991).

1.16.4. Heparin-binding epidermal growth factor (HB-EGF)

HB-EGF is a pleiotropic growth factor that plays a vital role in fertility (Raab and Klagsbrun, 1997). It belongs to the EGF family and can be found as a soluble form (Iwamoto and Mekada, 2000). It binds to both EGFR and ErbB4 (HER4) (Elenius *et al*., 1997). It is expressed in uterine epithelial and stromal cells, and its highest expression by epithelial cells coincides with the mid-secretory phase of the endometrial cycle (Simón *et al*., 2000). The deletion of HB-EGF in mouse uterus delays embryo implantation (Xie *et al*., 2007, Lim and Dey, 2009). It has been found that HB-EGF transcription is under the control of E2 and P (Gechtman *et al*., 1999). Decidualisation through cAMP enhances the expression of both forms of HB-EGF as well as one or both receptors (Chobotova *et al*., 2005, Schwenke *et al*., 2013). However, EGFR expression was reported to be more highly expressed in decidual cells in spontaneous abortion specimens (Balci and Özdemir, 2019). Chobotova *et al* (2005) indicate that HB-EGF may play a role in the differentiation of endometrium and abolishing TNF-α-induced apoptosis of hESC, as well as inhibition of HB-EGF, can reduce IGFBP-1 and PRL production. HB-EGF has powerful cytoprotective activity and is an essential signalling protein that control trophoblast invasion during early placentation. Reduced level of HB-EGF has been detected in pre-eclamptic pregnancies (Leach *et al*., 2002).

1.16.5. Activin, Inhibin, and Follistatin

Activin, inhibin and follistatin also belong to the TGF-β family that have crucial roles in the decidualisation of the endometrium (Jones *et al*., 2006b). Activin enhances the decidual MMP-2 production (Jones *et al*., 2006c) and is upregulated in parallel with PRL upon cAMP stimulation of hESC. It also stimulates decidual marker expression while activin function inhibited by follistatin (Jones *et al*., 2002).

1.16.6. Tumour necrosis factor (TNF-α).

TNF-α is a pleiotropic cytokine that is found in trophoblasts as well as in the endometrium (Staun-Ram and Shalev, 2005). It is produced by activated macrophages, monocytes, as well as T-lymphocytes (Vassalli, 1992). TNF-α has a broad range of activities extending between two extreme borders of tissue regeneration and apoptosis. This is shown in its different physiological and pathological roles. Physiologically, it is speculated that TNF-α is involved in cell survival, migration, and differentiation functions (Haider and Knöfler, 2009). In women with regular cycles, TNF-α is upregulated in the late luteal phase indicating that it might be involved in the shedding of the endometrium (Hunt *et al*., 1996). Clear discrimination between the physiological and pathological function of TNF-α has not yet been established; although, its concentration is assumed to be a determiner of its harmful or helpful role (Haider and Knöfler, 2009). TNF-α may affect both the endometrium and trophoblasts (Haider and Knöfler, 2009) as activated macrophages produce TNF-α that might alter trophoblastic invasion by enhancing apoptosis (Renaud *et al*., 2005) or activation of the plasminogen activator inhibitor pathway (Huber *et al*., 2006). Inoue *et al* (1994) and a number of other researchers have tried to explain the role of inflammation and TNF-α on implantation through exploring its role in the decidualisation and production of dPRL which was found to be inhibited (Inoue *et al*., 1994). Additionally, the robust reactive superoxide inducer activity of this cytokine in leukocytes, which massively infiltrate the endometrium during decidualisation, has drawn attention to link its effect on implantation with exerting an OS environment (Agarwal *et al*., 2005a, Vassalli, 1992). Sugino *et al* (2002) indicated that TNF-α induces SOD2 through protein kinase-C (PKC) mediated phosphorylation and involvement of nuclear factor-κB (NF- κB).

1.16.7. Platelet-derived growth factor (PDGF)

PDGF was purified in the late 1970s as a platelet-derived component that enhanced the proliferation of mesenchymal cells (Heldin *et al*., 1979). Further studies showed that there were two PDGF genes, which encode two polypeptides – the PDGF A and B chains. These produce AA, BB and AB proteins through homo and hetero-dimerisation of the A and B chains (Heldin *et al*., 1985) (Figure 1.5). In 2000 and 2001, two additional genes were identified that encode PDGF-CC (Tsai *et al*., 2000b) (Li *et al*., 2000) and PDGF-DD (Bergsten *et al*., 2001).

The PDGF A- and B-chains are produced as prototype molecules that undergo proteolytic processing in the NH2 and COOH termini respectively (Ostman *et al*., 1991). PDGF expression increases due to different stimuli such as hypoxia (Kourembanas *et al*., 1997), thrombin (Harlan *et al*., 1986), pregnancy, cytokines and growth factors (Betsholtz, 1992), resulting in chemotaxis, cell growth, survival, and actin reorganisation (Heldin and Westermark, 1990, Xiong *et al*., 2019).

1.17. PDGF receptors

PDGFs exerts their action through two tyrosine kinase receptors, PDGFR-α (Matsui *et al*., 1989) and PDGFR-β with molecular weights of 170 kDa and 180 kDa respectively (Yarden *et al*., 1986a). Ligand binding leads to receptor homo and heterodimerisation in αα, ββ, and αβ combinations.

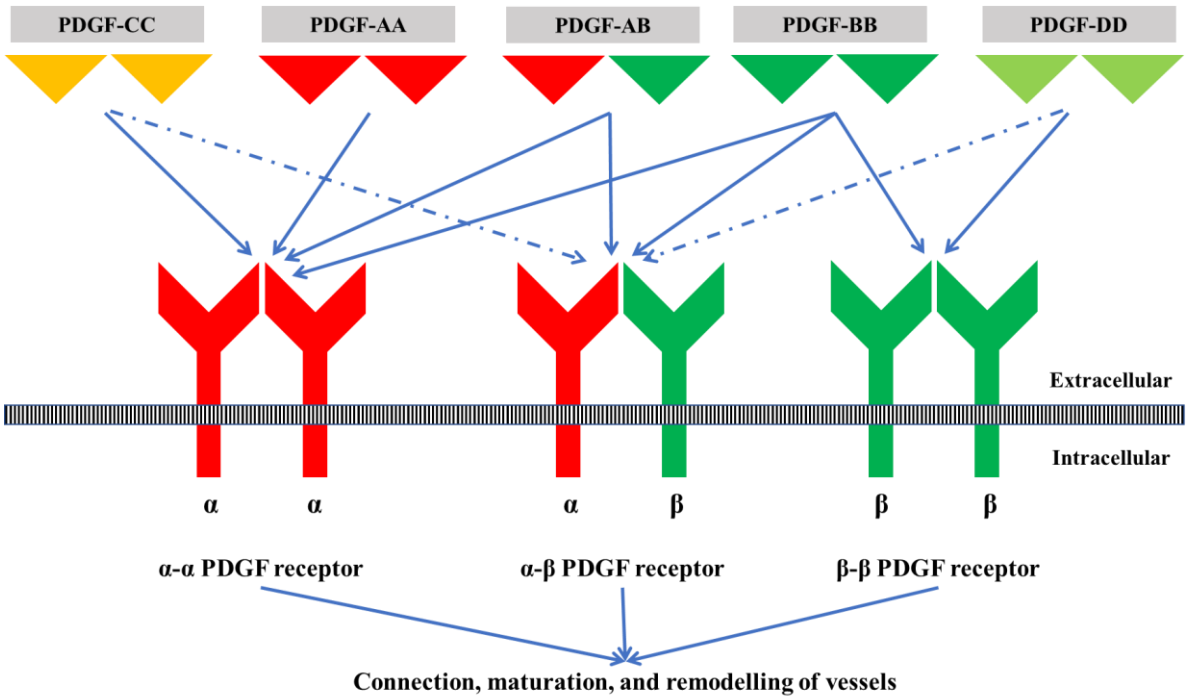


Figure 1.5: Receptor binding specificity of the PDGF isoforms. A diagram showing the binding of PDGF to their receptors as indicated by solid arrows. PDGFR-α binds PDGF-AA, BB, AB, and PDGF-CC. PDGFR-β can bind PDGF-BB and PDGF DD. The extracellular receptor region contains five immunoglobulin-like (Ig-like) domains. The ligands bind to the first three Ig-like domains. The intracellular region contains a tyrosine kinase domain. Dashed arrows indicated that the receptors may be activated. Adapted from Sadiq *et al* (2016)

PDGFR-α binds PDGF-AA, BB, AB (Heldin *et al.*, 1988, Seifert *et al.*, 1989) and PDGF-CC (Tallquist and Kazlauskas, 2004), while PDGFR-β binds PDGF-BB (Heldin *et al.*, 1988) and PDGF-DD (Seifert *et al.*, 1989, Tallquist and Kazlauskas, 2004). PDGFR-β has a higher binding affinity towards PDGF-BB (Heldin *et al.*, 1988) (Figure 1.5). The extracellular region of the receptors contains five immunoglobulin-like (Ig-like) domains and ligands bind to the first three Ig-like domains.

The fourth Ig-like domain is involved in direct receptor-receptor interactions and the intracellular region has a tyrosine kinase domain (Claesson-Welsh *et al*., 1989, Yarden *et al*., 1986a). The difference in mitogen potency between the A and B isoforms may be explained in part by the relative receptor expression with the PDGFR-α less prevalent than the PDGFR-β in most cell types. This makes PDGF-BB and PDGF-AB more potent mitogens for fibroblasts than PDGF-AA (Schatteman *et al.*, 1992).

1.17.1. PDGFR signal transduction

When the ligand binds the PDGFRs, they dimerise and transactivate the tyrosine kinase domains in the cytoplasm. A number of downstream cell signalling pathways are activated, including PI3K, MAPK and the Src tyrosine kinase family (Heldin and Westermark, 1999).

1.17.2. The role of PDGF in decidualisation and embryo implantation

PDGF is essential for embryonic development (Yarden *et al*., 1986b). It directly enhances epithelial-mesenchymal transformation (EMT) by lowering E-cadherin and increasing vimentin expression (Chen *et al*., 2017). PDGF plays an essential role in wound healing, promotes angiogenesis, tumour progression, and enhances ESC motility during decidualisation (Schwenke *et al*., 2013). PDGF-AA, PDGFR-α, and PDGFR-β mRNAs were expressed whilst PDGF-BB was absent in human (Österlund *et al*., 1996) and mouse blastocysts as identified by

reverse transcriptase polymerase chain reaction (RT-PCR) (Palmieri *et al*., 1992). In mouse embryos, Hellstrom *et al* (1999) identified high PDGF-BB and PDGFR-β expression in the sprouting artery endothelium and PDGF-BB knock-out mice die in the perinatal period due to cardiovascular and renal problems (Leveen *et al*., 1994).

1.18. Endosialin (CD248)

Endosialin (a.k.a. CD248, TEM1) is an 80.9 kDa cell surface glycoprotein (type 1). Although it was first described as a tumour-endothelial marker (Rettig *et al*., 1992, Croix *et al.*, 2000), the endosialin expression is limited to perivascular cells, e.g. pericytes and smooth muscle cells in the vascular bed (Lax *et al.*, 2007). Endosialin is mostly expressed during development and down-regulated in the adult (MacFadyen *et al*., 2007).

Stromal fibroblasts are the predominant source of endosialin, which has been used as a selective marker for the isolation of fibroblast subsets (MacFadyen *et al*., 2005). Endosialin can stimulate mesenchymal cell proliferation through its enhancing PDGF signalling via PDGFR-β (Tomkowicz *et al*., 2010, Wilhelm *et al*., 2016). Endosialin knockout mice lack a proliferative response towards PDGF-BB (Wilhelm *et al*., 2016) and reduced growth, invasion and angiogenesis in human tumour xenografts (Tomkowicz *et al*., 2007).

1.19. VEGF

VEGF is a potent angiogenic factor that stimulates endothelial cell migration and proliferation and survival. It was initially identified as a vascular permeability factor secreted by tumours (Simons *et al*., 2016). VEGF undergoes alternative mRNA splicing at the C-terminal leading to the formation of different VEGF isoforms, which are recognised by having or lacking exons 6, 7 and 8a/8b sequences with specific roles in haemostasis and vasculogenesis (Simons *et al*.,

2016, Ali *et al.*, 2019). In humans, there are five VEGF family members: VEGF-A (VEGF), -B, -C, -D, and placenta growth factor (PlGF). VEGF ligands bind to three receptor tyrosine kinases: Flt-1 (VEGFR-1) (De Vries *et al*., 1992), KDR (VEGFR-2) (Terman *et al*., 1992) and Flt-4 (VEGFR-3) (see Figure 1.6). The receptors are composed of an extracellular region of seven immunoglobulin-like domains, a transmembrane region, and the intracellular region. Furthermore, the kinase domain area is broken by kinase-domain insert leading to a C-terminal sequence (Pechan *et al.*, 2019). Despite the high binding affinity of VEGFR1 to VEGF, however, KDR mediates most of the VEGF biological activity in endothelial cells (Simons *et al*., 2016). Flt-1 is considered as the major VEGF receptor in placental trophoblasts, whereas KDR regulates the majority of VEGF-mediated activities in vascular endothelial cells (Li *et al*., 2016) (Figure **1.6**A & B). The third structurally related receptor is Flt-4 (VEGFR-3), which mediates the action of VEGF-C and VEGF-D in lymphatic and endothelial cells (Secker and Harvey, 2015). Additionally, VEGF co-receptors (neuropilins) enhance the binding of VEGF to its receptors (Mercurio, 2019). Most of the VEGF isoforms and receptors are found in the endometrium. However, the prevailing isoforms are $VEGF_{121}$ and $VEGF_{165}$ (Smith, 1995). VEGF expression is markedly upregulated upon hypoxia (Goteri *et al*., 2009) as well as many growth factors and cytokines, including PDGF and TNF-α (Yoshino *et al*., 2006).

Several authors have reported the upregulation of VEGF expression in the blastocyst (Kapiteijn *et al*., 2006) as well as human decidua as in ESC (Matsui *et al*., 2004), macrophage, epithelial cells and trophoblasts (Cooper *et al*., 1995). It is speculated that VEGF has important roles in implantation. Firstly, by stimulating angiogenesis and spiral artery remodelling (Ancelin *et al*., 2002). Secondly, by being an autocrine trophoblast mitogen as well as influencing their migratory and invasive potential (Yoshino *et al*., 2006).

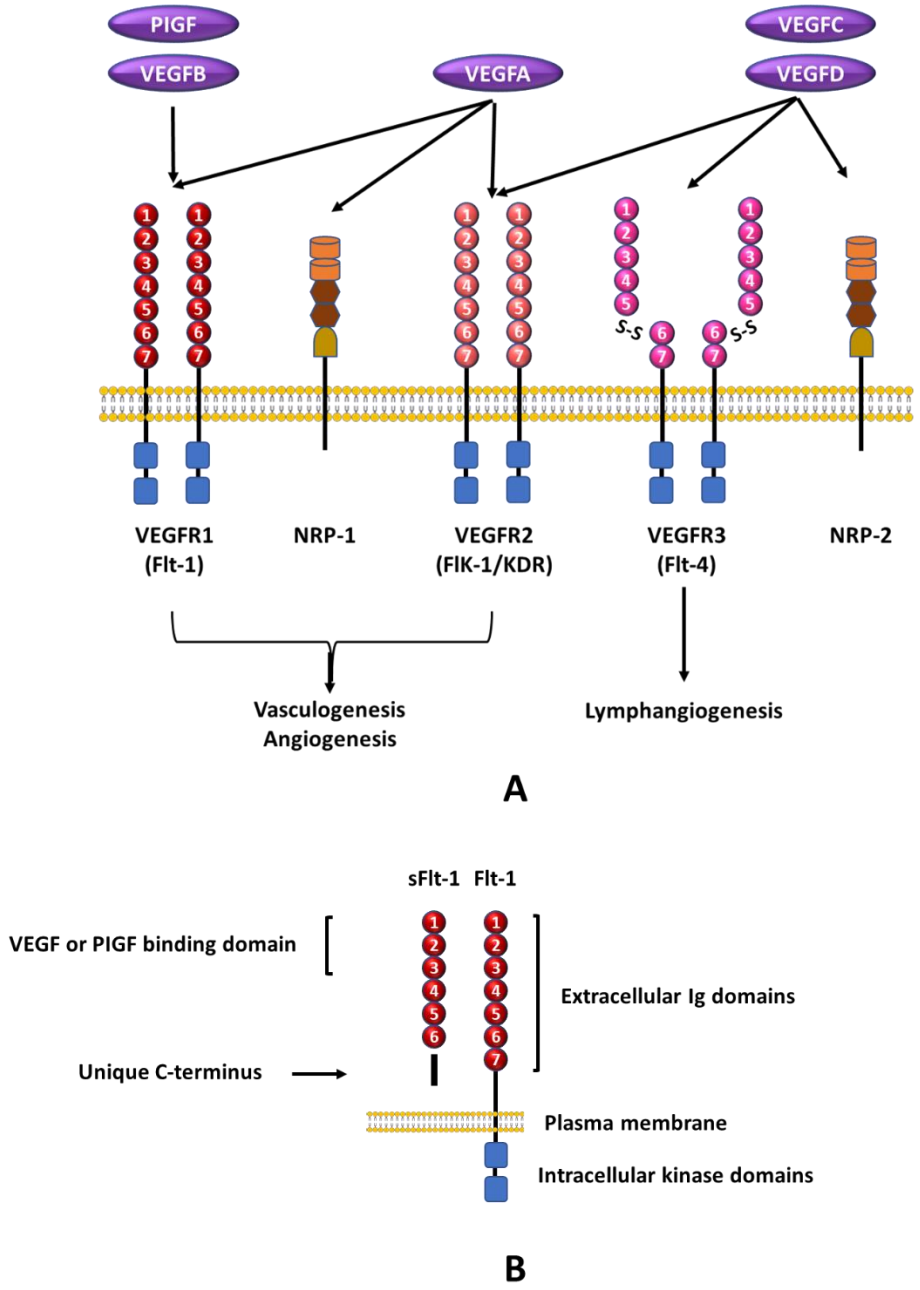


Figure 1.6: VEGF signalling system. (A) The binding preference of different VEGF family members and their receptors. The VEGF family composed of seven ligands for different genes transcript; VEGF-A, -B, -C, -D, and -E, placenta growth factor (PIGF) -1 and -2. VEGF isoforms have certain binding affinities to the VEGF receptor (VEGFR) -1, VEGFR-2 and VEGFR-3 tyrosine kinase receptors as shown. In addition, co-receptors, which are neuropilin (NRP)-1 and NRP-2 that enhance the binding affinity of these ligands to their respective receptors. (**B**) Flt-1 and sFlt-1 protein structure illustration. sFlt-1 protein lacks the cytoplasmic and transmembrane domains. Adapted from Shibuya (2011).

Sharma *et al* (2016) reported that VEGF increases trophoblast invasion, whereas Lash *et al* (1999) reported a reduced trophoblast invasion and increased motility with VEGF treatment. It has been found by Krüssel *et al* (2003) that VEGF is one of the first active genes during the pre-implantation period. The paramount role of VEGF and its receptor in gestational vascular remodelling has been highlighted by the finding that single VEGF allelic deletion results in intrauterine death (Carmeliet *et al*., 1996). Fong *et al* (1995) found that VEGF receptor deletion leads to abnormal vasculature development. Additionally, Danastas *et al* (2019) have reported reduced VEGF and its receptor expression in the receptive phase in the ovarian hyper-stimulated rat. Varas-Godoy *et al* (2019) found impaired angiogenic potential and reduced expression of VEGF in menstrual stem cells obtained from women with a history of pre-eclampsia. Furthermore, Zhou *et al* (2010) found that VEGF circulating levels are low in women with pre-eclampsia with endothelial dysfunction.

1.20. sFlt-1

Alternative mRNA splicing of the *Flt-1* gene generates sFlt-1, the soluble truncated form of the receptor (Kendall and Thomas, 1993, Kendall *et al*., 1996). sFlt-1 possesses only the extracellular region (first six Ig-like domains containing the ligand-binding domains) and lacks the transmembrane and intracellular domains (Figure **1.6**B). Thereby, it is a freely soluble secreted form of the receptor (Kendall *et al*., 1996). sFlt-1 works as potent anti-angiogenic through the sequestration of free VEGF and antagonising PlGF (Kendall and Thomas, 1993). The placenta is the predominant source of gestational sFlt-1 as proved by the dramatic reduction in maternal circulatory levels following placenta delivery (Maynard *et al*., 2003). VEGF can increase alternative Flt-1 splicing and sFlt-1 release (Fan *et al*., 2014).

Four alternatively spliced variants have been identified which differ only in their C-terminal regions. Two main variants of sFlt-1 have been implicated in infertility complications and pre-eclampsia: sFlt-i13 and sFlt-e15a (a.k.a sFlt-1 v2 or sFlt-1 14) sFlt-i13 is produced in the placenta, kidney, brain, heart, and endothelium (Palmer *et al*., 2015). sFlt-e15 is a human and simian primate-specific variant that is the predominant sFlt-1 isoform expressed in the placenta (Jebbink *et al*., 2011, Thomas *et al*., 2009). Cottrell *et al* (2017) have reported that increased sFlt-1 is negatively correlated with dPRL and VEGF expression in endometrial cells and sFlt-1 appears to suppress trophoblast invasion in humans (McMahon *et al*., 2014).

1.21. Antioxidants and reproduction

In a normal physiological state, ROS and their scavenger antioxidants are in balance. However, imbalance occurs in the presence of excess ROS or depletion of antioxidants (Agarwal *et al*., 2005b). OS is not only seen in pathological processes but has a normal physiological role in pregnancy (Myatt and Cui, 2004) and labour (Fainaru *et al*., 2002). ROS can exert their effects through their role in protein expression, DNA damage, and abnormal pathways in energy production (Ray *et al*., 2004).

ROS are a group of small molecules formed as a by-product of molecular oxygen metabolism, e.g. the superoxide radical (O_2), which forms within the respiratory chain in the mitochondria, hydroxyl radicals and hydrogen peroxide (H_2O_2) (Chen *et al*., 2010). Specific metallic ions, like iron ($Fe^{2+/3+}$) and copper (Cu) may lead to extensive ROS generation. In contrast, chelators such as transferrin and ethylene diamine tetra-acetic acid (EDTA), can bind these cations and neutralise their effects (Halliwell and Gutteridge, 1990). Antioxidants are one of the basic defence mechanisms in aerobic life and can be enzymes such as superoxide dismutase (SOD), glutathione catalase, and other peroxidases (Sies, 1991, Budni *et al*., 2013) and heme

oxygenase-1 (HO-1). The non-enzymatic types include vitamins E, C, and resveratrol. Oxidative stress both male and female fertility through increased ROS production due to infection, defective antioxidant defence mechanisms, toxin exposure (Sikka, 2001) and poor endometrial decidualisation (Gupta *et al.*, 2010). This can lead to implantation failure, reduced placental invasion (Wu *et al*., 2016), spontaneous abortion, unexplained infertility, recurrent pregnancy loss (Gupta *et al*., 2007), endometriosis, preeclampsia, and polycystic ovarian syndrome (Murri *et al.*, 2013).

1.22. HO-1

There are three heme oxygenase (HO) isoforms: HO-1, which is inducible and found mainly in the liver and spleen; HO-2, which is constitutively expressed and presents mainly in the testis and brain (Maines *et al*., 1986), and HO-3 is the inactive form and can be found in the brain (Shaman *et al*., 2013). HO-1 was firstly characterised in 1968 by Tenhunen (1969). It is a critical microsomal rate-limiting catalyst in the breakdown of heme to form carbon monoxide (CO), biliverdin, and free iron (Ghio *et al.*, 2018). It is considered a protective mechanism in many diseases (Maines, 1997, McDaid *et al.*, 2005) as HO-1 metabolites protect against hypoxia, cytotoxicity, inflammation, and other cellular stresses (Soares and Bach, 2009).

Different factors that can induce HO-1 production include heme compounds, drugs such as cimetidine, chemical solvents such as bromobenzene, hormones as insulin and adrenaline, metals as Cobalt, bacterial toxins, oxidative stress, fever and heat shock, skin injury, alkylating agents, neoplasms, and partial hepatectomy (Maines, 1988), inflammatory cytokines, and ultraviolet radiation (Terry *et al.*, 1999).

The critical role of HO-1 in cell function has been identified through the use of HO inhibitors. For instance, administration of HO-1 inhibitors to pregnant rats resulted in fetal resorption (Alexandreanu and Lawson, 2002).

1.21.1. The role of HO-1 in reproduction and pregnancy

In reproduction, HO-1 and its iron metabolism by-products are involved in the regulation of anti-inflammatory and anti-apoptotic mechanisms in the trophoblast and maintenance of the balance of oxidant-antioxidants in the placenta (Bainbridge and Smith, 2005). It was found that HO-1 increased the successful pregnancy outcomes by enhancing the function of regulatory T-Cells (TReg) and keeping dendritic cells at an immature stage (Schumacher *et al.*, 2012). HO-1 might affect cytokine and chemokine production in trophoblasts and lymphocytes through the production of CO, and the alteration of this delicate balance can affect placental angiogenesis (Wong *et al.*, 2012). Zhao *et al* (2008) showed that in heterozygote mice HO-$1^{+/-}$ had low weight offspring and low placental HO-1 RNA expression with a regular HO-1 placental activity and high expression of endothelial nitric oxide (NO) synthase (eNOS) and inducible NO synthase (iNOS). HO-1 induction shifts macrophage polarisation towards the anti-inflammatory M2 subtypes and this gives HO-1 a potential immune-modulating therapeutic approach for different diseases (Naito *et al.*, 2014). Many transcriptional pathways are involved in HO-1 upregulation, including the nuclear factor erythroid-2-related factor 2 (Nrf2)/Batch1 system (Naito *et al.*, 2014). The HO-1 immune modulating (Zenclussen *et al.*, 2007) effect has been considered in respect of the assumption that shifting towards type-TH2 cytokine expression (IL4 and IL10) favours successful pregnancy (Piccinni *et al.*, 2001) while shifting towards the TH1 type cytokines (IL-2, IL-6, TNF-α, and interferon-γ) favours an inflammatory state, immune rejection and miscarriage (Kwak-Kim *et al.*, 2005). The over-expression of HO-1 in a pregnant

mouse model increased the percentage of successful pregnancy outcomes (Zenclussen *et al*., 2006).

Serum HO-1 measurements show a strong inverse correlation with both systolic and diastolic blood pressures in hypertensive mothers 24 h before delivery. Therefore, HO-1 is considered as a biomarker for pregnancy-induced hypertension (Anderson *et al*., 2018). Reduced HO-1 levels lead to decreased CO levels and excess free heme, which binds to NO, leading to vasoconstriction – a prominent finding in preeclampsia (Hansson *et al*., 2015).

Using HTR-8 cells, it has been demonstrated that HO-1 reduces the migration of trophoblastic cells due to the stimulation of peroxisome proliferator-activated receptor–gamma (PPAR-γ) system signalling (Bilban *et al*., 2009). CO inhalation can minimise the development of IUGR and results *in situ* uNK cell proliferation (Zenclussen *et al*., 2015) whilst excess free heme application can cause fetal death in mouse models. This could be similar to the effect of inhibition of HO-1 action in pregnancy (Zenclussen *et al*., 2011). Uterine HO-1 mRNA expression was found to be higher in the estrus phase of the cycle where the P is highly expressed. Treatment of murine uterine cells with a combination of E2 and P caused increased HO-1 expression (Zenclussen *et al*., 2014). Ahmed *et al* (2000) found that two hours heme pre-treatment of the human villous explant tissue results in elevated HO-1 protein expression and reduced TNF-α toxicity, indicating a cyto-protective role of HO-1. Reduced HO-1 levels were detected with elevated levels of sFlt-1 and soluble endoglin in pre-eclamptic patient villous explants (Cudmore *et al*., 2007). Treatment with statins was found to increase levels of HO-1 and reduce sFlt1 and soluble endoglin secretion which led to the suggestion that statins could be used in the treatment of preeclampsia (Cudmore *et al*., 2007).

1.23. Modelling of human embryo implantation

A clear understanding of implantation failure and defective placental development is hindered by the limited availability of the human tissue and embryos for research purposes, as well as tight ethical constraints and the failure of animal models to fully recapitulate human implantation. Therefore, the use of cell lines provides an alternative approach to those working in this area. Cell lines offer the convenience of being able to be sub-cultured many times without changes in phenotype. However, cell immortalisation carries the risk of an abnormal karyotype and a loss of key characteristics of the primary parent cell leading to atypical function. Krikun *et al*. (2009) have partially solved this problem by introducing the human telomerase enzyme into primary hESC to produce an immortal stromal cell line (St-T1b) with a classical decidual response to progestational stimulants during *in vitro* work. Endometrial adenocarcinoma is the primary source for the majority of the epithelial cell lines used in embryo implantation e.g. HEC1 cells which are often used to represent a hostile endometrium as they have low adhesive potential and RL95-2 cells, which are used to represent the receptive endometrium in implantation model duo to their excellent adhesiveness. A range of immortalised trophoblasts has been used to represent the trophoblast. The HTR-8 trophoblast cell line (HTR-8/SVneo) was selected to simulate villus and EVT. The cells have matrix invading activity and express a range of different cytokine and growth factor receptors (Hannan *et al.*, 2010), which makes them an appropriate choice for studying the role of the trophoblast in embryo implantation (Table **1.1** & Table **1.2**).

Table 1.1: The expression of trophoblast subpopulation markers in human trophoblast cell lines. Adapted from Hannan *et al* (2010).

General markers[b]	KRT7	hCG	CGR	HLAG	CD9
Trophoblast subtypes, *in vivo*					
Trophectoderm	+	+	+		
Villous cytotrophoblast	+	+	+	-	-
Syncytiotrophoblast	+	+	+	-	-
Cell column	+	+distal	+	-	-
Extra Villous Trophoblast	+	+[c]	+	+	+
Primary trophoblast *in vitro*	+	+	+	+[d]	+[d]
CELL LINES					
HTR-8/Svneo	+	+	+	+[d]	
AC1M-88	+	+		+	+
AC1M-32	+	+		+	+
JEG-3	+	+	+	+	
JAR	+	+		_	
BeWo	+	+		±	+
SGHPL-4	+	+		+	+

[a] Presence or absence of selected phenotypic characteristics represented as follows: present (+) symbol; absent, (-); conflicting data, plus/minus (±). Blanks spaces represent the absence of data. [b]KRT7 = cytokeratin 7; hCG, human chorionic gonadotrophic hormone; CGR, human chorionic gonadotrophic hormone receptor; HLAG, human leucocyte antigen-G; CD9, cluster of differentiation antigen 9. [c] Hyper-glycosylated CG. [d] If grown on Matrigel.

Table 1.2: Trophoblast cell line characteristics with respect to implantation/placentation. Adapted from Hannan *et al* (2010).

Cell line	Origin	Function	Phenotype
HTR-8/Svneo	First trimester villus explants	Adhesion Migration Invasion Proliferation	EVT, VT
AC1M-88	Fusion of JEG-3 and term trophoblast	Adhesion Migration	EVT
JEG-3	Choriocarcinoma explant	Invasion	EVT
JAR	Gestational Choriocarcinoma	Invasion	VT/EVT
BeWo	Choriocarcinoma	Syncitialisation invasion	EVT
SGHPL-4	Primary extra-villus trophoblast	Adhesion Migration Invasion Proliferation	EVT
EVT, extra villus trophoblast; VT, villus cytotrophoblast.			

1.24. Aims and objectives

The interaction between the trophoblast and endometrium requires coordinated cross-talk between an invasive competent blastocyst and a receptive endometrium. As it is not possible to study these interactions in women due to ethical constraints as well as practical difficulties, we aimed to develop robust and reproducible *in vitro* models based on cell lines that would allow the investigation of hESC decidualisation and growth factors on trophoblast activity. We hypothesised that PDGFs and HO-1 are regulated during hESC decidualisation, lead to changes in VEGF activity and affect trophoblast function.

The specific objectives of this project were:

1. To establish three-dimensional *in vitro* models of implantation to investigate the role of the decidualised endometrium, growth factors, and cytokines on hESC interaction with the trophoblast.

2. To determine the effect of hESC decidualisation on trophoblast migration, spreading, and invasion.

3. To investigate the impact of decidualisation of hESC on PDGF expression and activity on hESC and trophoblasts.

4. To determine whether decidualisation leads to changes in HO-1 expression in hESC and if these account for shift in VEGF activity that accompanies implantation.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

All chemicals and reagents are supplied from Sigma-Aldrich (Dorset, UK) unless stated otherwise.

2.2. Cells and growth medium

2.2.1. HTR-8/ SVneo cells

HTR-8/ SVneo cells were derived from first trimester human trophoblasts immortalised by transduction with SV40 large T antigen and kindly provided by Dr Charles Graham (Sunnybrook Health Science Centre, Toronto, Ontario, Canada) (Graham *et al*., 1993). Cells were maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco – Fischer Scientific, Wilmington, UK) containing 5% (v/v) fetal bovine serum (FBS) with 200 μM L-glutamine (Weber *et al*., 2013, Graham *et al*., 1993).

2.2.2. The human endometrial St-T1b stromal cell line

St-T1b cell line was a kind gift from Dr Birgit Gellersen, Endokrinologikum Hamburg (B.G.) Hamburg, Germany. St-T1b cells were immortalised by retroviral transduction of human telomerase reverse transcriptase (hTERT) under the control of the SV40 promoter (Samalecos *et al*., 2009b). St-T1b cells were cultured in phenol red-free Dulbecco's modified Eagle medium (DMEM/Ham F12) (Gibco, Paisley, UK) and supplemented with 10% (v/v) FBS, 100 U/ml Penicillin, 100 μg/ml Streptomycin (Pen/Strep), 1 μg/ml insulin (Invitrogen), and 1 nM E2 (Inoue *et al*., 1994). This medium will be called E2 medium in the project.

2.2.3. Primary human endometrial stromal cells (hESC)

Endometrial tissue was obtained under informed consent from premenopausal women undergoing routine biopsy procedures at the Birmingham Women's Hospital in accordance with the University of Birmingham Biobank ethical approval (10-014). Primary hESC were isolated based on the modification of previous methods (Zhang *et al*., 1995; Chen and Roan, 2015) and kindly provided by Dr Ling Ting. The primary hESC were maintained in the growth medium used for St-T1b Cells. Primary cells in the experiments were not used beyond 15 passages to avoid phenotypic changes.

2.2.4. The human dermal microvascular endothelial cell line (HMEC-1)

The HMEC-1 cell line was purchased from ATCC (CRL-3243). These cells were immortalised through the retroviral mediated integration of the SV40 large T-antigen (Ades *et al*., 1992). The cells were maintained in MCDB131 containing 10% FBS (V/V) and Penicillin /Streptomycin

2.2.5. Human umbilical vein endothelial cells (HUVEC)

HUVEC were purchased from Promo Cell as donor pools. They were grown on flasks coated with 0.2% (w/v) gelatin in PBS for 20-30 min and maintained in endothelial growth medium-2 (EGM-2) (Promo Cell, Heidelberg, Germany).

2.3. Maintenance of cell growth

All cells were cultured under sterile conditions using a class II biological safety cabinet (type A2) (Holten laminar air). Cells were cultured at 37^{o}C in a humidified atmosphere with 5% carbon dioxide (CO_2) incubator. The culture medium was replaced with fresh medium every 2-3 days. Once cells had reached 80% - 90% confluence, the medium was aspirated, and they were washed with calcium and magnesium-free Dulbecco's modified phosphate-buffered saline

(PBS), and ~1ml of 0.25% -Trypsin-/1 mM EDTA (Gibco) added for 1-2 min at 37ºC. Fresh growth medium was added as required for seeding the cells into tissue culture flasks. All cell types were passaged at a ratio of 1:3 except HTR-8, which was 1:10.

2.4. Cryopreservation of cells

The cells were washed with PBS and detached with a trypsin-EDTA solution as described above and centrifuged at 200 x g for 5 min. The cell pellet was resuspended in 10% (v/v) dimethyl sulphoxide (DMSO) in their respective complete growth medium. The cells in ~1.5 ml of medium was dispensed into cryovials and frozen down to -80ºC in a Mr Frosty overnight. For long-term storage, the cells were transferred to liquid nitrogen. When required, frozen cells were thawed rapidly in a 37ºC water bath and added to ~10 ml of fresh medium and spun at 200g for 5 min. The pellet was resuspended in a fresh complete growth medium in a T-75 flask.

2.5. Mycoplasma test

To ensure the absence of Mycoplasma contamination, which would adversely affect the results, the cells were checked by EZ-PCR Mycoplasma test kit following the manufacturer's protocol. Briefly, the cells were grown over 72 h and the supernatant collected. The supernatant was centrifuged at 16,000g to precipitate mycoplasma. A 50 µl of buffer was added to the pellet followed by heating to 95ºC for three minutes. PCR was performed according to the manufacturer's conditions, and the products separated on 2% agarose gels. The cycling conditions were as follows: denaturation 30 sec, 94ºC, followed by 35 cycles of amplification, 30 sec at 94ºC, annealing for 2 min at 60ºC, and 60 sec at 72ºC.

2.6. Decidualisation of hESC

The St-T1b cells and primary hESC were grown to confluence in 6-well plates at a seeding density of $3x10^5$ cell/well. In order to induce differentiation, the cells were stimulated in decidualisation medium consisting of DMEM/Ham F12 (with HEPES) supplemented with 2% (v/v) charcoal-stripped FBS (Biosera, C/O Labtech International Ltd,/UK), 100 U/ml penicillin and 100 µg/ml streptomycin, 1 µM medroxyprogesterone (MPA), and 0.5 mM *8*-Bromoadenosine 3',5'-cyclic adenosine monophosphate (8-Br-cAMP) (Enzo Life Sciences, Exeter, UK). This medium is subsequently referred to as MPC medium throughout this thesis. The medium was replaced every 48 or 72 h and 24 h prior to harvesting the culture supernatants and cells (Figure 2.1). Morphological changes following stimulation of the cells were observed using 10x objective of a phase-contrast microscope (Evos XL, Fischer Scientific).

2.7. Phalloidin staining

To show changes in the actin cytoskeleton of decidualisation in hESC, the cells were stained with Phalloidin-iFluor 488 (Abcam, Cambridge, UK) and counterstained with Hoechst 33342 (AAT Bioquest, Sunnyvale, UK). The cells were plated at $6x10^5$ cells/well in E2 medium in 8-well culture slides. After 24 h, the cells were stimulated to decidualise. The cells were washed with PBS and fixed with 3.7% formaldehyde for 30 min. Each well was washed twice with PBS, and the cells permeabilised with 0.1% Triton-X100 in PBS for 3 min. The cells were washed with PBS and stained with 100 µl of 1x Phalloidin-iFluor 488. The dye was prepared by mixing 1 µl of the Phalloidin conjugate stock solution in 1 ml of PBS + 1% (w/v) bovine serum albumin (BSA) with Hoechst stain (5 µg/ml) for 30 min in the dark in a fume hood. The cells were washed and immediately mounted using a drop of mounting medium (Invitrogen), and a coverslip applied.

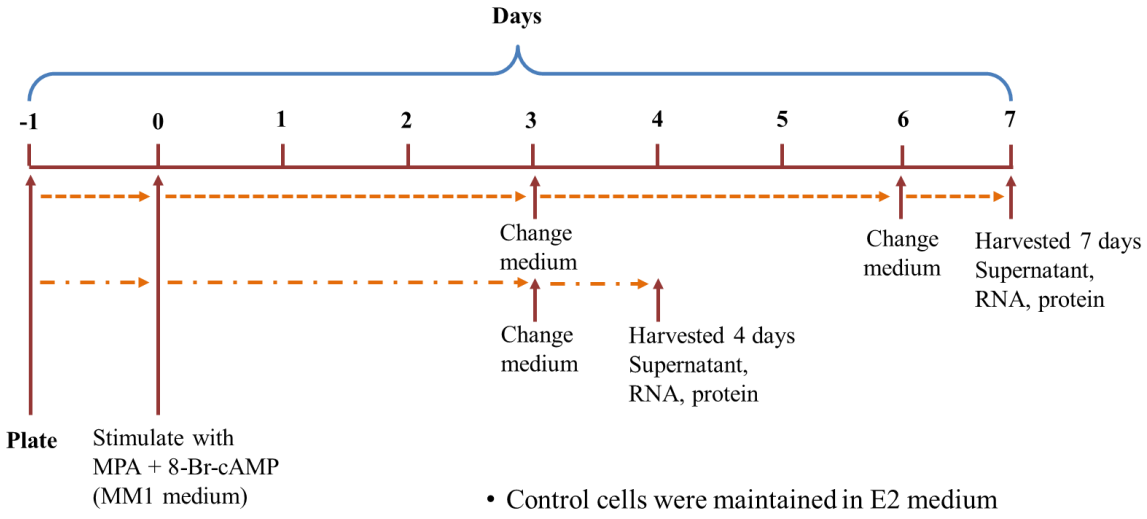


Figure 2.1: Decidualisation of St-T1b cells. A diagram illustrating the stimulation of hESC with MPA (1 µM) and 8-Br-cAMP (0.5 mM) on day 0, 3, and 6. At time points 4 and 7 days, supernatant, RNA, and protein were collected.

The cells were examined using the 10 x objective of a microscope (Olympus, Tokyo, Japan) equipped with epifluorescence at 493/517 nm for Phalloidin, and ultraviolet fluorescence (545/546 nm) for Hoechst staining.

2.8. Collection of cell lysate for RNA purification

hESC lysates for each sample/condition were harvested and pooled from three wells of a 6-well plate. The medium was aspirated, followed by a wash with 1 ml of PBS before175 µl of cell lysis buffer was added to each well. The plate was gently swirled to lyse the cells and the lysate harvested within 5 min. Total RNA was purified immediately, or the cell lysate stored at -20^{o}C, or -80^{o}C for future purification.

2.9. Total RNA purification

Total RNA extraction was performed using the Norgen total RNA plus kit according to the manufacturer's instructions (Norgen Biotek Corp., Thorold, Canada). Cell lysates were first passed through genomic DNA removal spin columns and spun at 14,000g. The flow-through was retained and molecular grade ethanol added (60 µl ethanol/100 µl of flow-through) and passed through RNA purification spin columns. The column was washed three times with the wash solution. The RNA was eluted from the column by adding 50 µl of elution buffer and spinning at 200g for 2 min, followed by 1 min at 14000g at 4^{o}C. Immediately, purified RNA was chilled on ice to prevent degradation and then stored at -80^{o}C

2.10. Reverse transcription

A Tetro cDNA Synthesis Kit (Bioline, London, UK) was used to prepare single-stranded complementary DNA (cDNA) from 1-2 µg of total RNA according to the manufacturer's instructions. The RNA concentration was measured with a NanoDrop spectrophotometer

(Labtech, East Sussex, UK). The same amount of RNA was added for a given set of samples. A Master mix was prepared as described in Table 2.1.

Table 2.1: Master Mix components used in reverse transcription reactions.

Reagent	Amount for one sample (µl)
Primer: oligo (dt) $_{18}$	1
10 mM dNTP mix	1
5 x RT buffer	4
Ribosafe RNase inhibitor (10 U/µl)	1
Tetro Reverse Transcriptase (200 U/µl)	1

A total volume of 20 µl was placed in each sample tube. This comprised 1-2 µg of total RNA in a total volume of 12 µl molecular grade and 8 µl of master mix. The amplification conditions were as followings; 42^{o}C for 30 min, 50^{o}C for 30 min, and 55^{o}C for 30 min. The resulting cDNA samples were stored at -20^{o}C.

2.11. Reverse transcription-polymerase chain reaction (RT-PCR)

The RT-PCR involved the preparation of reagents and primer master mix followed by running the prepared sample with the master mix on an agarose gel as follows; A master mix was prepared as follows: 0.3 µl of 100 mM dNTP mix, 4.0 µl of 5x reaction buffer, 12.1 µl of molecular grade water H_2O, 2.0 µl of 50 mM $MgCl_2$ solution, 0.1 µl of primers, and 0.3 µl Mango Taq DNA polymerase (5U/ µl) (Bioline). Sample amplification was performed in a SensoQuest lab thermal cycler machine (SensoQuest, Gottingen, Germany) according to the specific cycling conditions of each primer (*see* Table 2.2 and Table 2.3).

Table 2.2: Standard temperature and timing of each stage of RT-PCR run in a thermal cycler.

Stage	Temperature (^{0}C)	Time	
Denaturation	95	5 min	
Denaturation	95	30 sec	Cycle number is target dependent
Annealing	According to primer	30 sec	
Polymerisation	72	30 sec	
Polymerisation	72	10 min	

Table 2.3: Oligonucleotide primer sequences, amplicon sizes, and cycling conditions used for RT-PCR.

Gene	Cycling conditions	Amplicon size (bp)	Oligonucleotide sequence	Reference
dPRL	25 -30 cycles 94°C, 45 sec; 57°C, 45 sec; 72°C, 45 sec.	416	5'-GAGACACCAAGAAGAATCGGAACATACAGG-3' 5'-TCGGGGGTGGCAAGGGAAGAA-3'	(Samalecos *et al*., 2009a)
GAPDH	35 cycles : 95°C, 30 sec; 65°C, 30 sec; 72°C, 30 sec;	159	5'-CAATGACCCCTTCATTAGCC-3' 5'-TTGATTTTGGAGGGATCTCG-3'	In house
IGFPB-1	25 cycles 95°C, 30 sec; 63°C, 30 sec; 72°C, 90 sec.	378	5'-TGCTGCAGAGGCAGGGAGCCC-3' 5'-AAGGATCCTCTTCCCATTCCA-3'	(Samalecos *et al*., 2009a)

2.11.1. Agarose gel electrophoresis

Agarose gel electrophoresis was employed to separate PCR products by size. The gel percentage used varied from 1%, in the case of dPRL and IGFPB-1, to 2% for GAPDH. Gels were prepared using the molecular biology grade agarose powder (Bioline) in 1 x Tris-Acetate EDTA (TAE;1 mM EDTA, 20 mM acetic acid, and 40 mM Tris) buffer by heating in a microwave oven until dissolved. The gel was cooled to around 50^{o}C, then 5 µl/100 ml of Midori green safe DNA stain (Geneflow, Staffordshire, UK) was added. The gels poured in appropriate casting trays and allowed to cool. The gels were immersed in 1x TAE buffer. Samples of ~15 µl/well were then loaded along with 10 µl of the Hyperladder 100 base pair (bp) gel DNA marker (Bioline). A DNA separation was performed over 30-40 min at 80-120 V. The gels were imaged using a Gene Genius Bio-Imaging system with Genesnap analysis software (Synagen, Cambridge, UK).

2.12. Quantitative real-time PCR (qPCR)

The qPCR was performed to quantify the expression of genes of interest using the SensiMix SYBR Kit (Bioline). cDNAs (Section 2. 10) were diluted within molecular grade H_2O 1:3. Each reaction mix contained 7.5 µl from 2x SensiMix, 1 µl of diluted, 0.1 µl primer of primer mix (each primer at 50 pmol/µl) made up to a total volume of 15 µl with molecular grade water. β-actin was used as a housekeeping gene. Samples were amplified using a Rotor-gene Q (Qiagen) cycler. The relative target gene expression was calculated by the normalisation of the expression in comparison to the β-actin endogenous control using the ΔΔCT method (ΔΔCT = ΔCT sample - ΔCT housekeeping gene) (Mane *et al*., 2008, Pfaffl *et al*., 2004). The sequences of primers used in qPCR and the assay conditions are shown in Table 2.4.

Table 2.4: The forward and reverse primer sequences, annealing temperatures and references for genes involved in qPCR.

Primer	Forward sequence (5' – 3')	Reverse sequence (5' – 3')	°C	Reference
B-actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	58	(Mälarstig *et al*., 2003)
Endosialin	ACCTCGGAGATGAGTTGCTG	TTCCAGGCCTCGTCTTCA	58	(Wilhelm *et al*., 2016)
hFOXO1	TTGAATTCACCCAGCCCAAACT	GCTACCCCAGGATCAACTGGTG	58	(Tanaka *et al*., 2009)
IRF4	ACCCGGAAATCCCGTACCA	GGCAACCATTTTCACAAGCTG	58	(Vasquez *et al*., 2015)
KAI1	CGGCACAAGCAGATGGACAGG	CGGCAACAGGACCCAGAGTG	62	(Zhang *et al*., 2014)
LTBR	AGATGAAGTTGGGAAGGGTAACA	GCAGTGGCTCTAATGGATTTTTG	57	(Rajput *et al*., 2013)
OGG1	GTGGACTCCCACTTCCAAGA	GAGATGAGCCTCCACCTCTG	56	(Radak *et al*., 2011)
PDGF-A	TACTGAATTTCGCCGCCACA	CCAAAGAATCCTCACTCCCTACG	57	(Juliachs *et al*., 2014)
PDGF-B	TGTGCGGAAGAAGCCAATCT	AATAACCCTGCCCACACACTC	57	(Juliachs *et al*., 2014)
PDGFR-α	AGTTCCTTCATCCATTCTGGACT	CCGTCTGTCCCCCAGTT		(Juliachs *et al*., 2014)
PDGFR-β	CATCACCGTGGTTGAGAGC	AATTGTAGTGTGCCCACCTCTC		(Juliachs *et al*., 2014)
PLZF	TAGGGTGCACACAGGTGAGA	GTGCAGATGGTGCACTGGTA	58	(Kommagani *et al*., 2016)
sFlt-1- 15e	CTCCTGCGAAACCTCAGTG-	GACGATGGTGACGTTGATGT	54	(Whitehead *et al*., 2011)
sFlt-i13	ACAATCAGAGGTGAGCACTGCAA	TCCGAGCCTGAAAGTTAGCAA	56	(Whitehead *et al*., 2011)
Flt-1	TGGCAGCGAGAAACATTCTTTTATC	CAGCAATACTCCGTAAGACCACAC	58	In house

SOD2	TCAGCGGTAGCACCAGC ACT	ATCTGCGCGTTGATGTGA GG	64	(Araujo *et al*., 2011)

2.13. Assessing PCR primer efficacy

The efficacy of each qPCR primer set was tested before performing PCR. Primers were either designed using Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) or selected from publications and analysed using this programme to check for specificity, complementarity, and annealing temperature. After performing qPCR, melt curves were checked and the products separated on agarose gels to confirm the correct product/amplicon size. The efficiency of the primers was checked using a standard generated with ten-fold dilution series of the PCR product from 1:100 to 1:100,000,000 dilution.

2.14. *In vitro* cell migration assays

Scratch-wound cell migration assays based on the methods of Jovanović and Vićovac (2009), were performed with hESC (both primary and St-T1b cells) and HTR-8 to determine their migratory activity. The cells were passaged using Trypsin/EDTA and plated in 6-well plates at a seeding density of $3x10^5$ cells/well for primary hESC and $2.8x10^5$ cells/well for St-T1b cells. Control proliferative cells were seeded at the same density in a separate plate and maintained in a complete hESC growth medium. The next day scratches were made in the confluent cell monolayers using 100 µl pipette tips. The cells were washed twice with complete PBS to remove any cell debris and to ensure smooth wound edges (Liang *et al*., 2007). DMEM/Ham F12 medium supplemented with 1% FBS (v/v) (2 ml/well) was then added.

Images were assigned at ten reference points (Figure 2.2) along with the scratch initially at time zero and after 18 h incubation using the 10 x objective of an EVOS XL inverted-phase microscope (Fisher Scientific, Loughborough, UK) or 4 x objective (Olympus, Tokyo, Japan).

The degree of migration was determined based on the percentage of the scratch area remaining:

% of migration = [(initial scratch area – scratch area at 18 h) / initial scratch area)]*100.The scratch-wounded areas were determined using the ImageJ Trainable-Weka segmentation plugin. The results calculated using the Voxel counter plugin, which is applying to count the pixels of an image stack (Arganda-Carreras *et al.*, 2017). Essentially, the same procedure was performed using HTR-8 cells. HTR-8 cells were plated at $3x10^5$ cells/well in the complete HTR-8 growth medium, which was replaced with 2 ml of DMEM/Ham F12 medium supplemented with 1% FBS following scratch-wounding the cell monolayer.

2.15. Trophoblast spheroid production

HTR-8 cells were used to generate trophoblast spheroids according to the method of Korff and Augustin (Korff and Augustin, 1998). A 0.25% (w/v) stock of methylcellulose solution was produced by dissolving 3 g of autoclaved methylcellulose (4000 centipoise (cP)) in 125 ml of pre-heated RPMI-1640 at 60^{o}C for 20 min on a magnetic stirrer. A further 125 ml of RPMI-1640 was added and the solution kept on a magnetic stirrer overnight at 4°C in a cold room. The solution was then centrifuged at 2500g for 4 h and the supernatant removed and stored at 4°C. Spheroids were formed in 96-well non-adherent round bottom plates (Sarstedt, Newton, USA) using either 3000 or 750 cells/well (Korff *et al.*, 2004).

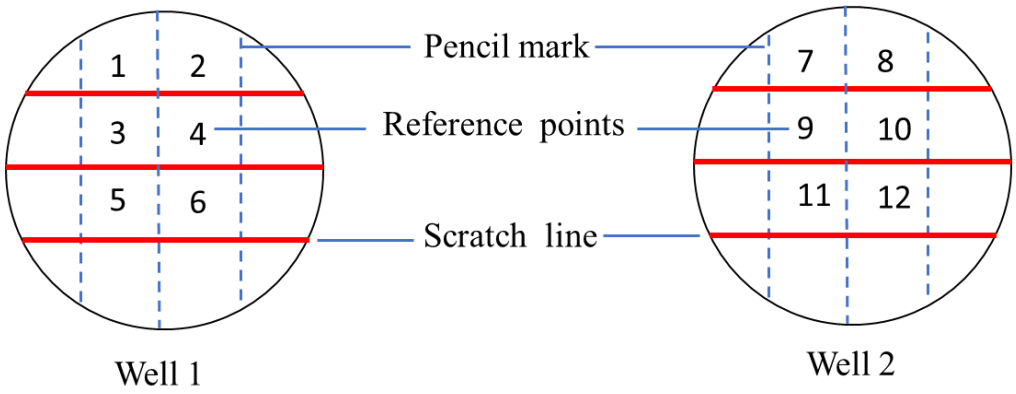


Figure 2.2: The *in vitro* scratch-wound assay plate template. Confluent monolayers of cells were scratched wounded according to reference template points using 100 µl pipette tips.

2.16. Spheroid expansion assay

Spheroid expansion assays were performed based on the method of Gonzalez *et al* (Gonzalez *et al*., 2011) with modifications. HTR-8 trophoblast cells were used to prepare 3000 cell spheroids using the methylcellulose method (Korff and Augustin, 1998). After 24 h, the spheroids were harvested using a 100 µl pipette and collected in a petri dish containing 2 ml of fresh medium. The spheroids were labelled with 10 µM Vybrant green Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) cell tracer dye (Invitrogen) in PBS for 15 min at 37ºC and 5% CO_2 in a humidified incubator. Decidualised or proliferative stromal cells were seeded at a density of $9x10^4$ cells/well in a 24-well plate. Once confluent, hESC were labelled with 10 µM Syto64 dye (Invitrogen) for 2 h in 0.5 ml of DMEM/Ham F12 containing 1% FBS. Three spheroids were then added to each well. Ten spheroids per treatment were monitored. Fluorescent and brightfield images captured at 0, 48 and 72 h time points using the 4x objective. The total area occupied by the HTR-8 cells from each spheroid was measured using ImageJ software at 0 h, 24 h and 48 h.

2.17. Spheroid invasion assay

Trophoblast spheroid invasion assays were performed essentially as described by Gellersen *et al* (2010). HTR-8 cells were used to generate trophoblast spheroids as described above (Section 2.15), with each spheroid consisting of ~750 cells (Korff *et al*., 2004). After 24 h, the spheroids were harvested using a 100 µl pipette and collected in a Petri dish containing 2 ml of a fresh medium. The spheroids were labelled with 10 µM Vybrant green CFDA SE cell tracer dye (Invitrogen) in PBS for 15 min at 37ºC and 5% CO_2 in a humidified incubator. A further washing step was then carried out in a separate Petri dish.

A 24-well plate was seeded with St-T1b cells that when confluent was stimulated to decidualise or maintained in a proliferative state. To each well, fibrinogen (3 mg/ml) in DMEM/Ham F12 medium was mixed with thrombin (4 U/ml). 200 µl/well added immediately either to overlay the cell monolayers or to empty wells of a 24-well plate and incubated at 37ºC CO_2 incubator for 15 min to allow the gel to set. This basal layer prevented spheroids from attaching directly to the bottom of the well. The spheroids (3-4/well) were then added in 300 µl of fibrinogen solution to each well. Thrombin (4 U/ml) was added and the plate gently rocked to allow even distribution of spheroids in the gel as it set. The plate was incubated at 37ºC in a 5% CO_2 incubator for 30 min to ensure uniform gel setting. Finally, 0.5 ml DMEM/Ham F12 medium containing 1% FBS and any treatment was added, and the plate incubated overnight at 37ºC. The next day, 3 mg/ml /well of Tranexamic acid was added to prevent gel degradation by proteolytic enzymes. Fluorescence imaging was performed at 490/20 nm wavelength (4 x magnification) at 48 h and 72 h.

2.18. Image analysis

Computer-Based image analysis was performed using ImageJ software on images captured for the migration, expansion and sprouting assays. Initially, the acquired images for each experimental condition were loaded individually into ImageJ then grouped into a single image stack. The acquisition noise was eliminated with a median filter. The image contrast was enhanced to achieve maximum discrimination between image background and foreground. An automatic image segmentation process was applied to identify scratch (Appendix 1), expansion (Appendix 2), and spheroid core (Appendix 3) using the Trainable Weka plugin (Jaccard *et al*., 2017) as shown in Figure 2.3. The plugin is primarily designed to segment objects into different colours. An automatic threshold was then applied to produce binary images with a black background and white foreground for quantification.

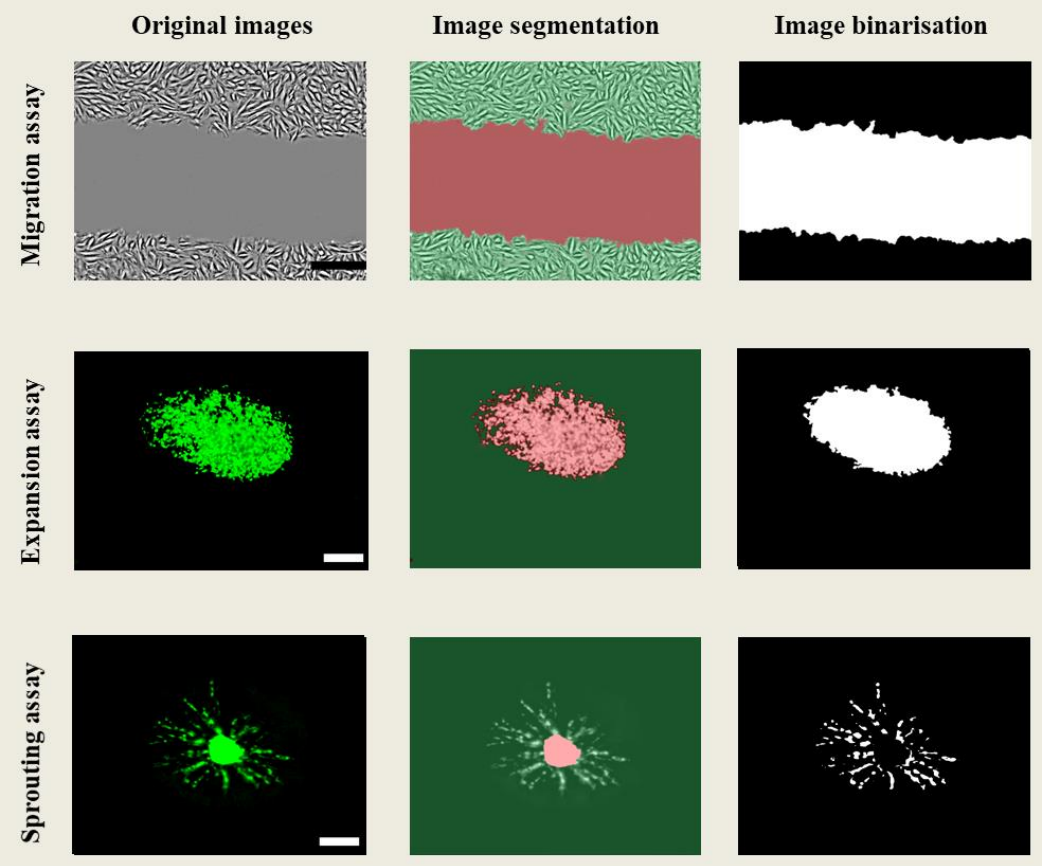


Figure 2.3: Image analysis of migration, expansion, and sprouting assays using ImageJ. The original images were segmented using the Trainable Weka segmentation plugin then binarised to calculate scratch, expansion, and sprout white areas. Scale bars = 400 µm for migration images, and 250 µm for expansion and sprouting images.

The degree of spheroid sprouting was analysed on captured images. The core was subtracted from the original image leaving only the spheroid sprouts. This novel approach readily facilitated the measurement of spheroid sprouting densities. The longest sprout in each spheroid quadrant was measured with an ImageJ vertical scale according to the previously established protocol used by Korff and Augustin (1999). An automatic threshold was then applied to quantify the whole sprout density (Figure 2.3).

2.19. MTT assay

The MTT (3-(4, 5-dimethylthiazol-2-y1) 2, 5-diphenyl tetrazolium bromide) assay was performed based on the method of Wan *et al* (1994). Briefly, St-T1b and HTR-8 cells were seeded at $3x10^4$ cells/well in a 96-well plate in 100 µl of complete growth medium and incubated overnight (Yu *et al.*, 2017). After 24 h, the test treatments were applied in DMEM/Ham F12 containing 1% (v/v) FBS in triplicate and the plate incubated for 72 h. The MTT solution (~10 µg/well) was added for 4 h (Table 2.5). The precipitated MTT was then dissolved in 150 µl/well of MTT solvent for 2 h at room temperature with mild agitation in the dark.

Table 2.5: Solutions used in the MTT test

Reagent	Components
MTT solution	1x PBS 5 mg/ml MTT (w/v)
MTT solvent	4 mM HCl, 10% (v/v) Nondet P-40 (NP40) in Isopropanol

The absorbance at 570 nm and background at 670 nm was measured using a microplate reader (VersaMax^{tm} ELISA, Molecular Devices, California, USA). Following the subtraction of the background at 670 nm, cell viability was calculated as a percentage of the untreated controls, which were set as 100%.

2.20. Collection of whole cell protein lysate

Cells were plated according to the required experimental protocol into 6-well plates. Three wells were used per treatment condition. The wells were washed with cold PBS, then with cold Radioimmunoprecipitation Assay (RIPA) lysis buffer (Pierce-Thermo scientific, Leicestershire, UK) containing Roche mini EDTA-free protease inhibitor cocktail (1 tablet per 10 ml) (Roche, Mannheim, Germany). The amount of RIPA buffer was determined according to the cell count with 125µl/well was routinely used. The plate was kept on ice for 3 min before protein lysates were collected using a cell scraper and incubated on ice for 15 min. The clearing of insoluble cellular debris was performed by centrifugation at 14000 g at 4^{o}C for 10-15 min. The supernatant was collected carefully and decanted into new Eppendorf tubes and stored at -20^{o}C or -80^{o}C.

2.21. Measurement of protein concentration

The Bicinchoninic acid (BCA) kit was used to measure protein concentration. This assay estimates the reduction of Cu^{+2} into Cu^{+1} ion (Smith, 1989). The number of ions liberated is proportional to the protein concentration through the formation of a stable purple product, which allows the calculation of protein concentration depending on the absorbance at 562 nm in a spectrophotometric plate reader.

Protein samples were diluted with PBS at 1:1, 1:2, and 1:4 ratios in order to ensure that at least one would fall within the range of the standard curve. BSA protein standards were set up in

PBS at the following concentrations: 1000, 800, 600, 400, 200, and 0 µg/ml. 10 µl of each standard and samples were added in triplicate to a flat-bottomed 96-well plate. 200 µl of BCA solution consisting of reagent A and reagent B mixed in a 1:50 ratio, was added to each well. The plate was incubated in the dark at 37^{o}C for 30 min, followed by measurement of the absorbance at 562 nm using a spectrophotometric plate reader. The average concentration of each protein sample was calculated from a standard curve following the subtraction of the background.

2.22. Preparation of protein samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Following protein concentration measurement, the volume of protein sample required for a total of 20 µg was calculated and the total volume adjusted to 15 µl with RIPA buffer. The protein samples separated under reducing conditions; therefore 9% (v/v) of 2-mercaptoethanol was added to the 4 x loading buffer (Invitrogen). Loading buffer 5 µl (4x) was added to 15 µl of each protein sample and heated to 95-100^{o}C for 5 min. On cooling to room temperature, samples were centrifuged at 12,000g for 5 min to remove any precipitated material.

2.22.1. Preparation of SDS-PAGE gel

SDS-PAGE gels were cast using 1 mm thickness disposable gel cassettes (Novex-life technologies, Nupage, Fischer Scientific, Loughborough, UK). Ten to 15% SDS-PAGE was used to separate protein samples depending on the molecular weight of the protein of interest. The recipe for the preparation of buffer solutions used to prepare SDS-PAGE gels is shown in Table 2.6.

Table 2.6: The recipes for SDS gel preparation.

Buffer	Contents
10%-15% Resolving SDS gel	0.1% (v/v) SDS 375 mM Tris pH 8.8 10%-15% (w/v) acrylamide; 0.26% (w/v) bis-acrylamide 0.1% (v/v) Tetramethyl ethylenediamine (TEMED) 0.1% (w/v) ammonium persulphate (APS)
Stacking SDS gel	0.1%(v/v) SDS 125 mM Tris; pH 6.8 5% (w/v) acrylamide; 0.13% (w/v) bis-acrylamide 0.1% (v/v) TEMED 0.1% (w/v) (APS)

The resolving gel was poured into the gel cassette to about ¾ full. Immediately, butanol or isopropanol was dropped on the upper margin of the poured gel to achieve a smooth upper edge and to prevent further gel oxygenation, which could interfere with polymerisation. Once the resolving gel had set, the stacking gel was poured, and an appropriate comb inserted.

2.22.2. Separation of protein by molecular weight

Prior to assembling the cassette in the electrophoresis tank (Geneflow), the inserted comb was removed gently and 1x running buffer (prepared from commercially available 10 x Tris/Glycine/SDS, Geneflow) poured to cover the wells of the gel cassette. Then, 5 µl of protein marker was added to a well (CSL-BBL pre-stained protein ladder, Geneflow), while the protein sample was loaded as 15 µl/well into the stacking gel. The gel then runs at 100 mV for 45- 60 min or until the bromophenol blue dye had arrived at the bottom of the gel to ensure a good protein separation.

2.22.3. Transfer of protein from SDS-PAGE gel to PVDF membrane

The separated proteins were transferred to the polyvinylidene difluoride (PVDF) membrane (Merck-Millipore, Darmstadt, Germany) using a wet gel transfer apparatus (Geneflow) as follows: The PVDF membrane was activated with methanol for 30 sec, washed with ultrapure water (3 min), then equilibrated with 1x transfer buffer for 5 min (Table 2.7).

Table 2.7: Buffer solutions used in the protein transfer to PVDF membrane.

Transfer solution	Components
10 x Western blot transfer buffer	142.60 g/l of 190 mM glycine, 30.31 g/l of 25 mM tris base, made up to 1 L in ultrapure water
1x Western blot transfer buffer	100 ml 10 x transfer buffer, 200 ml methanol, 700 ml ddH_2O

The stacking gel was removed from the resolving gel. A transfer module was prepared from sequential layers, including; sponges and filter paper pre-soaked in transfer buffer, SDS-gel, and PVDF membrane assembled closest to the positive plate of the module and another layer of pre-soaked sponge and filter paper. Protein transfer performed at constant voltage (100 V) for 2 h at 4^{o}C. Successful protein transfer was determined by soaking the membrane briefly in 0.1% Ponceau stain.

2.22.4. Western Blotting

To prevent non-specific protein binding, the membrane was blocked with 20% (w/v) skimmed milk powder (Marvel) in PBS and 0.1% Tween-20 (PBS-T) for 2 h at room temperature with gentle agitation. The membrane was washed three times (10 min each) with 1 x PBS-T with mild agitation followed by incubation with the appropriate dilution of the primary antibody in 5% BSA and 0.02% sodium azide in PBS-T at 4^{o}C overnight (Table 2.8). The membrane was

washed three times in PBS-T and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Table 2.9) in 5% (w/v) skimmed milk in PBS-T for 1h at room temperature. The membrane was washed three times in PBST and blotted to remove the excess buffer and soaked in enhanced chemiluminescence (ECL) Western blotting detection solution (Biological Industries, Beit Haemek, Israel) for 1 min. Excess ECL was removed by touching the edge of the membrane onto a dry tissue paper prior to wrapping with SaranWrap. An exposure to ECL film (Amersham Hyper film ECL, Fisher-scientific) was performed in the dark room at various exposure times, starting at 30 sec to develop the chemiluminescent signal. When required, the membrane was stripped in a mild stripping buffer to remove the bound antibody and re-probed with a different antibody (Table **2.10**).

Table 2.8: Primary antibodies used.

Antibody	Manufacturer and catalogue number	Species raised in	dilution
HO-1 monoclonal	Abcam (ab13324) Cell Signaling Technology (5853)	Rabbit	1:3000
PDGF-AA monoclonal	Santa Cruz Biotech. (sc-9974)	Mouse	1:500
PDGF-BB monoclonal	Santa Cruz Biotech. (sc-365805)	Mouse	1:1000
PDGFR-α monoclonal	Santa Cruz Biotech. (sc-398206)	Mouse	1:500
PDGFR-B monoclonal	Santa Cruz Biotech. (sc-374573)	Mouse	1:500
FOXO1 monoclonal	Cell Signaling Technology (C29H4)	Rabbit	1:1000
α and β-tubulin polyclonal	Cell Signaling Technology (2148S)	Rabbit	1:1000
sFlt-1 monoclonal	Sigma-Aldrich (101M4800)	Mouse	1:1000
Endosialin monoclonal	Santa Cruz (sc-377221)	Mouse	1:500

Table 2.9: Secondary antibodies used.

Antibody	Manufacturer and catalogue number	dilution
Anti-mouse IgG HRP-linked antibody	Cell Signaling Technology (7076S)	1:5000
Anti-rabbit IgG HRP-linked antibody	Cell Signaling Technology (7074S)	1:5000

Table 2.10: Mild stripping buffer.

Item	Ingredient
Mild stripping buffer	15 g glycine, 1 g SDS,10 ml Tween-20, and 1 L ultrapure water with pH adjustment to 2.2

2.23. ELISA

Enzyme-linked immunosorbent assays (ELISA) were employed to determine changes in human VEGF and sFlt-1 production in cell supernatants. The human VEGF and VEGF-R1 DuoSet ELISA kits were used according to manufacturers' protocol (R&D Systems, Minnesota, USA). ELISA plates were prepared by coating 96-well Nunc Immunosorb strip-well plates (Immuno^{TM}-Nunc) with 100 µl of a capture antibody at the recommended concentration in PBS overnight at room temperature. The capture antibody was aspirated, followed by three washes with 400 µl/well of the wash buffer, 0.05% (v/v) TWEEN-20 in PBS. The final wash was followed by tapping the plate over a clean tissue to remove the excess buffer. Blocking of non-specific binding was achieved using 300 µl/well of reagent diluent (1% BSA in PBS (w/v 0.22 µm-filtered) for 1 h. The wells were then washed three times with wash buffer as described above. The standards and samples were then added to the plate (100 µl/well) in duplicate and incubated for 2 h at room temperature then aspirated and the washing steps were repeated.

Subsequently, 100 µl of biotinylated kit-specific secondary antibody was added to each well and the plate incubated for 2 h. After further washing steps, the wells were incubated with 100 µl of Streptavidin-horseradish peroxidase conjugate in the reagent diluent for 20 min. The plates were washed and 100 µl of freshly prepared substrate solution added and incubated in the dark for 20 min. The reaction was stopped with 50 µl of 2 N sulphuric acid (H_2SO_4). Immediately, the optical density was measured at 450 nm with wavelength correction at 540 nm using a spectrophotometric plate reader. The concentration of the samples was calculated from the standard curve using Microsoft Excel.

2.24. siRNA-mediated knockdown in hESC

The Viromer® BLUE transfection kit was used to knockdown FOXO1 and HO-1 in hESC according to manufacturer's guidelines (Lipocalyx, Weinberger, Germany). The cells were plated at $2.2x10^5$ cells/ml in a 6-well plate to reach 65- 85% confluency over 24 h. Transfection was performed with 200 µl of siRNA complex/1.5ml of the complete growth medium/well. This transfection complex contained 37.5 nM siRNA in 2 µl Viromer blue. After 24 h, the cells were either stimulated to decidualise or maintained as proliferative for 24 h prior to the harvesting cell supernatants and protein or RNA lysates. The siRNA sequences used are shown in Table 2.11.

Table 2.11: siRNA duplex used in the current project.

Target	Forward sequence	Reverse sequence	Reference
FOXO1	5'-GAGCGUGCCCUACUUCAAGTT-3'	5'-UCUCCUAGGAGAAGAGCUGTT-3'	(Potente *et al*., 2005)
HO-1	5'-GGCAGAGGGUGAUAGAAGATT-3'	5'-UCUUCUAUCACCCUCUGCCTT-3'	(Cudmore *et al*., 2007)

2.25. Cell conditioned medium

2.25.1. hESC conditioned medium

St-T1b cells were plated at $10x10^5$ cells in 12 ml of E2 medium in a T-75 flask. Once confluent, they were stimulated to decidualise for 5 days with the MPC medium changed at 3 days. The cells were harvested and re-plated in 6-well plates and stimulated with MPC medium for a further 24 h. The conditioned medium was then collected. The same procedure was performed with proliferating cells in E2 medium. The conditioned medium was centrifuged at 200g for 10 min to remove any cells present and the supernatant stored at -20^{o}C.

2.25.2. HTR-8 cell conditioned medium

HTR-8 cells ($1x10^6$) were plated in 12 ml of the growth medium in a T-75 flask. The next day when the cells had reached confluence, fresh medium was added. After 24 h, the conditioned medium was harvested, centrifuged at 200g for 10 min and the supernatant stored at -20^{o}C.

2.26. Statistical analysis

Data were analysed using the SPSS statistics software package version 24. The Schapiro-Wilk test was applied to show whether the data are normally distributed. Two-tailed Student's T-Tests were used to compare the differences between two data sets when appropriate. For multiple comparisons, one-way or two-way ANOVA was used to find any significant difference among groups with *post hoc* tests, including the least significant difference (LSD) or Dunnet, as appropriate. For the abnormal (non-parametric) distribution of the data, the Mann-Whitney test was applied to calculate the significant difference. The levels of significance were set at $*p< 0.05$ $**p< 0.001$ in all comparisons.

CHAPTER 3

AN *IN VITRO* MODEL TO INVESTIGATE THE ROLE OF DECIDUALISATION ON STROMAL -TROPHOBLAST INTERACTION

3.1 Introduction

Successful human embryo implantation depends on a highly synchronised interaction of a competent blastocyst with a receptive endometrium. The molecular mechanisms that underpin this dialogue are still poorly understood. One key component is endometrial decidualisation which occurs spontaneously under the influence of the post ovulatory increase in P and local cAMP elevation even in the absence of an embryo (2012). Two typical decidual products, namely, dPRL and IGBP-1 are considered to be key molecular markers of decidualisation (Gellersen and Brosens, 2003b). The major regulator/driver of decidualisation of hESC is FOXO1, which is a transcription factor that induced with decidualisation and its inhibition can result in IGFBP-1 and PRL inhibition (Grinius *et al*., 2006). Decidual cells expressed can express other genes such as KAI1, PLZF, IRF4, and SOD2 (Kajihara *et al*., 2011).

The perturbation of endometrial decidualisation can result in abnormal trophoblastic invasion (Lei *et al*., 2007, Godbole *et al*., 2011, Suman *et al*., 2012), embryo implantation failure (Paria *et al*., 2002), recurrent miscarriage (Salker *et al*., 2010), pre-eclampsia, IUGR (Garrido-Gomez *et al*., 2017), and infertility problems such as endometriosis (Brosens *et al*., 2013). There is also an increased risk of cardiovascular problems in both mothers and offspring in later life (Ros *et al*., 2001, Smith *et al*., 2001).

Some studies suggest that decidualisation causes a physical barrier that inhibits trophoblastic invasion depending on the fact that trophoblastic invasion does not extend beyond the inner layer of the myometrium. Some studies suggest that the decidua inhibits invasion through secreting factors that increase trophoblast adhesion (Graham *et al*., 1992), as well as tissue factor that neutralises the proteolytic activity of trophoblastic metalloprotease (Lala and Graham, 1990). Menkhorst (2019) found that decidualisation significantly reduced the MMP production from ESC. Paiva *et al* (2009) found that the decidual limiting of trophoblast invasion

is due to the effect of IL-11 through its effect on STAT3. According to these findings, the decidua can be considered as a chemical barrier for invasion (Sharma *et al*., 2016). Other studies agree that the decidua has a regulating role on trophoblastic invasion through a fine balance between invasion activating and inhibitory substances (Kliman, 2000). Godbole *et al* (2011) found that the decidual conditioned medium enhances trophoblastic invasion through increasing the production of MMPs and the reduction of TIMMPs in trophoblast cell lines. Despite all these studies, the role of hESC decidualisation in trophoblasts invasion is still not definitely elucidated so far. Investigation of the role of decidualisation in the process of human blastocyst invasion is often hampered due to various constraints. For instance, difficulties in obtaining a primary endometrial tissue due to ethical and practical limitations, as well as the inability to passage primary cells *in vitro* for a long time (Krikun *et al*., 2004). Human embryos are relatively inaccessible and only a few labs in the world have access to donated embryos for research purposes (Brosens and Gellersen, 2010). This has necessitated the development of *in vitro* models to mimic human stromal cell trophoblast interactions. Consequently, immortalised endometrial and trophoblast cell lines have been utilised. Different types of endometrial stromal and trophoblastic cell lines have been introduced to date. Telomerase immortalised endometrial stromal cells have been developed and called St-T1b cells (Rinehart *et al*., 1993, Samalecos *et al*., 2009c). These cells have been used in the current *in vitro* models of embryo implantation as the endometrial side of maternal-trophoblast interface. To represent the trophoblast part of the interface, Gonzales *et al* (2011) used the AC-IM88 cell line. These cells might not be representative of embryo implantation or early pregnancy as it is a fusion of term placental cells with mutant JEG-3 choriocarcinoma, which has a tumour-like invasive behaviour more than the expected in a normal embryo implantation. Therefore, in the current study, another trophoblastic cell line, HTR-8/SVneo cells, were chosen. These cells are immortalised cells

derived from first trimester villus explants. The cells show adhesion, invasion, migration, and proliferative abilities, and the expression of KRT7, chorionic gonadotrophin receptor, and HLAG marker. These criteria enable them to be used as implantation and EVT models (Hannan *et al*., 2010). Grewal *et al* (2010) developed a heterologous two dimensional co-culture model composed of a mouse embryo over a layer of hESC with the modulation of hESC Rho GTPases expression to explore the role of endometrial stromal motility on spreading of the embryo over endometrium during implantation. Gonzalez *et al* (2011) developed an expansion co-culture model composed of T-hESC and AC-IM88 trophoblastic cell lines to evaluate the role of decidualisation in trophoblastic invasion. An essential requirement in model development is the establishing of accurate analysis protocol and preferably easy and automated and this what I was aiming during the project course.

3.1.1. Aim and objectives

The aim of this chapter was to develop suitable *in vitro* models to facilitate the investigation of the role of endometrial decidualisation, growth factors, and cytokines in trophoblast-endometrial interactions. Trophoblast invasion has been shown to be influenced by various cytokines and growth factors such as TNF-α, interleukins, LIF, and EGF in a paracrine or autocrine manner (Staun-Ram and Shalev, 2005).

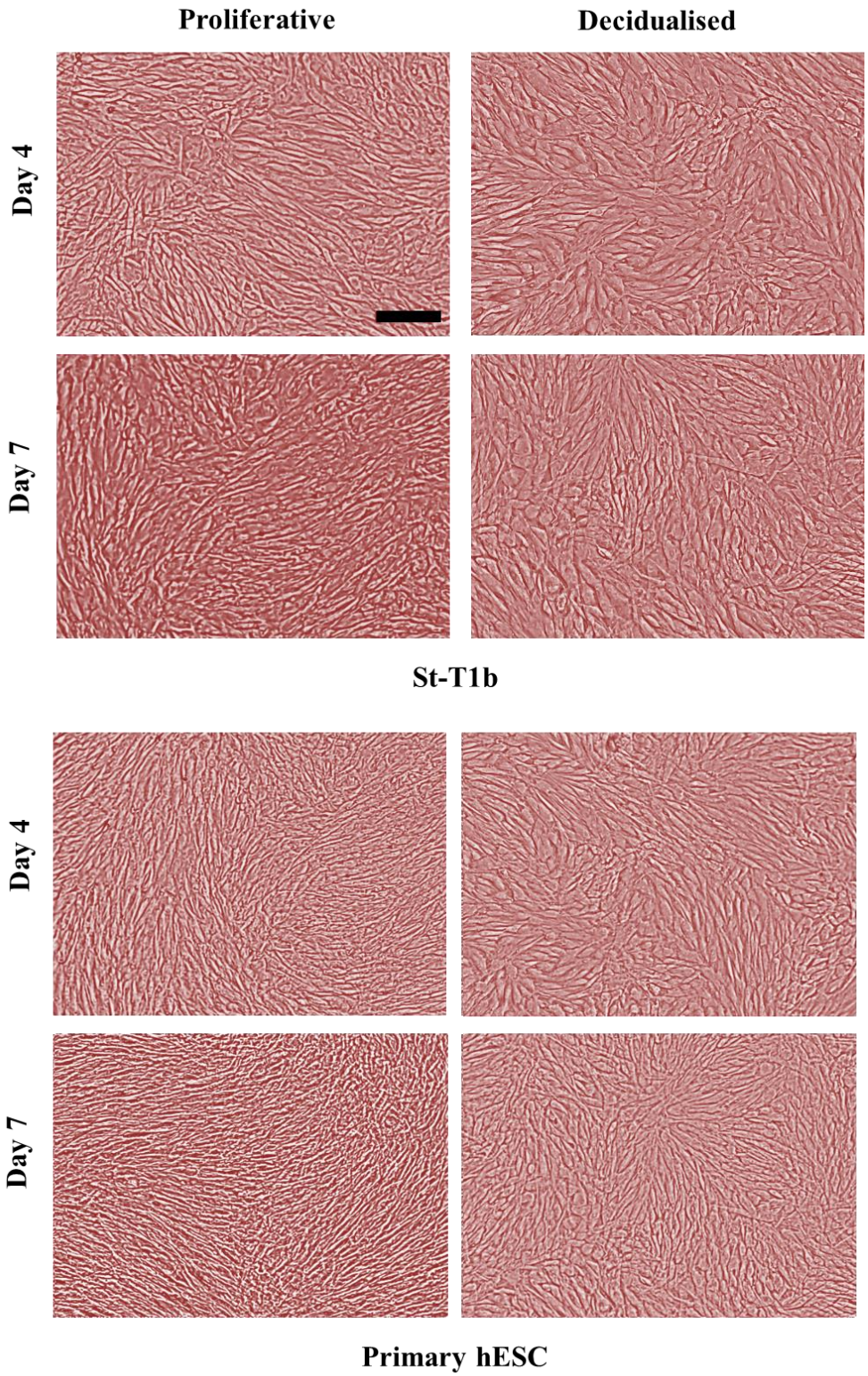
3.2. Results

3.2.1. *In vitro* characterisation of human endometrial stromal cell line (St-T1b) decidualisation

The human St-T1b endometrial stromal cell line (Samalecos *et al*., 2009) was used to investigate the effect of decidualisation on growth factor expression and cell function. Initially the ability of St-T1b to decidualise was confirmed to validate their use as a suitable alternative to primary hESC. The *in vitro* decidualisation of the cells was achieved using a decidualisation protocol based on that described by Samalecos *et al* (2009c) for 4 to 7 days. In order to induce differentiation, the cells were stimulated in decidualisation medium (MPC medium). The medium was replaced every 48 – 72 h and 24 h prior to harvesting the culture supernatants and cells (Figure 2.1).

3.2.2. Morphological characterisation of St-T1b cells and primary hESC decidualisation

St-T1b cells and primary hESC were plated at a seeding density of $3x10^5$cell/well in a six-well plate and induced for differentiation at 4 and 7 days. Differentiation was judged by the morphological reorganisation of elongated spindle shape St-T1b fibroblasts in the proliferative stage into epithelioid-like, enlarged, more cuboidal decidualised cells (Figure **3.1**A), which continue to proliferate and appear elongated and more densely packed on day 7 of the experiments. The cells were stained with Phalloidin-iFluor 488 and counterstained Hoechst 33342 to visualise their cytoskeleton. Cells were plated at $0.6x10^6$cells/well in E2 medium in an 8-well culture slide. Next day, the cells were induced to decidualisation as usual. The merged images of Phalloidin nuclear Hoescht 333432 staining confirmed the same reorganisation of actin cytoskeleton to the polygonal shape in decidualised cells with the formation of stress fibres (Figure 3.1A & B).


Proliferative
Decidualised
Day 4
Day 7
St-T1b
Day 4
Day 7
Primary hESC

A

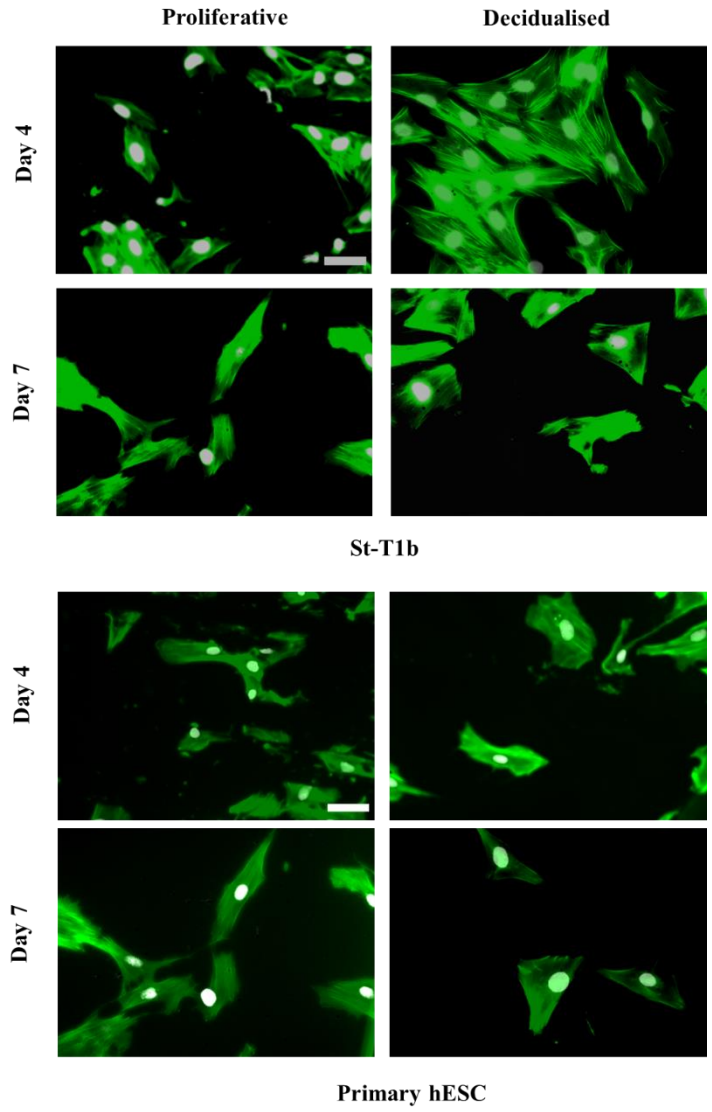


Figure 3.1: Morphological decidualistion of hESC. Confluent monolayers of St-T1b and primary hESC were incubated in E2 medium or MPC for 4 and 7 days. (**A**) Representative bright field images of proliferative and decidualised cells (10x objective). Scale bar = 100 µm. (**B**) Representative fluorescent images of proliferative and decidualised cells cytoskeleton and nuclei after staining with Phalloidin and Hoescht stain (20 x objective). Scale bar = 50 µm.

3.2.3. Biochemical characterisation of St-T1b cells decidualisation by qPCR and Western blotting

Further characterisation of St-T1b cell decidualisation was performed by examining the expression of important decidual genes including FOXO1, KAI1, PLZF, SOD2, and IRF4 by real-time qPCR with β-actin as a house-keeping gene. Following decidualisation, a significant upregulation ($p<0.05$) of FOXO1 mRNA was observed (Figure 3.2A). Western blotting was used to confirm the upregulation of FOXO1 protein in decidualised St-T1b cells (Figure 3.2B). Other decidual products including SOD2, IRF4, KAI1, and PLZF were upregulated significantly in the decidualised cells at day 4 and day 7 compared to the proliferative cells at the same time points (Figure 3.2C, D, and E). qPCR and Western blots confirm the normal decidualisation response of St-T1b to progestational and cAMP stimuli and allowed for further progress in using these cells as a model for primary stromal cells. An interesting finding was the significant upregulation of LTBR mRNA with decidualisation at 4 and at 7 days of stimulation (Figure 3.2G).

3.2.4. Characterisation of St-T1b cell marker gene expression

St-T1b cells were compared with primary hESC to examine their ability to show typical decidualisation biomarker genes coding for dPRL and IGFBP-1 by RT-PCR. The PCR products were separated on agarose gels. The results showed the dramatic induction of dPRL and IGFBP-1 in St-T1b cells following 4 days of decidualisation, which was maintained at 7 days of stimulation. The dPRL and IGFBP-1 bands were not detected in the proliferative St-T1b cells (Figure 3.3). GAPDH was used as a house keeping control gene for these studies.

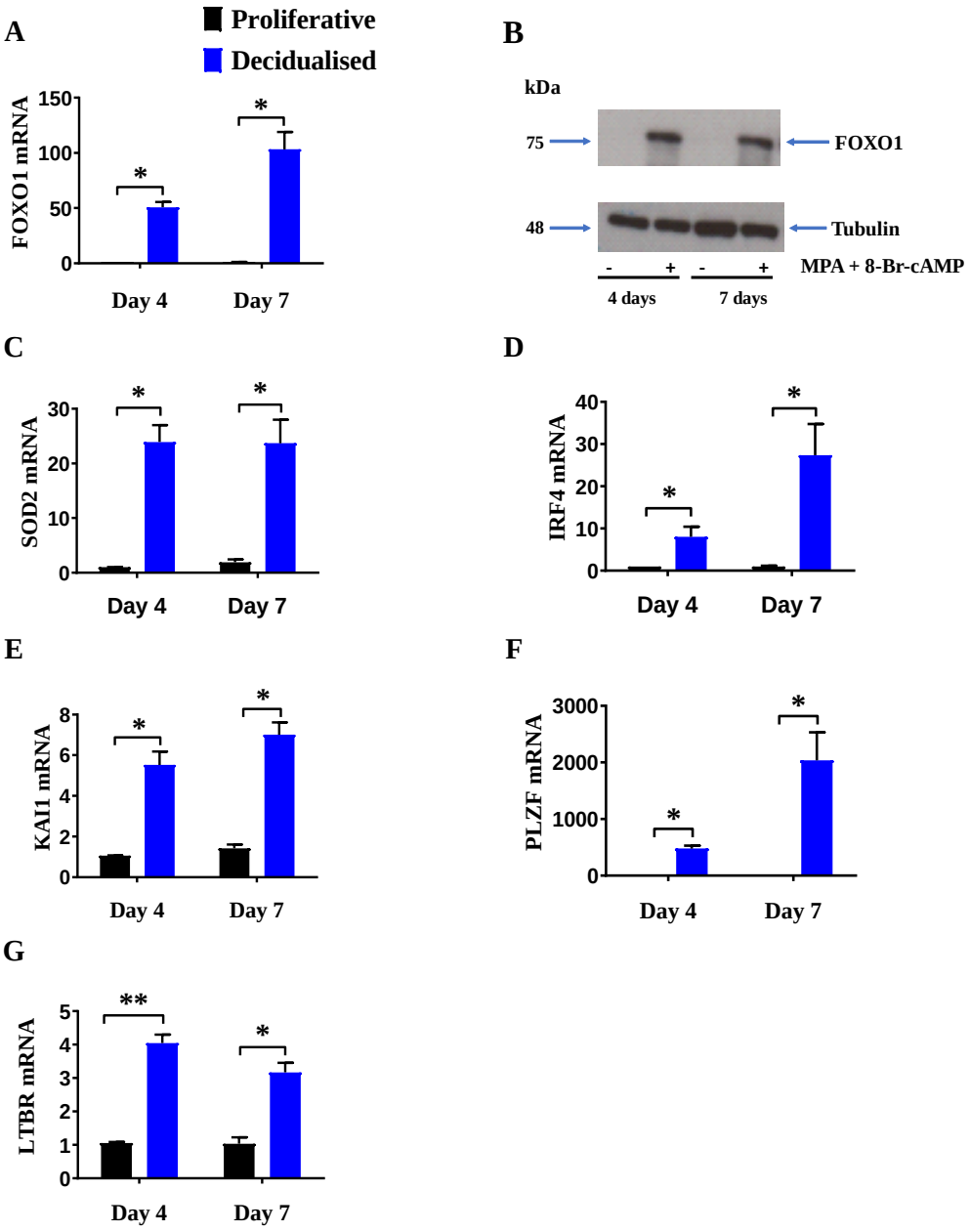


Figure 3.2: Biochemical characterisation of St-T1b cells by qPCR and Western blot. Confluent monolayers of St-T1b were incubated in MPC or in E2 for 4 and 7 days. Relative mRNA fold changes of (**A**) FOXO1, (**C**) SOD2, (**D**) IRF4, (**E**) KAI1, (**F**), PLZF, and (**G**) LTBR expressions normalised to β-actin. (**B**) Representative Western blots showing FOXO1 protein expression with Tubulin used as a loading control. Results are the mean (± SEM) of five independent experiments. Statistically significant differences (*$P<0.05$, **$P<0.001$).

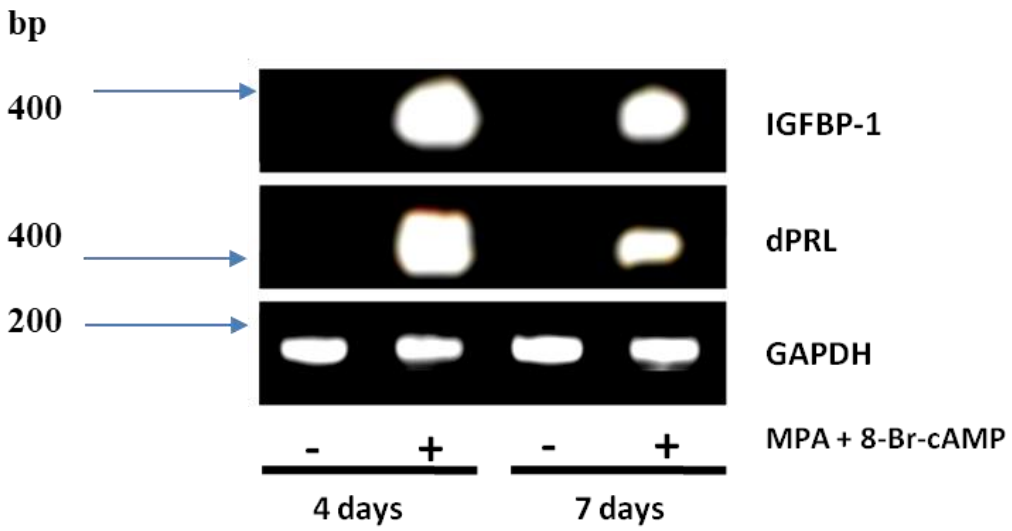


Figure 3.3: Characterisation of St-T1b cell IGFBP-1 and dPRL induction. St-T1b cells were induced to decidualise or maintained as proliferative cells for 4 and 7 days. RT-PCR used to detect the expression of IGFBP-1 (product size 380 bp) and dPRL (product size 416 bp) with GAPDH (product size 159 bp) as a housekeeping control. The products were separated on agarose gels RT-PCR in the decidualised day 4 and day 7 cells.

3.2.5. Biochemical characterisation of primary human endometrial stromal decidualisation

Primary hESC were derived from endometrial tissues obtained from three individuals. To examine their decidualisation response and compare with those of St-T1b cells, the same protocol of St-T1b decidualisation was applied. qPCR for FOXO1 and PLZF was performed alongside Western blotting for FOXO1 protein. The result revealed a significant upregulation ($p<0.001$) of FOXO1 mRNA genes in the decidualised cells at day 4 and day 7 of the experiments (Figure 3.4A). This upregulation of FOXO1 in decidualised cells was further confirmed by Western blot (Figure 3.4B). FOXO1 was strongly upregulated at both 4 and 7 days of decidualisation stimulation. PLZF mRNA was significantly upregulated ($p<0.05$) in day 4 and day 7 decidualised cells compared to proliferative cells (Figure 3.4C).

3.2.6. Development of St-T1b and HTR-8 cells *in vitro* scratch assay

Trophoblast and stromal cell migration is imprtant for embryo implantion (Grewal *et al*., 2008, Gellersen *et al*., 2013, Gellersen *et al*., 2010). In order to examine the role of hESC decidualisation and growth factors on hESC and trophoblast migratory and invasive activities, we developed a number of assays. Initially we used the scratch wound assay according to method develpoed by Liang *et al* (2007) to determine the migratory ability of stromal and trophoblast cells. A confluent T-75 flask of cells was stimulated for decidualisation with MMI medium for 72 h and then re-plated into a 6-well plate at a seeding density of $3x10^{5}$/well.

The *in vitro* scratch assay allows the monitoring of cell migration in a two dimensional view into a space created in a cell monolayer (Cory, 2011). This assay was selected because it is a relatively simple, straightforward and reproducible assay that does not require gradient (Boyden) chambers, and the cells can be monitored continuously under the light microscope.

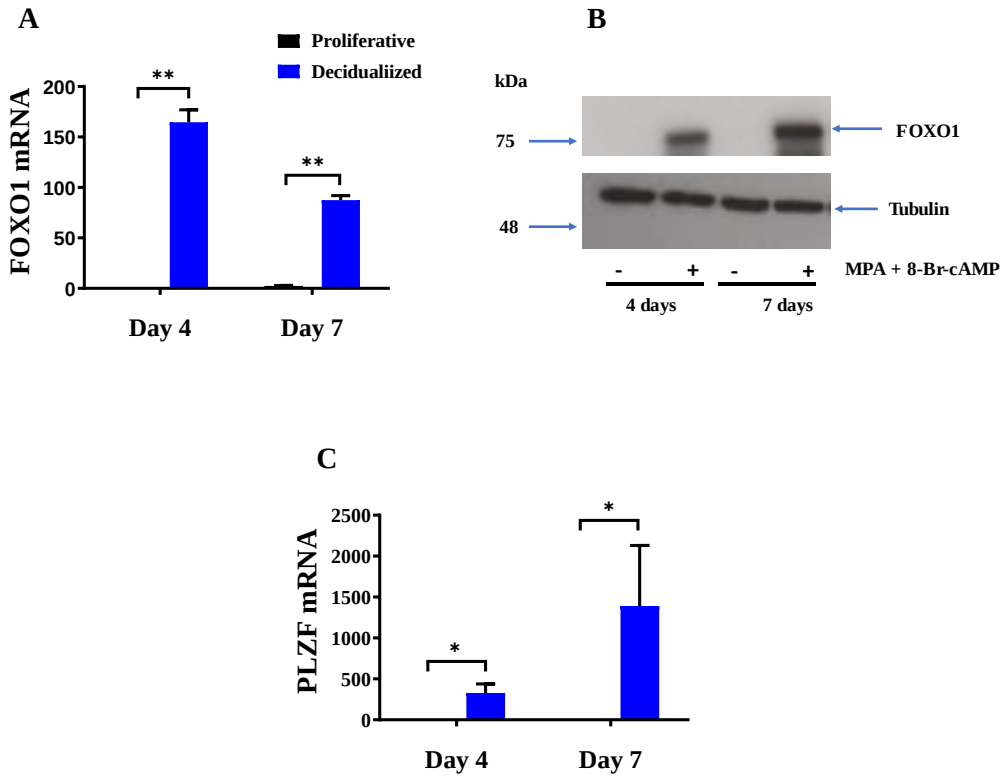


Figure 3.4: Biochemical decidualisation of primary hESC by qPCR and Western blot. Confluent monolayers of primary hESC were incubated in E2 medium or MPC for 4 and 7 days. Relative mRNA fold changes (**A**) FOXO1 and (**C**) PLZF expressions normalised to β-actin. (**B**) Representative Western blots showing FOXO1 protein with tubulin used as a loading control. Results are the mean (± SEM) of three independent experiments. Statistically significant differences (**P<0.05, **P<0.001)*.

During the development of the assay for the hESC and HTR-8 trophoblast cells, six different criteria were considered: The type of the pipette tip used to create the scratch, cell seeding density, the medium used, the time point at which migration is assessed and the use of fluorescent cell tracer dye to facilitate quantitation of migration, and the number of measurements/treatment condition to achieve reproducible results (Table 3.1).

Table 3.1: Development criteria followed in migration assay.

Criteria	Optimisation measure
Pipette tip used to generate scratch	100 µl tip
Cells seeding density	Equal seeding densities in all groups
Type of medium used	1% FBS medium
Follow up timing	18 h
Type of imaging	Bright field
Number of measurements	10 fields/ treatment condition

We found that 100 µl pipette tips gave a nice sharp scratch with an appropriate size (~ 600 µm) to allow visualisation in a single field under a 4 x objective lens. Some authors used different seeding density between decidualised St-T1b cells and proliferative groups of experiments (Schwenke *et al*., 2013). However, the current project assumed that it could be more representative of migration potential differences if the same seeding density were used in both groups. To achieve this, St-T1b decidualisation stimulation were performed in T-75 cm flasks followed by trypsinisation and reseeding in 6-well plates in equal densities. Indicator-free DMEM/F12 containing a range of serum concentrations from 0.5% to 10% were assessed and 1% FBS was selected for cell maintenance after scratch-wounding. This medium supported cell viability without stimulating much proliferation to reflect the situation *in vivo.* The optimal time

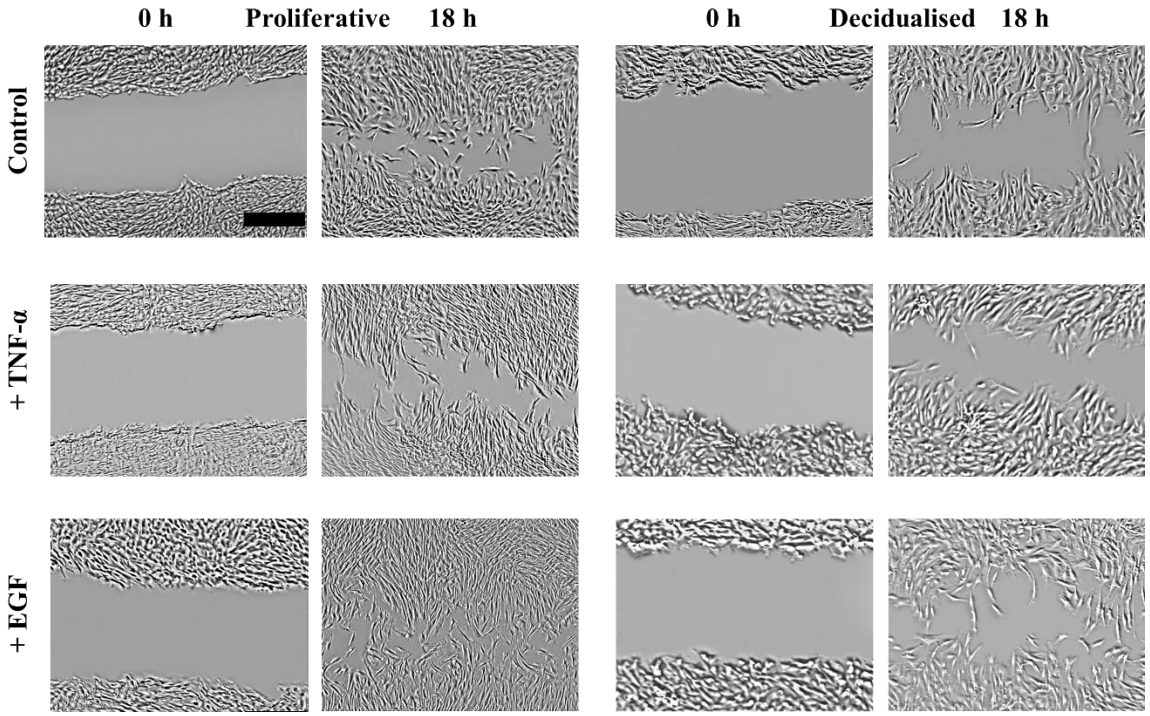
to assess St-T1b migration was determined to be 18 h following making the scratch-wound (0 h). At this time point, approximately 40% closure of the wound was observed in groups. It also does not exceed 24 h, so the results are not confounded by the cell proliferation.

Initially, bright field imaging was used for the migration assay and a new software analysis protocol was developed using ImageJ software. The images initially undergo segmentation using the trainable weka plugin, followed by thresholding and automated measurement of the area of the wound remaining. Ten points/condition were followed at zero time and 18 h using 4 x lens. Although fluorescent cell tracer dyes combined with fluorescence microscopy were trialled to determine whether a greater contrast of areas containing cells versus plastic would results in an easier image analysis. However, the use of trainable weka allowed the analysis of bright field images without the need for complicating the assay with the use of a fluorescent dye. The migration was calculated as:

$$\%\ \text{of migration} = \left(\frac{\text{scratch area at 0 h} - \text{scratch area at 18 h}}{\text{scratch area at 0 h}}\right) * 100$$

3.2.7. Decidualisation reduces hESC migration

To determine the effect of decidualisation on hESC cell migration, St-T1b and primary hESC were plated at $3x10^5$cell/well to obtain confluent monolayers of proliferative and decidualised hESC on 6-well dishes. Cells were scratched /wounded, and their migration assessed at 18 h. As HB-EGF/ EGF has been shown previously to promote hESC migration (Schwenke *et al*., 2013, Gentilini *et al.*, 2007), it was used as a positive control for these studies. Most treatment groups showed a more than 50 % of wound closure at 18 h (Figure 3.5A &B; Figure 3.6A & B). Decidualisation of hESC reduced their migration, irrespective of the conditions, compared with the proliferative cells.



A

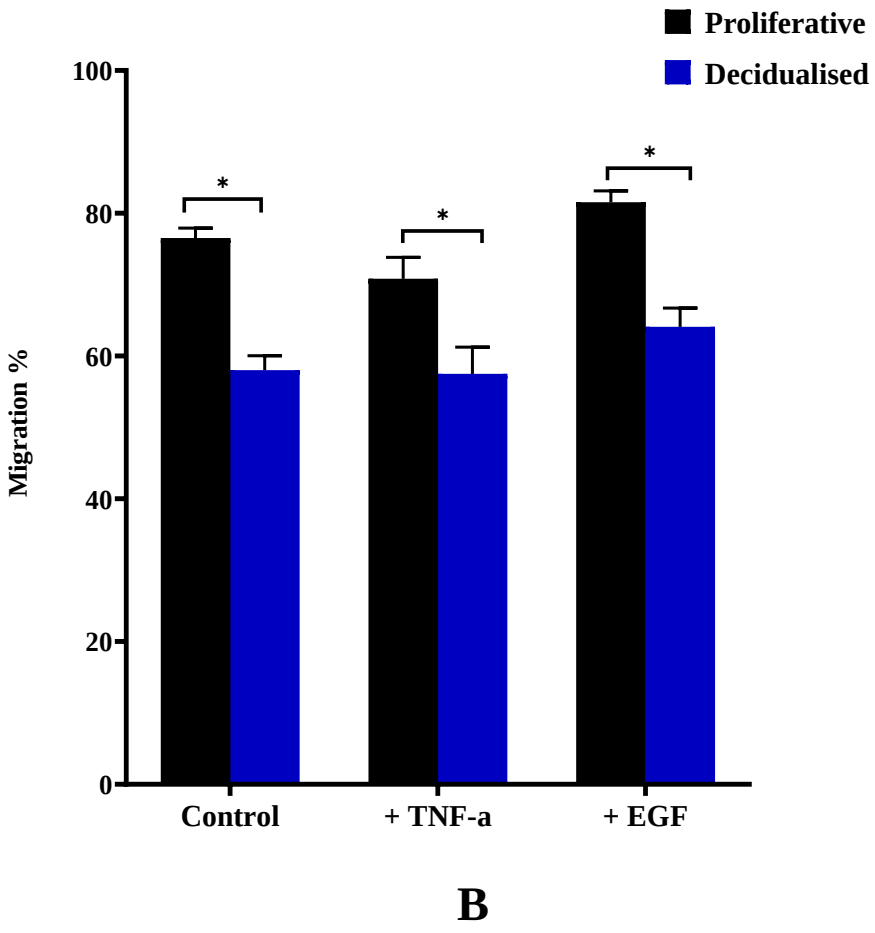
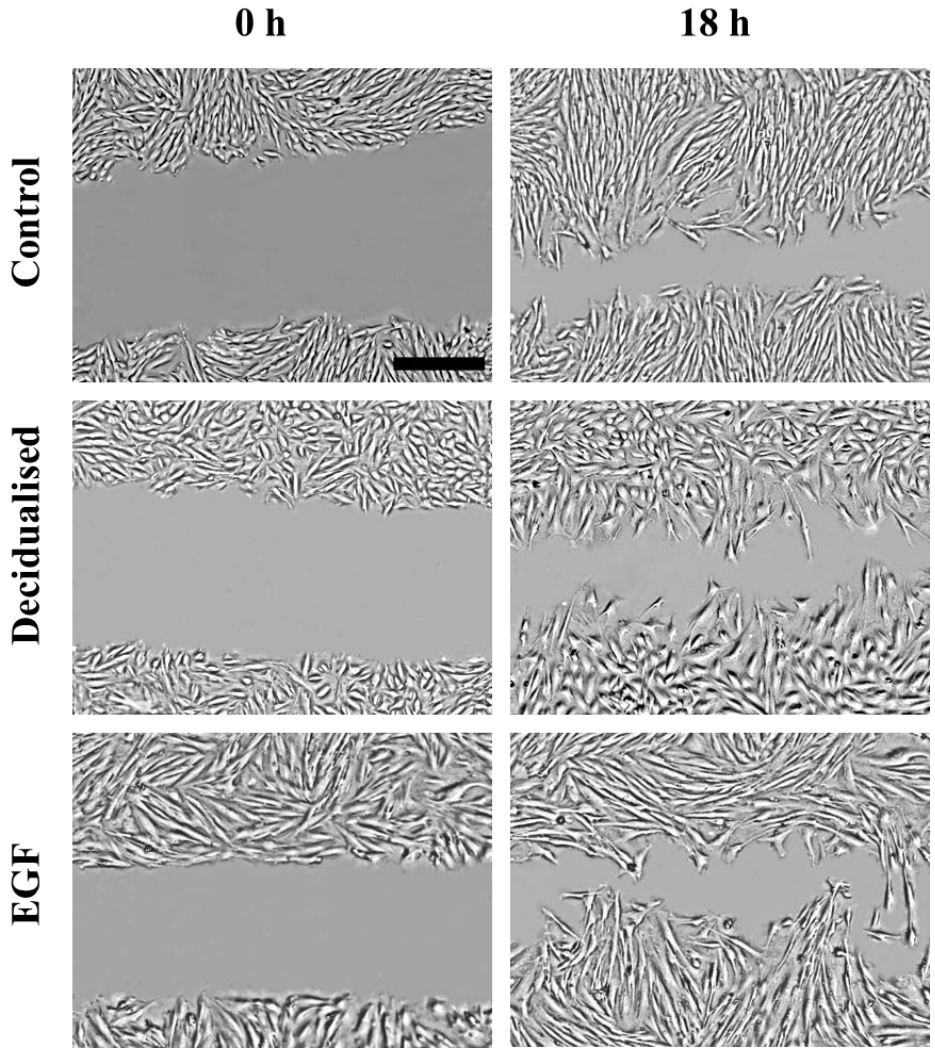


Figure 3.5: Decidualisation reduces St-T1b hESC migration. Confluent monolayers of St-T1b proliferative (control – black bars) or hESC decidualised (blue bars) for 4 and 7 days were scratch-wounded and stimulated with TNF-α (50 ng/ml) or EGF (200 ng/ml) in 2 ml of 1% FBS medium. Cell migration was assessed after 18 h at 10 points /treatment condition in 2 wells and calculated as migration % = (Scratch area at 0 h - scratch area at 18 h / scratch area at 0 h) *100. (**A**) Representative bright field images (4 x objective) showing the scratch areas at 0 and 18 h. (**B**) Results are means (± SEM) of the migration percentage at 18 h relative to the zero time point of three independent experiments. Statistically significant differences (*$P<0.05$). Scale bar = 400 µm.



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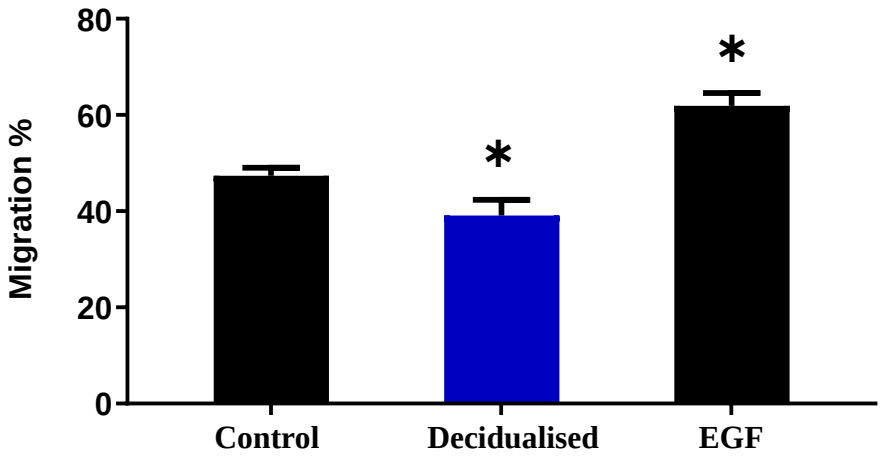


B

Figure 3.6: Decidualisation reduces primary hESC migration. Confluent monolayers of primary proliferative (control – black bars) or hESC decidualised (blue bar) for 4 and 7 days were scratch-wounded and stimulated with EGF (200 ng/ml in 2 ml of 1% FBS medium. Cell migration was assessed after 18 h at 10 points / treatment condition in 2 wells and calculated as migration % = (Scratch area at 0 h-scratch area at 18 h/scratch area at 0 h)*100. (**A**) Representative bright field images (4 x objective) showing the scratch areas at 0 and 18 h. (**B**) Results are mean (± SEM) of the migration percentage at 18 h relative to the zero time point of three independent experiments. Statistically significant differences (*$P<0.05$).

TNF-α (50 ng/ml) was found to have no effect on the migration of either the proliferative or decidualised cells in comparison with the untreated controls. EGF (200 ng/ml) stimulated both proliferative and decidual cell migration. Additionally, the EGF treated decidualised cells showed a faster migration in comparison with decidualised cells of the control and TNF-α treated groups (Figure 3.5A & B; Figure 3.6A & B).

TNF-α can cause apoptosis in some cell types (Prins *et al*., 1997). To ensure that any effects observed with TNF-α treatment are not due to cytotoxic effects or loss of cells, MTT assays (Ciapetti *et al*., 1993) were used to analyse cell viability. St-T1b and HTR-8 cells were seeded at $3x10^4$ cells/well in 96-well plates in 100 µl of the complete growth medium and incubated for 24 h. TNF-α (50 ng/ml) was added to the cells in DMEM/Ham F12 containing 1 % FBS for 72 h and the MTT assay performed. The viability of St-T1b cells (Figure **3.7**A) and HTR-8 cells (Figure 3.7B) was not significantly different to the untreated controls.

3.2.8. EGF stimulates trophoblast migration while decidualised hESC-conditioned medium does not alter it.

It was reported that treating trophoblasts with a proliferative or decidual conditioned medium could change their migratory potential (Yong *et al*., 2018). The HTR-8 cells were plated at $3x10^5$ cell/well in a six-well plate. The cells were scratched, and their migration were followed after 18 h while treated with EGF (200 ng/ml) or decidualised and proliferative St-T1b cells' conditioned medium. The result showed that EGF stimulates HTR-8 migration (Figure 3.8A & B) represented by migration percentage which represent the wound closure. The proliferative or decidualised St-T1b cells conditioned medium did not affect HTR-8 cells migration in comparison with the control cells in which 1% FBS medium was used (Figure 3.9A & B).

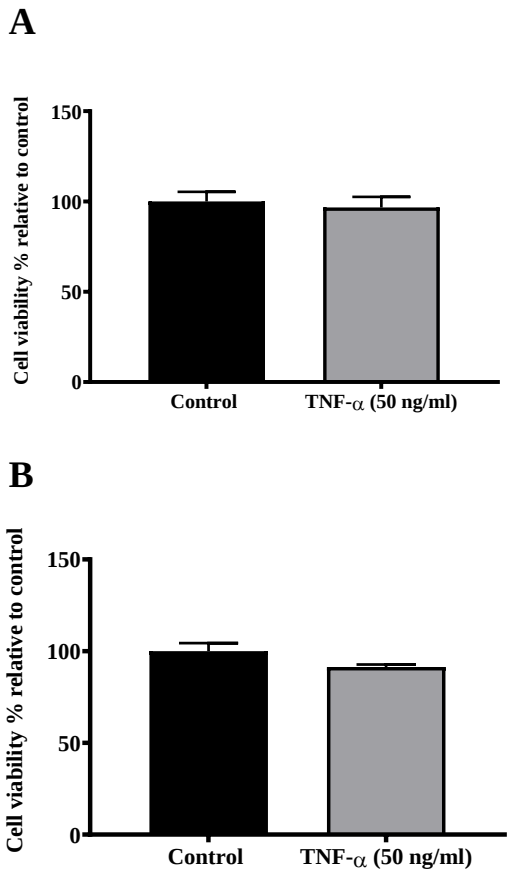
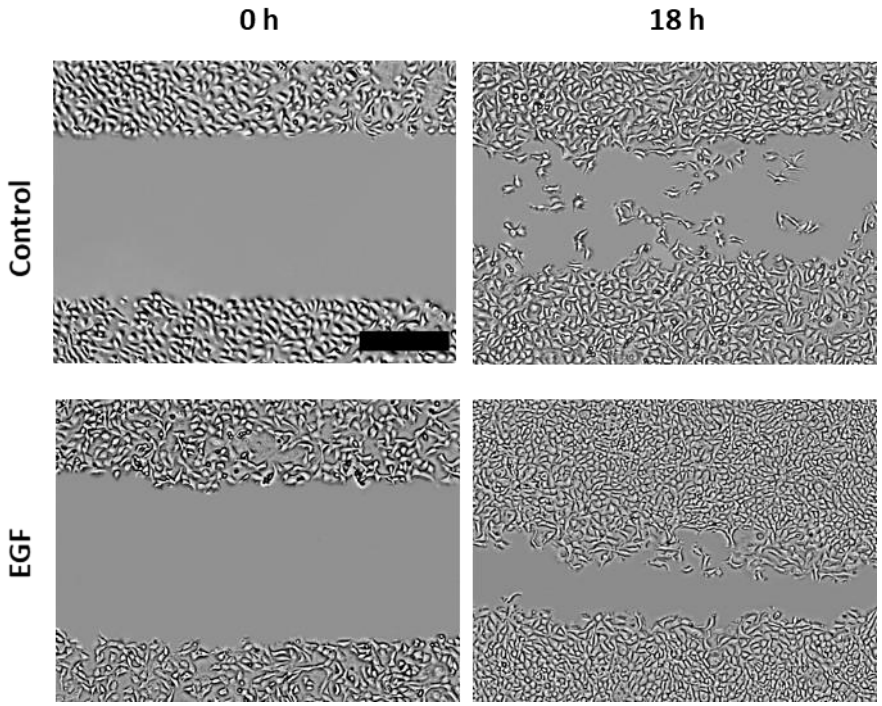


Figure 3.7: **TNF-α treatment does not affect hESC or trophoblast viability.** St-T1b and HTR-8 cells were seeded at $3x10^4$ cells/well in a 96-well plate. 24 h later, 50 ng/ml of TNF-α in DMEM ham F12 (1% FBS) was added for 72 h period using the MTT assay. The results are mean % (± SEM) of cell viability with respect to the untreated controls of three experiments in (**A**) St-T1b cells and (**B**) HTR-8 cells.



A

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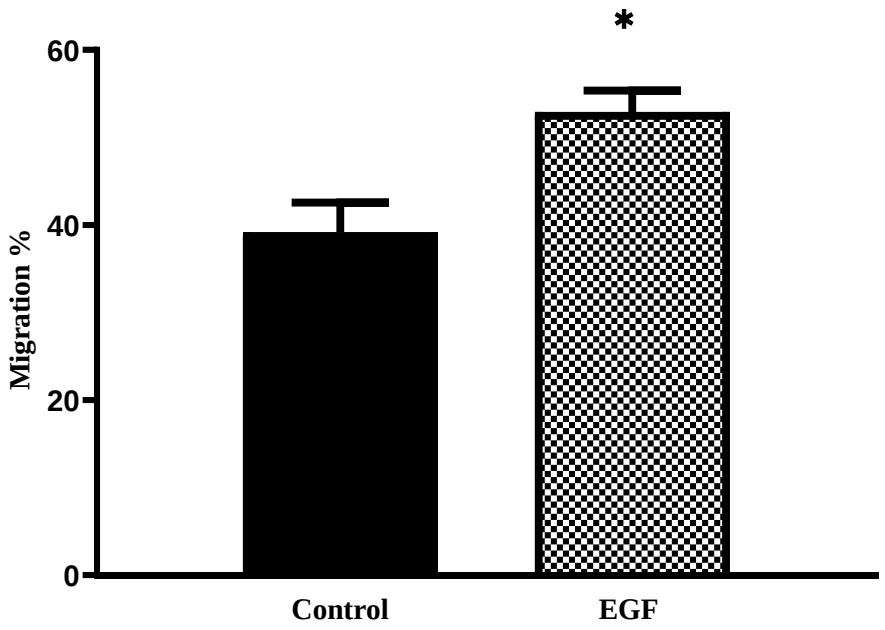
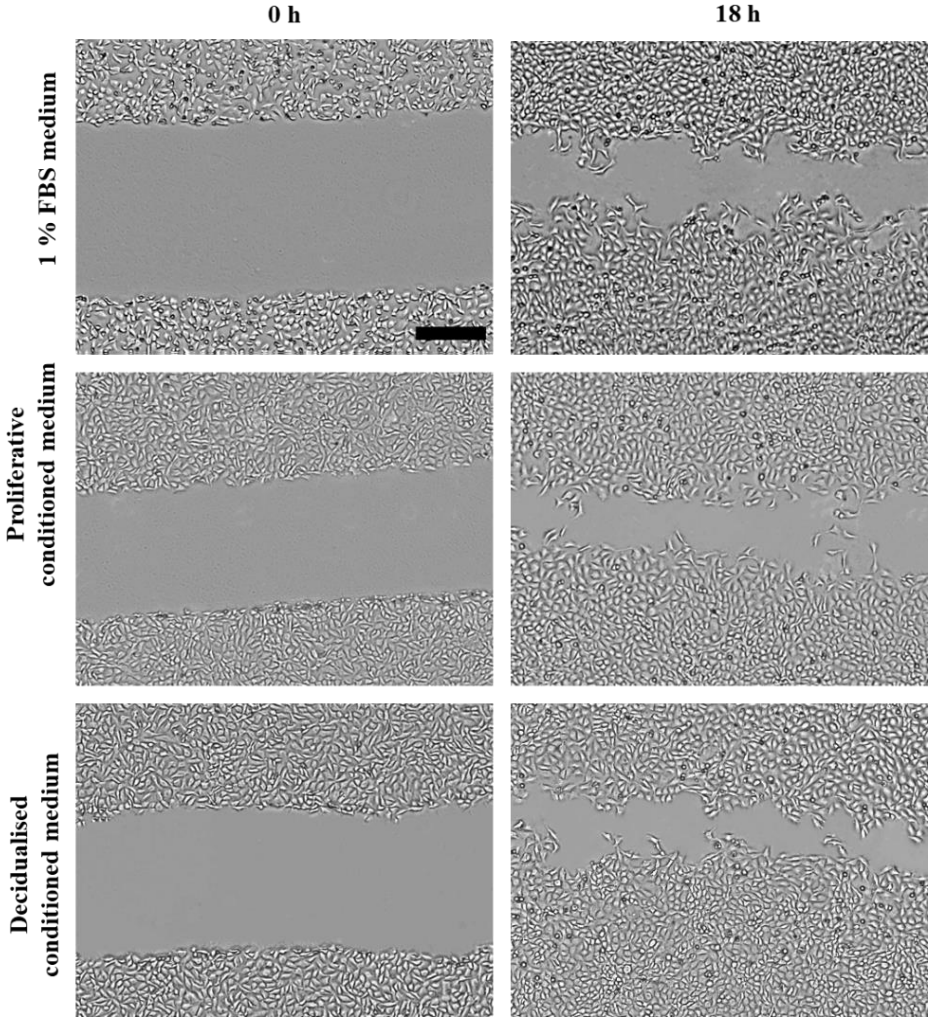


B

Figure 3.8: EGF stimulates HTR-8 cells *in vitro* migration. Confluent monolayers of HTR-8 cells were scratch/wounded. The migration was assessed in the presence of 2 ml of 1% FBS medium (control) and treatment with EGF (200 ng/ml). Cell migration was assessed after 18 h at 10 points /treatment condition in 2 wells and calculated as migration % = [(Scratch area at 0 h - scratch area at 18 h)/scratch area at 0 h)]*100. (**A**) Representative bright field images (10 x objective) showing the scratch areas at 0 and 18 h. Results are mean (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$).


0 h
18 h
1 % FBS medium
Proliferative conditioned medium
Decidualised conditioned medium

A

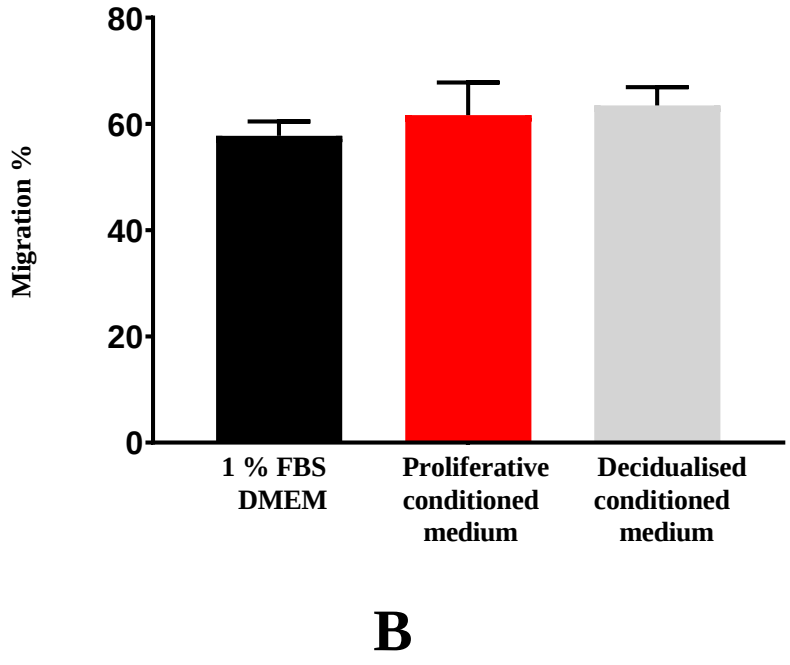


Figure 3.9: Differences in conditioned media have on effect in HTR-8 in vitro migration. Confluent monolayers of HTR-8 cells were scratch/wounded. Their migration was assessed after 18 h in the presence of 2 ml of 1% FBS medium (control – black bars), proliferative ST-T1b conditioned medium (red bar), and decidualised St-T1b conditioned medium (grey bar). Cell migration was assessed after 18 h at 10 points /treatment condition in 2 wells and calculated as migration % = [(Scratch area at 0 h - scratch area at 18 h)/scratch area at 0 h)]*100. (**A**) Representative bright field images (10 x objective) showing the scratch areas at 0 and 18 h. (**B**) Results are means (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$). Scale bar = 200 µm.

3.2.9. Development of HTR-8 spheroid expansion assay

Gonzalez *et al* (2011) described a trophoblast spheroid expansion model, which aims to simulate blastocyst spreading/invading over the decidua in the early stages of implantation. This model was further developed using HTR-8 cells cultured as spheroids to represent the implanting blastocyst, and St-T1b cells to mimic the endometrial side of interface.

During the development of spheroid expansion assay, different criteria were considered including; methods of spheroid cell preparations, the size of each spheroid, the number of spheroids placed in each well, how to follow the cells in using continuous real time analysis, and the type of florescent dye to optimise visualisation (Table 3.2).

Table 3.2: Spheroid criteria followed in HTR-8 spheroid expansion assay development.

Criteria	Optimisation measure
Spheroid preparation method	Methyl cellulose method
Spheroid size	3000 HTR-8 cells /spheroid
Spheroid number	3 spheroids/well in 24 well plates
Fluorescent dye	Vibrant green cell tracer dye

The project adopted the method of Korff and Augustin (1998), which used to generate trophoblast spheroids in 0.24 % (w/v) methyl cellulose. To optimise the size of the HTR-8 spheroids, different sizes ranging from 750, 1500, 3000, and 7500 HTR-8 cells/spheroid were tried. 3000 HTR-8 cells/spheroid was used following the method of Gonzalez *et al* (2011). This generated a spheroid of approximately 200 µm, which mimics blastocyst size (~70-200 µm), as well as yielding a good analysable expansion size without the risk of cells taking apart and not keep compact body or the dying risk of spheroid core cells death in case of a larger size (Figure 3.10).

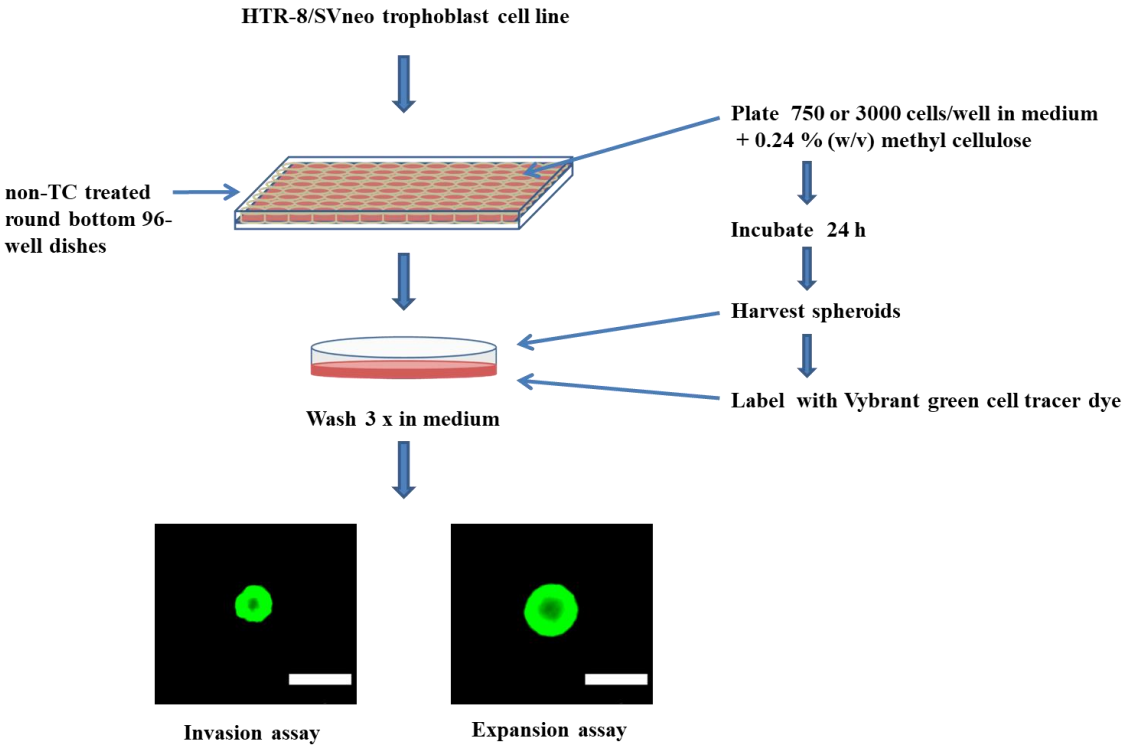


Figure 3.10: HTR-8 spheroid production. A flow-chart showing the generation of HTR-8 spheroids using methylcellulose in a non-tissue treated 96 wells-plate over 24h. The harvested cells were used either in the spheroid sprouting or spheroid expansion assay. Scale bar = 250 µm.

Three spheroids were placed in each well to avoid the clumping of spheroids to each other and to give a space for spheroid expansion that can be visualised with 4 x lenses in a manner that could allow calculation of individual expansion.

Gonzalez *et al* (2011) used an immunohistochemistry stain to visualise the spheroid after fixing the slides. In the current study, Vybrant green cell tracer dye was used, which allowed continuous monitoring of the assay and facilitated the observation of the same group of cells under the same experimental conditions. During assay optimisation, different dyes were tried calcein green-AM and Vybrant green cell tracer. The first dye was continuously leaking from spheroid cells and lead to fluorescence fading over 72 h. Vybrant green dye provided a better visualisation and analysis of spheroid expansion using a novel adopted protocol. The protocol allowed automatic measurement of the expansion area by segmentation with a trainable weka plugin followed by thresholding and area measurement in ImageJ. The spheroid expansion was relatively calculated according to the following equation:

$$\text{Spheroid expansion} = \frac{\text{spheroid size at 48 h or 72 h}}{\text{spheoid size at 0 h}}$$

3.2.10. HTR-8 spheroid expansion is stimulated with the decidualisation of a feeder underneath hESC monolayer and treatment with EGF and TNF-α

Three HTR-8 spheroid cells were placed in each well of 24 well/plate and labelled with Vybrant green cell tracer dye. The spheroids were placed on St-T1b cells, which were grown as monolayers on the well dishes and labelled with Syto64 dye. Using St-T1b cells labelled with Syto64 dye, the cell monolayer appeared intact and unaffected by the spreading HTR-8 spheroid/cells, which appeared to spread over them rather than integrating in the monolayer on the surface of the flask (Figure 3.11).

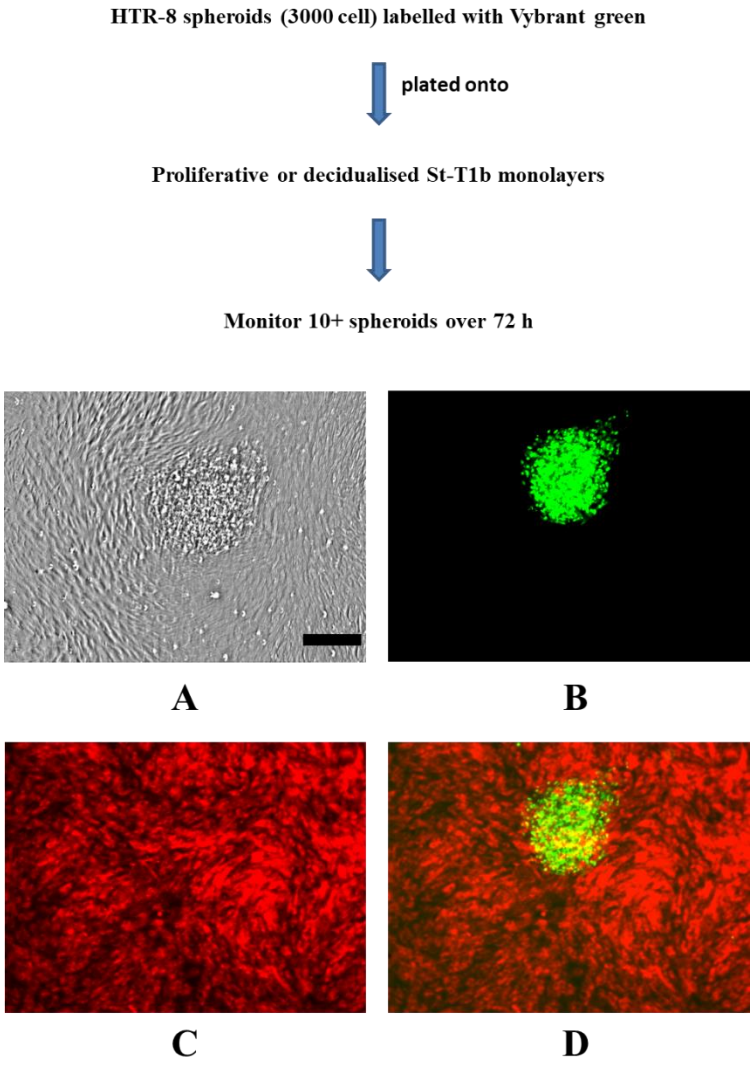
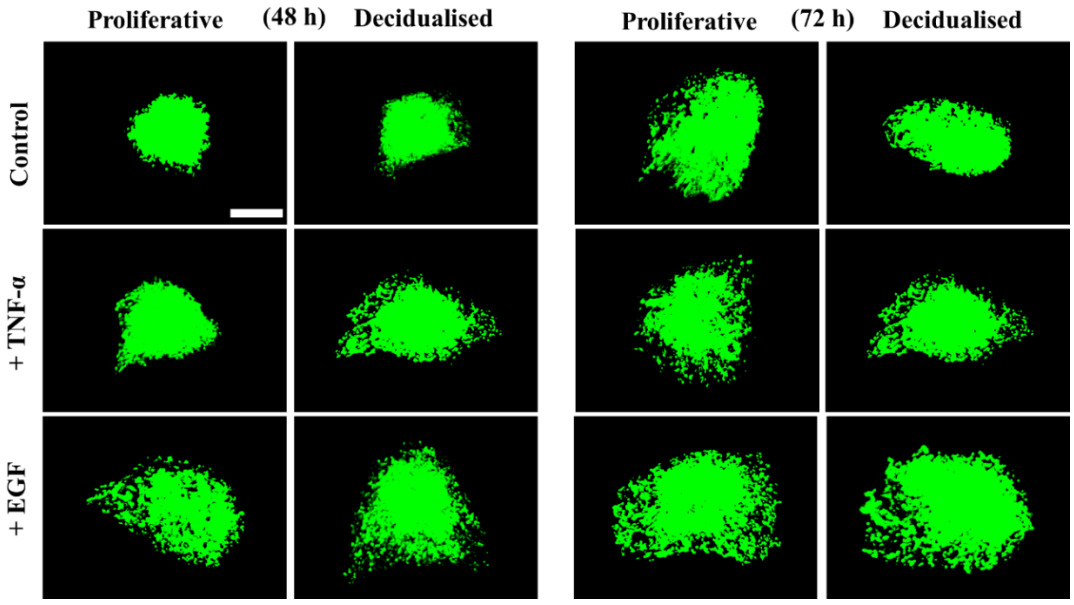


Figure 3.11: Fluorescent labelling of a trophoblast spheroid cultured on hESC monolayer. Representative images showing (**A**) HTR-8 spheroid bright-field image, (**B**) an HTR-8 spheroid labelled with Vybrant green CFDA-SE, (**C**) a St-T1b cell monolayer labelled with Syto64 dye at 48 h time point, and (**D**) merged image (40x magnification). Scale bar = 300 µm.

HTR-8 spheroids were added in 1% FBS DMEM HAM F12 medium and monitored over three days using 4 x lenses. The spheroids expanded and migrated out over the hESC with an increase in the area of > 4-fold at 48 h and ~ 8-fold at 72 h (Figure 3.12A & B) compared with the initial adherent spheroid size. The rate of spheroid expansion was greater on TC plastic than hESC. Spheroid expansion was evaluated in response to endometrial decidualisation and to a range of growth factors and cytokines that have been implicated in embryo implantation including EGF and TNF-α. The HTR-8 spheroid expansion was enhanced in the TNF-α treated proliferative cells and in both proliferative and decidualised EGF-treated hESC when compared with the control cells at 48 h (Figure 3.12A & B). At 72 h, the EGF treated co-culture spheroids displayed marked ($P<0.05$) trophoblastic outgrowth and spreading over proliferative and decidualised hESC in comparison with the corresponding control cells. TNF-α treatment also increased ($P<0.05$) spheroid expansion in both proliferative and decidualised groups in comparison with the control cells. Decidualised TNF-α treated cells showed a greater increase ($P<0.05$) in spheroid expansion than the corresponding proliferative cells and the control decidualised cells (Figure 3.12A & B).

3.2.11. Development of HTR-8 spheroid sprouting assay.

Abnormal trophoblast invasion can result in the failure of embryo implantation, recurrent miscarriage, intrauterine growth restrictions, and pre-eclampsia (Maynard *et al.*, 2003). To evaluate the role of endometrial decidualisation on trophoblast activity, a spheroid sprouting/invasion assay was developed by adopting the versatile three dimensional cytotrophoblast spheroidal model of Korff *et al* (2004) into a co-culture model. The wells were already plated with a confluent monolayer of St-T1b cells either in a decidualised or in a proliferative state.


Proliferative
(48 h)
Decidualised
Proliferative
(72 h)
Decidualised
Control
+ TNF-α
+ EGF

A

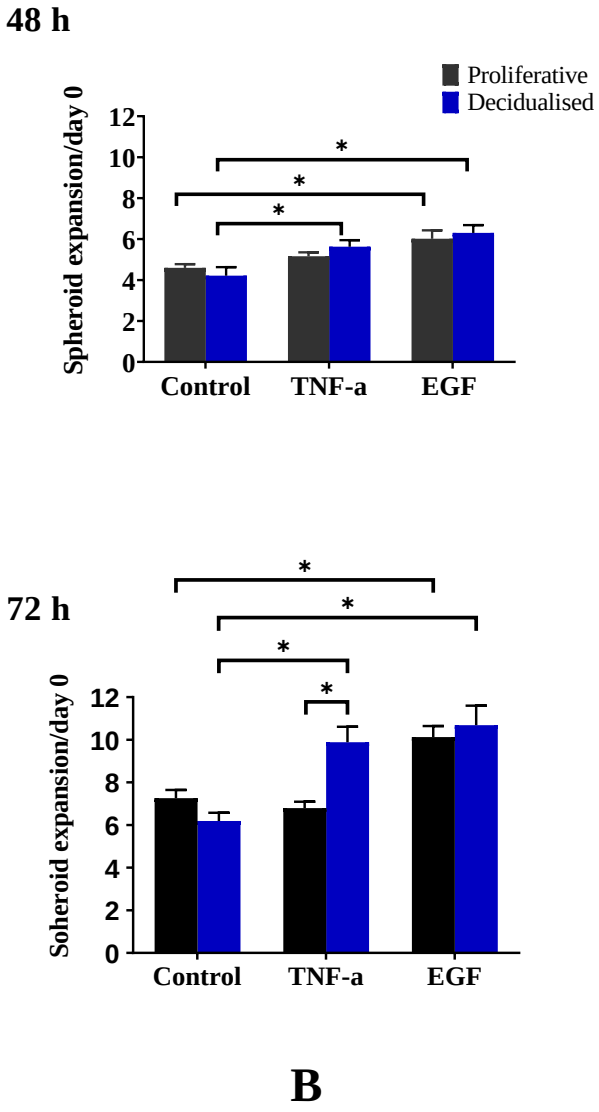


Figure 3.12: EGF and TNF-α enhanced HTR-8 spheroid expansion. HTR-8 spheroids were labelled with Vybrant green dye and seeded onto proliferating (black bar) or decidualised (blue bar) monolayers of St-T1b cells. The co-cultures were then treated with TNF-α (50 ng/ml) or EGF (200 ng/ml) in 1% FBS medium. The HTR-8 spheroid expansion was calculated as spheroid size at 48 h or 72 h /spheroid size at 0 h using ImageJ. Ten spheroids/treatment condition were measured in 4 wells. (**A**) Representative fluorescent images using Vybrant green dye (4x objective) showing spheroid expansion areas at 48 and 72 h. (**B**) Results are the means (± SEM) of three independent experiments. Statistically significant differences *(*P<0.05*). Scale bar = 200 µm.

Three to four labelled spheroids (750 HTR-8 cells/spheroid) were added to each well. 0.5 ml DMEM/Ham F12 medium containing 1% FBS and EGF (200 ng/ml) or TNF (50 ng/ml) was added. Fluorescence imaging was performed at 48 h and 72 h. During the early trials of model, several criteria were included as the size of spheroids, type of gel used, fluorescent dye, and the methods of embedding the spheroids into the gel (Table 3.3).

Table 3.3: Spheroid criteria used in HTR-8 spheroid sprouting assay development

Criteria	Optimisation measure
Size of spheroid cell	750 HTR-8 cells/spheroid
Gel	Fibrin
Imaging	Florescent using vibrant green-labelled cells
Method of embedding spheroids in the gel	Spheroids suspended in fibrinogen applied/set over a thin preformed gel

To select a suitable size of spheroids, 750, 1500, and 3000 HTR-8 cell/spheroid were tried. 750 cell/spheroid was the most appropriate size as it enabled the incubation of three spheroids (~ 500 µm) per well in the 24-wells plate without clumping, as well as a capability to visualise spheroid cell sprouting by 4 x lens. Collagen (Korff *et al.*, 2004) or fibrin gels (Cartwright *et al.*, 1999) have been used to study the spheroid sprouting or invasion before. In the current project, fibrin gels were used as they resemble the physiologic matrix at the embryo implantation site (Bischof and Campana, 1996). Initially, the spheroids were introduced directly into the wells in fibrinogen (3 mg/ml) which was then cross-linked (Figure 3.13). Unfortunately, this often resulted in the spheroids resting on the tissue culture plate at the bottom of the well. This interfered with the sprouting/invasion of cells as well as the analysis.

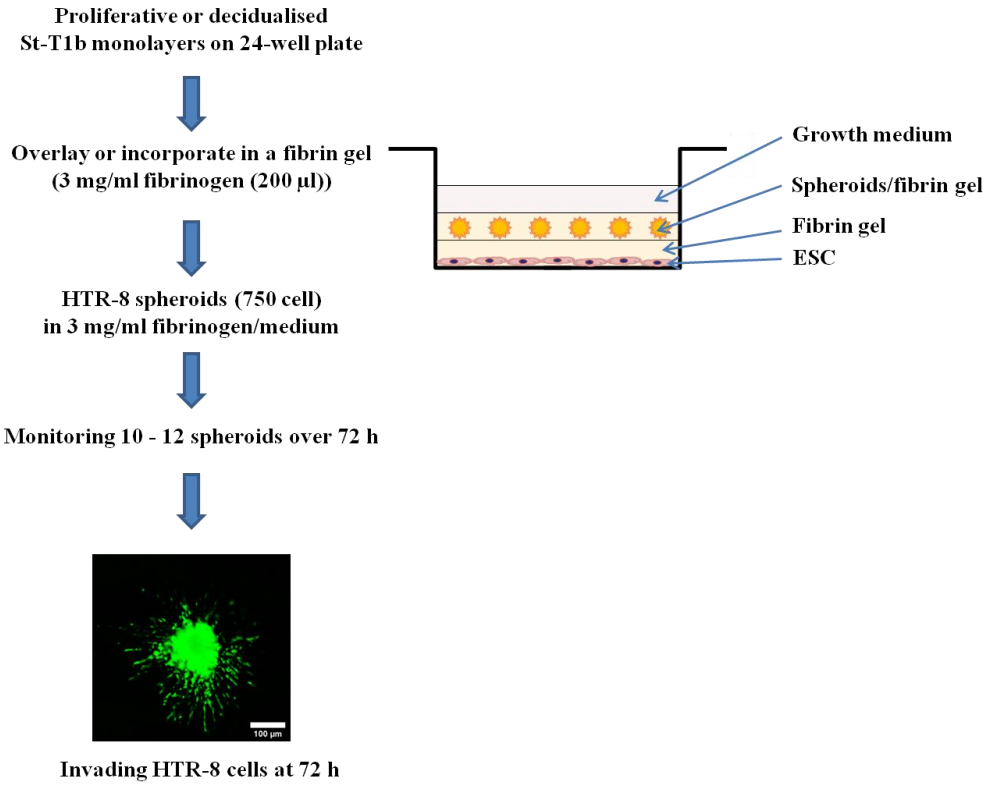


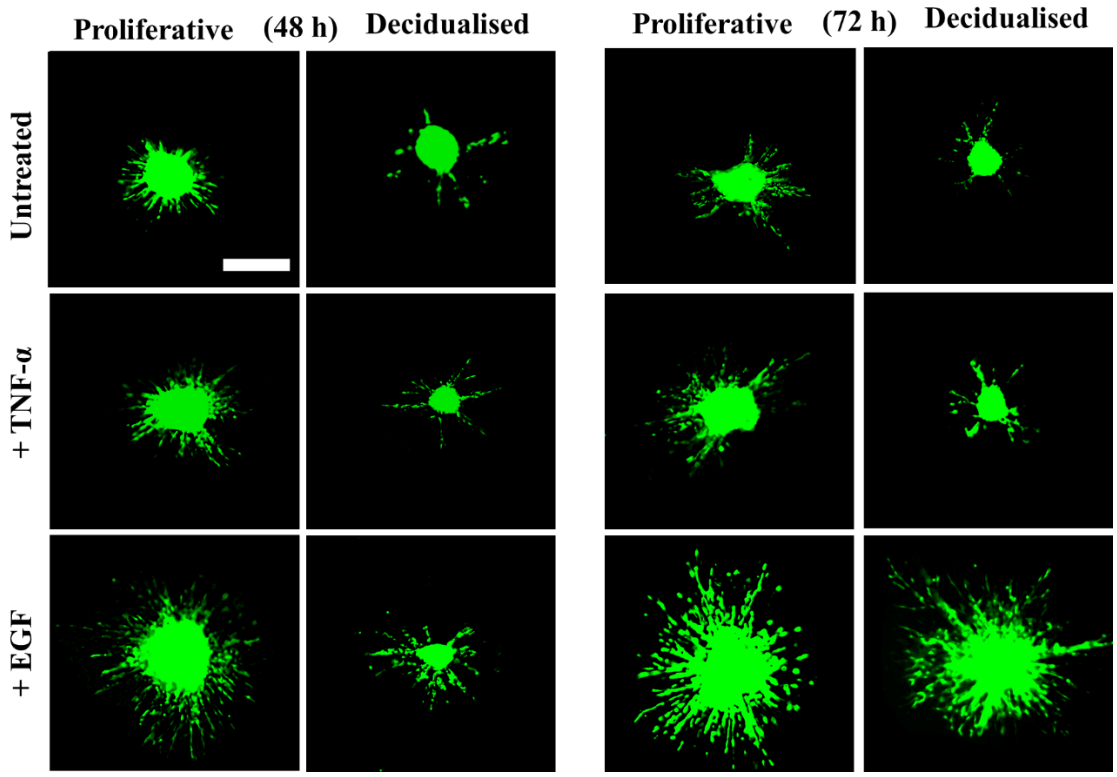
Figure 3.13: Spheroid invasion assay. A diagram showing the generation of spheroid from 750 HTR-8 cells using methylcellulose in a non-tissue treated 96-wells plate. The cells were followed using a fluorescent microscope (4x magnification). Scale bar = 100 µm.

The problem was resolved by applying a thin layer of fibrin gel first, over which the spheroid containing gel was set. Observations showed that HTR-8 spheroids embedded in fibrin gels started to form sprouts after ~ 24 h of embedding, indicating that HTR-8 cells were a good choice as a representative model of invasive trophoblasts. However, the degree of sprouting at 24 h was minimal, therefore imaging the HTR-8 spheroids was performed at 48 h and 72 h. The HTR-8 spheroids were labelled with Vybrant green cell tracer dye to enable the monitoring of the sprouts over 72 h and facilitate the capture and analysis of images. A new analysis protocol was developed using a trainable weka plugin. This allowed segmentation of the spheroid and to perform automated subtraction of the core area followed by thresholding and measurement of sprouting density and length using ImageJ.

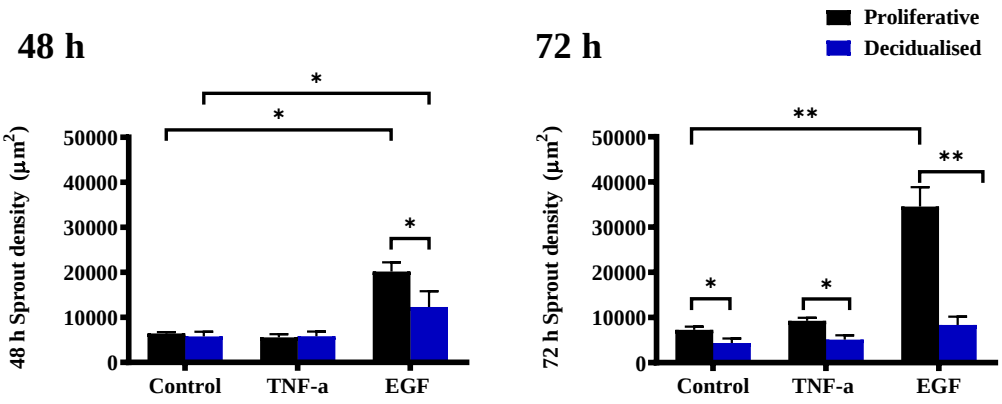
3.2.12. Reduction of HTR-8 spheroids sprouting with decidualisation of underlying stromal monolayer and stimulated with EGF treatment.

The spheroid sprouting/invasion assay was assessed by the three dimensional cytotrophoblast spheroidal model of Korff *et al* (2004). Three HTR-8 spheroids/well of 750 HTR-8 cell /spheroid were placed in a fibrin gel in a 24-well plate over a monolayer of decidualised or proliferative St-T1b cells. The cells were followed over 48 h and 72 h using a 4 x lens. The density of spheroid sprouting was found to be lower in spheroids set over an EGF treated decidualised hESC monolayer than those set over a proliferative one at 48 h.

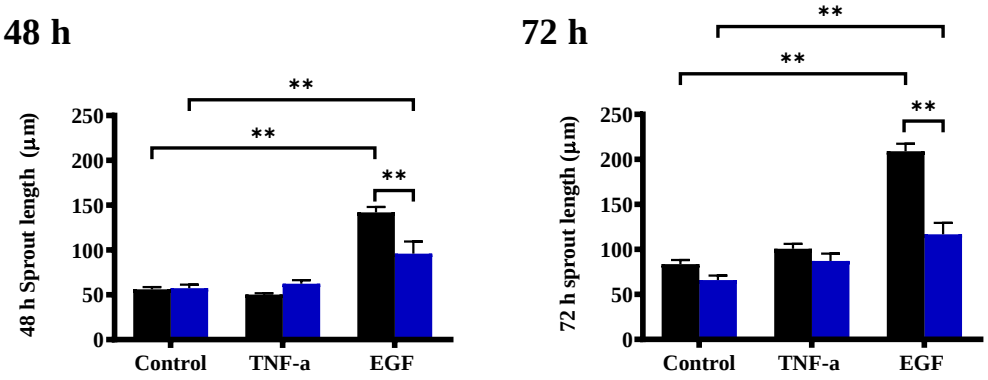
The EGF treated group at this time period showed the maximum sprouting density and length of invasion extending into the fibrin gel (Figure 3.14A & B). At 72 h, the decidualised cells invasion was lower than the proliferative group in all type of treatments. EGF treated HTR-8 spheroid cells, whether seeded over a decidualised or proliferative monolayer showed the maximum sprouting density and length (Figure 3.14A & B).


Proliferative (48 h)
Decidualised
Proliferative (72 h)
Decidualised
Untreated
+ TNF-α
+ EGF

A



Sprout density



Sprout length

B

Figure 3.14: Reduced HTR-8 spheroid invasion over a decidualised St-T1b layer in comparison with proliferative cells. Labelled HTR-8 spheroids were set in a fibrin gel over a proliferating or decidualised labelled monolayer of St-T1b hESC. The co-cultures were then treated with TNF-α (50 ng/ml) or EGF (200 ng/ml) in 1% FBS medium. The sprout density and sprout length were calculated for 10 spheroids / condition in 4 wells using ImageJ. (**A**) Representative fluorescent images of HTR-8 spheroid invasion into fibrin gel at 48 h and 72 h (4x objective). (**B**) Results are means (± SEM) of 3 independent experiments. Statistically significant differences (**P<0.05, **P<0.001*). Scale bar = 200 µm.

3.3 Discussion

3.3.1. *In vitro* characterisation of St-T1b decidualisation

In order to study the effects of decidualisation on gene expression and function, we selected a Telomerase-immortalised endometrial stromal cell (St-T1b) cell line (Rinehart *et al*., 1993, Samalecos *et al*., 2009c). This cell line has been used to study endometrial stromal cell decidualisation (Samalecos *et al*., 2009c), invasiveness (Gellersen *et al*., 2010), motility (Schwenke *et al*., 2013) , and an *in vitro* modelling of embryo implantation (Gonzalez *et al*., 2011).

In order to be used successfully in *in vitro* embryo implantation models, endometrial stromal cell lines need to possess two essential characteristics. First, the ability to retain the key phenotypic properties of the original primary cells. Second, the ability to respond and undergo a progestational response to the appropriate stimuli and express the morphological and biochemical changes characteristic of decidualisation. The process of differentiation of endometrial fibroblasts into enlarged polygonal cells, rich with lipid and glycogen, is a part of MET typical of decidualisation (Zhang *et al*., 2013b) to create a receptive implantation environment. For these studies we examined the cells at two-time intervals, 4 and 7 days. These were selected to the time leading up to implantation cover the implantation window, which normally spans the 20-24th day of the menstrual cycle (Kao *et al*., 2002)

Collectively, the findings indicate that upon induction with MMI medium containing MPA and 8-Br-cAMP, the St-T1b cells showed morphological transformation from small elongated fibroblast cells into large rounded epithelioid cells over 7 days. This transformation of the decidualised St-T1b cells was similar to those seen in primary cells used in the project and obtained from three different individuals. The merged images of stained decidualised cells

showed a reorganisation in the cytoskeleton and the appearance of stress fibres similar to that reported by Samalecos *et al* (2009c). The cytoskeleton plays a fundamental role in mitosis, motility, apoptosis, and contractility (Ayscough and Winder, 2004). Cytoskeleton reorganisation is controlled by the phosphorylation of myosin light chain and actin dynamics. The down-regulation of α-smooth muscle actin is seen *in vitro* decidualisation in baboon (Strakova *et al*., 2005), while upregulation of myosin phosphorylation inhibits decidualisation (Ihnatovych *et al*., 2007).

3.3.2. Summary of hESC decidualisation.

The strong up-regulation of key decidual markers in St-T1b cells such as dPRL IGFBP-1 FOXO1, KAI1, PLZF, SOD2, and IRF4 indicates that they have undergone successful decidualisation. The results obtained for the St-T1b cells over many passages (20- 40) were similar to the primary hESC confirming the original study by Samalecos *et al* (2009c) and validated their use as a suitable surrogate for primary hESC in the development of *in vitro* models throughout this study. Samalecos *et al* (2009c) found that cAMP can induce transient decidualisation and IGFPB-1 expression, but the use of MPA on cAMP-primed hESC led to a robust sustained decidual response. hTERT generated St-T1b cells have advantages over T-hESC fibroblast immortalized with hTERT or hESC immortalised with SV40 large T antigen, such as the St-2 cell line (Brosens *et al*., 1996), in their ability to retain their decidualisation response over prolonged periods in culture. Beside P, the decidualisation of hESC is built on cAMP and the continuous effect of the PKA pathway. cAMP exerts its P sensitisation effect via the Protein kinase A (PKA) pathway (Gellersen and Brosens, 2003b).

The MPA response depends on P as well as on androgen receptors (Samalecos *et al*., 2009c). FOXO1 has been identified as the initial induced target of the PKA pathway. cAMP and

progestin increase nuclear FOXO1 levels and attenuate phosphorylated AKT levels in hESC (Feroze-Zaidi *et al.*, 2007, Labied *et al*., 2006). Different studies elucidate the transcription of decidual products and markers. Knockout of FOXO1 in primary hESC resulted in the inhibition of 75% of IGFBP-1 and dPRL RNA (Grinius *et al*., 2006). SOD2, which is ROS scavenger during decidualisation is a direct target of FOXO1 and confers the decidua an OS resistance by direct regulation of mitochondrial protection antioxidant enzymes (Kajihara *et al*., 2011). Gellersen *et al* (2007a) found that KAI1 protein in primary hESC is stimulated by cAMP and not by P and on silencing of KAI1, IGFBP-1 protein inhibition occurs. PLZF is another decidual product that has been recognised with Chromatin immunoprecipitation RNA sequence (ChIP-DNA Seq) as having approximately ten PGR response binding elements, which suggest that PLZF is a direct target of P (Kommagani *et al*., 2016). Chip-qPCR study has identified that there is a co-binding of FOXO1 at most of PGR binding sites in 10 Kb genomic boundaries including the highly enriched motif of an important decidual product which is IRF4. IRF4 is a direct target of FOXO1 and PR. The knockdown of either progesterone receptor or FOXO1 can ablate IRF4. IRF4 ablation can alter the decidualisation morphological transformation and secretion of dPRL and IGFBP-1 (Vasquez *et al*., 2015).

Interestingly, this study found for the first time the upregulation of Lymphotoxin Beta Receptor (LTBR) mRNA in hESC decidualisation, a member of the TNF-α receptor family (McDERMOTT, 2001), LTBR is critical for secondary lymphoid tissue generation and peripheral natural killer maturation (Fu and Chaplin, 1999). The decidual LTBR upregulation can lead us to suggest further studies to investigate the role of the decidualisation process in innate immunity and in the increased immunological protection of the fetus or the mother such as against viral infection.

Following decidualisation, St-T1b cells were found to have a significantly lower migration rate than the proliferative controls. The long standing concept of the passive decidua and invasive embryo has more recently been widely challenged by the observation that the endometrial decidua has a propensity to migrate during trophoblast invasion (Grewal *et al*., 2008) and encapsulate or engulf the invading blastocyst (Brosens and Gellersen, 2010). This decidual motility may even act as a selective tool of good embryos. Weimar *et al* (2012) demonstrated that the decidual stromal cells of women with recurrent miscarriage have an active migratory potential even in the presence of compromised embryos whereas the decidual cells of fertile women can discriminate by reduced migration towards bad embryos. Salker *et al* (2010) might explain this poor discrimination due to the extended receptive period, which allows the implantation of even a compromised embryo. These results are in a broad agreement with Fabi *et al* (2017) who did an in vitro scratch assay in primary hESC and found a reduced motility in decidualisation and MET due to the inhibition of the AKT/PI3K survival pathway. In contrast, the activation of the AKT/PI3K pathway, in epithelial to mesenchymal transition (EMT), generally increases cell proliferation, motility and invasiveness (Grille *et al*., 2003). Furthermore, the increased expression of KAI1 with decidualisation as proved earlier in this chapter can contribute to the reduced decidual motility. KAI1 inhibits cell invasion and tumour metastasis (He *et al*., 2005).

It had been reported that EGF stimulates hESC migration in a transwell migration assay (Gentilini *et al*., 2007). However, Schwenke *et al* (2013) reported that decidualised St-T1b cells were more mobile than proliferative cells in a chemoattractant manner due to the concentration gradient difference in HB-EGF treatment between the upper and lower chambers in the trans well migration assay. This difference may be attributed to the different assay techniques used by Schwenke *et al*, who used a higher seeding density for decidualised cells than proliferative

cells. In this study I used the same seeding density with the decidualised and proliferative cells and looked for trans-kinetic migration. The current finding of reduced migration of decidualised cells in comparison with proliferative cells might be explained based on the knowledge that decidualised cells have a larger size, with rich lipid and glycogen contents to provide nutritional privilege to the growing embryo. EGF was used in the scratch wound assays to study the effect of local growth factor as HB-EGF on hESC motility. HB-EGF plays a critical role in embryo implantion as it induces the differentation of trophoblasts (Leach *et al.*, 2004). The deletion of HB-EGF in mouse results in a compromised implantion (Xie *et al.*, 2007). Its mRNA expressed in hESC (Leach *et al*., 2004) and by stimulating an incresed level of dPRL and IGFBP-1, EGF enhances decidualisation (Chobotova *et al*., 2002, Chobotova *et al*., 2005).

3.3.2. TNF-α did not affect hESC migration in comparison with the control.

TNF-α is produced by both stromal and trophoblastic cells (Todt *et al*., 1996). Increased TNF-α levels have been reported in endometriosis (Kyama *et al.*, 2006). Peng *et al* (2019) found that TNF-α increases the motility of ectopic endometrium via the upregulation of CXC chemokine 16 (CXCL16), which stimulates phosphorylation of Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2). One of the common symptoms of endometriosis is infertility, which makes this disease possesses a critical burden to the society and patient quality of life (Bulun, 2009). TNF-α can be considered as one of the causes of reproductive failure. The use of anti-TNF-α medication can help in the treatment of infertility and improve fetal survival (Chaouat *et al*., 1990), also, inhibit stress- induced abortion in mice (Arck *et al*., 1997). The results showed that TNF-α treatment did not alter stromal cell migration during *in vitro* wound scratch assay. To my knowledge, no previous evaluation of the effect of TNF-α on normal stromal cell migration has been performed. However, TNF-α has been reported to have a negative effect on HTR-8 and JEG3 migration (Todt *et al.*, 1996). Furthermore, TNF-α enhances endometrial

stromal contractility using an *in vitro* collagen gel contraction assay. This supports the hypothesis that TNF-α takes part in peri-menstrual endometrial remodelling and repair

3.3.3. Treatment with decidualised conditioned medium does not affect HTR-8 migration.

Protein array studies performed in our lab have shown that decidualisation of St-T1b leads to changes in the profile of secreted growth factors and cytokines. In order to determine the implication of these changes for blastocyst invasion and implantation, proliferative or decidualised St-T1b cell conditioned medium was used in scratch-wounded assays to investigate their activity on HTR-8 cells migration. The result revealed no difference in HTR-8 cell migration following treatment with conditioned medium from decidualised or proliferative St-T1b cells in comparison with the control cells in which 1% FBS medium was used.

Whilst these investigations were ongoing a study was published by Yong *et al* (2018), which reported the effects of St-T1b cells decidualised condition medium on HTR-8 cell migration. They found a reduced migration of HTR-8 treated with decidualised St-T1b cell conditioned medium. This discrepancy with our results might be attributed to the different evaluation techniques of cell migration. They used the xCELLigence assay, which is a real time analysis that counts cell numbers rather than the scratch area depending on a change in the electrical signal. This signal is used an indicator of cellular status, such as cell number without repeating time points and multiple imaging. Therefore, it followed the same cell group throughout the experiment (Scrace *et al*., 2013). Their studies used 25% conditioned medium ours used 100% conditioned medium. In addition, decidualisation of St-T1b cells was induced using only cAMP for 72 h, in contrast, this project used 5 days of stimulation with cAMP and MPA (MPC

medium) followed by replating in in MPC medium 24 h prior to the conditioned medium harvesting.

3.3.4. Development of the trophoblast expansion assay

The HTR-8 spheroid expansion assay was developed to examine the spreading of HTR-8 spheroids on the hESC monolayer following decidualisation. The results revealed an enhanced HTR-8 spheroid expansion with the decidualisation of the underlying stromal monolayer as well as treatment with EGF and TNF-α.

The original spheroid expansion assay developed by Gonzalez *et al* (2011) used the AC-1M88 cells grown as spheroids and cultured over a T-hESC layer. At 48 and 72 h, the cells were fixed, and immunohistochemical staining used to allow the discrimination of AC-1M88 trophoblasts from the hESC monolayer. In this chapter, the assay has been further developed using HTR-8 spheroids and optimised to allow continuous monitoring of the same spheroids over the test period instead of fixing the cells at multiple time points. Following the decision to use HTR-8 cells as trophoblast cells in the assay, the type of spheroid preparation was determined. Previously, different techniques have been used to create trophoblast spheroids. For instance, the widely used hanging drop method (Timmins and Nielsen, 2007). Although this method is relatively easy and time saving, its major disadvantage is the difficulty of obtaining uniform spheroids of similar sizes. The method of Korff and Augustin (1998) was used to generate HTR-8 cell spheroids by culturing the cells in 0.25% (w/v) methyl cellulose stock in HTR-8 medium (1:5) in non-TC treated round bottom plates, which yielded spheroids of an approximately equal size and a uniform shape.

Our finding of similar HTR-8-spheroid expansion, whether they were seeded over decidualised or proliferative stromal cells, contradicts the stimulated expansion by decidualisation in the

Gonzalez *et al* (2011) study. This may be explained by the different cell line they used, AC-188 cells, which might have more invasion potential.

The enhanced expansion with TNF-α as well as EGF treatment in the assay is similar to the stimulated expansion in the Gonzalez *et al* (2011) study, although they found more expansion with HB-EGF in the HTR-8 spheroids seeded over a proliferative monolayer. This contradiction may be that the decidual cells already secrete HB-EGF, which makes the proliferative cells more sensitive to the exogenous growth factor. Alternatively, the discrepancy can be explained based on the Grewal *et al* (2008) co-culture model of donated human embryo and primary hESC which reported that decidualised endometrial cells move in an opposite direction from the implantation site leading to more trophoblast expansion. Impaired trophoblast spheroid expansion during the implantation window was seen in co-cultures of endometrial tissues from recurrent miscarriage patients due to the overexpression of cellular retinoic acid-binding protein 2 (CRABP2), which may inhibit endometrial cell growth (Lee *et al.*, 2011b).

3.3.5. Optimization of image processing

Image analytic procedures are largely dependent on the quality of processed images. Variations in pixel sizes and contrast produce images with variable qualities, which affect experiment findings that are based on comparative evaluations. As image pixel size is the function of the imaging microscope type and magnification, the standardisation of contrast has been guaranteed here by developing macros on ImageJ to ensure uniform adjustment procedures for all images in the same experiment.

Image segmentation is a key process in image analysis to isolate a specific object within a high quality image. The trainable weka segmentation plugin has been demonstrated as a precise tool to differentiate between image objects (Vyas, 2017). In this project, the application of that

plugin has enabled a clear differentiation between the scratched area and cells. This allowed for estimating the total remaining scratch area rather than measuring the distance between scratch margins at different points (Abdulkareem, 2017). Also, the plugin eliminated the need for the manual determination of spheroid boundaries during the calculation of spheroid expansion.

The extent and magnitude of sprouting are essential criteria to determine the invasiveness of a spheroid to the surrounding tissue (Laschke and Menger, 2017). Therefore, both sprout length and sprout density were estimated in this study. Previous sprout models relied on automatic skeleton analysis of the spheroid after extracting the core area (Hermant *et al*., 2007). During preliminary studies, the visual comparative assessment of the skeleton analysis method indicated that the thick sprout can be represented in more than one skeletonized line. Obviously, this will add further length to the actual sprout length, the number of sprouts will be more than the actual number. Therefore, direct measurement of individual sprout length seems to be more reliable than the skeleton method. However, sprout overlapping makes measuring the length of every single sprout unachievable. Accordingly, we followed the method of Korff and Augustin (1999) who measured the longest sprout at each spheroid quadrant. To evaluate the sprout density, we measured the sprout area, which involves all invading sprouts, as a means to avoid errors that can be encountered during the non-automatic counting of sprout numbers.

3.3.6. Reduction of HTR-8 spheroids sprouting with decidualisation of the underlying stromal monolayer and stimulated with EGF treatment

The results revealed that HTR-8 spheroids invasion density is reduced in the control spheroids embedded in the gel over a decidualised monolayer of St-T1b cells. In contrast, adding EGF during HTR-8 and St-T1b co-culture assays resulted in maximum HTR-8 spheroids sprouting density and length when these spheroids were seeded over a proliferative monolayer of stromal

cells than those in the decidualised state. Enhancement of HTR-8 spheroid invasion with treatment with EGF is in agreement with Bass *et al* (1994) and Staun-Ram *et al* (2004) who found that EGF increased the invasion of primary human trophoblast cells through the increased expression of MMP2 and 9.

The finding of reduced trophoblast invasion in the presence of the decidualised hESC monolayer might be attributed to the fine tuning of different factor influences, such as IGFPB-1, which is a decidualisation marker that increases the invasion (Chakraborty *et al*., 2002), and to the interaction between metalloproteinase inhibitors and metalloproteinase to regulate trophoblastic invasion (Staun-Ram *et al.*, 2004). Accordingly, this project supports the notion that the decidua works as a physiological barrier, which controls trophoblastic invasion to the physiological limit without invading into the inner layer of myometrium. In contrast, pregnancy without proper decidualisation, as in ectopic pregnancy, can reflect uncontrolled invasiveness leading to the expulsion of the gestational sac (Refaat *et al*., 2008, Lala and Graham, 1990, Billington, 1971)

In conclusion; St-T1b cell line showed a decidual response similar to that of primary hESC, therefore, can be considered as an appropriate robust model for primary hESC. Also, the HTR-8 cell line cultured as spheroids showed expansion and invasive properties, which indicate they are a good choice to represent the trophoblasts of the invading blastocyst. Functionally, stromal cell motility increased with EGF treatment and reduced following decidualisation. HTR-8 cell migration did not change with decidualised hESC conditioned medium. HTR-8 spheroid expansion increases with decidualisation while the invasion was controlled. The established assays can serve as platforms to further investigate the factors affecting the maternal-fetal interface at the time of implantation. These assays are simple, reproducible, and can be analysed with freely available software using the developed protocol.

CHAPTER 4

THE ROLE OF DECIDUALISATION ON PDGF FAMILY EXPRESSION

4.1. Introduction

PDGF can affect different cell functions such as proliferation, matrix synthesis, chemotaxis, vascularization, antiapoptosis, and wound healing (Islam *et al*., 2016). PDGFR-α was originally identified as a mitogen for cells of mesenchymal origin such as fibroblasts (Pierce *et al*., 1991). Different roles have been reported for PDGF in female reproductive function such as ovarian function, embryo implantation, decidualisation, trophoblast invasion, angiogenesis, and infertility problems such as endometriosis. Piscale *et al* (2015) found that injection of PDGF receptor inhibitors to the female rat can inhibit folliculogenesis as PDGF is required for the transition from primordial to primary follicle in the rat (Nilsson *et al*., 2006). Higher levels of PDGF were detected in the peritoneal fluid of the fertile women in comparison with nulliparous individuals (Overton *et al*., 1996). Lee *et al* (2007b) reported a low level of PDGF-AA mRNA expression in the euotopic endometrium during the secretory phase in women with excessive endometriosis and infertility. In the uterus, PDGF stimulates the proliferation of hESC (Surrey and Halme, 1991) and decidual cells (Saji *et al*., 1997). It can modulate the expression of LIF during decidualisation (Arici *et al*., 1995). PDGF was shown to stimulate the migration of hESC (Jeong *et al.*, 2015) in both *in vitro* scratch and trans-well migration assays (Matsumoto *et al*., 2005). PDGF-BB was reported to enhance the chemokinesis and chemotactic migration of St-T1b and primary hESC, whilst PDGF-AA increases their chemotactic migration only (Schwenke *et al*., 2013). PDGF has been reported not to affect the invasive ability of cyto-trophoblasts (Bass *et al* (1994), whilst other studies as Ferretti *et al* (2007) indicated that PDGF enhances trophoblastic invasion.

Different studies have investigated the localisation of PDGF isoforms and their receptors in the blastocyst and endometrium in order to explore whether it works in a paracrine or autocrine manner on the affected cells. In the endometrium, Boehm *et al* (1990) reported the presence of

PDGF-B transcripts in late proliferative human endometrium tissue (which is the only type used) whilst PDGF-AA was rarely detected by northern blotting. In contrast, Chegini *et al* (1992) recognised that primary hESC expressed immunoreactive PDGF-AB and PDGFR-β only. Immunohistochemical staining of human fetal-maternal interface shows an intense immunostaining of PDGFR-β and to a lesser extent PDGFR-α in decidual cells, whilst trophoblasts and endothelial cells fail to show staining (Jokhi *et al*., 1997). An additional study performed by Mazur *et al* (2015) using RNA analysis in primary hESC reported that PDGF-CC, PDGF-AA, and PDGFR-α are differentially expressed before or after decidualisation stimulation. In mice, an immunohistochemistry study showed intense immunostaining for PDGF in the peri-implanting decidual endometrial zone during pregnancy (Jaber and Kan, 1998).

4.1.1. Aims and objectives

Although PDGF isoform and receptor expression, the expression and potential activity of these growth factors, ligands, or their effect on hESC–trophoblast interactions has not been demonstrated comprehensively. We hypothesised that decidualisation would alter the expression of PDGFs and their receptors in hESC, and that PDGF would affect the interaction, migration and invasion of hESC and trophoblasts.

4.2 Results

To investigate the potential role of PDGFs in hESC and trophoblast function, the expression of PDF isoforms and their receptors were first investigated in hESC and the HTR-8 cell line.

4.2.1. Expression of PDGF isoforms by HTR-8 and St-T1b cells

PDGFs are reported to be expressed by endothelial cells (Heldin and Westermark, 1999). HUVEC and the HMEC-1 cells were used as a control for these studies. The cells were grown to confluence on 6-well dishes and the mRNA and protein harvested, and qPCR and Western blotting performed.

As expected, endothelial cells showed a clear PDGF-AA mRNA and protein expression (Figure 4.1A). The HTR-8 trophoblast cell line expressed a high level ($p<0.001$) of PDGF-AA mRNA in comparison with HUVEC, HMEC-1 and proliferative St-T1b cells. Likewise, protein expression in HUVEC was higher ($p<0.05$) than those in St-T1b cells. Recombinant PDGF-AA (R-AA) produced in E. coli was used as a positive control. Western blotting produced a band of 17 kDa, whereas the endogenous PDGF-A chain produced a band of ~ 20 kDa (Figure 4.1B).

PDGF-BB mRNA was not detected in hESC (Figure 4.1C). PDGF-BB was detected in HTR-8 cells, although levels were significantly lower than HUVEC (Figure 4.1A). The lack of PDGF-BB expression in hESC was confirmed by Western blot analysis (Figure 4.1D). In HTR-8 and endothelial cells, a positive band (<20 kDa) was detected and Recombinant human PDGF-BB (R-BB) (2 ng) produced in E. coli was used as a positive control which ran as a single band at ~17 kDa.

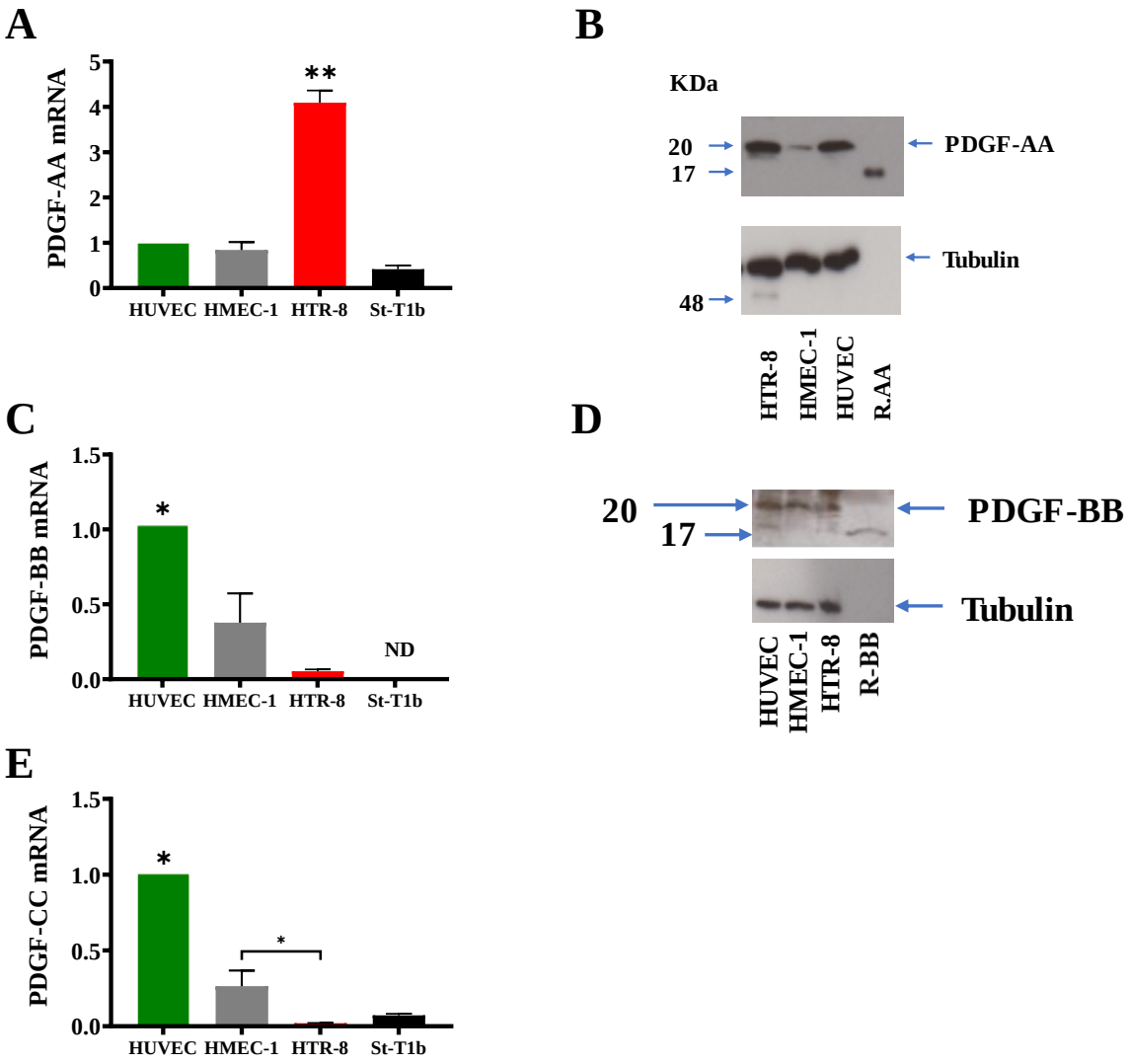


Figure 4.1: HTR-8 express PDGF-AA and PDGF-BB and PDGF-CC while hESC didn't express PDGF-BB. Confluent monolayers of HUVECs, HMEC-1, HTR-8, and St-T1b cells were incubated in their complete growth medium for 24 h. Relative mRNA fold changes of (**A**) PDGF-AA (**C**) PDGF-BB and (**E**) PDGF-CC expression normalised to β-actin. Representative Western blot showing (**B**) PDGF-AA and (**D**) PDGF-BB protein expression with Tubulin used as a loading control. Results are the mean (± SEM) of three independent experiments. Statistically significant differences (**P<0.05, **P<0.001*). Recombinant PDGF-AA (R-AA) and PDGF-BB (R-BB).

By comparing the expression of PDGF-CC mRNA expression in endothelial, HTR-8, and St-T1b cells, qPCR analyses revealed that HUVEC significantly expressed a higher level of PDGF-CC mRNA (Figure 4.1.E)

4.2.2. Expression of PDGF receptors and endosialine by HTR-8 and stromal cells

The results revealed significantly greater PDGFR-α mRNA expression in St-T1b cells in comparison with HTR-8 cells and endothelial cells (Figure 4.2A). This finding was confirmed with a strong band protein expression corresponding to PDGFR-α in St-T1b cells in comparison with HTR-8 and endothelial cells (Figure 4.2B).

As expected, PDGFR-β was detected in hESC with similar levels, also it was present in HTR-8 cells (Figure 4.2C). Although PDGFR-β mRNA was significantly lower than St-T1b cells, no PDGFR-β protein was detected by Western blotting in HUVEC and HMEC-1 cells (Figure 4.2C & D).

As endosialin can affect fibroblast cells through its binding to PDGFR-β (Tomkowicz *et al*., 2010, Wilhelm *et al*., 2016), its expression in HTR-8 and hESC was investigated. This result revealed that HTR-8 and endothelial cell lack this protein which is strongly expressed by St-T1b cells (Figure 4.2E).

4.2.3. Reduction of PDGF-AA expression during hESC decidualisation

In St-T1b cells, PDGF-AA mRNA was significantly higher in the proliferative group than the decidualised group at day 4 (Figure 4.3A). In parallel, Western blotting revealed a stronger band (20 kDa) in proliferative cells on day 4 and 7 than the decidual hESC (Figure 4.3B).

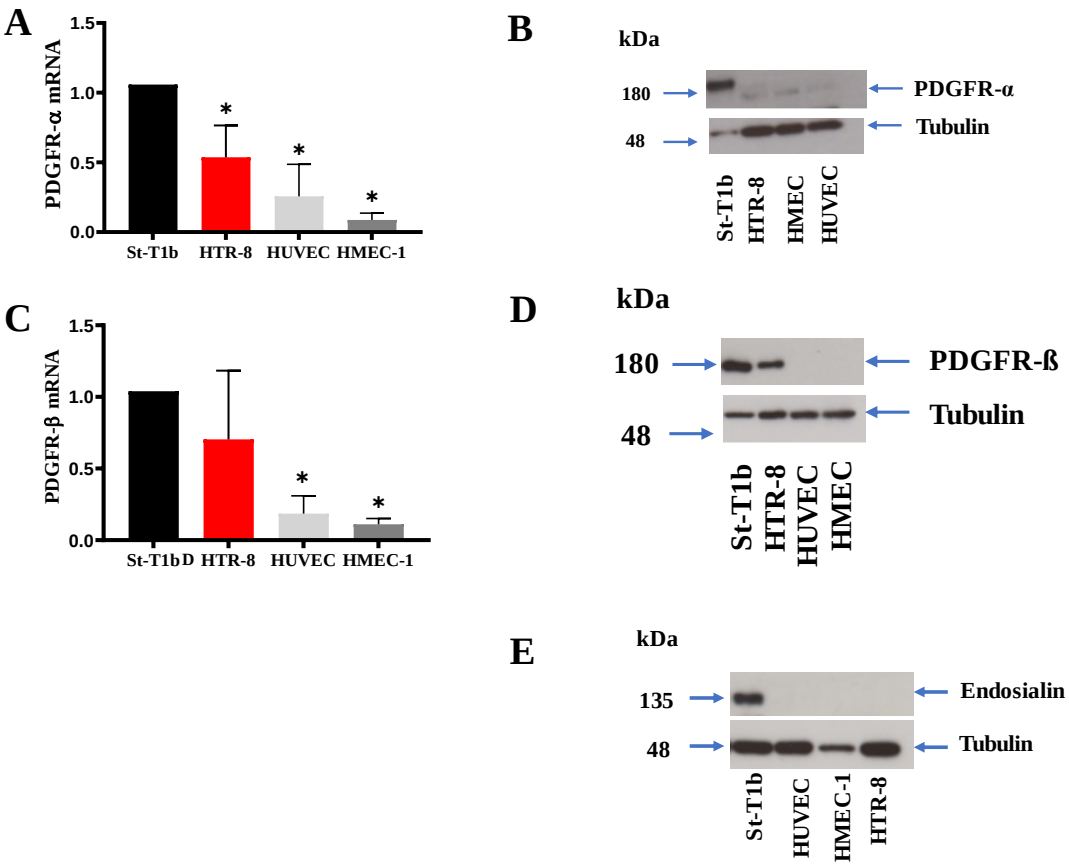


Figure 4.2: HTR-8 express PDGFR-α and PDGFR-ß and lack endosialin expression. Confluent monolayers of HUVEC, HMEC-1, HTR-8, and St-T1b cells were incubated in their complete growth medium for 24 h. Relative mRNA fold changes of **(A)** PDGFR-α and (**C)** PDGFR-ß expression normalised to β-actin. Representative Western blot showing **(B)** PDGFR-α, **(D)** PDGFR-ß, and **(E)** endosialin protein expression with Tubulin used as a loading control. Results are the mean (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$).

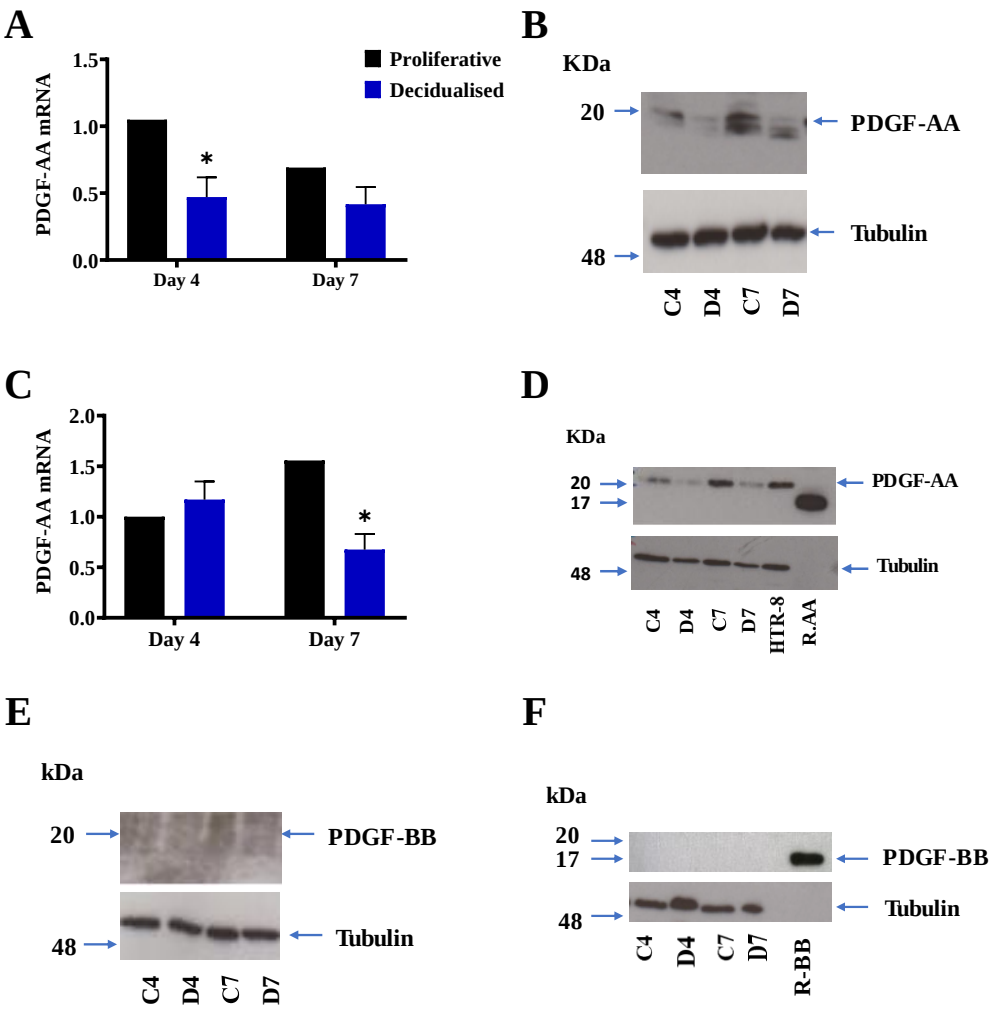


Figure 4.3: PDGF-BB is not expressed in hESC. Confluent monolayers of St-T1b and primary hESC cells were incubated in E2 or in MPC medium for 4 and 7 days. Relative mRNA of PDGF-AA in (**A**) St-T1b cells and (**C**) in primary hESC normalised to β-actin. Representative Western blots showing PDGF-AA protein expression in (**B**) St-T1b cells and (**D**) Primary hESC; and PDGF-BB protein expression in (**E**) St-T1b cells and (**F**) primary hESC with Tubulin used as a loading control. Results are mean (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$). C4 (control day 4), C7 (control day 4), D4 (decidualised day 4), D7 (decidualised day 7), R-AA (Recombinant PDGF-AA), and recombinant PDGF-BB (R-BB).

In primary hESC, PDGF-AA mRNA was also significantly higher in proliferative in comparison with decidualised cells on day 7 (Figure 4.3C). Likewise, Western blotting revealed a strong PDGF-AA protein expression on proliferative day 4, day 7, and HTR-8 cells (Figure 4.3D).

4.2.4. hESC does not express PDGF-BB

As the PDGF-BB gene was reported to be regulated during hESC decidualisation (Mazur *et al.*, 2015), its expression by hESC was evaluated. qPCR and Western blotting were performed following decidualisation induction in hESC at 4- and 7-days intervals. PDGF-BB mRNA was undetected by qPCR (Figure 4.1C), and Western blotting showed the absence of PDGF-BB protein in St-T1b and primary hESC (Figure 4.3E & F).

4.2.5. Increased PDGF-CC expression during hESC decidualisation

PDGF-CC was reported to be regulated during hESC decidualisation (Mazur *et al*., 2015). This experiment evaluated the PDGF-CC mRNA expression during hESC decidualisation. The result revealed for the first time that PDGF-CC mRNA is significantly upregulated during the early decidualisation of primary hESC (Figure 4.4A & B).

4.2.6. Upregulation of PDGFR-α expression during hESC decidualisation

In order to confirm the expression of PDGFR-α in hESC and assess the effect of decidualisation on its mRNA and protein expression, we performed qPCR and Western blotting. Upon decidualisation of hESC, the mRNA expression of PDGFR-α was found to be significantly upregulated at day 4 and day 7 in both St-T1b and primary hESC (Figure 4.5A & C). This result was confirmed in both types of hESC by Western blots, which showed a strong induction of PDGF receptor particularly in primary hESC with decidualisation (Figure 4.5B & D).

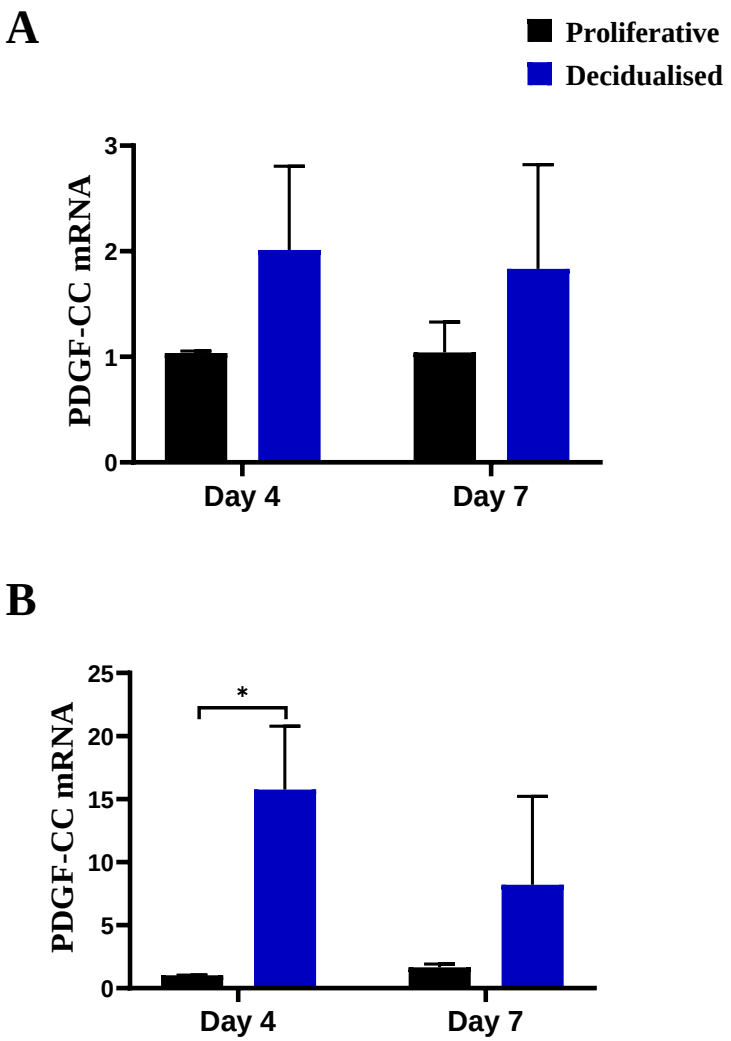


Figure 4.4: Upregulation of PDGF-CC expression during primary hESC decidualisation. Confluent monolayers of St-T1b and primary hESC were incubated in E2 or in MPC medium for 4 and 7 days. Relative mRNA fold changes of PDGF-CC in (**A**) St-T1b cells and (**B**) primary hESC. Results are mean (± SEM) of three independent experiments. Statistically significant differences (**P<0.05**P<0.001*).

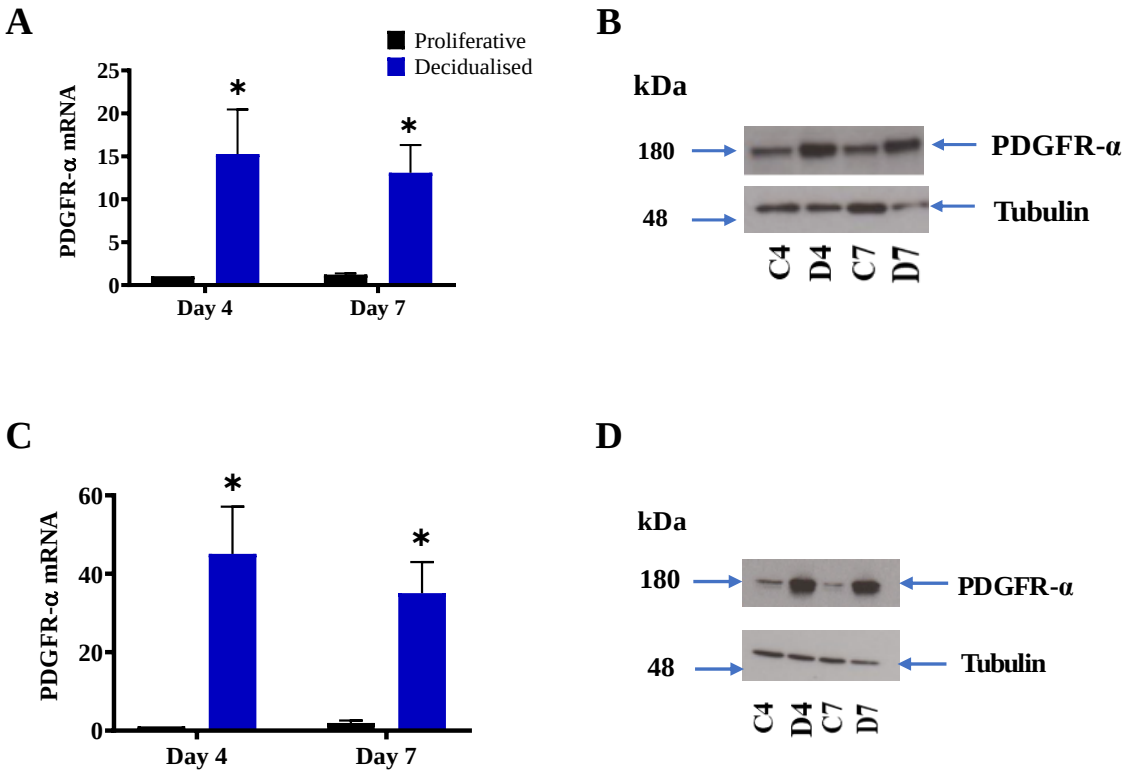


Figure 4.5: Upregulation of PDGFR- α expression during hESC decidualisation. Confluent monolayers of St-T1b and primary hESC were incubated in E2 or in MPC medium for 4 and 7 days. Relative mRNA of PDGFR-**α** in (**A**) St-T1b cells and (**C**) primary hESC. Representative Western blot showing PDGFR-α protein expression in (**B**) St-T1b cells and (**D**) primary hESC with Tubulin used as a loading control. Results are mean (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$). C4 (control day 4), C7 (control day 4), D4 (decidualised day 4), D7 (decidualised day 7)

4.2.7. Upregulation of PDGFR-ß during hESC decidualisation

To further evaluate the expression of PDGFR-ß by hESC, the effect of decidualisation on mRNA and protein expression was determined in these cells. Upon the decidualisation of St-T1b and primary hESC, PDGFR-ß mRNA expression was significantly upregulated on day 4 decidualised St-T1b cells (Figure 4.6A & C). However, although PDGFR-ß was readily detected by Western blotting in hESC, there were no observable differences between the decidual and proliferative cells (Figure 4.6B & D).

4.2.8. Upregulation of endosialin expression during primary hESC decidualisation

As the expression of endosialin had not been explored previously in hESC, we determined the relative level of endosialin in St-T1b cells and primary hESC and its regulation by decidualisation. The results revealed that endosialin mRNA and protein are expressed in St-t1b cells as well as primary hESC (Figure 4.7A & C). Whilst in St- T1b cells, decidualisation did not significantly affect endosialin expression, in primary cells, endosialin mRNA was significantly upregulated in day 4 decidualised primary hESC. Western blotting showed a strong band of endosialine in St-T1b cells (Figure 4.7B). In primary cells, protein bands were more intense in day 4 and day 7 decidualised cells (Figure 4.7D).

4.2.9. The effect of FOXO1 on PDGF expression: Positive correlation between FOXO1 and PDGFR-α mRNA expression during hESC decidualisation

Mazur *et al* (2015) found that PDGFR-α and FOXO1 were differentially expressed during decidualisation of hESC by RNA analysis. To determine whether there was an association between FOXO1 and PDGF expression, changes in the levels of gene expression in St-T1b and hESC were correlated **(**Figure 4.8**).**

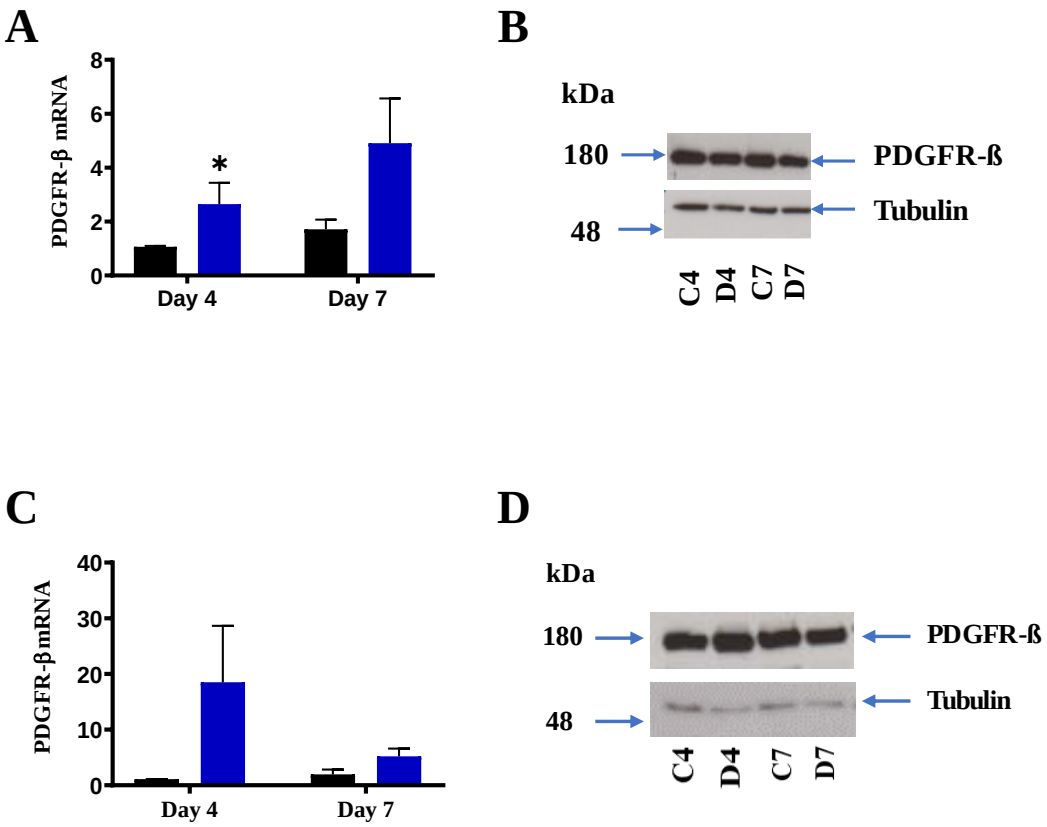


Figure 4.6: Upregulation of PDGFR-β expression during hESC decidualisation. Confluent monolayers of St-T1b and primary hESC were incubated in E2 or in MPC medium for 4 and 7 days. Relative mRNA fold changes of PDGFR-ß in (**A**) St-T1b cells and (**C**) primary hESC. Representative Western blots showing PDGFR-ß protein expression in (**B**) St-T1b cells and (**D**) Primary hESC with Tubulin used as a loading control. Results are mean (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$). C4 (control day 4), C7 (control day 4), D4 (decidualised day 4), D7 (decidualised day 7)

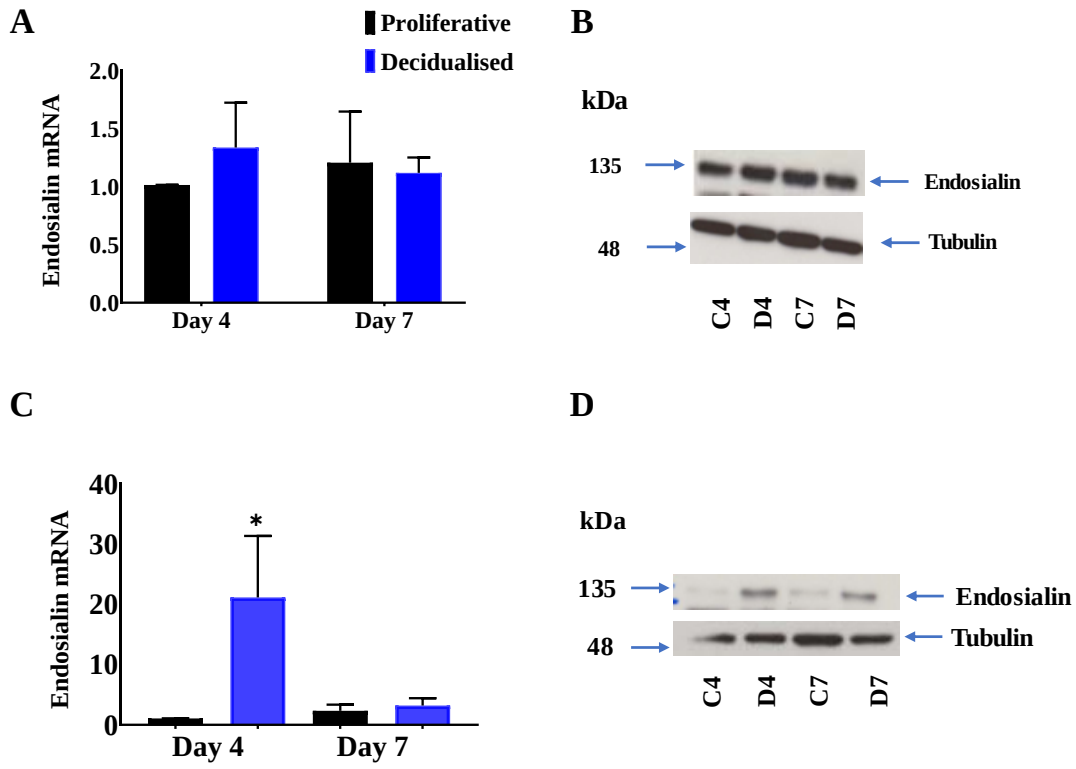


Figure 4.7: Upregulation of endosialin expression during primary hESC decidualisation. Confluent monolayers of St-T1b and primary hESC were incubated in E2 or in MPC medium for 4 and 7 days. Relative mRNA of endosialine in (**A**) St-T1b cells and (**C**) primary hESC. Representative Western blot showing endosialin protein expression in (B) St-T1b cells and (**D**) primary hESC. Tubulin used as a loading control. Results are mean (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$). C4 (control day 4), C7 (control day 4), D4 (decidualised day 4), D7 (decidualised day 7).

A positive correlation of mRNA expression levels between PDGFR-α and FOXO1 for both St-T1b and primary hESC upon decidualisation was found using Pearson's correlation coefficients (r^2) for statistical analysis (Figure 4.8).

To determine whether upregulation of PDGFR-α during decidualisation was regulated by FOXO1, FOXO1 knockdown was performed in St-T1b cells using Viromer blue transfection reagent. The levels of PDGFR-α mRNA were reduced following FOXO1 knockdown; however, the differences did not reach significance (Figure 4.9A). Additionally, we were unable to detect a difference in PDGFR-α protein expression between the decidualised FOXO1 transfected cells (siFOXO1) and non-targeted decidualised group (siControl) (Figure 4.9D).

4.2.10. Expression of PDGF-AA with TNF-α treatment in St-T1b cells

HTR-8 spheroid expansion was enhanced in the TNF-α treated ST-T1b cells. To investigate the effects of TNF on endometrial stromal cell PDGF and receptor expression, St-T1b cells were treated with TNF-α (50 ng/ml) for 16 h or 24 h. qPCR and Western blot analyses of PDGF-AA, PDGFR-α, and PDGFR-β were performed. PDGF-AA mRNA expression was significantly induced with TNF-α at both treatment intervals (Figure 4.10A). However, no obvious differences in protein expression were detected by Western blot (Figure 4.10B). PDGFR-α and PDGFR-β RNA and protein expressions were not significantly changed with TNF-α treatment. (Figure 4.10C- F).

4.2.11. PDGF-BB stimulates hESC migration

PDGF-BB and PDGF-AA were reported to enhance the migration of hESC (Schwenke *et al*., 2013). To elucidate the effect of PDGF on stromal cell migration during decidualisation, St-T1b were grown to confluence on 6-well dishes, induced to decidualise in MMI medium, followed by scratch-wound and treatment with PDGF-BB (10 ng/ml).

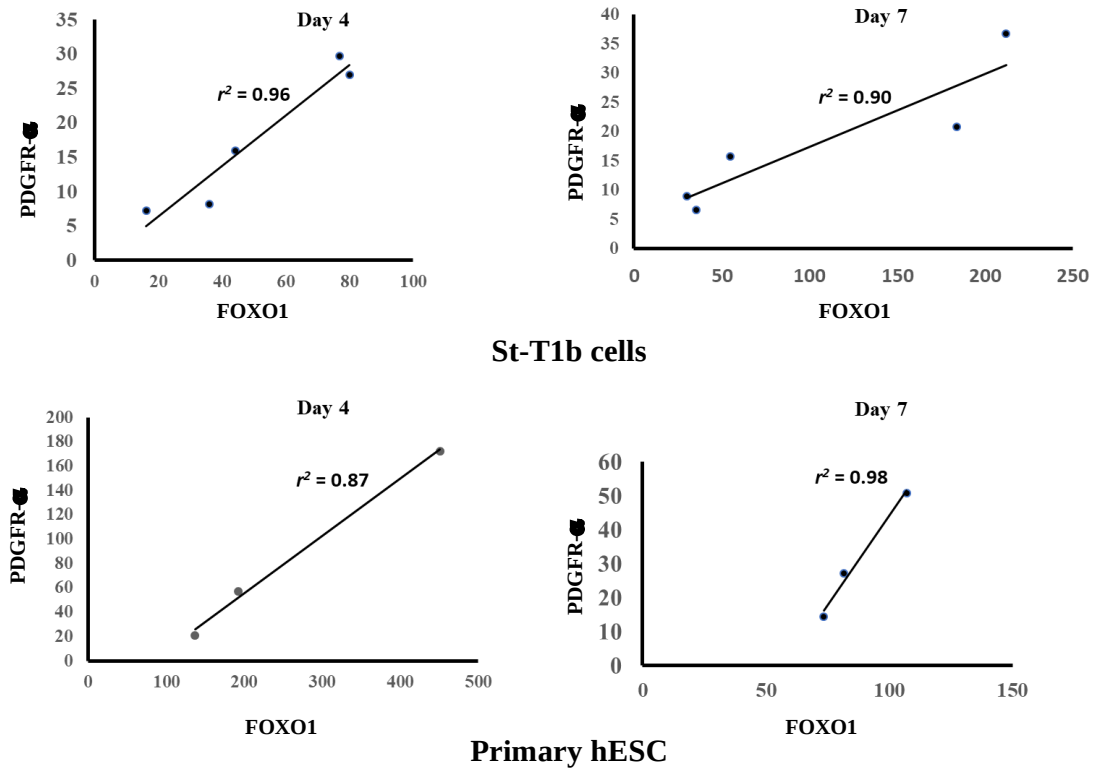


Figure 4.8: Positive correlation between FOXO1 and PDGFR-α expression during hESC decidualisation. Confluent monolayers of St-T1b cells and primary hESC were incubated in an E2 or MPC medium for 4 and 7 days. Relative mRNA expressions of decidualised PDGFR-α and FOXO1 of five independent experiments in St-T1b cells, and three independent experiments in primary hESC were determined by qPCR. Pearson correlation (r^2) analyses were applied between FOXO1 and PDGFR- α during day 4 and day 7 hESC decidualisation.

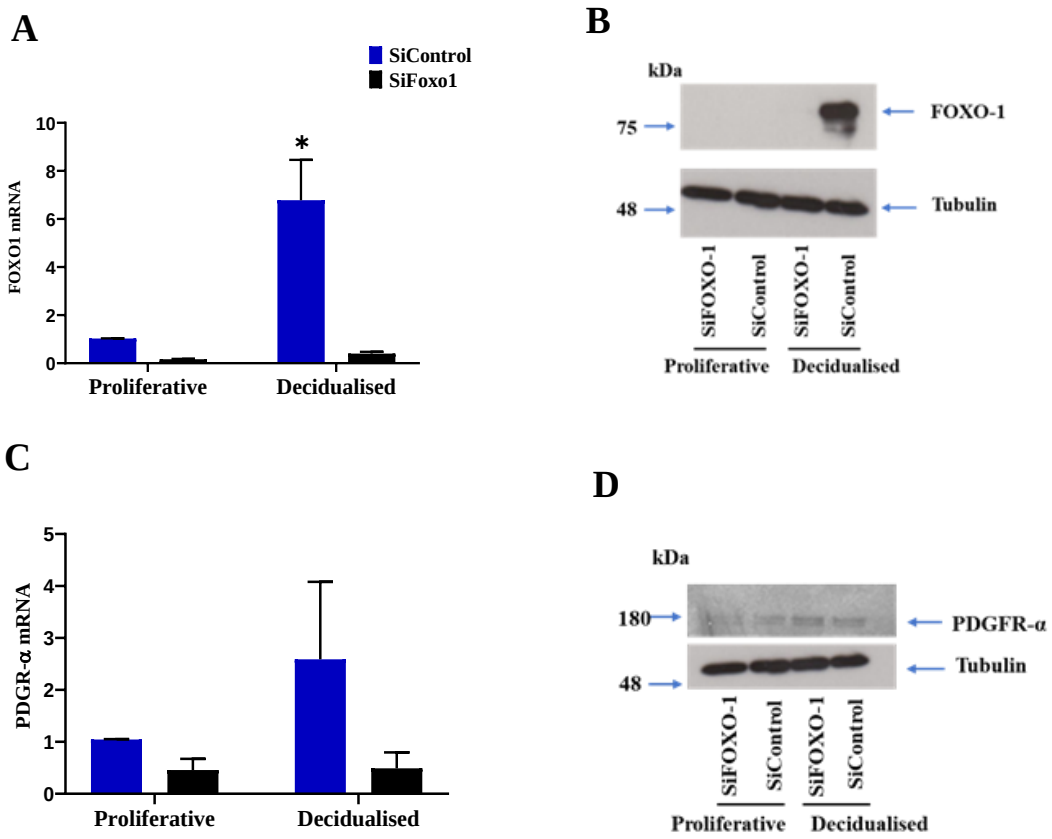


Figure 4.9: Effect of FOXO1 knockdown on PDGFR-α expression in hESC. Confluent monolayers of St-T1b were treated with transfection siRNA (37.5 nM) for 24 h followed by incubation in a E2 or in MPC medium for 24 h. qPCR and Western blot analyses were performed. Relative mRNA of (**A**) FOXO1 and (**C**) normalised to β-actin. Representative Western blot of (**B**) FOXO1 and (**D**) PDGFR-α protein expression. Tubulin used as a loading control. Results are the mean (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$).

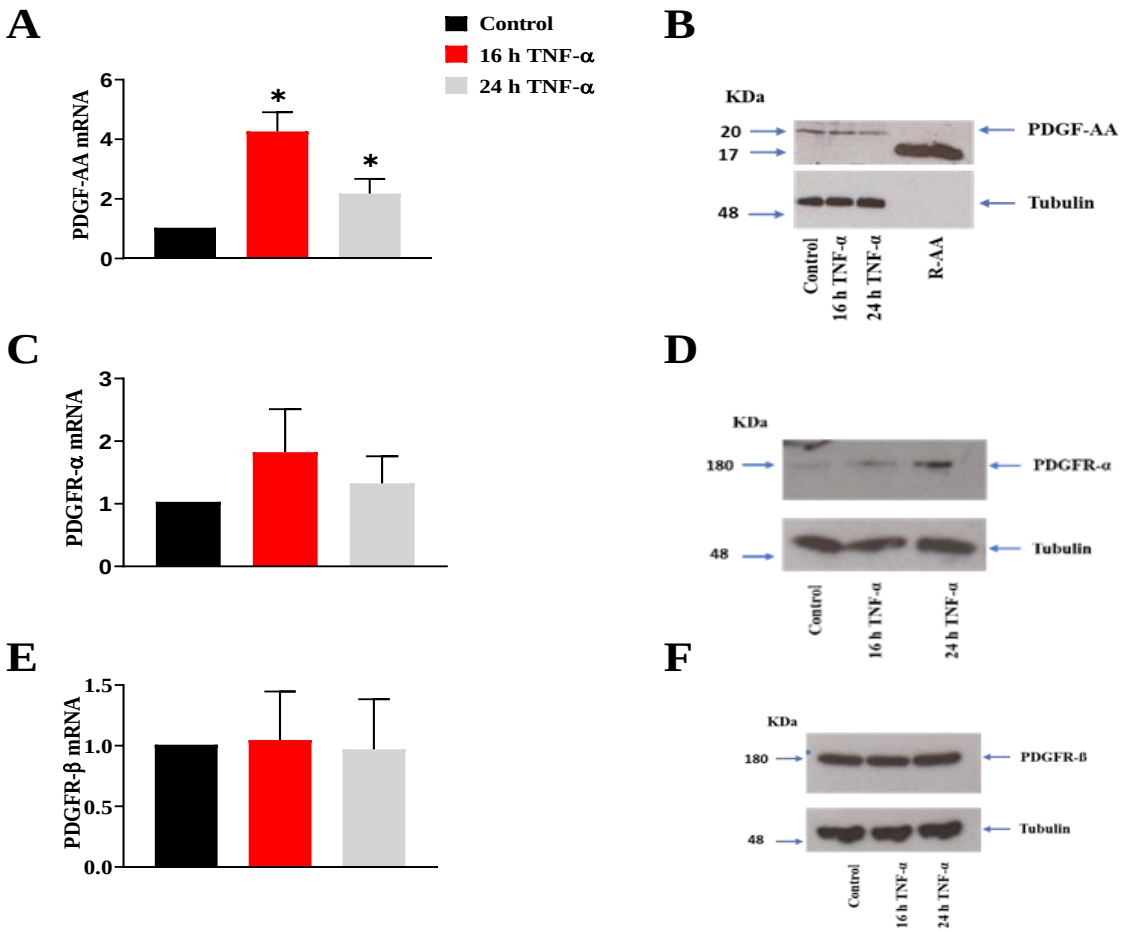


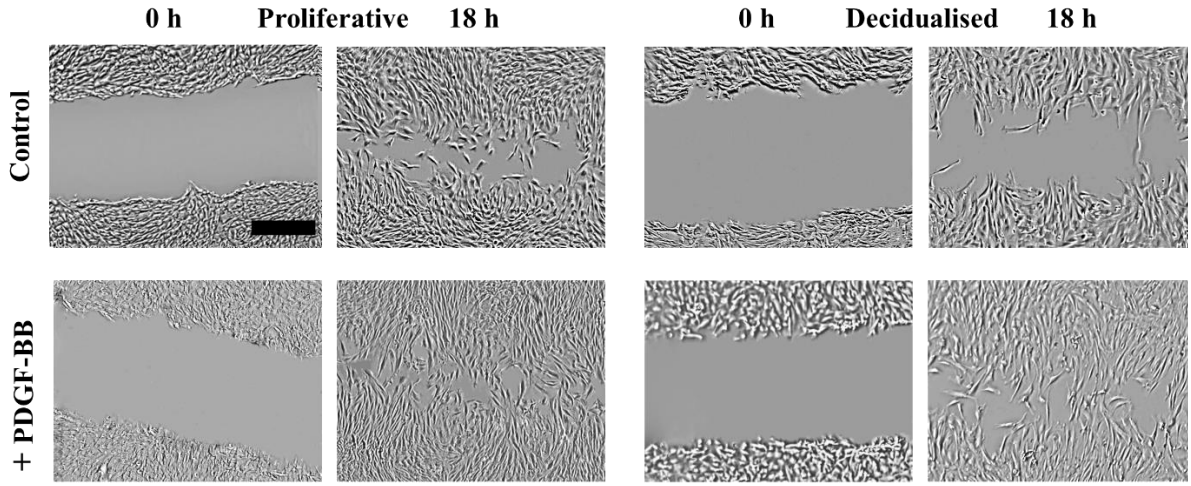
Figure 4.10: Effect of TNF-α treatment on PDGF-AA and cognate receptors in hESC. A confluent monolayer of St-T1b cells were treated for 16 h or 24 h with TNF-α (50 ng/ml) in 1% FBS DMEM/HAM F12 medium. Relative mRNA of (**A**) PDGF-AA, (**C**) PDGFR-α, and (**E**) PDGFR-β normalized to β-actin. Representative Western blot of (**B**) PDGF-AA, (**D**) PDGFR-α, (**F**) and PDGFR-β. Tubulin used as a loading control. Results are the mean (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$).

hESC migration was significantly increased with PDGF-BB treatment in both proliferative as well as decidualised cells (Figure 4.11B). The migration of decidualised cells was significantly lower than that of the corresponding proliferative cells. The images showed that the remaining scratched areas were smaller in the proliferative than in the decidualised cells (Figure 4.11A). Additionally, in the PDGF-BB-treatment group, the scratched regions were smaller than in the untreated controls.

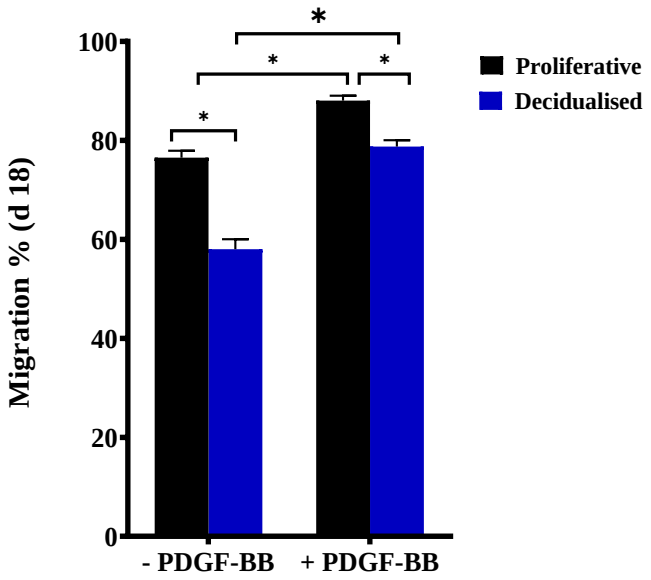
Additional evaluation of stromal cell migration was performed using PDGF-AA (100 ng/ml) or HTR-8 cell-conditioned medium. For this purpose, confluent monolayers of St-T1b cells growing on 6-well dishes were scratch-wounded and treated with either PDGF-AA or HTR-8 cell conditioned medium. PDGF-BB-treated cells were used as a positive control. PDGF-AA treatment or adding HTR-8 conditioned medium had no effect on St-T1b migration in comparison with untreated cells (Figure 4.12B). The photomicrographs showed that PDGF-AA or HTR-8 conditioned medium does not promote hESC migration (Figure 4.12A).

4.2.12. PDGF-BB promotes HTR-8 spheroid expansion

Decidualisation and growth factors have been reported to affect the expansion of spheroid cells over the endometrium during embryo implantation (Gonzalez *et al.*, 2011); however, the role of PDGF-BB on hESC has not been evaluated yet. Therefore, a spheroid expansion assay was performed under PDGF-BB treatment. In this assay, Vybrant green labelled HTR-8 spheroids were seeded over proliferative or decidualised St-T1b monolayers that were treated with PDGF-BB. The spheroid expansion was followed for 48 h and 72 h and analysed in relation to 0 h spheroid using ImageJ ($Spheroid\ expansion = \frac{Spheroid\ area\ (48\ h\ or\ 72\ h)}{Spheroid\ area\ (0\ h)}$).

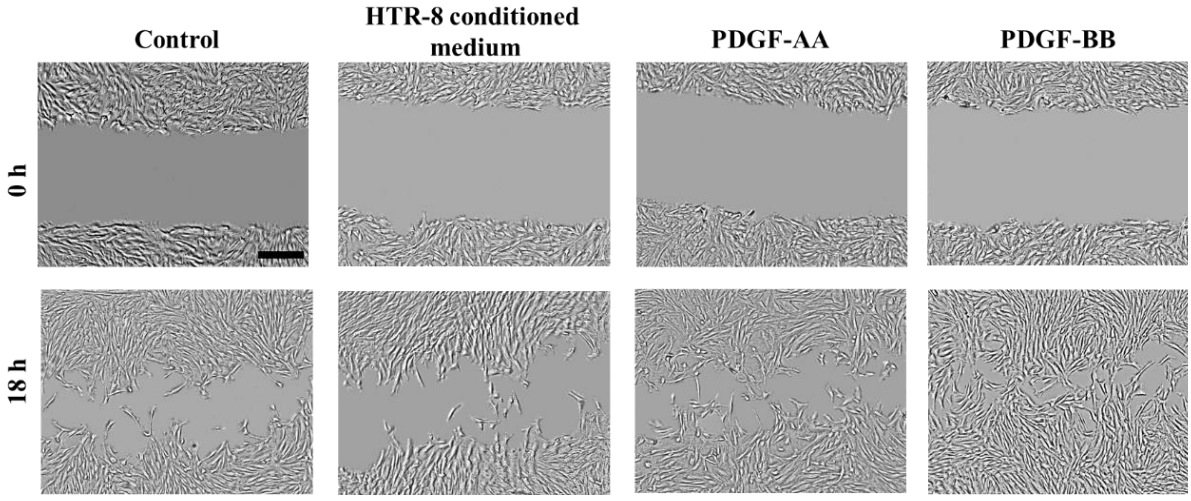


A



B

Figure 4.11.: PDGF-BB stimuli effect on St-T1b *in vitro* migration. Confluent monolayers of St-T1b hESC were induced for decidualisation or left as proliferative cells and scratch-wounded. Their migration assessed after 18 h in the presence of PDGF-BB (10 ng/ml) in 1% FBS Ham F12 DMEM medium. Cell migration was assessed after 18 h at 10 points/treatment conditions in 2 wells and calculated as migration % = (Scratch area at 0 h - scratch area at 18 h/scratch area at 0 h)*100. (**A**) Representative bright field images (4 x objective) showing the scratch areas at 0 and 18 h. (**B**) Results are means (± SEM) of the migration percentage at 18 h relative to zero time point of three independent experiments. Statistically significant differences (*$P<0.05$). Scale bar = 400 µm.



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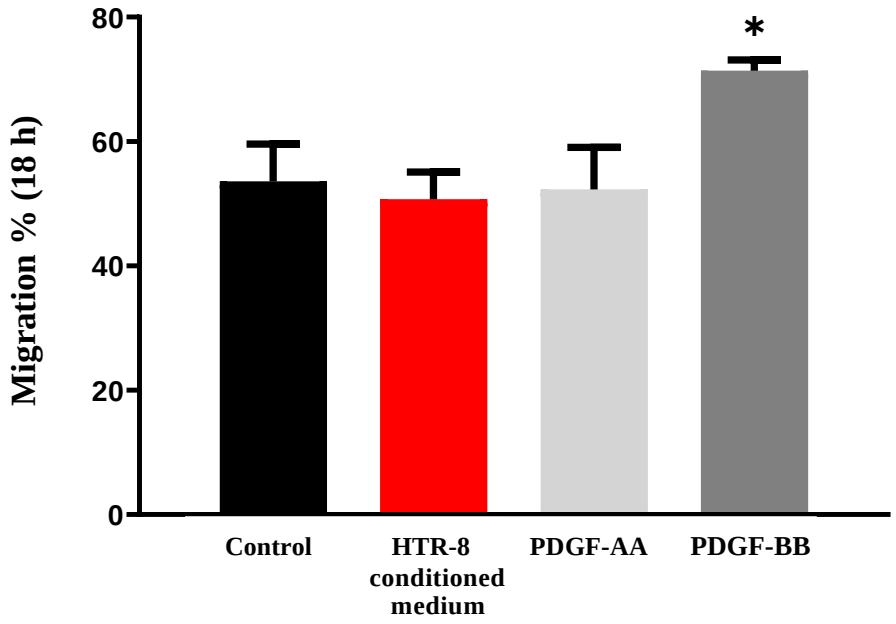


B

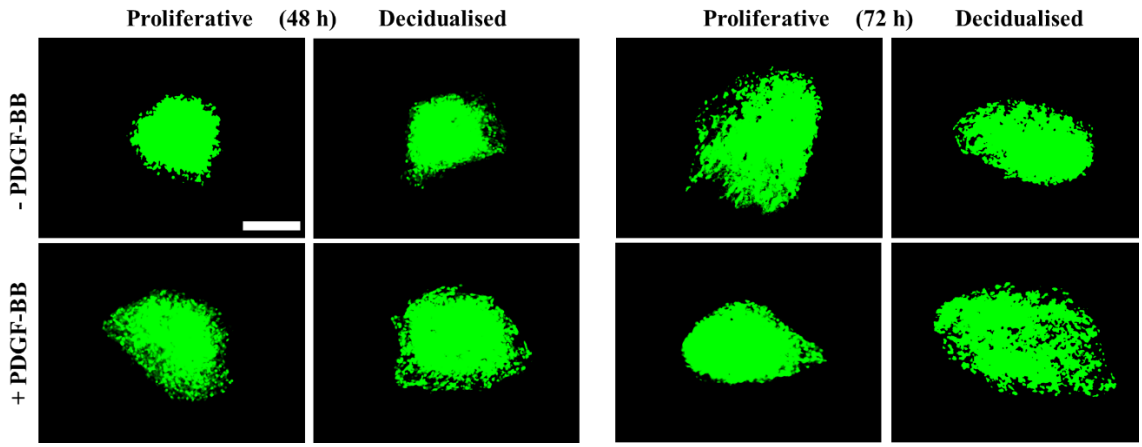
Figure 4.12. Effect of PDGF-AA or HTR-8 on ST-T1b migration. Confluent monolayers of proliferative St-T1b hESC were scratch-wounded. Their migration assessed after 18 h in the presence of HTR-8 conditioned medium, PDGF-BB (10 ng/ml), and PDGF-AA (10 ng/ml) in 1% FBS medium. Cell migration was assessed after 18 h at 10 points /treatment condition in 2 wells and calculated as migration % = (Scratch area at 0 h - scratch area at 18 h/scratch area at 0 h)*100. (**A**) Representative bright field images (4 x objective) showing the scratch areas at 0 and 18 h. Scale bar = 400 µm. (**B**) Results are means (± SEM) of the migration percentage at 18 h relative to zero time point of three independent experiments. Statistically significant differences (*$P<0.05$).

PDGF-BB treatment was found to enhance the expansion of HTR-8 spheroids over a proliferative St-T1b monolayers at 48 h (Figure 4.13B). By 72 h the spheroids continue to expand as seen in the images (Figure 4.13A). The PDGF-BB treated decidualised St-T1b cell monolayers promoted HTR-8 spheroid expansion to a significantly greater extent in comparison with PDGF-BB treated proliferative hESC and untreated decidualised hESC (Figure 4.13B & A).

4.2.13. PDGF-BB enhanced the invasion of HTR-8 spheroids

Trophoblastic invasion was reported to be stimulated by PDGF-BB (Ferretti *et al.*, 2007); however, a clear role of PDGF-BB on trophoblastic invasion in the context of decidualisation is not yet explored. Thus, HTR-8 spheroid invasion assay was utilised. The assay performed by embedding the Vybrant green-labelled HTR-8 spheroids in fibrin gels set over a layer of proliferative or decidualised St-T1b cells. The findings show that spheroid sprouting density and length at 48 h of the experiment were enhanced in PDGF-BB treatment whether seeded on proliferative or decidualised monolayers. However, the sprouting density was lower in the spheroids in the presence of decidualised St-T1b monolayers (Figure 4.14B). The images show enhanced trophoblast spheroid sprouting in the presence PDGF-BB treated decidualised hESC groups (Figure 4.14A).

At 72 h of the experiment, the sprouting density of PDGF-BB treated group was higher ($p<0.001$) in the spheroids seeded over the proliferative monolayers than those seeded over the decidualised monolayers. The sprouting length was also increased ($p<0.05$). In comparison with the untreated cells, the sprouting density and length were lower than those of PDGF-BB treated cells (Figure 4.14B). The images of labelled HTR-8 spheroids showed reduced invasion in the cells seeded over decidualised St-T1b monolayers (Figure 4.14A).


Proliferative (48 h)
Decidualised
Proliferative (72 h)
Decidualised
- PDGF-BB
+ PDGF-BB

A

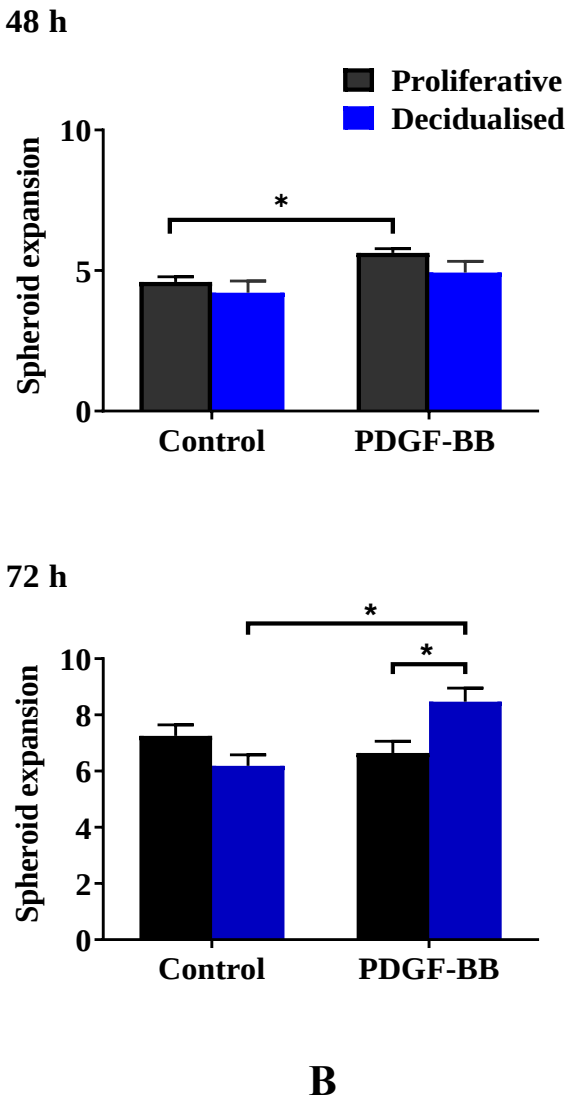
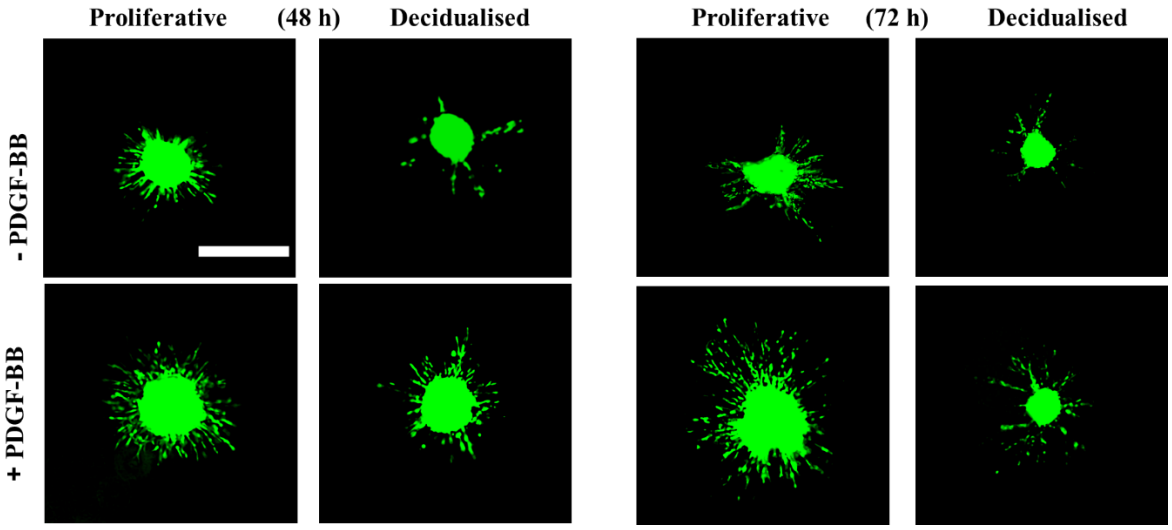
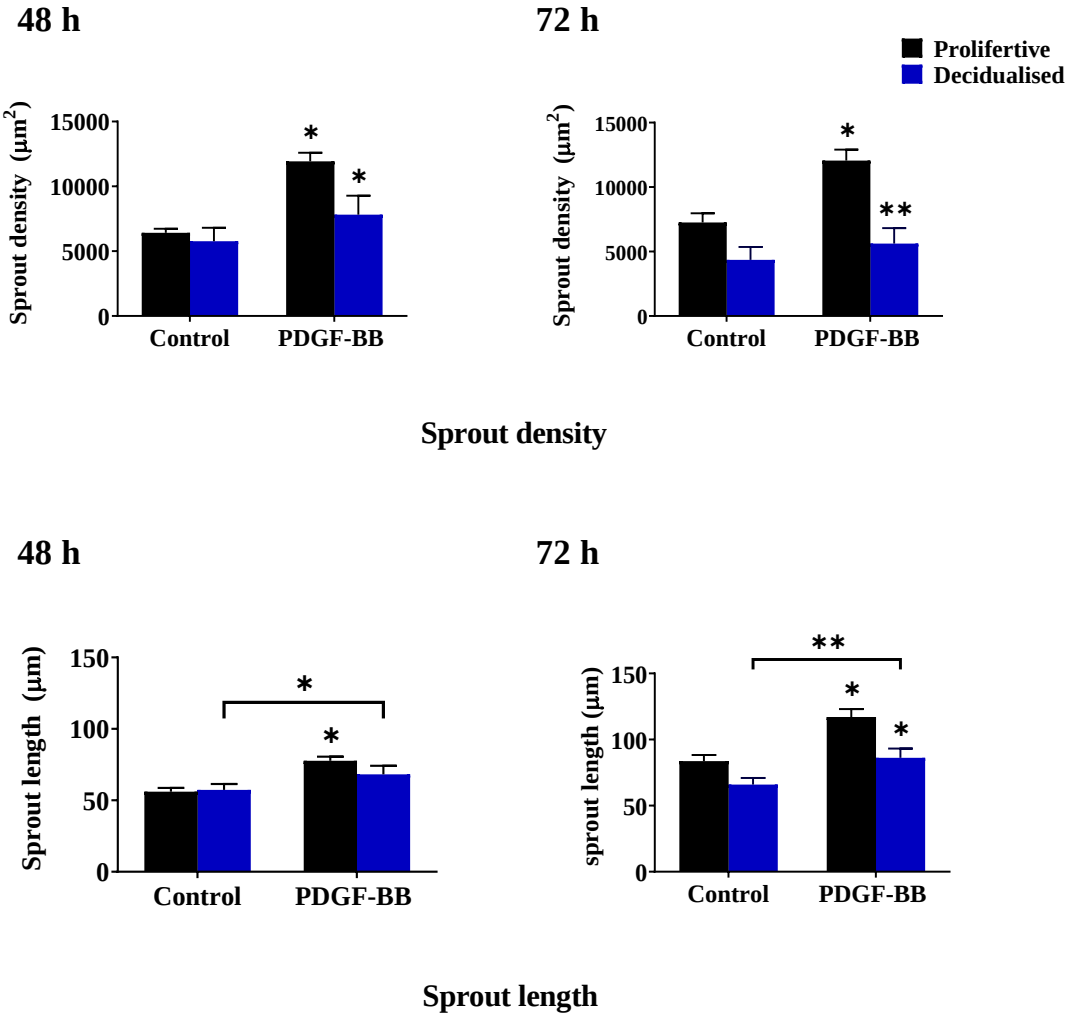


Figure 4.13: PDGF-BB increased HTR-8 spheroid expansion. HTR-8 spheroids were labelled with Vybrant green dye and seeded onto proliferating (black bar) or decidualised (blue bar) monolayers of St-T1b cells. The co-cultures were then treated with PDGF-BB (10 ng/ml) in 1% FBS medium. HTR-8 spheroid expansion was calculated as spheroid size at 48 h or 72 h /spheroid size at 0 h using ImageJ. Ten spheroid/treatment condition were measured in 4 wells. (**A**) Representative fluorescent images using Vybrant green dye (4x objective) showing spheroid expansion areas at 48 and 72 h (**B**) Results are the means (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$). Scale bar = 400 µm.


Proliferative
(48 h)
Decidualised
Proliferative
(72 h)
Decidualised
- PDGF-BB
+ PDGF-BB

A



B

Figure 4.14: PDGF-BB stimulated hESC promotes the invasion of HTR-8 spheroids. Labelled HTR-8 spheroids were set in a fibrin gel over proliferating or decidualised labelled monolayer of St-T1b hESC. The co-cultures were then treated with PDGF-BB (10 ng/ml) in 1% FBS medium. The sprout density and sprout length were calculated for 10 spheroids / condition in 4 wells from 3 independent experiments using ImageJ. (**A**) Representative fluorescent images of HTR-8 spheroid invasion into the fibrin gel at 48 h and 72 h (4x objective). (**B**) Results are means (± SEM) of 3 independent experiments. Statistically significant differences (*$P<0.05$, **$P<0.001$). Scale bar = 200 µm.

4.3. Discussion

4.3.1. Expression of PDGFs in hESC.

It is well known that endothelial cells such as HUVEC and HMEC-1 are expressers of PDGF angiogenic factors. Therefore, they have been used in this project as a positive control to investigate PDGF expression by trophoblastic cell lines and hESC. The result revealed that PDGF-AA is expressed by hESC and HTR-8 at mRNA and protein levels. The PDGF-AA expression in HTR-8 cells were even higher than in endothelial cells. While PDGF-BB was expressed in HTR-8 both at an mRNA and protein level, however it was undetected in St-T1b cells.

The results of this chapter revealed that PDGF-AA mRNA expression in St-T1b cells was lower in decidualised day 4 cells than in proliferative cells. Similar expression was seen in day 7 primary hESC. PDGF-AA can bind to PDGFR-α on stromal cells and exerts its effect on the maternal fetal interface. PDGF-BB exerts its effect through PDGFR- α or with a higher binding affinity to PDGFR-β. Both receptors have been proved to be expressed in hESC and HTR-8 cells. In Western blots of PDGF-AA and PDGF-BB, the recombinant PDGF-AA and PDGF-BB bands appeared at a lower level than the endogenous PDGF bands detected in the hESC endothelial or HTR-8 cells. Dai *et al* (2015) has recognised the same discrepancy in band levels when using PDGF recombinant protein produced in yeast due to post translation modifications. The recombinant PDGF-AA and PDGF-BB proteins used in this thesis were produced in *E. coli,* which is not capable of post translation modifications. Both PDGF-B and PDGF-A possess pro-peptide sequences (~60 amino acids in length), which are cleaved from the mature PDGF by proprotein convertases such as furin (Fredriksson *et al*., 2004). The recombinant protein is formed from disulphide-linked homodimers of two mature forms of PDGF-B chains of ~16

kDa size. When performing the Western blotting under denaturing and reducing conditions the homodimers of PDGF-BB will be destabilised and only the PDGF-B monomers are detected. On the other hand, the protein band detected in the cell lysates most likely is the mature form, which in eukaryotic cells will be in proprotein sequence and runs above 20 kDa.

The low expression of PDGF-AA with decidualisation goes in agreement with Lee *et al* (2007b) who found a low expression of PDGF-AA mRNA in the eutopic endometrial tissue obtained either by curettage or following hysterectomy during the secretory phase of the menstrual cycle. Lash *et al* (2011) reported the absence of PDGF-BB immunoreactivity in primary hESC at any stage of the menstrual cycle which is in agreement with our findings of qPCR and Western blotting. Additionally, Gray *et al* (1995) by an immunohistochemistry study found a steroid related regulation of PDGF ligands and receptors in a pregnant mice, also found that Diethylstilbestrol (DES) treatment can reduce PDGF ligands and receptors. The steroid regulation of PDGF ligands might explain the current finding of changed PDGF-AA expression during decidualisation on treatment with the progestational compound. Likewise, other authors showed the absence or the rarely detection of PDGF-AA and PDGF-BB in hESC. Schwenke *et al* (2013) showed the absence of PDGF-AA and PDGF-BB during St-T1b decidualisation in a microarray study. In contrast, Boehm *et al* (1990) reported the presence of PDGF-BB and rare detection of PDGF-AA transcripts by Northern blotting of primary hESC mRNA. Chegini *et al* (1992) recognised the absence of immunoreactivity of both PDGF-AA and PDGF-BB and the presence of PDGFR-β immunoreactivity in primary hESC.

4.3.2. PDGF-CC upregulation during decidualisation in primary cells

PDGF-CC is a polypeptide which has C-terminal and N-terminal with a Complement subcomponents C1r/C1s, Urchin EGF-like protein, and Bone marrow protein 1 (CUB) domain,

which found almost exclusively in plasma membrane-associated and extracellular proteins. The possession of this domain gives the PDGF-CC a similarity to VEGF family. Furthermore, PDG-CC expressed the cysteine motif of growth family similar to PDGF family (Tsai *et al*., 2000a) (Li *et al*., 2000). PDGF-CC binds to PDGFR-α and PDGFR- β (Cao *et al*., 2002). PDGF-CC is highly expressed in the platelets, prostate, testis and uterus. It plays an important role in the embryo development as well as in wound healing through improving proliferation, remodelling, survival and chemotaxis (Grazul-Bilska *et al*., 2003).

PDGF-CC mRNA was detected in HTR-8 cells, but the expression was less than that seen in endothelial and stromal cells. A significant up-regulation of PDGF-CC mRNA was detected in decidualised primary hESC. These results disagree with Mazur *et al*. (2015) who found in a microarray study that PDGF-CC is one of the genes upregulated during the decidualisation of hESC through a PGR binding mechanism. PDGF-CC was first recognized in human endometrium and fallopian tubes and initially termed Fallotein (Tsai *et al*., 2000a). Li *et al* (2000) found through a northern blot analysis that PDGF-CC is also highly expressed in placenta. Vasquez *et al* (2015) found that PDGF-CC has a binding site for FOXO1 and PGRs in a 10 Kb region upstream of the transcriptional start site. The finding of PDGF-CC upregulation during decidualisation needs further research particularly in the context of the effect on the cell functions at the maternal-fetal interface by knockdown and see whether this affects cell migration.

4.3.3. Increased expression of PDGF receptor during decidualisation of hESC

The result revealed a greater expression of PDGHFR-α in St-T1b cells when compared with the trophoblast cell line and HTR-8 cells. PDFR-ß was also expressed in St-T1b and HTR-8 cells but its protein was not detected in endothelial cells. Also, the results revealed a consistent

upregulation of PDGFR-α mRNA and protein during decidualisation in both types of hESC. These findings imply a steroid regulation role in PDGF receptor expression, which can further lead to functional differences in these cells. Lash *et al* (2011) indicated a reduction in those receptors' immunostaining in the secretory phase of women with recurrent miscarriage as well as following diethylstilbestrol (DES) administration in mice (Gray *et al*., 1995). Mazure *et al* (2015) supported our finding as it recognised PDGFR-α as a decidualisation differentiated gene regulated by the PGR, whilst FOXO1 was not identified as a regulator of PDGFR-α (Vasquez *et al*., 2015). Additionally, Mazure *et al* (2015) reported that PDGFR-β is not regulated during primary hESC decidualisation and this supports our finding in primary cells.

PDGF Ligands binding to PDGF tyrosine kinase receptors stimulates receptor dimerisation and initiates different signalling. Ligands-receptors binding is a complex process which functionally limited *in vivo* mainly to the interaction with PDGFR-α and PDGFR-β (Andrae *et al.*, 2008), while heterodimers such as PDGFR-αß receptor also exists as have been described in mice *in vitro (Klinghoffer et al., 2001)*. For this reason, this project focused on the expression of PDGFR-α and PDGFR-β only.

According to our finding it can be suggested that PDGF-AA can work in an autocrine manner on hESC, also in a paracrine manner through its release from endothelial and trophoblastic cells. The absence of PDGF-BB in the stromal cells can indicate that it works through a paracrine manner through its release from endothelial and trophoblastic cells.

4.3.4. Upregulation of endosialin in primary hESC

Endosialin is a stromal cell surface receptor that has been shown to be highly expressed in the embryo. It has lower expression levels in the adult life except where its expressed in normal blood vessels, the corpus luteum, and during wound healing (Croix *et al.*, 2000), or in a

pathological conditions as tumours, arthritis (Maia *et al*., 2010), and atherosclerosis (Hasanov *et al*., 2017). Endosialin can stimulate mesenchymal cell proliferation through the PDGFR-β activity (Tomkowicz *et al*., 2010, Wilhelm *et al*., 2016). Interestingly, the results show for the first time the intense endosialin protein expression by St-T1b and upregulation in its mRNA and protein expression in the decidualised primary hESC. The intense endosialine expression by the St-T1b cell line may be explained based on the knowledge that endosialin is associated with tumour and is not expressed in normal cells. Further evaluation of the endosialine role in the embryo-trophoblast interactions is required. For instance, by using endosialin overexpressed or knockdown experiments to investigate its effect on the migration, proliferation, and invasion of cells in embryo implantation. If any effect can be proved then further evaluation to map the effect pathway whether it is through the modulation of PDGF-BB, ERK, or PDGFR-ß as seen in human pericytes (Tomkowicz *et al*., 2010)

4.3.5. Regulation of PDGFRs in hESC

A positive correlation between PDGFR-α and FOXO1 has been found in this project. However, on FOXO1 knocked-down there was no significant change in PDGFR-α expression. This positive correlation supports the study of Mazur *et al* (2015), who recognised the PDGFR-α and FOXO1 as both differentially expressed in decidualisation of primary hESC with the P has a regulatory effect on PDGFR- α. Mei *et al* (2012) found that triple knockdown of FOXO1/3/and 4 in human neuroblastoma cell line resulted in the ablation of PDGFR-α. However, the isolated knockdown of any of these did not abolish PDGFR- α transcription. More evaluation of PDGFR- α regulation through the use of dual knock down of FOXO1 and FOXO3 is suggested.

4.3.6. The effect of TNF-α treatment on PDGF expression

In this project, PDGF-AA and PDGF-BB and their receptors were investigated in the context of their possibility to be expressed under the influence of TNF- α. This cytokine was applied at two intervals to explore any difference, which might be missed by the length of treatment. The results revealed that PDGF-AA mRNA increased with this treatment. Despite this increase, the protein expression of PDGF-AA or the receptors mRNA and proteins were not changed. This might link with the finding in nulliparous women with endometriosis who expressed a high level of TNF-α and a low level of PDGF in comparison with fertile women who expressed a higher level of PDGF in the peritoneal fluid (Overton *et al.,* 1996)

4.3.7. PDGF-BB has a stimulatory functional role over the cells in the maternal fetal interface.

It is generally agreed that trophoblast invasion is driven by gradients of chemo-attractants to invade the endometrial decidual stroma and blood vessels (Salamonsen *et al*., 2007). This project gives a new evidence to support the concept that the decidua is an active tissue during embryo implantation and not only a passive one. This notion is further supported by our finding that endometrial stromal cells have a motile response towards the angiogenic factor PDGF-BB as previously reported by Schwenke *et al* (2013). However, the migration is slower in the decidualised cells in comparison with the proliferative cells. Although it is proved that PDGFR-α receptors are upregulated during decidualisation, but the PDGF-BB has a strong affinity towards PDGFR-ß. Additionally, slower migration might be explained again due to the larger sizes of the decidualised cells, which make their movement slower and mask receptors upregulation. The presence of PDGF-BB response in the St-T1b cells, with the absence of its secretion, indicates that PDGF-BB acts in a paracrine manner in hESC

Further examination of the effect of PDGF on the endometrial stromal cell movement were performed by using PDGF-AA treatment or the HTR-8 cells conditioned medium, which were shown to express PDGF-AA. The use of condition medium was to investigate the presence of any other secretion from the HTR-8 cells that might affect stromal cell motility during implantation window. However, the result revealed no difference in the chemokinesis migration with the use of any of these treatments. This agrees with the finding of Schwenke *et al* (2013) who demonstrated an effect of PDGF-AA on chemoattractant stromal migration and not on chemokinetic migration.

The fetal-maternal interface functions were evaluated by measuring HTR-8 trophoblast expansion and invasion following the treatment of the co-culture of trophoblast and stromal monolayer with PDGF-BB. The results revealed that PDGF-BB increase the expansion rate. Additionally, PDGF-BB stimulates the invasion of HTR-8 spheroid. This may be explained by the PDGFR upregulation as proved in this project. As well as, by the high expression of integrin B1 in hESC and trophoblastic cells. The integrin B1 binds to collagen, fibronectin, and laminin, those are highly expressed during decidualisation (Aplin *et al*., 2008). On the use of integrin B1 antibody, the expansion was suppressed (Shiokawa *et al*., 2000)

The result of enhanced invasion of HTR-8 spheroid with PDGF-BB treatment may be attributed to the presence of PDGF-BB receptors in both trophoblast and stromal cells. This finding agrees with the Ferretti *et al* (2007), who demonstrated that PDGF-BB was a potent stimulator of trophoblast invasion. They also show a similarity between trophoblast cells and an invasive tumour through the expression of integrin, low expression of E-cadherin, high expression of matrix metalloprotease 9, and activation of PI3K/AKT axis which enhance the migratory and invasive abilities. These factors enhance the invasive attitude of tumour and trophoblastic cells. Primac *et al* (2019) recognised an interaction between integrin α11 and PDGFR-β that led to

support the invasion in breast cancer fibroblasts with PDGF-BB through the use of human and mouse models. However, Bass *et al* (1994), using human placental cell in Matrigel coated trans well inserts invasion assay, reported that EGF can stimulate trophoblastic invasion duo to the presence of its receptor in the trophoblastic cells, while PDGF-BB showed no effect.

In conclusion, the expression of PDGF-AA, PDGF-CC, PDGFR-α, PDGR-β, and endosialin have been expressed differentially with decidualisation in hESC, while PDGF-BB was not detected. TNF-α treatment upregulates the PDGF-AA mRNA expression. PDGF-BB enhances the migration of stromal cells as well as the expansion and invasion of trophoblastic cells and this supports the proposed hypothesis that assumes that PDGF-BB can have an effect on migration.

Table 1.1 Table Summary of PDGF isoform and receptors expression in hESC.

PDGF Isoform or receptor	St-T1b cell		Primary hESC	
	Proliferative type	Decidualised type	Proliferative type	Decidualised type
PDGF-AA	++	+	++	+
PDGF-BB	_	_	_	_
PDGF-CC	+	++	+	++
PDGFR-α	+	++	+	++
PDGFR-β	+	++	+	++

-undetected, + detected, ++ increased

CHAPTER 5

INVESTIGATING HO-1 EXPRESSION AND REGULATION IN HESC DECIDUALISATION

5.1. Introduction

HO-1 plays an important role in protection against hypoxia, cytotoxicity, inflammation, and different types of oxidative stress (Maines and Panahian, 2001, Peng *et al.*, 2019). HOs and their by-products are physiologically active during pregnancy. They are considered master players in the establishment and maintenance of a healthy pregnancy (Wong *et al.*, 2012). They have been found to reduce uterine contraction (Acevedo and Ahmed, 1998), control the hemodynamic function, maintain the oxidative and antioxidative balance, regulate apoptotic and anti-inflammatory mechanisms, and control immunological tolerance to the allogenic fetus (Bainbridge and Smith, 2005). HO-1 expression is upregulated during pregnancy in both the human and mouse (Barber *et al.*, 2001, Zenclussen *et al*., 2003) and low HO-1 levels can result in a failure of implantation and have been linked with the development of pre-eclampsia (Zenclussen *et al*., 2015) and IUGR later in pregnancy (Barber *et al.*, 2001, Donnez *et al*., 2016)

Yoshiki *et al* (2001), reported that HO-1 is predominantly localised in the endometrial epithelia and macrophages by immunohistochemistry, and there was a constitutive expression of HO-1 throughout the menstrual cycle in these cells as observed by by RT-PCR and Western blotting. Dassen *et al* (2008) found through immunohistochemistry and qPCR of the human endometrial tissue that tissue obtained from mestrual phase expressed significantly higher HO-1 mRNA than those obtained during the proliferative phase. Zenclussen *et al* (2014) found in mice uteri that HO-1 mRNA was upregulated in the receptive period of the estrus phase. Low HO-1 levels were reported in aborted mouse decidual tissue (Zenclussen *et al*., 2002). Consistent with these findings, low HO-1 expression is associated with failed implantation, abnormal trophoblast invasion (McCaig and Lyall, 2009), recurrent miscarriage (Donnez *et al*., 2016), and endometriosis (Jamali *et al*., 2019). The upregulation of FOXO1 upon endometrial stromal cell

decidualisation is essential for the process of decidualisation and confers oxidative stress resistance in decidual cells (Kajihara *et al.*, 2006).

5.1.1. Aims and objectives

Previous studies reported that Foxo1 induces HO-1 expression in mouse muscle and hepatocytes through direct binding to the HO-1 promoter (Liu *et al*., 2013, Kang *et al.*, 2014). This suggests that, during decidualisation, HO-1 may be upregulated by FOXO1 and play a protective role in reducing the level of reactive oxygen species. It was therefore hypothesised that decidualisation would upregulate HO-1 expression in hESC and that HO-1 induction may affect hESC function and stromal-trophoblast interactions. We aimed to elucidate the sensitivity of HTR-8 cells migration to hemin.

5.2. Results

5.2.1. HO-1 is upregulated during hESC decidualisation

Although HO-1 levels were reported to increase during the receptive phase of the estrus cycle in mice (Zenclussen *et al*., 2014), its expression in hESC during decidualisation has yet to be examined. To investigate this, St-T1b and primary hESC were seeded in 6-wells plates at a density of $3x10^5$ cells/well. Cells were either maintained in E2 medium or induced to decidualise by treatment with MPC medium for 4 to 7 days. The results show for the first time, a significant increase in HO-1 mRNA expression in both St-T1b (Figure 5.1A) and primary hESC isolated from three different individuals (Figure 5.1C) following decidualisation at day 4 in comparison with the proliferative cells. Western blotting confirmed the increased HO-1 protein levels in the decidualised St-T1b cells (Figure 5.1B) and primary hESC (Figure 5.1D).

5.2.2. cAMP promotes HO-1 expression in hESC

In order to investigate the effect of either P or cAMP driven pathways on HO-1 expression in hESC, the effect of individual MPA and 8-Br-cAMP stimulation was examined over a 24 h period. Primary hESC and St-T1b cells were seeded in 6-well plates at a density of $3x10^5$ cells/well. The cells were maintained in E2 medium or induced with MPA (1 µM) or 8-Br-cAMP (0.5 mM) alone or in combination in MMI medium for 24 h. The results showed that HO-1 mRNA expression significantly increased following stimulation with 8-Br-cAMP or 8-Br-cAMP plus MPA compared with the untreated control (Figure 5.2A).

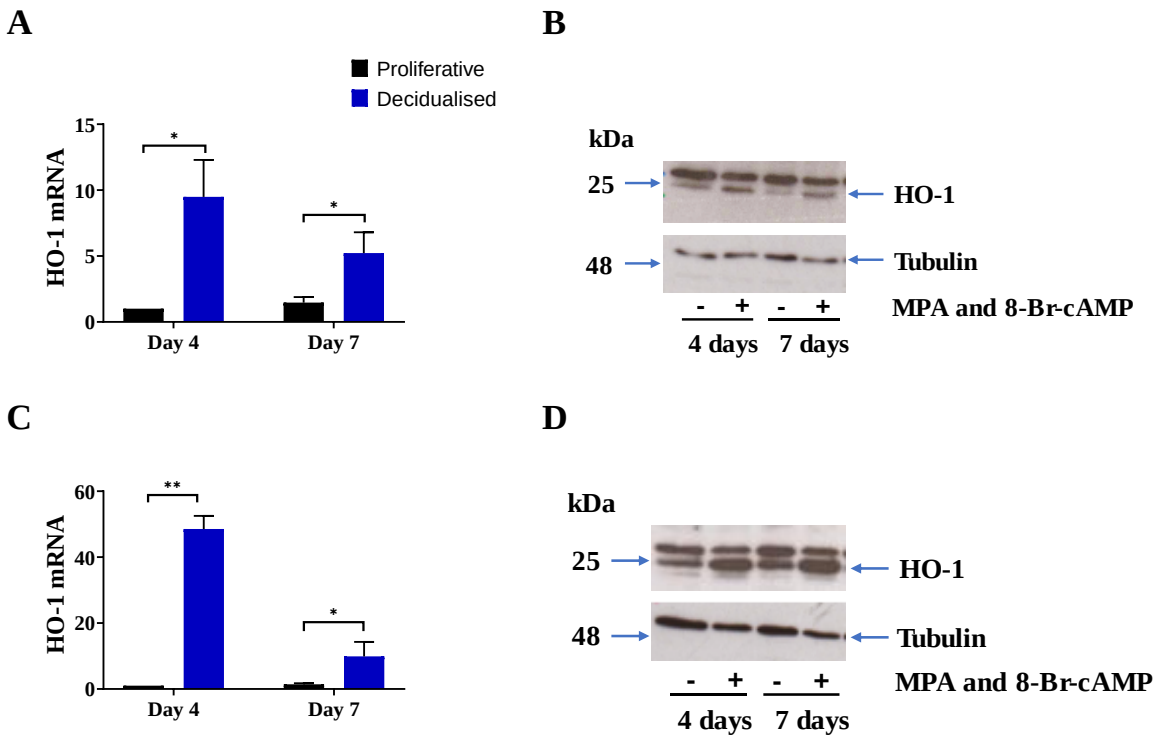


Figure 5.1: HO-1 is upregulated during hESC decidualisation. Confluent monolayers of St-T1b and primary hESC were incubated in E2 medium or MPC for 4 and 7 days. Relative HO-1 mRNA fold changes expression in (**A**) St-T1b cells and (**C**) primary hESC normalised to β-actin. Representative Western blots showing HO-1 protein expression in (**B**) St-T1b cells and (**D**) primary hESC with tubulin used as a loading control. Results are the mean (± SEM) of four (**A**) and three (**B**) independent experiments. Statistically significant differences (*$P<0.05$**$P<0.001$).

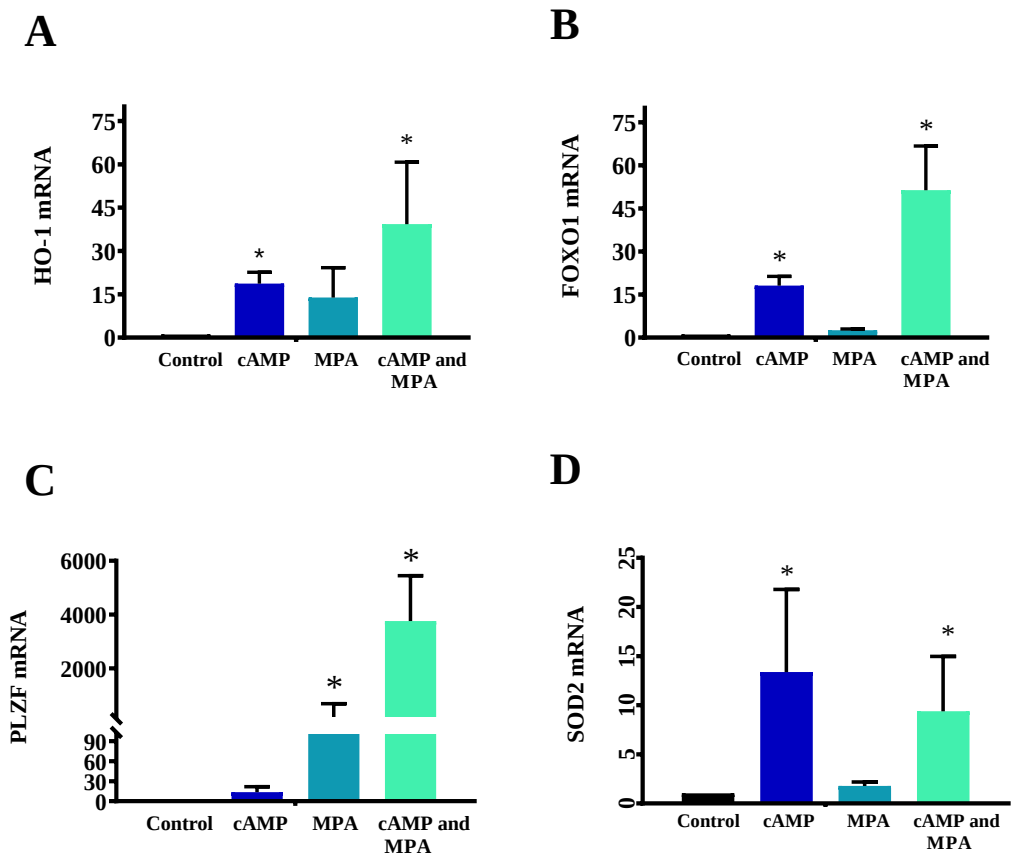


Figure 5.2: cAMP driven pathways upregulate HO-1 expression. Confluent monolayers of primary hESC were incubated in a complete growth medium with E2 (1 nM), insulin (1 µ/ml) or in MMI medium with 8-Br-cAMP (0.5 mM) or MPA (1 µM) alone, or in combination for 24 h. Relative mRNA fold changes (**A**) HO-1, (**B**) FOXO1, (**C**) PLZF, and (**D**) SOD2 expression normalised to β-actin. Results are means (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$).

To confirm the activation of relevant pathways in hESC following stimulation with cAMP or MPA, the expression of FOXO1 and PLZF were examined (Figure 5.2B & C). As expected, 8-Br-cAMP led to a significant increase in FOXO1 mRNA, which appeared to be potentiated in the presence of MPA, whilst as expected there was no change in FOXO1 levels in the presence of MPA alone (Figure **5.2**B). These findings were also supported by the examination of PLZF which is regulated by P. PLZF is very strongly upregulated by MPA (Figure 5.2C). SOD2 is recognised as FOXO1 mediated gene and also showed a significant increase in expression with cAMP (Figure 5.2D)

5.2.3. FOXO1 is a positive regulator of HO-1 expression in hESC

Although FOXO1 has been shown to directly regulate HO-1 expression in the liver, muscle, and pancreas (Kang *et al.*, 2014, Cheng *et al.*, 2009), to our knowledge, it has not been investigated in the regulation of HO-1 in hESC. To determine whether FOXO1 positively regulates HO-1 expression during decidualisation, hESC were plated at $2.2x10^5$ cells/well in 6-well plates to a 65%-85% confluency. The transfection of FOXO1 (siFOXO1) or universal control siRNA (siControl) was performed using Viromer Blue in 2 ml of E2 medium/well 24 h post transfection. The cells were either stimulated with MPC or maintained in E2 medium 24 h prior to the harvesting. The results showed a successful FOXO1 knockdown at an mRNA and protein level in both St-T1b and primary hESC. In St-T1b cells, qPCR revealed a six-fold reduction in FOXO1 mRNA expression in comparison with the control (Figure **5.3**A). Western blotting revealed a dramatic decrease in FOXO1 protein (Figure 5.3B) confirming the successful FOXO1 knockdown. HO-1 mRNA was significantly reduced following FOXO1 knockdown than in the control cells (Figure 5.3C). This result was confirmed by Western blot as shown in Figure 5.3D.

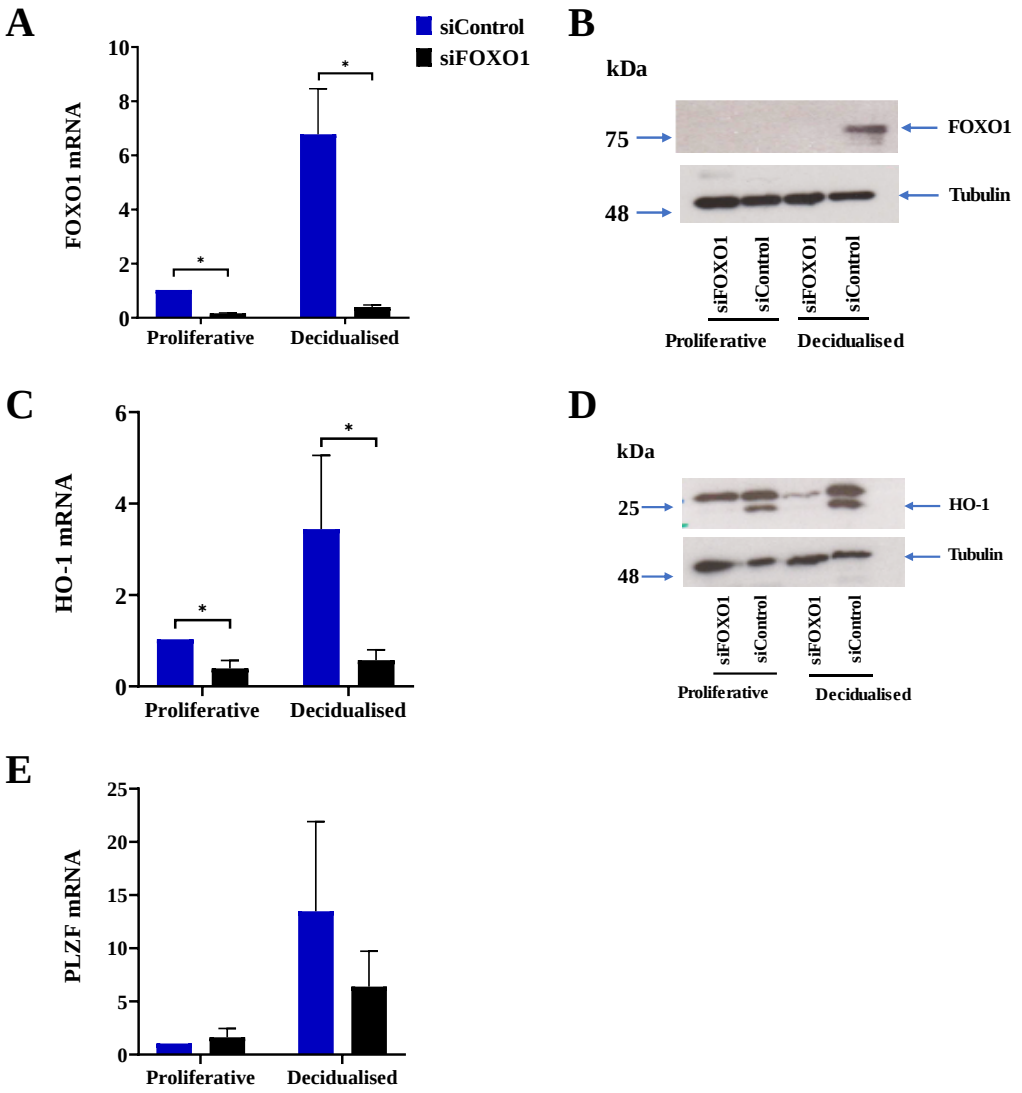


Figure 5.3: FOXO1 knockdown inhibits HO-1 expression in hESC. Confluent monolayers of St-T1b cells were transfected with siRNA targeting FOXO1 (siFOXO1) or control (siControl) siRNA for 24 h followed by incubation in E2 medium or in MPC medium for 24 h. Relative mRNA fold changes of (**A**) FOXO1 (**C**) HO-1 and (**E**) PLZF expression normalised to β-actin. Representative Western blot showing (**B**) FOXO1 and (**D**) HO-1 protein expression. Tubulin used as a loading control. Results are the mean (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$).

The decidual marker PLZF, which is not directly regulated by FOXO1 (Kommagani *et al*., 2016, Vasquez *et al*., 2015) was also investigated in FOXO1 knockdown cells. As expected, PLZF was not significantly affected by the loss of FOXO1 (Figure **5.3**E). Collectively, these results show that HO-1 is upregulated by the cAMP – PKA – FOXO1 pathway during hESC decidualisation.

5.2.4. HO-1 induction reduced the migration of primary hESC.

HO-1 is reported to be involved in aortic smooth muscle cell migration (Chen *et al*., 2012, Rodriguez *et al*., 2010) and endometrial repair following menstruation (Maybin and Critchley, 2012). However, to our knowledge, the evaluation of the effect of HO-1 expression on the motility of hESC has not been demonstrated yet. To investigate the effect of HO-1 on the functional behavior of hESC, an *in vitro* scratch assay was performed. Primary hESC were stimulated to decidualise with MPC medium or maintained in E2 medium in T75 flasks for 3 days. Cells were harvested, plated on 6-well dishes and stimulated under the same conditions for a further 24 h. The cell monolayers were then scratch-wounded and washed with PBS. Two ml of DMEM/Ham F12 medium supplemented with 1 % FBS was then added with either hemin (10 μM), or PDGF-BB (10 ng/ml). Cell migration into the scratch was assessed at 18 h and the % of migration calculated using the following equation:

$$\%\ \text{of migration} = \frac{\text{scratch area (0 h)} - \text{scratch area (18 h)}}{\text{scratch area (0 h)}}\ \text{x}\ 100$$

The proliferative primary hESC treated with hemin showed significantly reduced migration compared with the untreated cells, indicating that HO-1 may be acting as a negative regulator of their migration. In contrast, PDGF-BB significantly induced hESC migration (Figure 5.5A & B).

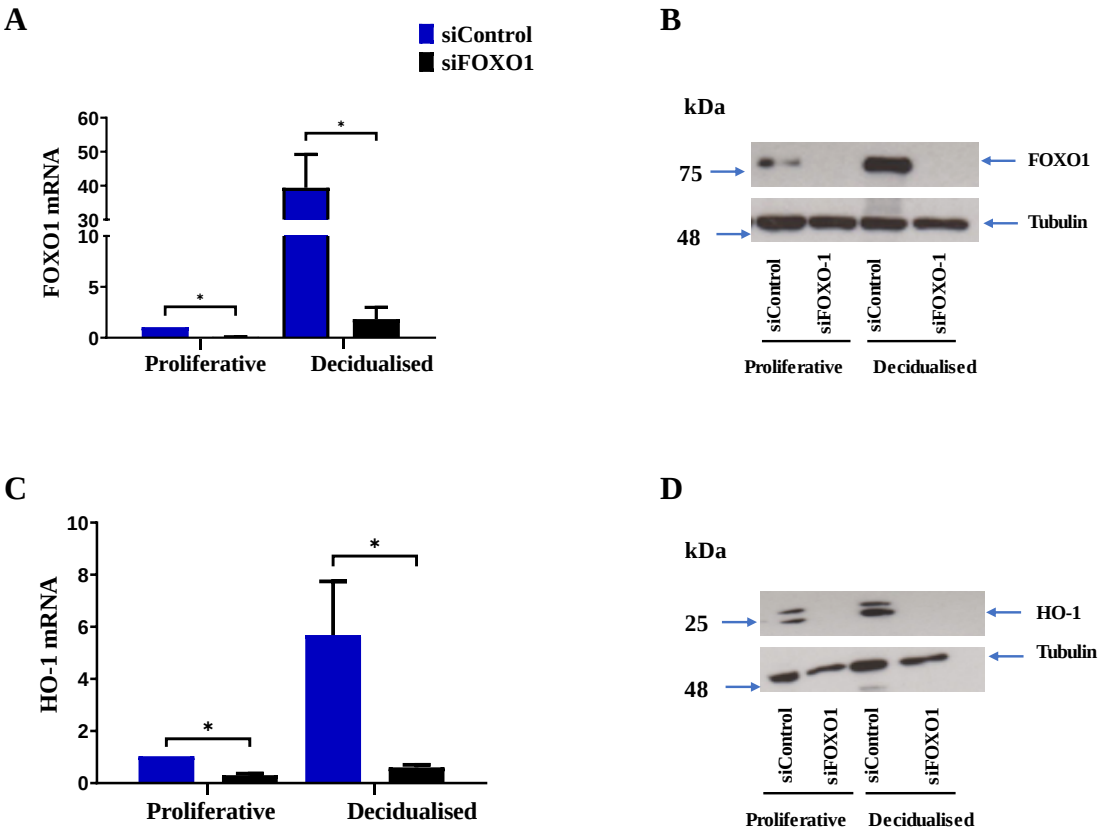
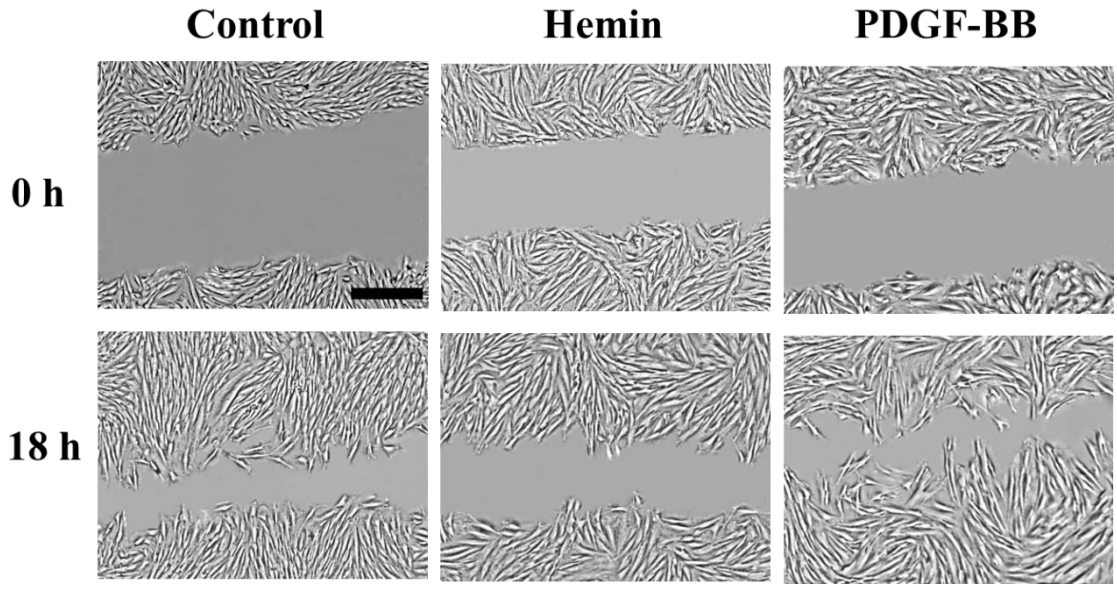


Figure 5.4: FOXO1 is a positive regulator of HO-1 expression in primary hESC. Confluent monolayers of primary hESC were transfected with siRNA targeting FOXO1 (siFOXO1) or control (siControl) siRNA for 24 h followed by incubation in E2 medium or in MPC medium for 24 h. Relative mRNA fold changes of (**A**) FOXO1 and (**C**) HO-1 expression normalised to β-actin. Representative Western blots showing (**B**) FOXO1 and (**D**) HO-1 protein expression. Tubulin used as a loading control. Results are the means (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$).



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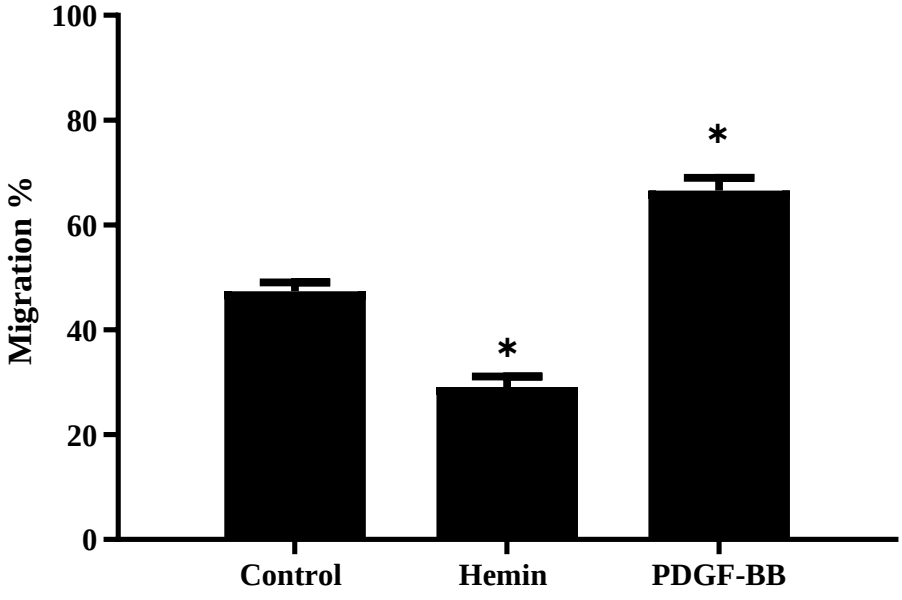


B

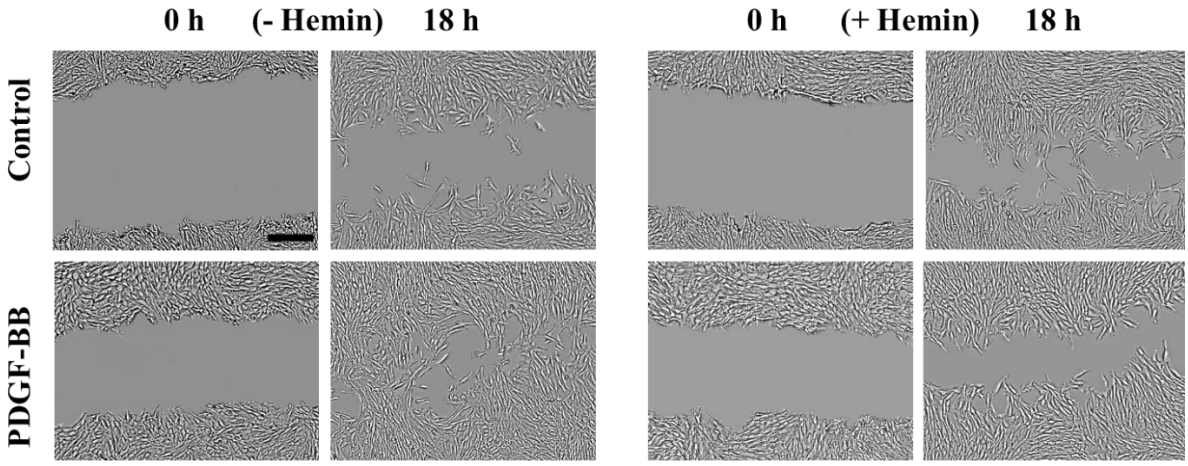
Figure 5.5: HO-1 induction diminished migration in primary hESC. Confluent monolayers of primary hESC were scratch-wounded and stimulated with PDGF-BB (10 ng/ml or hemin (10 μM). Cell migration was assessed after 18 h at 10 points /treatment condition in 2 wells and calculated as migration % = (Scratch area at 0 h-scratch area at 18 h/scratch area at 0 h)*100. (**A**) Representative bright field images (4 x objective) showing the scratch areas at 0 and 18 h. (**B**) Results are means (± SEM) of the migration percentage at 18 h relative to zero time point of three independent experiments. Statistically significant differences (*$P<0.05$). Scale bar = 400 μm.

5.2.5. HO-1 has no effect on St-T1b cell *in vitro* migration

An *in-vitro* scratch assay was performed in St-T1b cells with PDGF-BB stimulation used as a positive control. The St-T1b cells were seeded in 6-well plates at a density of $2.8x10^5$cells/well. The next day the cells were scratched and treated with PDGF-BB (10 ng/ml) or hemin (10 µM) (See HO-1 induction confirmed by Western blot in Figure 5.6C). The degree of migration was assessed at 18 h. The results showed that the hemin mediated-induction of HO-1 had no significant effect on the migration of the control cells (Figure 5.6A & B). As observed previously, PDGF-BB treatment of cells caused a significant increase in migration. Although, hemin treatment alone had no effect on St-T1b migration compared with the untreated control, it significantly reduced the level of PDGF-BB-induced St-T1b migration (Figure 5.6A & B).

5.2.6. Growth Factor treatment increases the migration of HTR-8 cells.

It was reported that the invasive and migratory ability of trophoblasts was affected by different growth factors and cytokines activity (Bischoff *et al*., 2000). To achieve this, the HTR-8 cells were plated at a density of $3x10^5$ cells/well in 6-well plates. Once confluent, the cell monolayers were scratch-wounded and hemin (10 µM), EGF (200 ng/ml) or PDGF-BB (10 ng/ml) were added. The reduction of the denuded scratch area was assessed at 18 h as mentioned before. Hemin had no effect on HTR-8 migration compared to untreated controls (Figure 5.7A & B). As expected, EGF significantly increased HTR-8 migration, which was inhibited with hemin treatment. PDGF-BB treatment alone or in combination with hemin did not affect HTR-8 migration (Figure 5.7A & B).



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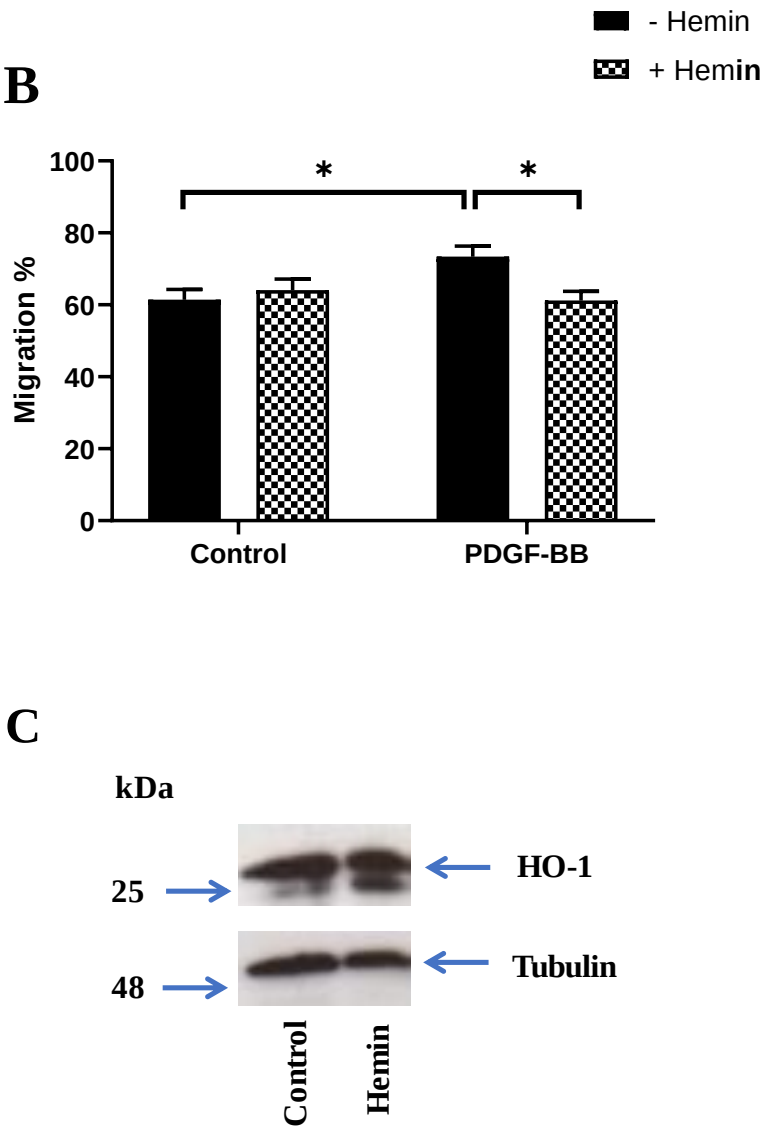
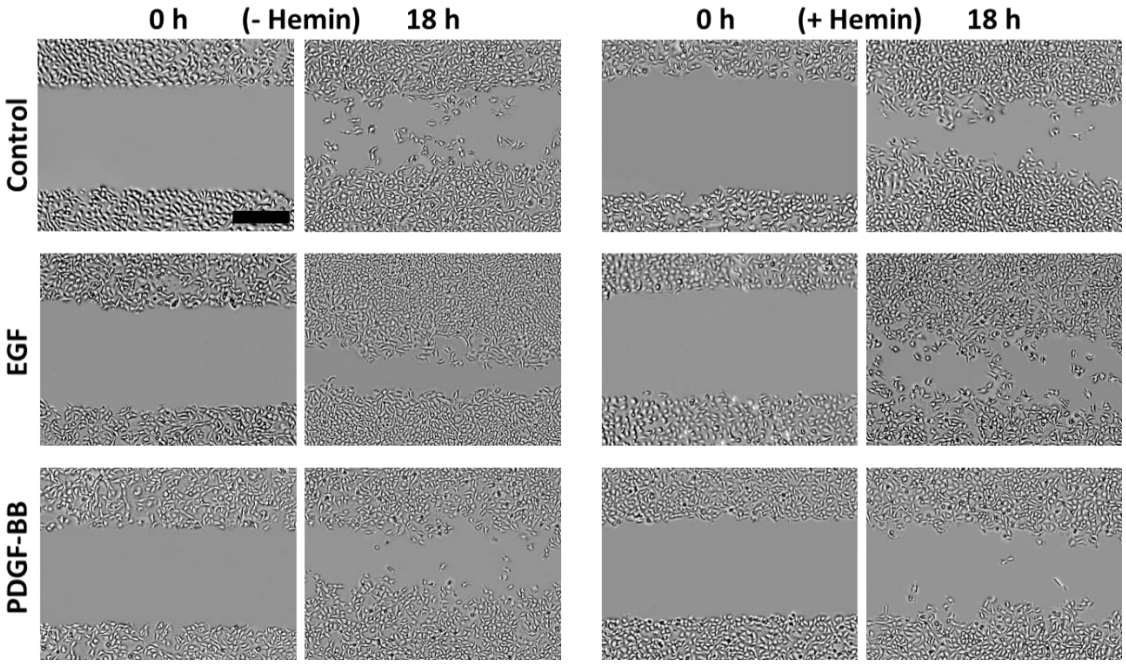


Figure 5.6: **HO-1 induction diminished migration in St-T1b hESC**. Confluent monolayers of St-T1b hESC were scratch-wounded and stimulated with PDGF-BB (10 ng/ml or hemin (10 µM). Cell migration was assessed after 18 h at 10 points /treatment condition in 2 wells and calculated as migration % = (Scratch area at 0 h-scratch area at 18 h/scratch area at 0 h)*100. (**A**) Representative bright field images (4 x objective) showing the scratch areas at 0 and 18 h. (**B**) Migration percentage at 18 h relative to zero time point. (**C**) Western blot of HO-1 induction. Results are means (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$). Scale bar = 400 µm.



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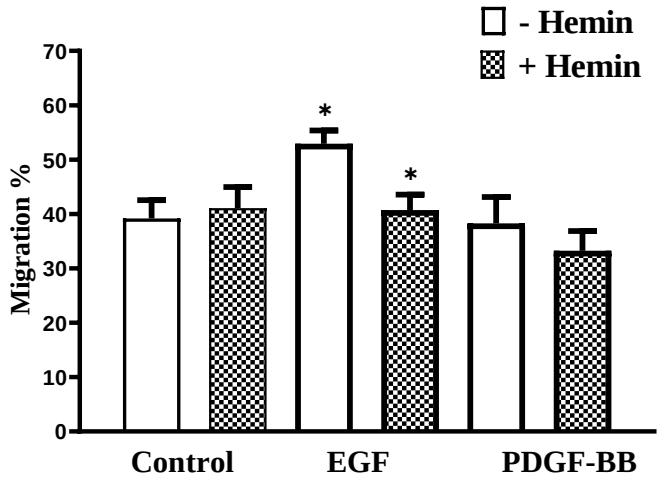


B

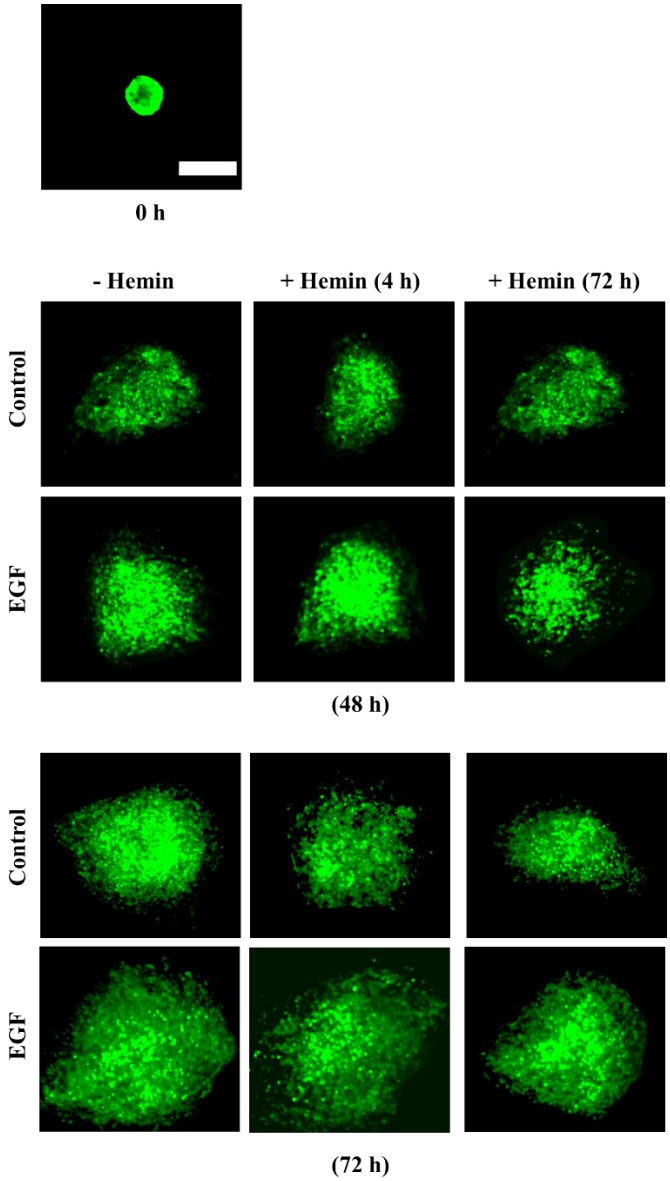
Figure 5.7: Hemin treatment has no effect on HTR-8 migration. Confluent HTR-8 cell monolayers were scratch-wounded and treated with EGF (200 ng/ml), PDGF-BB (10 ng/ml), and hemin (10 μM) in 2 ml of 1% FBS medium. Cell migration was assessed after 18 h at 10 points /treatment condition in 2 wells and calculated as migration %=(Scratch area at 0 h-scratch area at 18 h/scratch area at 0 h)*100. (**A**) Representative bright field images (4 x objective) showing the scratch areas at 0 and 18 h. (**B**) Results are means (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$). Scale bar = 400 μm.

5.2.7. Hemin-mediated HO-1 induction inhibits trophoblast spheroid expansion

The spheroid expansion co-culture model was employed to mimic a blastocyst spreading during embryo implantation to evaluate the effect of HO-1 induction using hemin in hESC on trophoblast-stromal cell interactions. These experiments were performed either in the presence of hemin (continuous hemin treatment) or with hemin added only for 4 h prior to co-culture of the spheroid. Hemin was added to the E2 medium for 4 h and then the hemin reapplied only to the "continuous hemin" treated cells in 1% FBS medium. The results showed that 4 h treatment for hESC monolayer has no effect on HTR-8 spheroid expansion. Continuous hemin treatment of the co-culture had no significant effect on HTR-8 spheroid expansion at 48 h (Figure **5.8**A & B). While, continuous hemin treatment reduced spheroid expansion at 72 h (Figure **5.8**A & B). Although EGF significantly increased the expansion rate at 72 h, a significant reduction of EGF-stimulated HTR-8 spheroid expansion was observed with continuous hemin treatment in the EGF in comparison with the control cells.

3.2.8. HO-1 has no direct effect on HTR-8 spheroid invasion in the absence of stromal cells

To further evaluate the effect of HO-1 on the invasive capacity of trophoblasts directly or through its activity in hESC, the HTR-8 spheroid invasion assay was used. HTR-8 spheroids were embedded in a fibrin gel. EGF (200 ng/ml) and PDGF-BB (10 ng/ml) were added alone or in a combination with hemin (10 μM) in 1% FBS ham F12 DMEM medium. The spheroid sprouting density was calculated by trainable weka plugin in ImageJ. The sprout length was calculated as the mean of the longest sprout in four quarters of the spheroid measured using ImageJ.



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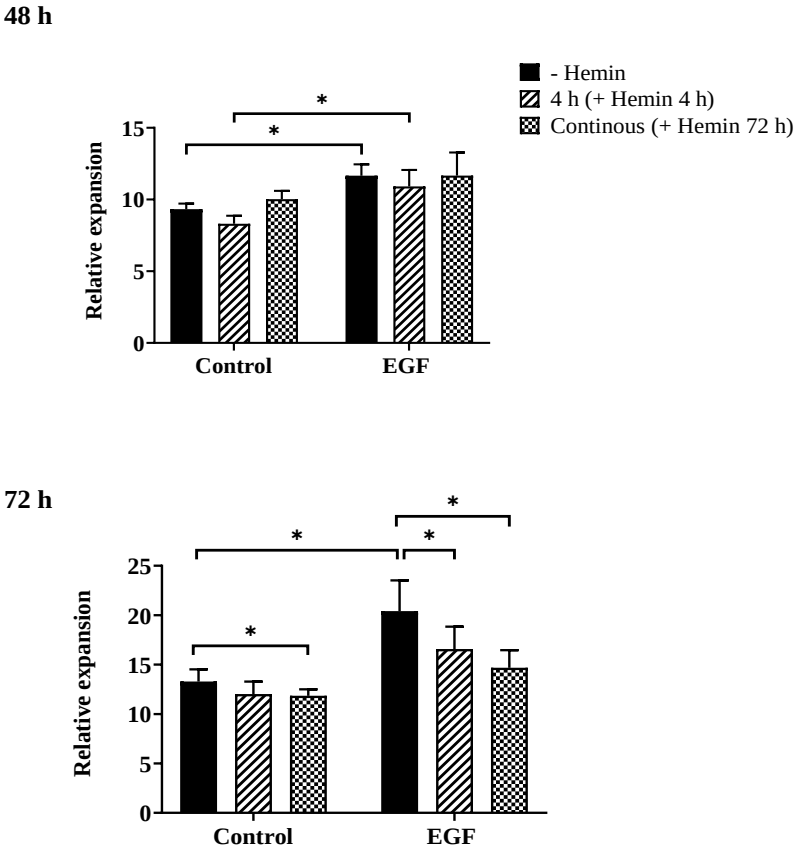


B

Figure 5.8: Hemin stimulation reduces trophoblast spheroid expansion. Labelled HTR-8 spheroids with Vybrant Green dye were seeded onto proliferating monolayers of St-T1b cells. The co-cultures were then treated with EGF (200 ng/ml) and hemin (10 μm) continuously or hemin applied only for 4 h (hemin 4 h) before spheroid embedding. HTR-8 spheroid expansion was calculated as spheroid size at 48 h or 72 h /spheroid size at 0 h using ImageJ. Ten spheroid/treatment conditions were measured in 4 wells. (**A**) Representative fluorescent images using Vybrant green dye (4x objective) showing spheroid expansion areas at 48 and 72 h. (**B**) Results are the means (± SEM) of three independent experiments. Statistically significant differences *(*P<0.05*). Scale bar = 200 μm.

The results revealed that EGF and PDGF-BB increased the HTR-8 spheroid sprout length significantly at 48 h and 72 h respectively ($p<0.05$). At 72 h of the experiment, hemin treatment made no difference in the length or the density of the sprouts except on 72 h of treatment when it upregulated upon combined treatment with PDGF-BB (Figure 5.9A & B).

5.2.9. Hemin treatment promotes trophoblast invasion in the presence of stromal cells

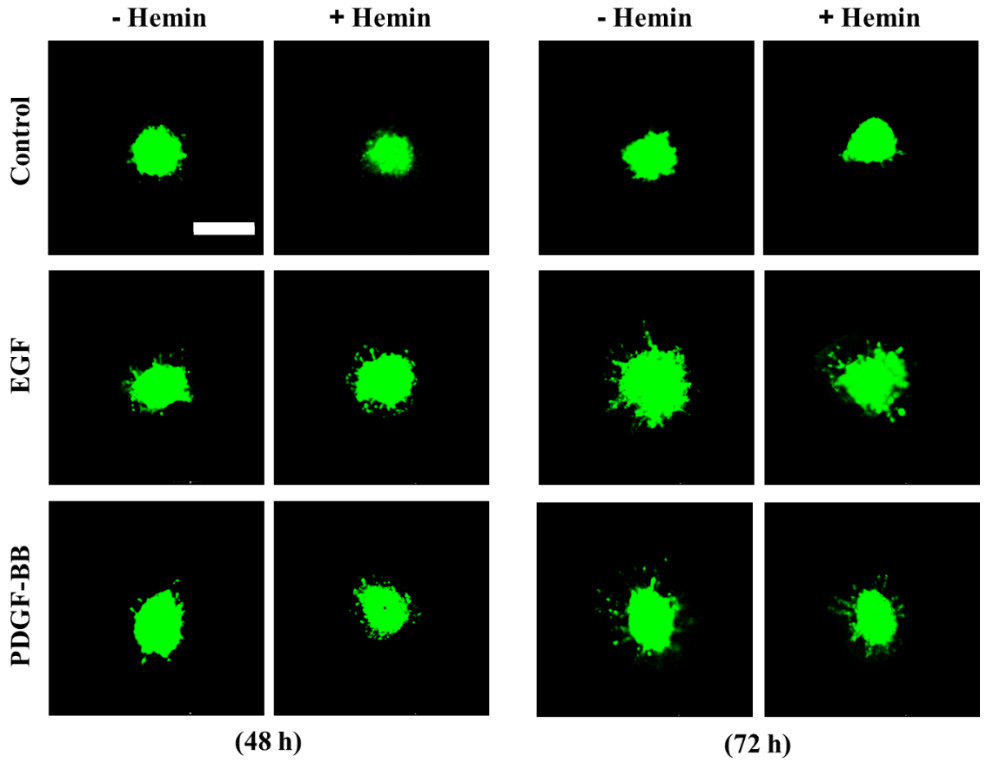
The HTR-8 spheroid co-culture model was used to determine whether hemin treatment of hESC affected the invasion of co-cultured trophoblast spheroids. For this purpose, HTR-8 spheroids were labelled with Vybrant Green dye and set in fibrin gels over a proliferative St-T1b cell monolayer. The co-culture then treated with hemin (10 μM), PDGF-BB (10 ng/ml), and EGF (200 ng/ml) in 1% FBS medium. The hemin added on two periods. First treatment for 4 h to the monolayer in E2 medium. The second treatment was applied for the co-culture. The HTR-8 spheroids were monitored with a fluorescent microscope at 48 h and 72 h. As previously observed, both trophoblast sprout density and length were enhanced with EGF and PDGF-BB treatment at 48 h and 72 h (

Figure **5.10**A & B). Hemin treatment caused a significant increase in sprout density and length in the control group. In the EGF and PDGF-BB treated group, HO-1 induction made no difference in sprouting density and length.

5.2.10. Upregulation of HO-1 mRNA in PDGF-BB treated St-T1b cells.

PDGF is reported as one of the HO-1 antioxidant inducer (Ryter and Choi, 2002).To further investigate the effect of PDGF on HO-1 as well cMYC, which is cell proliferation regulating gene reported to be expressed during hESC decidualisation (Popovici *et al*., 2000), the cells

were plated in 6-well plates then treated with PDGF-AA (100 ng/ml) and PDGF-BB (10 ng/ml) for 24 h. qPCR analyses were performed.



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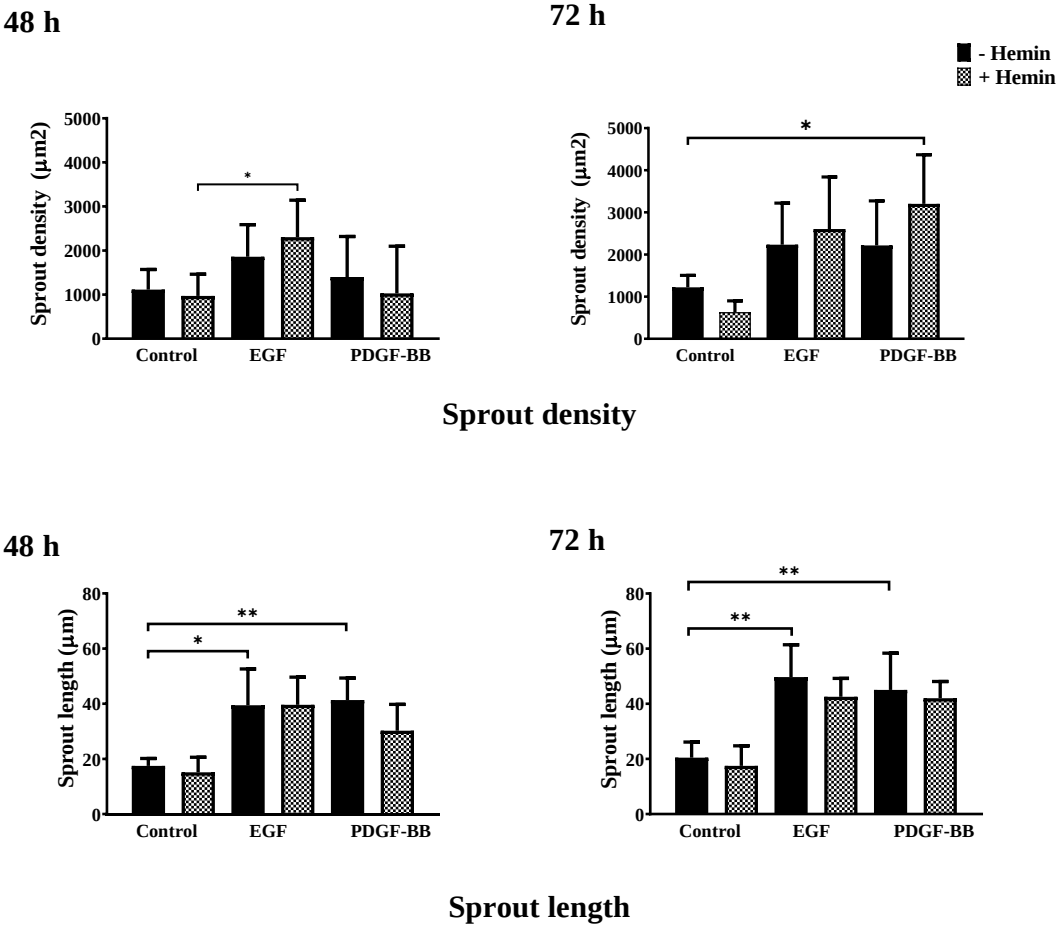
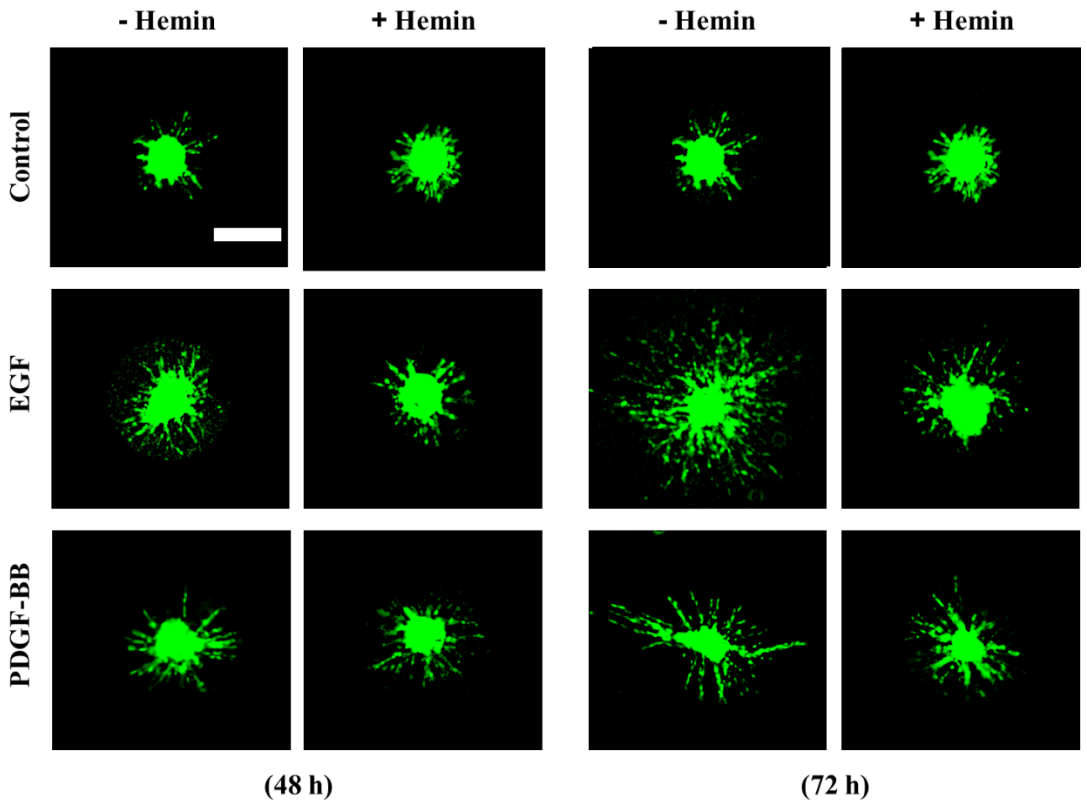


B

Figure 5.9: Hemin treatment has no effect on trophoblast spheroid invasion. HTR-8 spheroids were labelled with Vybrant Green dye and set in a fibrin gel with hemin (10 µM), PDGF-BB (10 ng/ml), and EGF (200 ng/ml) in 1% FBS medium. The sprout density and sprout length were calculated for 10 spheroids/conditions in 4 wells from 3 independent experiments using ImageJ (**A**) Representative fluorescent images of HTR-8 spheroid invasion into fibrin gel at 48 h and 72 h (4x objective). (**B**) Results are means (± SEM) of 3 independent experiments. Statistically significant differences (*$P<0.05$). Scale bar = 200 µm.



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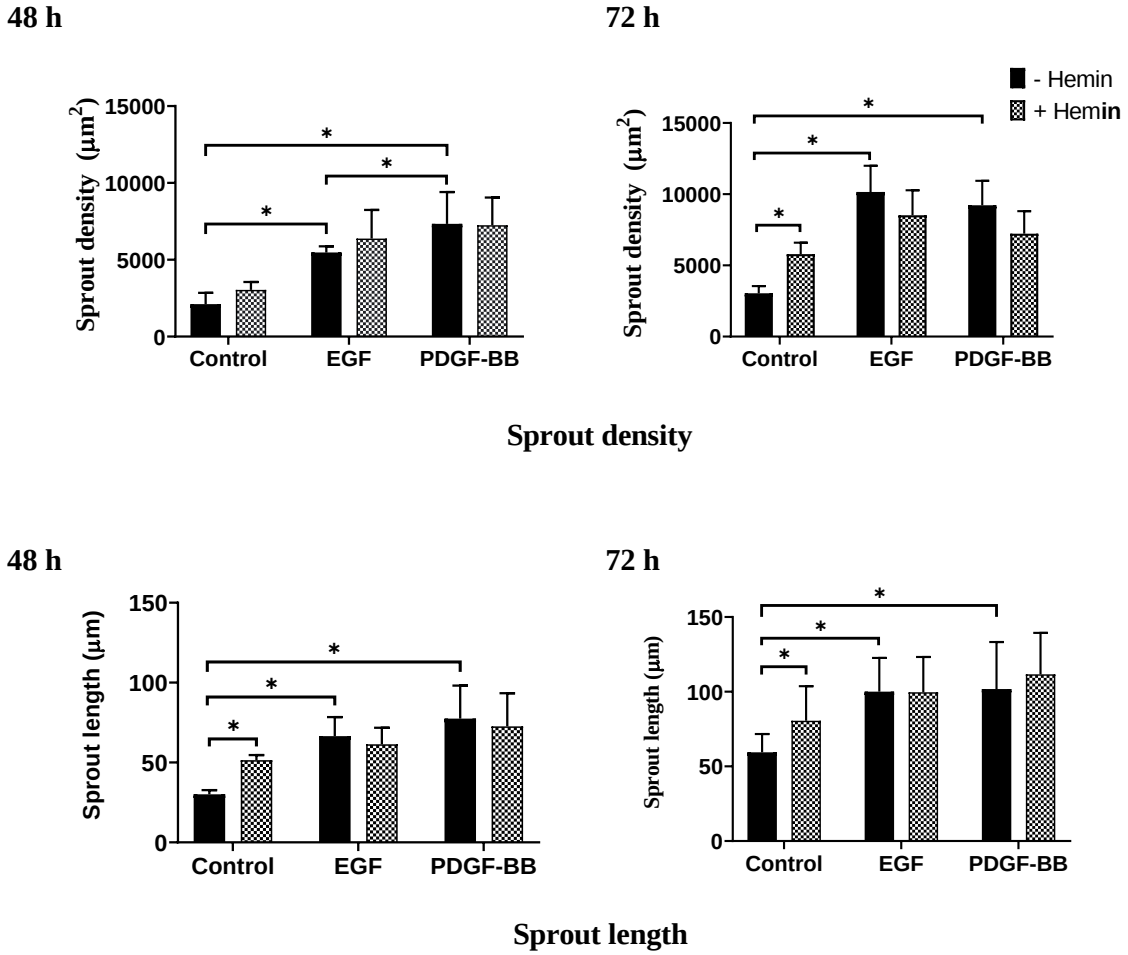


Figure 5.10: Hemin promotes trophoblast spheroid invasion in the presence of hESC. HTR-8 spheroids were labelled with Vybrant Green dye, embedded in fibrin gel set over a layer of St-T1b cells and treated with hemin (10 µM) for 4 h in E2 medium then treated with PDGF-BB (10 ng/ml), EGF (200 ng/ml), and hemin (10 µM) in 1% FBS medium. The sprout density and sprout length were calculated for 10 spheroids / condition in 4 wells from 3 independent experiments using ImageJ (A) Representative fluorescent images of HTR-8 spheroid invasion into fibrin gel at 48 h and 72 h (4x objective) (B). Results are means (± SEM) of 3 independent experiments. Statistically significant differences (*$P<0.05$, **$P<0.001$). Scale bar = 200 µm.

These results revealed that C-MYC mRNA was non-significantly changed with PDGF-treatment (Figure 5.11A). In the case of HO-1 mRNA expression, PDGF-BB treatment significantly induced its expression in comparison with the untreated cells, while PDGF-AA treatment did not induce a significant change (Figure 5.11B & C).

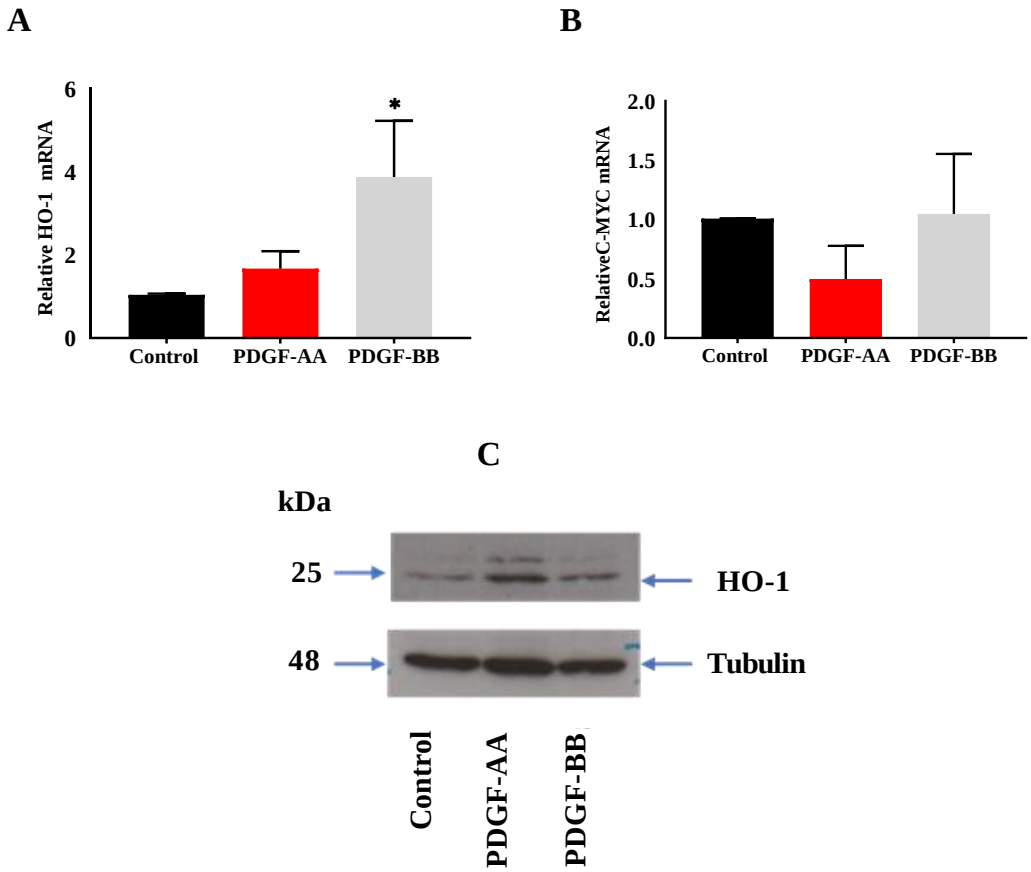


Figure 5.11: The Effect of PDGF treatment on HO-1 expression in St-T1b cells. A confluent monolayer of hESC treated for 24 h with PDGF-BB (10 ng/ml) or PDGF-AA (100 ng/ml) in 1% FBS DMEM/HAM F12 medium. Relative mRNA of (**A**) HO-1 and (**B**) cMYC mRNA normalised to β-actin. Representative Western blot of (C) HO-1 protein expression. Tubulin used as a loading control. Results are the mean (± SEM) of three independent experiments. Statistically significant differences (*$P<0.05$).

5.3. Discussion

5.3.1. HO-1 is upregulated during hESC decidualisation

Decidualisation is a reprogramming of the endometrium to enhance embryo implantation and protection against oxidative stress (Kajihara *et al*., 2006). HO-1 has various roles in pregnancy as a tissue-protective, anti-inflammatory, and antioxidant enzyme (Bainbridge and Smith, 2005). Our results demonstrate, for the first time, the up-regulation of HO-1 expression in both primary hESC and St-T1b cells at mRNA and protein levels during *in vitro* decidualisation. Oxidative stress might be one of the reasons that induce HO-1 as the decasualised tissue is more resistant to oxidative stress (Kajihara *et al*., 2006, Jamali *et al*., 2019, Clark *et al*., 2000). Moreover, this upregulation may be attributed to different expression during the phases of the menstrual cycle. This agrees with the finding of Zenclussen *et al* (2014) who found, with qPCR, increased uterine HO-1 mRNA expression during the estrus phase of mice cycles. However, Yoshiki *et al* (2001) found that HO-1 is constitutively expressed throughout the menstrual cycle in the uterus generally, but its main localisation was in the endometrial epithelia and macrophages and not in the endometrial stromal cells.

5.3.2. cAMP promotes HO-1 expression in hESC

In order to dissect the contribution of 8-Br-cAMP and MPA on HO-1 expression, hESC were stimulated with them individually or in combination for 24 h. The activation of the individual pathways with 8-Br-cAMP and MPA was confirmed using FOXO1 and PLZF as markers. As expected, FOXO1 was induced with cAMP and this supports the earlier finding of Brosens and Gellersen (2006), who indicated that FOXO1 is cAMP-inducible. As expected, PLZF was induced with MPA and this supports the finding of Kommagani *et al* (2016), who demonstrated that PLZF is a direct target of P by RNA expression analysis and chromatin

immunoprecipitation (ChIP) assay which identified several P response elements in the PLZF gene. We identified that HO-1 upregulation during decidualisation is dependent on the cAMP stimulated pathways.

cAMP causes nuclear accumulation of FOXO1 and the upregulation of pro-apoptotic anti-proliferative P53 protein. This means the enhancement of transcription of factors that induce cell cycle arrest and apoptosis, which occurs during P withdrawal before menstruation (Labied *et al*., 2006, Chatzaki *et al*., 2001). The active fraction of FOXO1 results in the transcription of differentiation-promoting genes such as PRL and IGFBP-1 (Brosens and Gellersen, 2006). This indicates the important role of P in reducing the active amount of FOXO1 during decidualisation to prevent decidual cell death (Labied *et al*., 2006). PLZF is considered as a pro-survival anti-apoptotic factor that is highly expressed in decidualised hESC and suppresses decidual marker transcription. PLZF is rapidly induced by P, but not with cAMP in hESC (Fahnenstich *et al*., 2003). PLZF balances the apoptotic action of P53 (Brosens and Gellersen, 2006, Maybin and Critchley, 2009, Szwarc *et al*., 2017). FOXO1 is an important factor in cell cycle regulation and this activity depends on its cellular localisation. Inactivation and phosphorylation of FOXO1 occurs with its release from the nucleus into the cytoplasm resulting in promoting cell survival and proliferation (Brunet *et al*., 1999).

Towards the end of luteal phase, P levels drop resulting in a reduction in PLZF expression. This again activates FOXO1 (The level of active FOXO1 will change, but there will always be some active), which coordinates with P53 toward upregulating the transcription of apoptotic factors such as BCL2 L11 (BIM). BIM is a target gene of FOXO1 activated by cAMP and inhibited by P. With P withdrawal, BIM expression increases, cell death occurs, and menstruation ensues (Brosens and Gellersen, 2006). The identification of FOXO1-mediated upregulation of HO-1

in hESC during the menstrual cycle would indicate that levels should be increased towards the end of the luteal phase prior to menstruation. This would be consistent with the involvement of HO-1 in limiting the haemorrhagic endometrial damage following menstruation (Maybin and Critchley, 2012).

5.3.3. FOXO1 is a positive regulator of HO-1 expression in hESC

The results indicated that the upregulation of FOXO1 during decidualisation was, at least in part, responsible for HO-1 upregulation. HO-1 maintains the oxidative and antioxdative balance, and regulates the apoptotic and anti-inflamatory mechanisms. The finding of HO-1 upregulation in decidual hESC and its regulation by cAMP led us to look further at the involvement of FOXO1 by applying FOXO1 knockdown. A successful FOXO1 knockdown was achieved in primary hESC and St-T1b cells**.** A significant reduction of HO-1 expression was observed following FOXO1 knockdown in primary hESC and St-T1b cells under both proliferative and decidual conditions. To our knowledge, this is the first time that Foxo1 has been identified as a positive regulator of HO-1 expression in hESC. Our findings agree with studies in other tissues/cell types in the mouse that showed that FOXO1 can positively regulate HO-1 expression (Cheng *et al*., 2009, Kang *et al.*, 2014). Kang *et al* (2014) found that Foxo1 regulates HO-1 expression in mouse muscle atrophy through direct binding to DNA binding sites in the HO-1 promoter using ChIP analysis. Cheng *et al.,* (2009) found that when double knockout of insulin receptors 1 and 2 in mice hepatic cells, Foxo1 has an HO-1 regulation activity and deletion of Foxo1 resulted in HO-1 expression reduction. However, Vasquez *et al* (2015), has not listed HO-1 as one of the direct targets of FOXO1 identified by DNA-Seq and ChIP seq analysis during hESC decidualisation. Further research might be suggested through FOXO1 over-expression in hESC followed by ChIP seq to elucidate any regulatory FOXO1 role in HO-1 expression.

5.3.4. HO-1 negatively affects the migration of hESC.

The migratory capacity of hESC is increasingly highlighted as an active contributor in the prominent uterine remodeling that occurs during embryo implantation (Petrie *et al.*, 2009) and endometrial regeneration following menstruation (Gentilini *et al.*, 2007). Dysregulated hESC migration can be seen in cases of implantation failure and pathological uterine conditions, such as endometriosis (Weimar *et al*., 2013). HO-1 is involved in embryo implantation and in limiting the hemorrhagic endometrial damage following menstruation (Maybin and Critchley, 2012). The current findings revealed that hemin has an inhibitory effect on the basal migration of primary hESC and PDGF-BB combined migration in St-T1b cells. There is only minimal information available on the direct effect of HO-1 in hESC migration. Rodriguez *et al* (2010) found that both HO-1 and CO can inhibit PDGF-BB-stimulated migration via the inhibition of nicotinamide adenine dinucleotide phosphate oxidase (NOX1) pathway in rat aortic smooth muscle cells. HO-1 knockdown in human leukemic cell line increases their motility (Abdelbaset-Ismail et al., 2017, Abdelbaset-Ismail et al., 2019). Additionally, HO-1 silencing in human retinal epithelial cells resulted in a reduced migration of co-cultured HUVEC cells (Zhang *et al*., 2013a)

5.3.5. Hemin treatment inhibits EGF-stimulated migration of HTR-8 cells

Using the *in vitro* scratch assay of HTR-8 cells, it was found that hemin significantly reduced the migration of EGF-stimulated HTR-8 cells. This agrees with the findings of Bilban *et al* (2009), who demonstrated reduced HTR-8 migration in transmigration assays following HO-1 over-expression. The authors used retroviral transduction of a doxycycline-inducible HO-1 and showed that HO-1 over-expression inhibits migration through an increase in peroxisome proliferator activated receptor-γ (PPARγ). PPARγ is a member of a large nuclear receptor

superfamily that is expressed in the placenta and regulates the expression of various genes (Zhao *et al.*, 2019). Chen *et al.*, (2012) also found that HO-1 induction in rat vascular smooth muscle cells inhibited their migration.

The result also showed that EGF has a stimulatory effect and this is in agreement with Haslinger *et al* (2013), who used primary trophoblasts, JEG-3, and HTR-8/SVneo. Interestingly, PDGF-BB treatment of HTR-8 cells made no difference to their *in vitro* scratch migration, although we showed that PDGF-BB stimulates trophoblast cell invasion in sprouting assays in the presence and absence of hESC. This led to a suggestion that the increased invasion by PDGF-BB is not related directly to the stimulation of their migration. Therefore, further study of changes in the stromal-trophoblast interaction, such as metalloprotease levels quantification using specific enzyme-linked immunosorbent assays, is required.

5.3.6. HO-1 reduces HTR-8 spheroid expansion but increases the invasion in the presence of St-T1b cells

We investigated the role of HO-1 on the interaction between trophoblasts and endometrial stroma. To achieve this, we employed our spheroid expansion and sprouting (invasion) co-culture models mimicking blastocyst spreading and invasion during embryo implantation. The results showed that HO-1 treatment reduced the expansion of HTR-8 spheroids on hESC and this effect was more pronounced with continuous hemin treatment for 72 h the than initial 4 h treatment.

HTR-8 spheroid invasion into the fibrin gel in the absence of stromal cells was not as pronounced as in the presence of hESC. This suggests that stromal cells act as an enhancer of trophoblast invasion. This may be explained by the secretion of factors from hESC that promote trophoblast invasion. In one study, trophoblasts were found to secrete integrins (Knöfler and

Pollheimer, 2012) and CXCL12 chemokines that in turn stimulate stromal cells to secrete CXCR4, which promotes their invasion (Ren *et al.*, 2011). The enhancement of HTR-8 invasion by hESC adds further evidence to support the prevailing hypothesis that decidual cells are important contributors to the highly coordinated embryo-uterine interactions which are essential for embryo implantation.

Hemin treatment caused increased sprout density and length under basal conditions at 72 h only in the presence of hESC. A study performed by Ha *et al* (2015) found that HO-1 products as CO and biliverdin increase HTR-8 and HUVEC invasion using trans-well migration assay, which investigates the invasion of each cell type individually and not as a co-culture. Li *et al* (2019) found that HO-1 increases invasion of HTR-8 and HUVEC under oxidative stress conditions by regulating the tight junction zonula occludens-1 (ZO-1) protein. This indicates the role of HO-1 in protecting pregnancy against oxidative stress. However, McCaig and Lyall (2009) found that using an HO-1 neutralising antibody has no effect on term cytotrophoblasts while the use of HO-2 inhibitors reduced the cell invasion into a Matrigel. Hence, we can further expand the HTR-8 spheroid sprouting assay by applying selective inhibitors of constitutive HO-2 as oxypentyl derivatives 29, 31, 48, 50 and the thiobutyl derivative 39 as a pharmacological tool suitable for investigating the biological roles of HO-1 by its own (Salerno *et al*., 2015). Additionally, as the application of HO-1 inducer was to the co-culture, so that the observed resulted affect can be due to its effect on each type of cells separately, or due to the factors released from both cells. To further dissect this effect, HO-1 knockdown in each types of cells can be investigated separately to understand what influential cells are involved in the effect of HO-1 on the invasion assay. Further study can be performed *in vivo* through HO-1 induction using cobalt protoporphyrin to investigate the migration and invasion of trophoblasts in

pregnant rats. The present finding can add a new affirmation to support the finding that HO-1 enhances embryo invasion

5.3.7. Upregulation of HO-1 mRNA in PDGF-BB treated St-T1b

HO-1 is the cellular sensor of oxidative stress which act as a cytoprotective through directly preventing cell damage. HO-1 is found to be reduced in pre-eclamptic pregnancy and intrauterine growth restriction. The result revealed that HO-1 mRNA level increased upon PDGF-BB treatment. This finding is in agreement with Ryter and Choi (2002), who recognised cytokines and growth factor such as PDGF as HO-1 induce through their effects on the cell surface receptor. The binding to the PDGF receptor leads to the alteration in the intracellular reactive oxygen species level and the activation of redox-signalling system and nuclear transcription factors as mitogen activated protein kinase (MAPK). This signalling pathway initiates the HO-1 inducing system. Chien *et al* (2015) found that the inhibition of PDGFR-α in human cardiomyocytes can inhibit HO-1 release.

The result also showed that exogenous PDGF did not change the C-MYC mRNA level. This agrees with Kelly *et al*. (1984), who recognised that c-sis, which is a putative structural oncogene of PDGF-BB, regulates the production of C-MYC. However, C-MYC is unstable genes which increases to a high level within three hours of fibroblast treatment with PDGF then return to quiescent level after that. Here, RNA harvesting was performed following 24 h of PDGF treatment. However, Goustin *et al* (1985) indicated a colocalisation of cMYC and PDGF-B in cytotrophoblast cells in the placenta that leads to the assumption that the proliferation of the placenta can shape an autocrine loop.

In conclusion, HO-1 mRNA and protein expression are upregulated during hESC decidualisation and cAMP is necessary for this upregulation. In addition, FOXO1 is a positive

regulator in this upregulation. HO-1 has a pronounced inhibitory effect on the primary hESC cell migration, and HTR-8 spheroid expansion, whereas it increases the invasion of HTR-8 spheroids over an endometrial stromal monolayer. These findings support our hypothesis that HO-1 can influence the function of interacting cells at the endometrial-trophoblast interface.

CHAPTER 6

INVESTIGATING THE ROLE OF HO-1 IN THE REGULATION OF SFLT-1 EXPRESSION IN HESC DECIDUALISATION

6.1. Introduction

Flt-1 undergoes alternative mRNA splicing producing sFlt-1, which works as a potent anti-VEGF factor through the sequestration of free VEGF and the formation of a complex that competes negatively with its receptors in endothelial cells (Kendall *et al*., 1996). VEGF and its receptors play an important role in the endometrial decidualisation, embryo implantation, trophoblastic invasion, and the maintenance of healthy pregnancy (Plaisier, 2011, Goldman-Wohl and Yagel, 2002). An imbalance in VEGF and sFlt-1 expression can be seen in different reproductive problems such as abnormal trophoblast invasion (McMahon *et al*., 2014), early recurrent spontaneous abortion (Pang *et al*., 2013), porcine peri-implantation fetal death (Tayade *et al*., 2007), endometriosis (Cho *et al*., 2012), and later in pregnancy in pre-eclampsia and IUGR (McKeeman *et al*., 2004). With RT-PCR, Krüssel *et al* (1999) found that sFlt-1 is highly expressed in the proliferative rather than the secretory phase of human endometrial tissues. Lockwood *et al* (2007) found that sFlt-1 mRNA expression is enhanced by thrombin in the first trimester decidua. The pre-eclamptic decidua shows thrombophilia and haemorrhage as thrombin is produced from decidual cells expressing tissue factor. A negative correlation between HO-1 expression and sFlt-1 expression was found in endothelial cells and placental explants (Cudmore *et al*., 2007); however, the relation between HO-1 and sFlt-1 expression in hESC has not yet been demonstrated.

sFlt-e15a and sFlt-i13 are two Flt-1 splice variants, which have been implicated in reproductive problems. sFlt-e15a is a recently discovered primate-specific variant (Thomas *et al*., 2009) produced in non-endothelial cell types, such as vascular smooth muscle cells (Sela *et al*., 2008) and trophoblasts. sFlt-i13 is produced in the placenta, kidney, brain, heart, and endothelium (Palmer *et al*., 2015). sFlt-1 isoforms have antiangiogenic properties as they inhibit endothelial cell invasion, sprout formation, and migration (Jebbink *et al*., 2011).

6.1.1. Aim and objectives.

In this chapter, the effect of HO-1 on the expression of sFlt-1 variants (sFlt-e15a and sFlt-i13), and VEGF during hESC decidualisation was investigated. sFlt-1 and VEGF expression were measured by qPCR and ELISA, in hESC stimulated to decidualise following HO-1 induction or inhibition.

6.2. Results

6.2.1. Decidualisation causes a significant reduction of sFlt-1 production in hESC

To evaluate the effect of decidualisation on sFlt-1 and VEGF release from hESC, an ELISA was used to measure the levels of sFlt-1 and VEGF in ST-T1b cell supernatants collected at day 4 and day 7 of decidualisation. The results revealed a significant reduction of sFlt-1 protein secretion on day 4 and day 7 compared with control cells maintained in E2 medium (Figure 6.1). In contrast VEGF secretion increased significantly *($p<0.001$)* on day 7 (Figure 6.2).

6.2.2. Downregulation of sFlt-1 protein expression on 24 h decidualisation stimulation in St-T1b hESC

To delineate the pathways involved in the downregulation of sFlt-1 during decidualisation, hESC were stimulated with 8-Br-cAMP and/or MPA. St-T1b cells and primary hESC were seeded in 6-well plates at a density of 3 $x10^5$ cells/well. The cells either maintained in E2 medium or treated with MMI medium containing MPA (1 µM) or 8-Br-cAMP (0.5 mM) alone or in combination for 24 h. Supernatants were collected and sFlt-1 measured by ELISA. The results showed a significant reduction of sFlt-1 protein release in all decidualised St-T1b groups in comparison to the controls (Figure 6.3A).

In the primary hESC, sFlt-1 protein expression was found to be lower in controls (~350 pg/ml) in comparison with the St-T1b cells controls (~3500 pg/ml). MPA and cAMP stimulation for 24 h was not found to significantly affect sFlt-1 production in primary hESC.

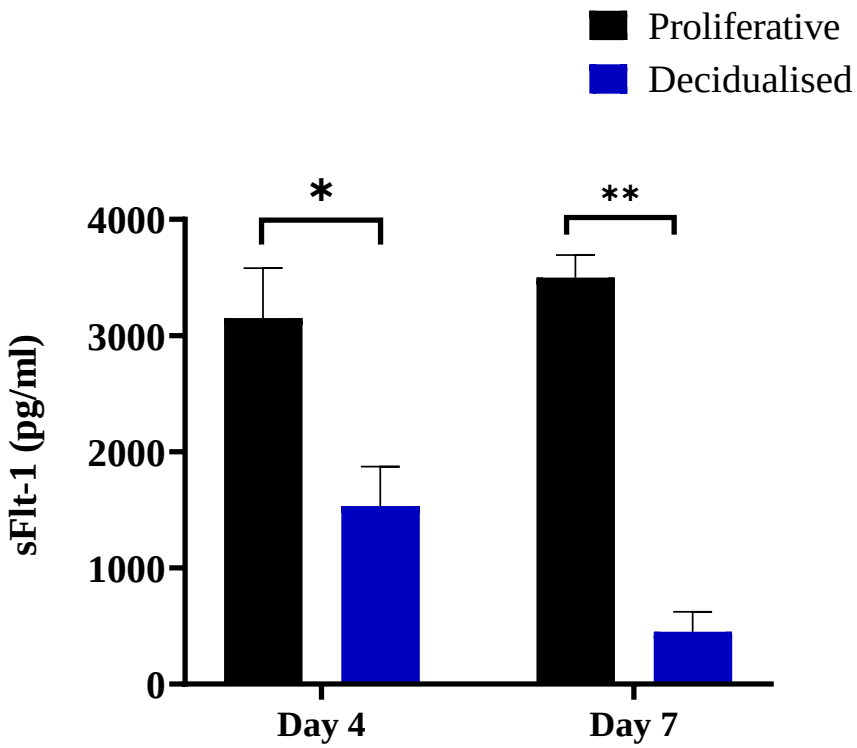


Figure 6.1: Reduction of sFlt-1 secretion in hESC during decidualisation. Confluent monolayers of St-T1b cells were incubated in E2 medium or MPC medium for 4 and 7 days. sFlt-1 was measured in the cell supernatants by ELISA. Results are mean (± SEM) of three independent experiments performed in duplicate. Statistically significant differences *(*p<0.05, **p<0.001).*

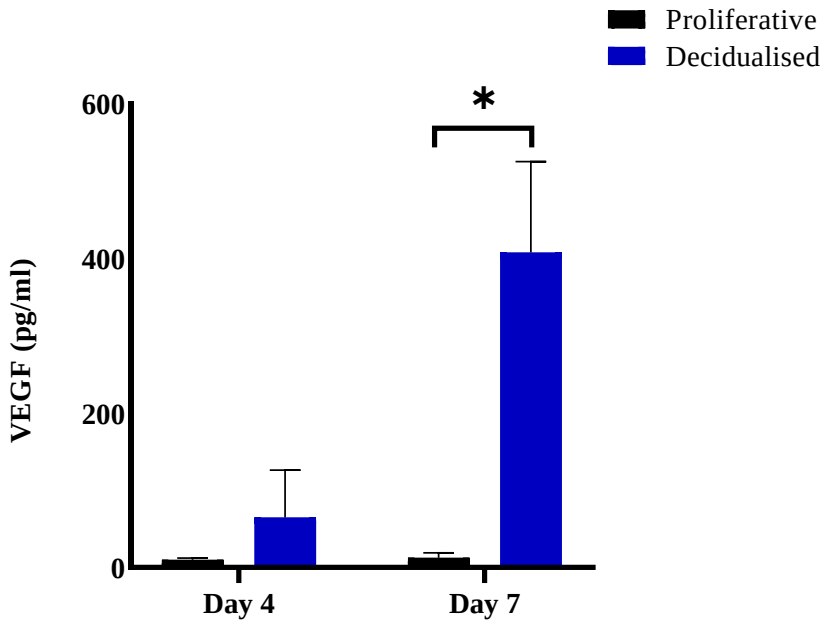


Figure 6.2: VEGF secretion increases in hESC during decidualisation. Confluent monolayers of St-T1b cells were incubated in E2 or MPC medium for 4 and 7 days. Measurement of sFlt-1 protein concentration was performed by ELISA. Results are mean (± SEM) in three independent experiments. Statistically significant differences (*$p<0.05$).

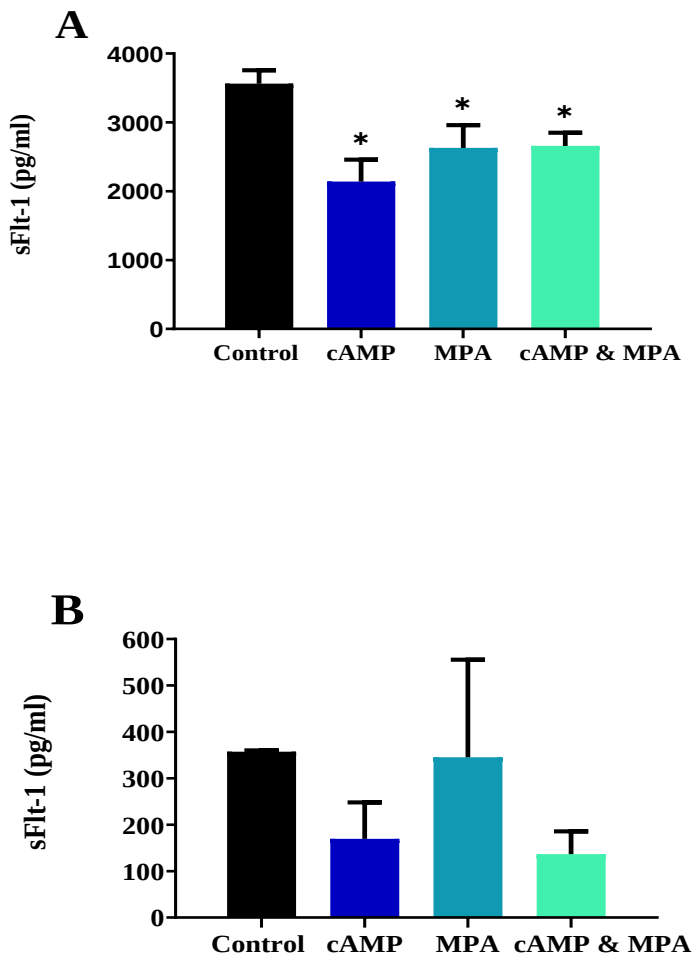


Figure 6.3: Effect of MPA and 8-Br-cAMP on sFlt-1 protein secretion in hESC. Confluent monolayers of St-T1b cells (**A**) and primary hESC (**B**) were incubated in a complete E2 or in MMI medium containing either 8-Br-cAMP (0.5 mM) or MPA (1 µM) alone or in combination for 24 h. sFlt-1 measured in the cell supernatants by ELISA. Results are the mean (± SEM) of three independent experiments performed in duplicate. Statistically significant differences (*$p<0.05$).

6.2.3. Reduction of FOXO1and Flt-1 mRNA expression in HO-1 induced hESC St-T1b and increased FOXO1 expression in primary hESC

A negative correlation between HO-1 expression and sFlt-1 expression was reported in human endothelial cells and placental explants (Cudmore *et al*., 2007). Here, we investigated the effect of HO-1 induction on hESC, focusing on changes of FOXO1 and *Flt-1* mRNA splice variant expression. In St-T1b cells and hESC, hemin (10 μM) resulted in a 70% increase HO-1 mRNA induction accompanied by a marked increase in protein expression as revealed by Western blotting (Figure 6.4A & B). In primary hESC, the same hemin concentration induced a marked (5-fold) increase in HO-1 mRNA in comparison with the controls. This mRNA upregulation was also confirmed by Western blotting (Figure 6.5A & B). Following confirmation of HO-1 induction with hemin, the associated changes in FOXO1, sFlt-1 variants, and SOD2 expression were investigated. The results showed a significant reduction in the full-length Flt-1 and sFlt-i13 mRNA in St-T1b cells and sFlt-1 protein was only detected weakly in the hemin treated cells (Figure 6.4D, E & G).

In primary hESC, the changes in Flt-1, sFlt-i13a, and sFlt-e15a variants were not significantly upregulated (Figure 6.5E, F, & G). In Western blots, a faint signal (sFlt-i13) was observed in the hemin treated cells (Figure 6.5H) of the same size as recombinant sFlt-1, which was used as a positive marker.

FOXO1, SOD2, and OGG1 were used to determine if there was an increase in oxidative stress induced using hemin. St-T1b cells showed a significant down-regulation of FOXO1 mRNA and no difference in SOD2 mRNA levels (Figure 6.4C & H). In contrast, primary hESC showed a significant upregulation of FOXO1 and SOD2 mRNA (Figure 6.5C & I).

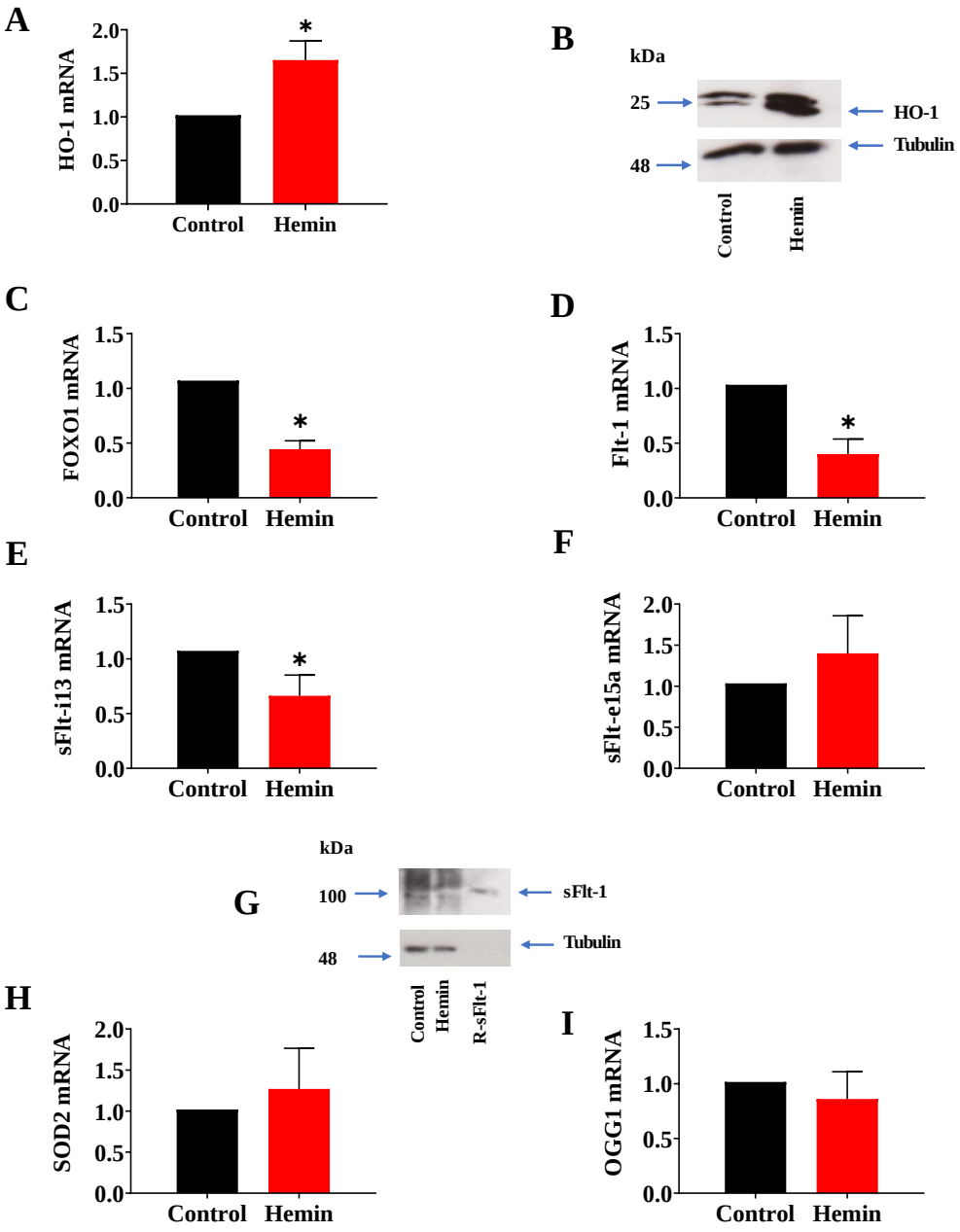


Figure 6.4: FOXO1 and Flt-1 mRNA expression reduced in HO-1 induced hESC. Confluent monolayers of St-T1b cells were treated for 24 h with hemin (10 µM) in E2 medium. Relative mRNA fold changes of (**A**) HO-1, (**C**) FOXO1, (**D**) full length Flt-1, (**E**) sFlt-i13, (**F**) sFlt-e15a, (**H**) SOD2 and (**I**) OGG1 normalised to β-actin. Representative Western blots showing (**B**) HO-1 and (**G**) sFlt-1 protein expression with recombinant sFlt-1 (R-sFlt-1) as a positive marker. Tubulin was used as a loading control. Results are the mean (± SEM*)* of four independent experiments. Statistically significant differences *(*p<0.05)*.

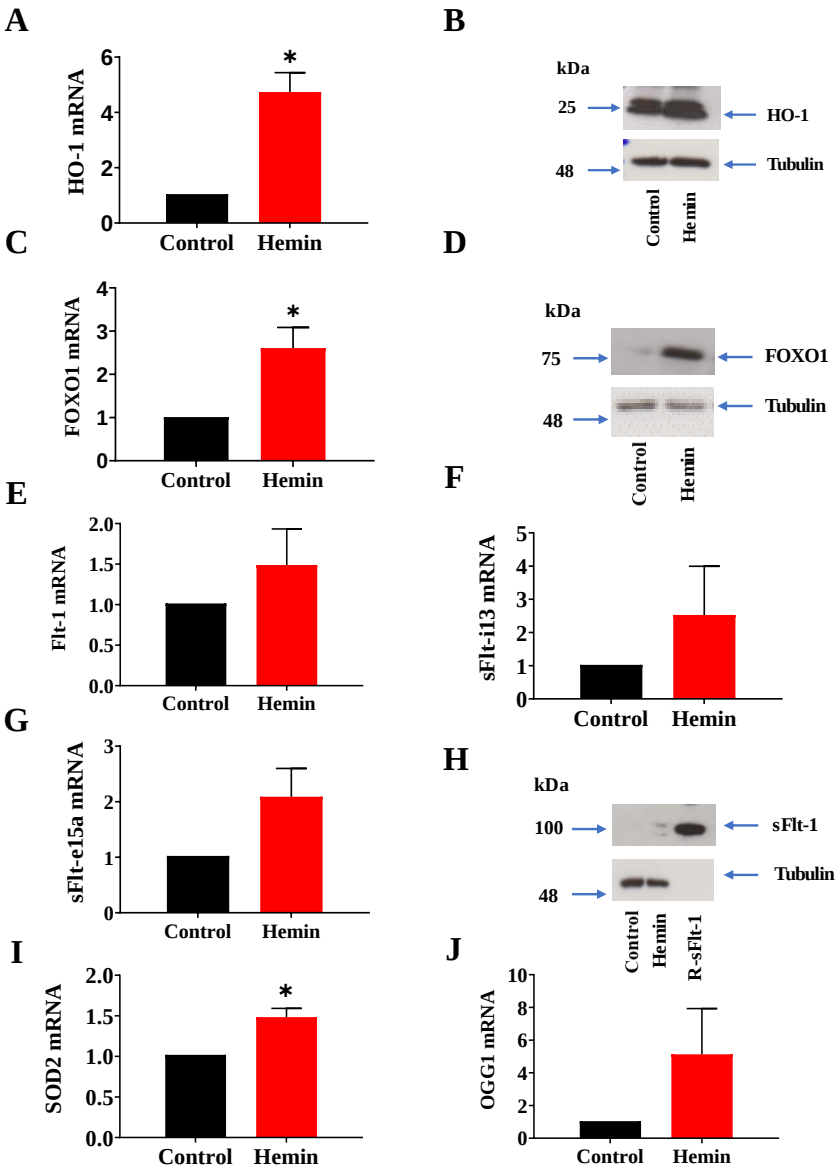


Figure 6.5: HO-1 induction upregulates FOXO1 in the primary hESC. Confluent monolayers of primary hESC were treated for 24 h with hemin (10 µM) in E2 medium. Relative mRNA fold changes of (**A**) HO-1, (**C**) FOXO1, (**E**) Flt-1, (**F**) sFlt-i13, (**G**) sFlt-e15a, (**I**) SOD2, and (**J**) OGG1 normalised to β-actin. Representative Western blots showing (**B**) HO-1, (**D**) FOXO1, and (**H**) sFlt-1 protein expression with recombinant sFlt-1 (R-sFlt-1). Tubulin was used as a loading control. Results are the means (± SEM*)* of three independent experiments. Statistically significant differences *(*p<0.05).*

The upregulation of FOXO1 was also seen at the protein level expression in primary hESC, whilst FOXO1 protein was undetected in St-T1b cells. OGG1 mRNA levels did not change in either St-T1b cells or primary hESC (Figure 6.4I; Figure 6.5D & J).

6.2.4. Reduction of sFlt-e15a mRNA in hemin induced HUVEC

As HO-1 was previously reported to down-regulate the sFlt-1 production in endothelial cells (Cudmore *et al.*, 2007), HUVEC were used to investigate the effect of hemin treatment on sFlt-1 variant expression and to compare with the results seen in hESC. HUVEC were seeded at $2.8x10^5$cells/well in a 6-well plate. Confluent monolayers of HUVEC were treated with hemin (10 µM) for 24 h and HO-1 mRNA and protein levels analysed. A 2.5-fold induction of HO-1 mRNA was detected upon hemin treatment (Figure 6.6A). HO-1 protein expression was strongly induced. Following confirmation of HO-1 induction, we investigated Flt-1, sFlt-1 mRNA expression levels. The results showed a non-significant reduction of sFlt-i13 mRNA and a significant reduction in sFlt-e15a expression (Figure 6.6E, C & D). In Western blots, three bands were detected (Figure 6.6F). The lower band is sFlt-i13, a less prominent band of approximately 100 kDa, sFlt-e15a is <135kDa, and the upper band of ~170 kDa was the full-length Flt-1 receptor. The intensity of all the bands increased following hemin treatment.

The sFlt-1 protein concentration in the hESC supernatants was analysed by ELISA. The results revealed a significant elevation of sFlt-1 expression in the hemin treated group (Figure 6.7).

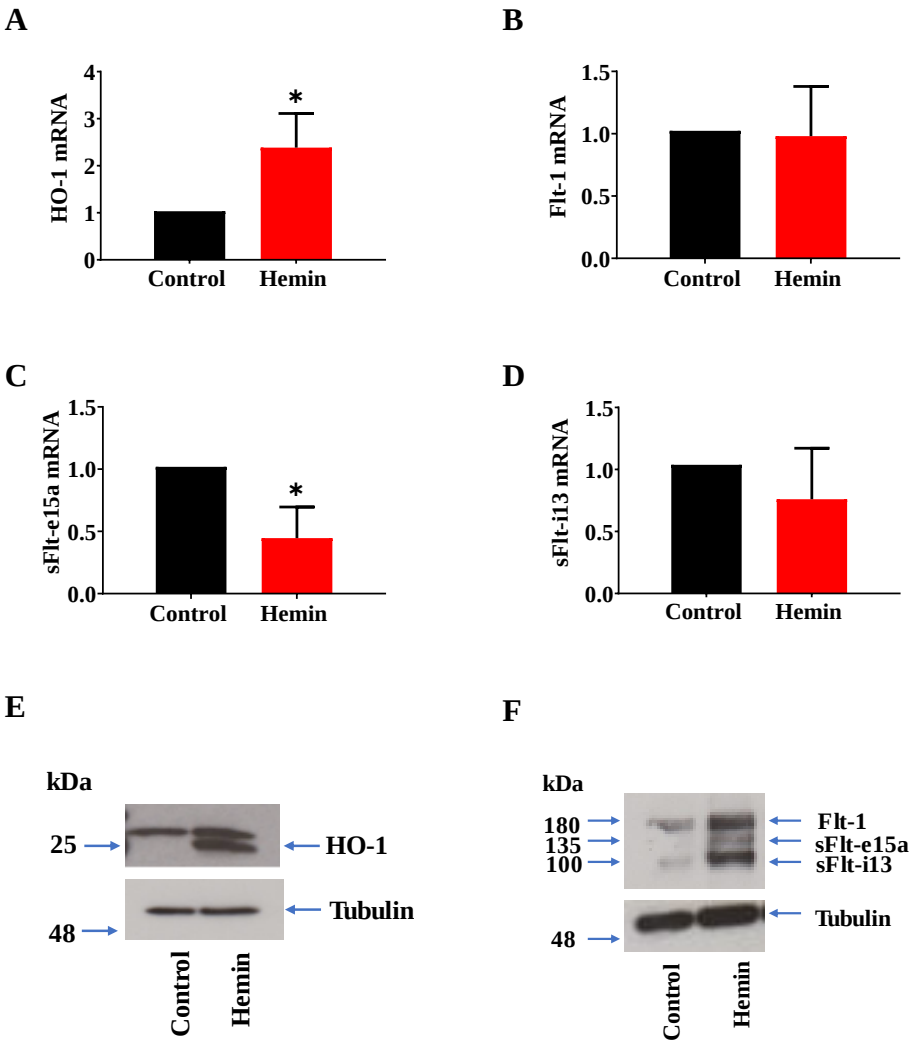


Figure 6.6: sFlt variant expression in endothelial cells following hemin treatment. Confluent monolayers of HUVEC were treated with hemin (10 µM) in for 24 h. Relative mRNA fold changes (**A**) HO-1, (**B**) Flt-1, (**C**) sFlt-e15, and (**D**) sFlt-i13 mRNA expression measured by qPCR and normalised to β-actin. Representative Western blots showing (**E**) HO-1 and (**F**) sFlt-1 protein. Tubulin used as a loading control. Results are the mean (± *SEM)* of four independent experiments. Statistically significant differences *(*p<0.05)*.

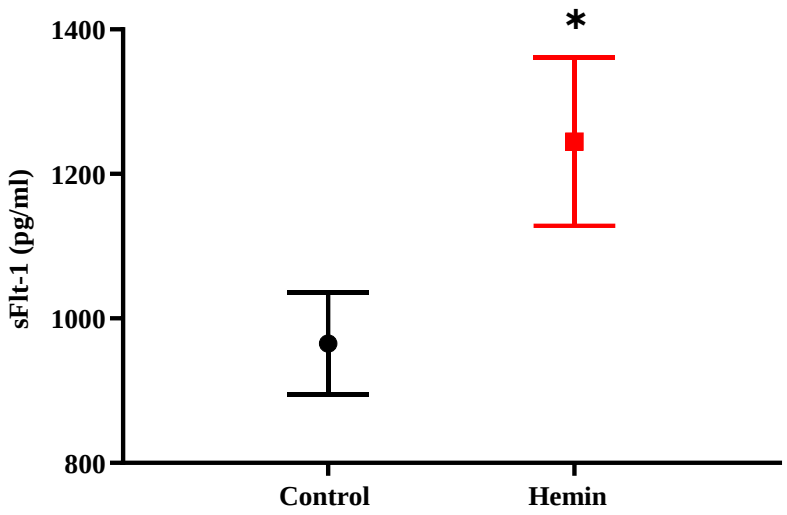


Figure 6.7: HO-1 induction increased sFlt-1 release in endothelial cells. Confluent monolayers of HUVECs were treated with hemin (10 µM) for 24 h. Supernatants were harvested, and sFlt-1 protein secretion determined by ELISA. Results are the mean (± SEM) of sFlt-1 protein relative to untreated control of three independent experiments performed in duplicate. Statistically significant differences *(*p<0.05)*.

6.2.5. Up-regulation of HO-1 increases sFlt-1 and VEGF expression in hESC

HO-1 regulates both VEGF and sFlt-1 expression in various cell types (Cudmore *et al.*, 2007). Here, we assessed the role of HO-1 using pharmacological stimulators and inhibitors; namely, hemin and OB24 hydrochloride (OB24 h) and tin protoporphyrin IX dichloride (SnPP) on sFlt-1 and VEGF release in hESC. hESC were plated at $1x10^5$ cells/well in a 24-well plate and incubated until they reached confluence. The cells were then treated with hemin (10 µM) for 4 h to ensure that HO-1 was induced. Then, OB24 (10 µM) and SnPP (10 µM) were added into E2 medium with hemin (10 µM) for a further 24 h. Hemin treatment caused an elevated expression of sFlt-1 in St-T1b cells and primary hESC (Figure 6.8). The up-regulation of HO-1 in these cells was confirmed by Western blotting (Figure 6.4B). A significant elevation of sFlt-1 expression was observed following hemin treatment, which was also observed in cells treated with OB24. However, sFlt-1 expression did not change in the SnPP treated hESC (Figure 6.8A).

In primary hESC, sFlt-1 secretion was significantly upregulated following hemin treatment and increased in HO-1 expression confirmed by Western blotting (Figure 6.5B). This response was not affected by the presence of the HO-1 inhibitors (Figure 6.8A, Figure 6.8B).

Interestingly, VEGF expression in St-T1b cells was found to be significantly upregulated following SnPP treatment in the absence of hemin induction (Figure 6.9A). Hemin caused a significant increase of VEGF expression in the OB24 treated group. In primary hESC, VEGF expression did not increase significantly following hemin treatment in hESC (Figure 6.9B).

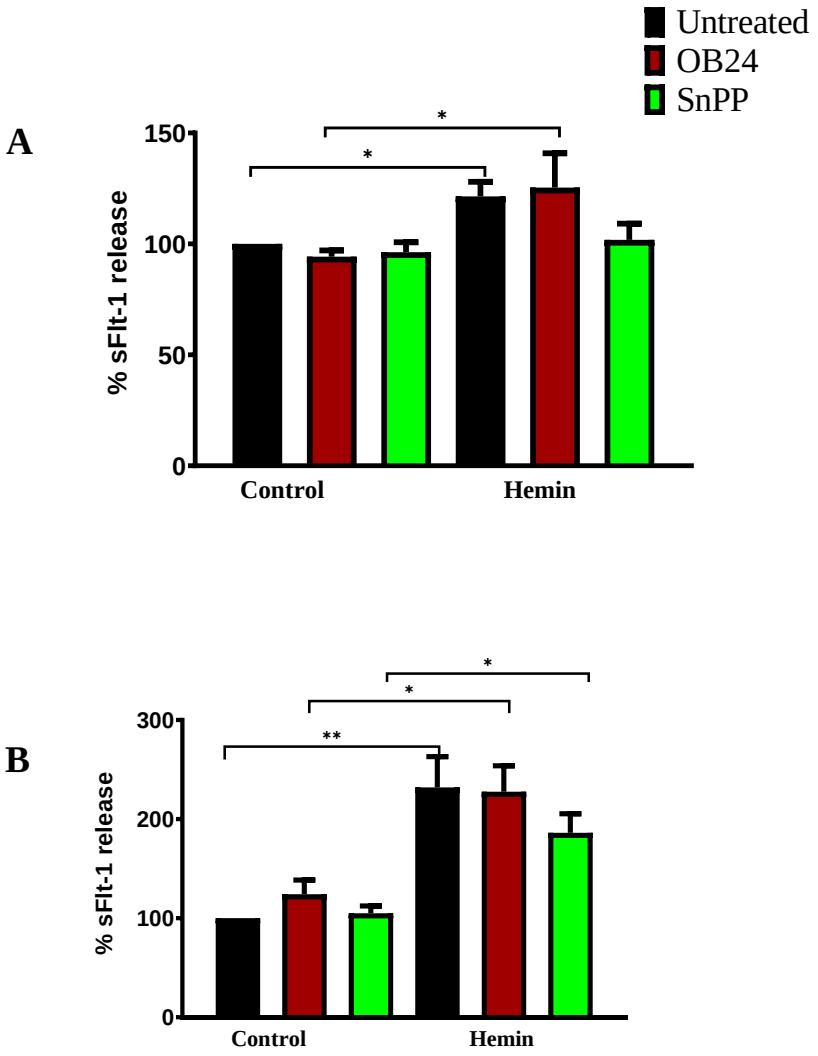


Figure 6.8: HO-1 induction increased sFlt-1 protein secretion in hESC. Confluent monolayers of St-T1b cells and primary hESC were incubated with hemin (10 µM) 4 h prior to or in combination with OB24 (10 µM) or SnPP (10 µM) for 24 h in E2 medium. ELISA measurements of sFlt-1 protein release were performed in (**A**) St-T1b cells and (**B**) primary hESC supernatants. Results are the mean (± SEM) of % sFlt-1 protein release relative to untreated control of three independent experiments performed in duplicates. Statistically significant differences (*$p<0.05$, **$p<0.001$).

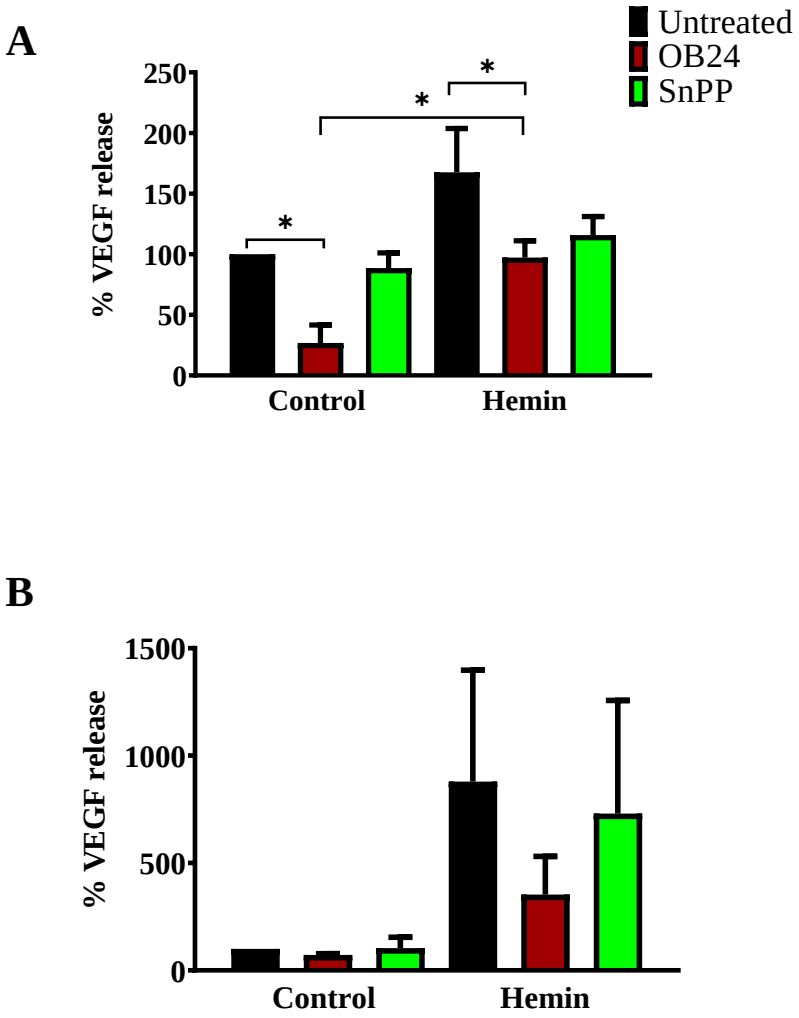


Figure 6.9: HO-1 induction affects VEGF protein expression in hESC. Confluent monolayers of St-T1b cells and primary hESC were incubated with hemin (10 µM) 4 h prior to and in combination with OB24 (10 µM) or SnPP (10 µM) in E2 medium for a further 24 h. ELISA measurements for VEGF protein release were performed in (**A**) St-T1b cells and (**B**) primary hESC supernatants. Results are the mean % VEGF release (± SEM) relative to untreated control of three independent experiments performed in duplicate. Statistically significant differences (*$p<0.05$, **$p<0.001$).

6.2.6. HO-1 knockdown in primary hESC

To further investigate whether the hESC HO-1 activity can affect sFlt-1 release, HO-1 was knocked down in primary hESC with the same protocol used for FOXO1 knockdown. mRNA, western blot protein expression, and ELISA analyses were performed. Unfortunately, the results showed only a partial knockdown of HO-1 in both mRNA and protein levels. Therefore, the results could not be inferred to a conclusion (Figure 6.10).

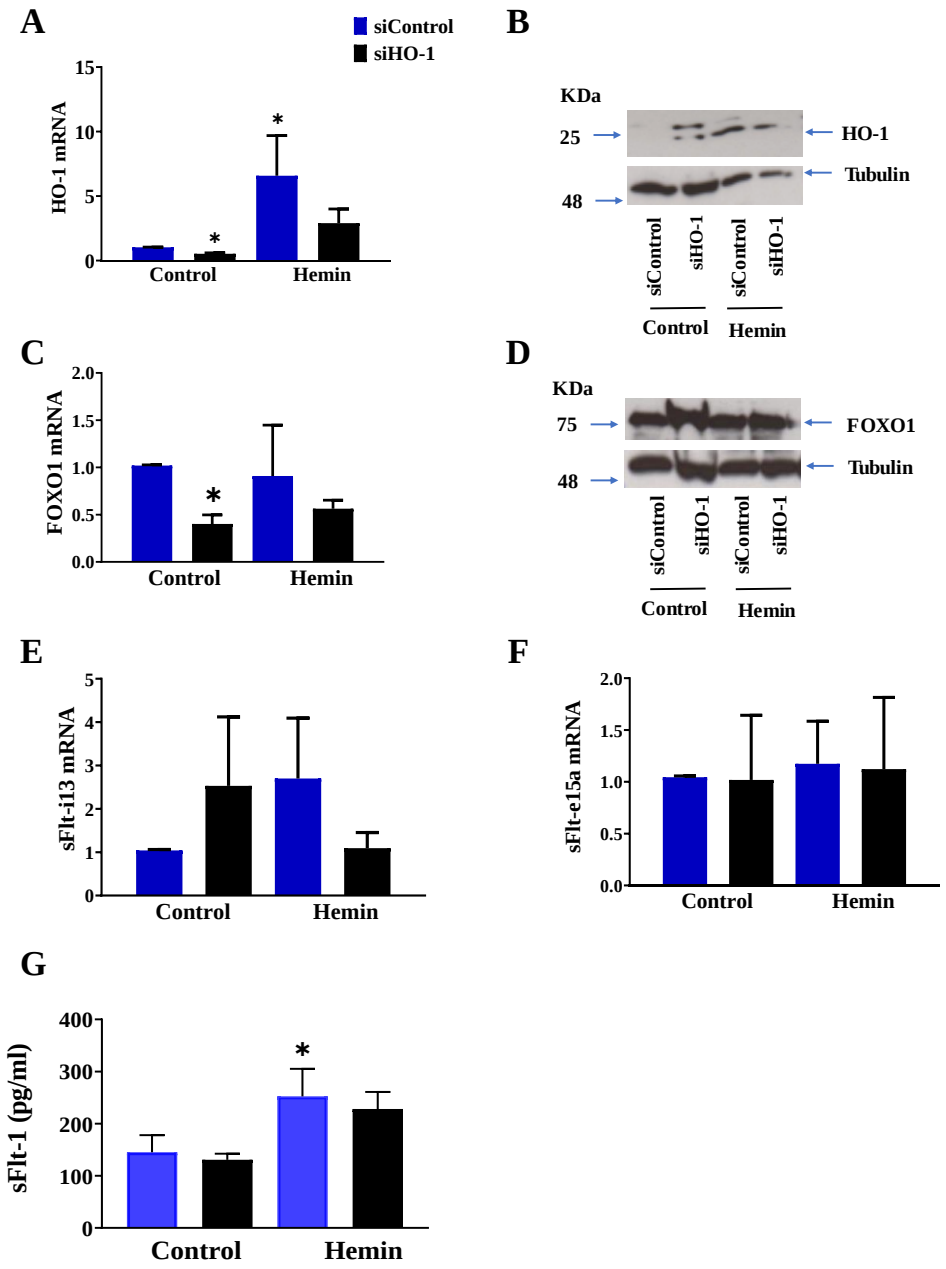


Figure 6.10: HO-1 knockdown in primary hESC. Confluent monolayers of primary hESC were transfected with siRNAs targeting HO-1 (siHO-1) or control (siControl) siRNA for 24 h followed by incubation in E2 medium in combination with hemin (10 μM) in for 24 h. Relative mRNA fold changes of (**A**) HO-1, (**C**) FOXO1, (**E**) sFlt-i13 and (**F**) sFlt-e15a normalised to β-actin. (**G**) ELISA analysis of sFlt-1 protein expression following HO-1 knockdown in primary hESC. Representative Western blots showing (**B**) HO-1, (**D**) FOXO1 protein expression. Tubulin was used as a loading control. Results are the means (± SEM) of three independent experiments. (G) ELISA measurements of sFlt-1 protein release. Statistically significant differences (*$p<0.05$).

6.3. Discussion

6.3.1. Elevated VEGF and reduced sFlt-1 release in hESC during decidualisation.

Here, we aimed to describe the control of sFlt-1 and VEGF in hESC during *in vitro* decidualisation, and the impact of HO-1 expression on them. Our results reveal the upregulation of VEGF and down-regulation of sFlt-1 in St-T1b cells following decidualisation. These findings are consistent with the recent report by Cottrell *et al* (2017), which was published during this study. They induced human endometrial cells from the proliferative stage to decidualise with a cocktail of E2, P, and cAMP at the initial stage of the experiment. Then, they maintained decidualisation with cAMP alone. They found an up-regulation of VEGF protein by ELISA with a reduced level of sFlt-1 by qPCR, western blot, and ELISA assay in decidualised cells with cAMP versus the control group. The findings show a reduction of sFlt-1 protein release over 24 h in hESC stimulated with cAMP or MPA alone or in combination. This may be related to a combined effect of FOXO1 (which depends on cAMP activity) and P activity, which induced decidualisation. Further *in vitro* studies by overexpressing FOXO1 in hESC can identify the role of FOXO1 in sFlt-1 release. Krüssel *et al* (1999) reported a constant mRNA expression of full length Flt-1 in the menstrual cycle, whilst sFlt-1 reduced in the secretory phase using endometrial biopsies from women at different stages of the menstrual cycle. These findings agree with our results in hESC decidualisation, which naturally occurs during the secretory phase of the menstrual cycle and could be explained on the basis of steroid hormone regulation of sFlt-1 expression. Furuyama *et al* (2000) found that Foxo deletion in mice can result in the reduction of Full length Flt-1in angiogenic cells. The reduction of sFlt-1 gives much-updated evidence to confirm the importance of decidualisation in protection against sFlt-1 related problems.

Our finding of the upregulation of VEGF during hESC decidualtion supports the previous finding of others (Matsui *et al*., 2004, Lockwood *et al*., 2009). Studies by Vasquez *et al* (2015) and Mazur *et al* (2015) found that FOXO1 and P regulate VEGF upregulation during decidualisation. Vasquez *et al* (2015) found by ChIP sequencing of decidualised hESC that VEGF and Flt-1 are among the genes regulated with stromal decidualisation and FOXO1 transcription. Mazur *et al* (2015) found that decidual VEGF expression is regulated with the PGR. Schatz *et al* (2003) also found an increase in VEGF mRNA and protein in hESC decidual production. In contrast to our finding of increased VEGF release on P and cAMP treatment, Okada *et al* (2010) found that E2 stimulation of hESC increases VEGF and reduces sFlt-1 release. In our study, we found that E2 in E2 medium does not increase the VEGF level, whilst MPC medium increases its release, which might be due to the activation of PI3/AKT signaling during decidualisation (Fabi *et al*., 2017).

6.3.2. HO-1 induction increases sFlt-1 protein expression in hESC

The ELISA results of sFlt-1 release following hemin treatment in hESC show that it is upregulated even in the presence of pharmacological HO-1 inhibitors. This upregulation was combined with a significant rise in VEGF levels produced by St-T1b cells. Fan *et al* (2014) reported that in women with pre-eclampsia, the overexpression of VEGF by placental trophoblasts is associated with sFlt-1 over-expression in endometrial cells. In cultured murine trophoblasts, VEGF was found to increase the expression of *sFlt1* mRNA.

OB24 is a new small molecule HO-1 inhibitor. It is an imidazole derivative that works as a potent selective HO-1 inhibitor with 50 % inhibitory activity of HO-1 when used at 10 µM in the prostate cell line (PC3M) (Alaoui-Jamali *et al*., 2009). SnPP is a competitive substrate inhibitor of HO-1 enzyme activity. It was reported that SnPP, at a level of 10 µM, can result in

>50 % inhibition of HO-1 activity (Drummond and Kappas, 1981, Grundemar and Ny, 1997). Cudmore *et al* (2007) found that the adenoviral HO-1 overexpression in HUVEC results in sFlt-1 down-regulation, and when HO-1 is inhibited with SnPP in the presence of VEGF, the sFlt-1 reduces. This may be attributed to the difference between endothelial cells and hESC or may be due to more complicated causes as this study was performed on a pre-eclamptic condition, which occurs later in pregnancy (after 20th week of gestation). Furthermore, we induced HO-1 in HUVEC with hemin; therefore, the induction of sFlt-1 may be either due to HO-1 induction itself or due to another effect of hemin on the hESC. Other approaches could be adopted using alternative HO-1 inducers such as Zinc protoporphyrin (ZnPP), Statins, Resveratrol, or adenoviral overexpression of HO-1 constructs.

Our findings may not be linked directly to the regulatory relation between VEGF and a sFlt-1 release in hESC as VEGF increases during decidualisation while sFlt-1 decreases. Hence, we need to indicate that there might be an autocrine function of endogenous VEGF in hESC as in the endothelial cells to maintain survival (Lee *et al*., 2007a, Domigan *et al*., 2015). Further experiments to measure sFlt-1 release following treatment with VEGF might be indicated. HO-1 knockdown was not successful, and due to time constraints, ordering of new siRNA to check what made the knockdown failed was not achievable. However, sFlt-1 was also elevated in the presence of hemin in the HO-1 knockdown trial.

6.3.3. Reduction of FOXO1 and Flt-1 mRNA expression in HO-1 induced hESC St-T1b.

The findings revealed that FOXO1 is a positive regulator of HO-1 in hESC and that HO-1 can affect levels of cellular oxidative stress, which is a known regulator of FOXO1. Accordingly, we planned to determine whether HO-1 activity might affect FOXO1 expression (*i.e.* can HO-

1 affect FOXO1 levels). Interestingly, HO-1 induction led to up-regulation of FOXO1 in primary hESC. To find an explanation, we looked to connect FOXO1 expression with an oxidative stress environment (Kuang *et al.*, 2014), which might be induced by hemin itself through measuring the levels of SOD2 and OGG1. OGG1 is upregulated in conditions of severe oxidative stress when DNA damage has occurred (Ruchko *et al*., 2005). The results showed that the OGG1 level was unchanged, and this may exclude the possibility of a severe oxidative stress environment in the induction of HO-1 with hemin. However, the rise in Foxo1 in primary cells was associated with raised SOD2. This may suggest that hemin treatment may be associated with a mild stressful environment (Souza *et al*., 1997) in the primary hESC that was not seen in the St-T1b cell line. It could be that St-T1b cells are immortalized and more resistant, with a higher threshold of coping with hemin than primary cells. Kang *et al* (2014) found that HO-1 induction using hemin in differentiated mouse myoblasts resulted in the induction of Foxo1 leading to elevated muscle degradation factors known as atrogenes and hemin administration to mice for 7 days caused mice muscle atrophy. Muscle atrophy and protein degradation were found to be caused by increased oxidative stress (Sacheck *et al*., 2007).

The second focus of hemin treatment was to check its effect on sFlt-1 expression. The results indicate the down-regulation of sFlit-i13 mRNA expression in St-T1b cells with hemin treatment, whilst the difference in primary hESC was non-significant. Very recent research by Palmer *et al* (2017) introduced a discriminatory tool to differentiate between early and late onsets pre-eclampsia. In the early onset, they found an increased protein expression of the placental predominate sFlt-1splice variant (sFlt-e15a), whilst the late onset, pre-eclampsia is mainly endothelial in origin and associated with endothelial cell production of their predominant sFlt-i13 isoform. In Western blots, the band was faint in comparison with the recombinant sFlt-1 band, which was used to indicate the right position of sFlt-1 band.

Therefore, an ELISA assay was used to investigate the sFlt-1 protein release, especially by hemin-treated hESC.

In conclusion, decidualisation reduces the sFlt-1 protein expression in both St-T1b cells and primary hESC with increases VEGF protein. 24 h decidualisation stimulation, whether by cAMP or MPA causes a reduction in sFlt-1 protein expression in St-T1b without any significant difference following treatment with them separately. hESC hemin treatment over 24 h increases sFlt-1 protein expression.

CHAPTER 7

GENERAL DISCUSSION

Embryo implantation is an essential stage in mammalian reproduction and cyclical hESC decidualisation, coordinated by ovarian steroids is a key component of this process (Nie and Dimitriadis, 2019). Aberrant decidualisation can lead to failed implantation (De Carolis *et al.*, 2019, Berkhout *et al.*, 2019), poor embryo selection (Teklenburg *et al.*, 2010a, Weimar *et al.*, 2012), pregnancy complications such as recurrent miscarriage (Medicine, 2012a), IUGR and pre-eclampsia (Leeman and Fontaine, 2008). Exploring the molecular events regulating decidualisation may identify pathways and factors that underlie endometrial-related infertility and pregnancy complications and provide novel targets for therapy.

7.1. Establishment of *in vitro* models of human embryo implantation

The study of human implantation is limited by a number of ethical and practical factors. Although both these issues obviously restrict the direct investigation of human implantation *in vivo*, the use of animal models has undoubtedly provided invaluable information but is often hampered by cost and the inherent physiological differences between species. Higher primates most closely represent the human situation, but their use has now largely been phased out on ethical grounds and due to the prohibitive costs involved. Rodent models are most frequently used with their advantage of short generation times and convenience of use. However, they cannot fully recapitulate the process of human implantation. For example, the short estrous cycle in comparison with the 28 day human menstrual cycle. Cell lines established using retroviral transduction human telomerase (Samalecos *et al.*, 2009c, Yuhki *et al.*, 2011) seem to retain more phenotypic features of the parent cells than those immortalised with SV40 large T antigen (Chapdelaine *et al.*, 2006) and hESC lines produced by both methods appear to yield lines with an appropriate progestational response (Yuhki *et al.*, 2011). In this project, the comparison of the St-T1b cells and primary hESC demonstrated that the St-T1b cell decidualisation response is very similar to primary hESC. Therefore, they represent a

convenient and viable alternative to the use of primary cells to establish *in vitro* models to investigate embryo implantation and the effects of hESC decidualisation. The use of cell lines offers an advantage in terms of consistency of response within an assay over time and the potential to adopt techniques such as CRISPR for the generation of mutant lines to examine particular aspects of gene regulation and function in the implantation process (Wyvekens *et al.*, 2015, Kinnear *et al.*, 2019, Marx, 2019)

Several *in vitro* implantation models have reported previously. The first co-culture implantation model was reported around 60 years ago (Glenister, 1961). Many models have been subsequently developed to mimic various aspects of embryo implantation (Rogers *et al.*, 1986, Ho *et al*., 2012, Bischof and Campana, 1996, Mardon *et al*., 2007, Berneau *et al*., 2019). Although similar models were developed previously (Gonzalez *et al*., 2011), significant refining modifications were introduced during this project. The result was the development of convenient, robust, and reproducible models that can be applied to study the improvement of embryo implantation. The use of fluorescent cell tracer dyes allows the opportunity to continuously monitor various aspects of implantation over 72 h and improved analysis.

To better mimic the effect of decidualisation on the invasion of HTR-8 spheroids, hESC could be seeded into the matrix to provide direct cell contact. This proved problematic when using fibrin gels because it was hard to control the initial cell distribution and prevent cell clumping and deformation of the fibrin gel with culture. Fibrin gels were used to embed spheroids for these studies as they are thought to be broadly representative of the matrix present in the decidua during trophoblast invasion (Bischof and Campana, 1996). However, they are easily degraded, which requires the addition of plasmin inhibitors, such as tranexamic acid, and limits their use in long-term culture (Cholewinski *et al*., 2009). Recently, there has been much interest in the development of 3D cell culture using new hydrogel matrices with controlled setting properties

based on materials such as modified alginate. These are more durable matrices overcoming the problems encountered with traditional gels.

The introduction of an epithelial cell monolayer would extend these models to enable examination of the initial interactions of the epithelium with ESC and trophoblasts using the HTR-8 spheroids. A model of attachment and invasion, including all three types with an epithelial monolayer overlying hESC embedded within a gel, has been reported (Wang *et al*., 2011). Recently, a new model of trophoblast invasion using a bioprinting technique has been reported (Ding *et al.*, 2019). We can incorporate the idea of this model in our co-culture through adding a 2D multi-ring model, which will form the front of HTR-8 trophoblast and can be used as an indicator of invasion and migration potential. Further expansion of our model could be made through the use of rabbit or mouse embryos instead of HTR-8 spheroids, as in the study performed by Grewal *et al* (2010) and Fischer *et al* (2012). Additionally, the effect of follicular fluid on trophoblast invasion could be examined.

7.2. Fluorescent image analysis protocols

Different analysis protocols have been previously reported to analyse images of the scratch-wound migration assay. Some depend on the manual measurement of the area between two points in the scratch or the distance between multiple points along the scratch (Abdulkareem, 2017). Manual measurement is very time consuming and can be subjective. The use of the ImageJ trainable weka plugin allows the measurement of the total scratch area rather than the measurement of the distance between multiple points along the scratch. It also carries the advantage of automated assessment of multiple fields along the scratch. Automated analysis was also possible with a fluorescently labelled spheroid expansion area without the need for manual demarcation of the trophoblast/spheroid boundaries.

To overcome the possibility of multiple-line representation of a thick trophoblast sprout in the analysis of spheroid invasion assay, the trainable weka plugin was applied as an alternative to the skeleton analysis method (Blacher *et al*., 2014, Salisbury, 2017). Automated subtraction of the spheroid core was performed, followed by thresholding and measurement of the total sprouting area. The three analytic protocols are computer-based processes performed via freely accessible ImageJ software. The protocols can be applied to image stacks saving investigator time and effort spent while analysing multiple single images. This protocol can be adopted by researchers and overcomes the difficulty of using new macros and maintaining the functionality of such macros.

7.3. The role of decidualisation on endometrial and trophoblast cell functions

The endometrium displays cyclical changes in its receptivity and these changes are the hallmark of decidualisation (Vergaro *et al*., 2019). However, the role of decidualisation in embryo invasion into the endometrium is not fully understood. Several studies have reported that decidualisation controls trophoblast invasion during implantation through different mechanisms (Sharma *et al*., 2016). They are proposed to present a physical or chemical barrier to the invading trophoblasts through the modulation of factor secretion, such as adhesion molecules and MMPs (Menkhorst *et al*., 2019). Our results indicate a reduced trophoblast sprout invasion into fibrin gel in the presence of decidualised stromal cells. Furthermore, we found an increase in the expansion of trophoblast spheroids over decidualised hESC monolayers. This may be interpreted as decidualisation first enhances the spreading of the embryo to offer more nutritional supply by simple diffusion, then restricts its further invasion. This controlled invasion may be a protective mechanism to prevent tumour-like embryo invasion, and subsequent harm to the mother, which can result from placenta accreta (Jauniaux *et al*., 2018, Miller *et al*., 1997). Although IGFBP-1, dPRL, and PDGF enhance trophoblast

invasion (Chakraborty *et al*., 2002, Stefanoska *et al*., 2013), reduced invasion was seen during decidualisation. This reduced invasion may be attributed to fibrinoid deposition by EVT during invasion (Pijnenborg *et al*., 2006, Pijnenborg and Brosens, 2010). Further measurement of factor, which might counteract the effect of IGFBP-1, can be performed, such as the investigation of α5β1, the fibronectin receptor, which binds to IGFBP-1 (Irwin and Giudice, 1998, Gu *et al*., 2019). My results showed reduced hESC migration during decidualisation, which might be attributed to inhibition of the PI3K/AKT pathway (Fabi *et al*., 2017).

7.4. The role of decidualisation on PDGF family expression

Various functions have been reported for PDGF family in the female reproductive system such as ovarian function (Carson *et al*., 1989), embryo implantation (Nie and Dimitriadis, 2019), decidualisation, trophoblast invasion, angiogenesis and infertility problems including endometriosis (Kobayashi *et al*., 2014, Simopoulou *et al*., 2019). The expression of the PDGFs and their receptors by the HTR-8 trophoblast cell line and hESC was characterised using endothelial cells as a positive control. This confirmed the expression of PDGF-AA, PDGF-CC, PDGFR-α, and PDGFR-β and the absence of PDGF-BB in hESC. HTR-8 cells expressed PDGF-AA, PDGF-BB, PDGF-CC, PDGFR-α, and PDGFR-β. As expected, both PDGFR-α and PDGFR-β were not detected in endothelial cells. These findings were in broad agreement with Lash *et al* (2011) and further support a protein array study performed previously in our lab (Ting & Hewett – unpublished results). Decidualisation with MPA and cAMP differentially effect of PDGR-α and PDGF-AA expression in hESC. Further investigation is needed to dissect which decidualisation stimulus was responsible for this change. PDGF-BB enhanced the migration of stromal cells, expansion and invasion of HTR-8 spheroids. Furthermore, this study revealed, for the first time, the upregulation of endosialin during primary hESC decidualisation. Endosialin has been shown to promote PDGF activity in pericyte cells and may enhance

PDGFR internalisation and signalling (Tomkowicz *et al.*, 2010). Further studies are required to investigate the function of endosialin in decidualisation. Endosialin knockdown experiments could be used to determine its effect in hESC in the scratch-wound migration assay in response to PDGF-BB and HTR-8 spheroid expansion assay and signalling studies. If successful, endosialin knock-out mice could be used to determine its effect following induction of decidualisation (Halhali *et al.*, 1991, Liu *et al.*, 2016).

7.5. HO-1 expression and function in hESC decidualisation and embryo trophoblast interaction

HO-1 plays a vital role in successful pregnancy (Bainbridge and Smith, 2005). Zenclussen and colleagues (2014) reported that HO-1 was upregulated in the receptive period of the estrus phase in mouse estrous cycle. In agreement with these studies we show that HO-1 expression is upregulated in hESC decidualisation *in vitro*. This may contribute in part to the oxidative stress resistance state of decidual and placental tissue (Kajihara *et al.*, 2006, Peng *et al.*, 2019) due to the steroid regulation and cAMP induction of decidualisation as confirmed on hESC treatment with cAMP and MPA individually. Hence, we showed that HO-1 upregulation during decidualisation is dependent on cAMP stimulated pathways. Here, we show that FOXO1 silencing is associated with a significant reduction of HO-1 expression. The demonstration of FOXO1-mediated up-regulation of HO-1 in hESC indicates that HO-1 should increase towards the end of the luteal phase before menstruation and therefore, may be involved in limiting the haemorrhagic endometrial damage following menstruation (Maybin and Critchley, 2012). Further research might be suggested through the use of endometrial-specific FOXO1 knockout in mice followed by an examination of putative FOXO1 transcription factor binding sites in the HO-1 promoter to further elucidate the regulatory FOXO1 role in HO-1 expression.

HO-1 is involved in embryo implantation and in limiting the hemorrhagic endometrial damage following menstruation (Maybin and Critchley, 2012). Rodriguez *et al* (2010) found that HO-1 inhibits migration in rat aortic smooth muscle cells via the inhibition of the nicotinamide adenine dinucleotide phosphate oxidase (NOX1) pathway. In agreement with these studies, we showed that hemin has an inhibitory effect on the migration of primary hESC. Bilban *et al* (2009) demonstrated a reduced HTR-8 migration in transmigration assays following HO-1 over-expression. Our results show a reduction of PDGF-BB stimulated HTR-8 cell migration on treatment with hemin. Further studies can be suggested to explore any effect of PPARγ, which is expressed in the placenta and can affect migration (Zhao *et al*., 2019).

In a study by Knöfler and Pollheimer (2012) study, trophoblasts were found to produce integrin and CXCL12 chemokines that in turn stimulate stromal cells to secrete CXCR4, which promotes trophoblast invasion (Ren *et al*., 2011). We showed that HTR-8 spheroid invasion into fibrin gels in the absence of stromal cells was not as pronounced as in the presence of hESC. Therefore, stromal cells may act as an enhancer of trophoblast invasion by the secretion of factors from hESC that promote trophoblast invasion. Ha *et al* (2015) found that HO-1 products increase HTR-8 and HUVEC invasion using a trans-well migration assay. The assay investigates the invasion of individual cell types and not as a co-culture model. However, our co-culture method showed that hemin treatment caused increased sprouting in the presence of hESC. McCaig and Lyall (2009) showed that HO-1 and HO-2 have different roles in cell invasion. Hence, we can further expand the HTR-8 spheroid sprouting assay by applying selective inhibitors of constitutive HO-2 such as analogues of 1-(4-chlorobenzyl)-2-(pyrrolidin-1-ylmethyl)-1H-benzimidazole (clemizole) (Vlahakis *et al*., 2013), for investigating the biological roles of HO-1 by its own or by *in vitro* or *in vivo* knockdown trials. Further study can be performed *in vivo* through HO-1 overexpression or knockdown to investigate the

migration and invasion of trophoblast in mice or rats, such as the study performed by Zenclussen *et al* (2003).

7.6. Regulation of VEGF and sFlt-1 by decidualisation

VEGF and its receptors play an important role in endometrial decidualisation and embryo implantation, driving the vascular and trophoblast responses (Plaisier, 2011, Goldman-Wohl and Yagel, 2002). Dysregulated VEGF and sFlt-1 expression can be seen in different reproductive problems such as the early recurrent spontaneous abortion (Pang *et al*., 2013), endometriosis (Cho *et al*., 2012), pre-eclampsia and IUGR (McKeeman *et al*., 2004). sFlt-e15a and sFlt-i13 are the two major Flt-1 splice variants that have been implicated in reproductive problems. As the decidual cells are a significant source of VEGF in the endometrium (Fan *et al*., 2014, Matsui *et al*., 2004), the regulation of its activity by sFlt-1 is likely to be critical for a healthy pregnancy. Cottrell *et al* (2017) found an upregulation of VEGF protein with a reduced level of sFlt-1 in decidualised hESC. Their findings agree with our findings of increased VEGF and reduced sFlt-1 release upon decidualisation of hESC. A study by Vasquez *et al* (2015) recently found that FOXO1 regulates VEGF in agreement with findings from our laboratory. Mazur *et al* (2015), who found that VEGF and Flt-1 are differently expressed with decidualisation in hESC. We found that the induction of decidualisation with both cAMP and MPA resulted in a significant reduction of sFlt-1 level.

7.7. HO-1 effect on sFlt-1 and VEGF expression

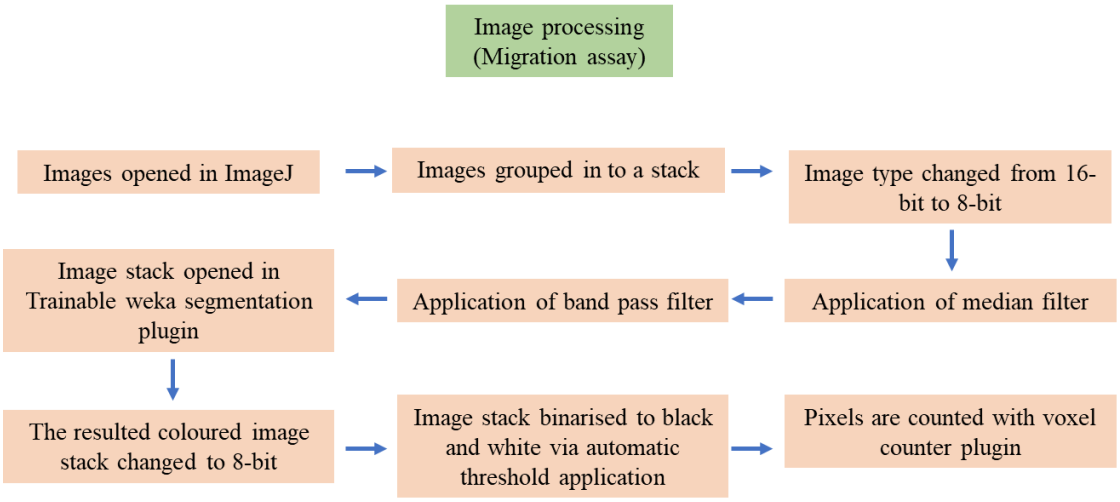
Hemin treatment of hESC shows that sFlt-1 is upregulated with treatment by HO-1 pharmacological inhibitors. The increased level of sFlt-1 may be due to the effect of HO-1 induction itself or due to the other effects of hemin on the hESC. To investigate this further,

another approach could be adopted using alternative HO-1 inducers such as Zinc protoporphyrin (ZnPP), Statins, Resveratrol, or adenoviral overexpression of HO-1 constructs.

7.8. Conclusion

The results of this PhD study contribute to our knowledge of human embryo implantation mechanisms through the establishment of *in vitro* models to evaluate the critical role of decidualisation in this limiting step of human reproduction. Essentially, the findings recognise the role of decidualisation in the expression of growth factors implicated with implantation and how the growth factor and decidualisation affect the stromal-trophoblast interaction. They identify for the first time that decidualisation increases the expression of PDGF receptors, HO-1, and endosialin in hESC. Furthermore, they confirm the concurrent findings of increased expression of VEGF and reduced sFlt-1 expression during hESC decidualisation.

APPENDICES



A

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    saturate");
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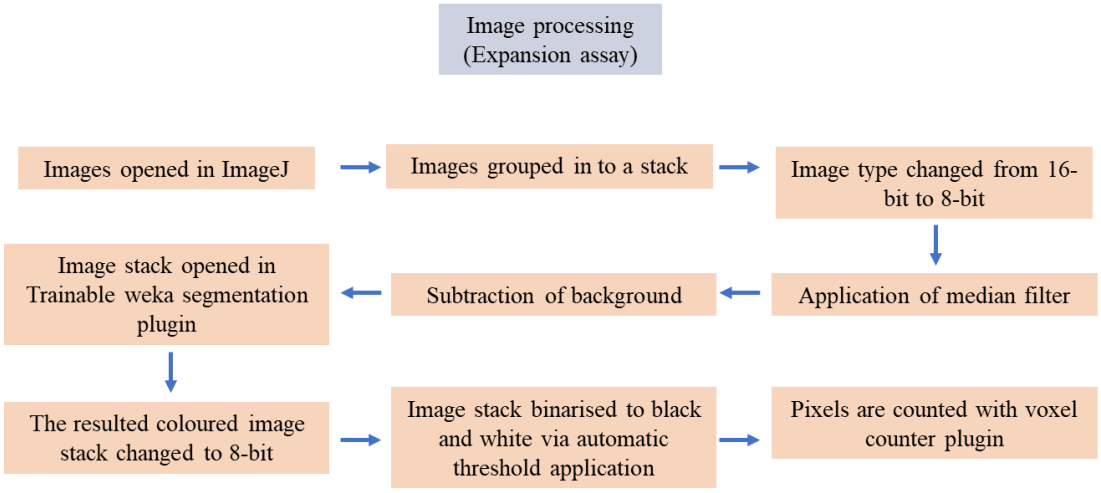
15. setAutoThreshold("Default dark");
16. //run("Threshold...");
17. setOption("BlackBackground", true);
18. run("Convert to Mask", "method=Default background=Dark black");
19. run("Voxel Counter");
```

B

Appendix 1: The image analytic protocol used for the migration assay. (**A**) A flow chart shows the processes used to measure the area of migration using ImageJ. (**B**) A macro developed in ImageJ to simplify and standardise image processing each time.



A

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11. call("trainableSegmentation.Weka_Segmentation.getResult");
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13. run("8-bit");
14. setAutoThreshold("Default dark");
15. //run("Threshold...");
16. setOption("BlackBackground", true);
17. run("Convert to Mask", "method=Default background=Dark black");
18. run("Voxel Counter");
```

B

Appendix 2: **The image analytic protocol used for the expansion assay.** (**A**) A flow chart shows the processes used to measure the area of migration using ImageJ. (**B**) A macro developed in ImageJ to simplify and standardise image processing each time.

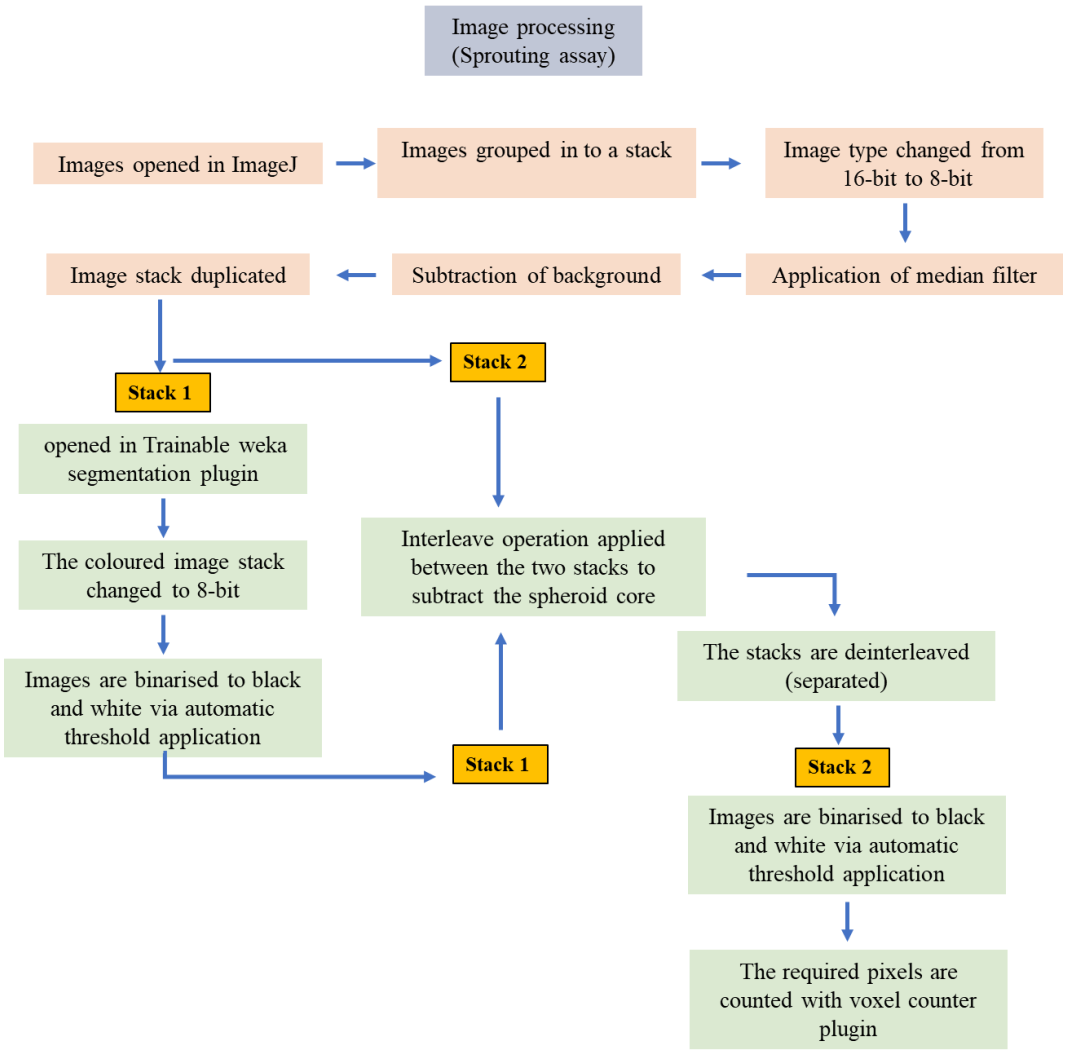
Image processing (Sprouting assay)
Images opened in ImageJ
Images grouped in to a stack
Image type changed from 16-bit to 8-bit
Application of median filter
Subtraction of background
Image stack duplicated
Stack 1
Stack 2
opened in Trainable weka segmentation plugin
The coloured image stack changed to 8-bit
Images are binarised to black and white via automatic threshold application
Stack 1
Interleave operation applied between the two stacks to subtract the spheroid core
The stacks are deinterleaved (separated)
Stack 2
Images are binarised to black and white via automatic threshold application
The required pixels are counted with voxel counter plugin

A

```
1.  selectWindow("Sprouting.png");
2.  run("Images to Stack", "name=Sprouting title=[] use");
3.  run("8-bit");
4.  run("Median...", "radius=2 stack");
5.  run("Subtract Background...", "rolling=50 stack");
6.  run("Duplicate...", "duplicate");
7.  //setTool("freeline");
8.  run("Trainable Weka Segmentation");
9.  selectWindow("Trainable Weka Segmentation v3.2.28");
10. call("trainableSegmentation.Weka_Segmentation.addTrace", "0", "1");
11. call("trainableSegmentation.Weka_Segmentation.addTrace", "1", "1");
12. call("trainableSegmentation.Weka_Segmentation.trainClassifier");
13. call("trainableSegmentation.Weka_Segmentation.getResult");
14. selectWindow("Classified image");
15. run("8-bit");
16. setAutoThreshold("Default dark");
17. //run("Threshold...");
18. run("Convert to Mask", "method=Default background=Dark black");
19. run("Interleave", "stack_1=Sprouting stack_2=[Classified image]");
20. //setTool("wand");
21. doWand(427, 372);
22. setBackgroundColor(255, 255, 255);
23. run("Clear", "slice");
24. //setTool("rectangle");
25. run("Deinterleave", "how=2");
26. selectWindow("Combined Stacks #1");
27. setAutoThreshold("Default dark");
28. //run("Threshold...");
29. setAutoThreshold("Default dark");
30. //setThreshold(69, 255);
31. run("Convert to Mask", "method=Default background=Dark black");
32. run("Voxel Counter");
```

B

Appendix 3: **The image analytic protocol used for the sprouting assay.** (**A**) A flow chart shows the processes used to measure the area of migration using ImageJ. (**B**) A macro developed in ImageJ to simplify and standardise image processing each time.

PRESENTATIONS AND POSTERS RELATED TO THIS THESIS

1. **Institute of Cardiovascular Science (ICVS) away day: Birmingham, 24th June 2016.**

   Oral presentation: Improving embryo implantation rate after assisted conception techniques.

2. **ICVS away day: Birmingham, 9 June 2017.**

   Three minutes thesis presentation.

3. **Fertility 2018 conference: Liverpool, 2018.**

   Poster presentation: *In vitro* modelling of human embryo implantation.

4. **Gordon Conference of Mammalian Reproduction: Barga-Italy, 2018.**

   Poster presentation: *In vitro* modelling of human embryo implantation.

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