Effects of Heparin on Mouse Pregnancy and Human Trophoblast Cell Lines

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Abstract

Severe preeclampsia (sPE) is a life threatening pregnancy disorder originating from the placenta. The exchange surface in the placenta is formed by a layer of syncytiotrophoblast (SCT) cells, which are formed and rejuvenated via terminal differentiation of the underlying proliferative villous cytotrophoblast (VCT) cells. The balance of VCT proliferation and differentiation is thought to be abnormal in sPE. Heparin, classically an anticoagulant drug, decreases recurrence in women at risk of sPE by 50% via unknown mechanisms. We studied the effects of heparin on proliferation and differentiation on trophoblast cells using human cell lines and mice. Heparin caused upregulated expression of $PCNA$, a proliferation marker, in choriocarcinoma BeWo cells as a model of VCT cells. Heparin in mice caused upregulated expression of suspected trophoblast progenitor markers ($Eomes, Sca1$), without altering syncytial differentiation. Hence we suspect that heparin improves pregnancy outcome by promoting VCT cell proliferation.
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<td>Antiphospholipid syndrome</td>
</tr>
<tr>
<td>AT</td>
<td>Anti-thrombin</td>
</tr>
<tr>
<td>BeWo</td>
<td>Choriocarcinoma cell line</td>
</tr>
<tr>
<td>B-HCG</td>
<td>Beta chain, human chorionic gonadotropin</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromo deoxy uridine</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep vein thrombosis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EVT</td>
<td>Extravillous trophoblast</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FIHC</td>
<td>Fluorescent immunohistochemistry</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>HTR8/SVneo</td>
<td>Extravillous trophoblast cell line</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
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<tr>
<td>LDH</td>
<td>Lactose dehydrogenase</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Preeclampsia</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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PLGF  Placental growth factor

qRT-PCR  Quantitative real-time polymerase chain reaction

SCT  Human Syncytiotrophoblast

sFLT1  Soluble fms-like tyrosine kinase-1

sPE  Severe early onset preeclampsia

S-TGC  Sinusoidal trophoblast giant cells

SynT  Mouse Syncytiotrophoblast

TS  Trophoblast stem cells

UFH  Unfractionated heparin

VCT  Villous cytotrophoblast

VEGF  Vascular endothelial growth factor
Chapter 1 Introduction
Preeclampsia (PE) is a life threatening disorder of pregnancy that currently can be reversed only by removal of the placenta and hence delivery of the fetus. PE is a multi-organ hypertensive disorder and the disease is thought to originate from the placenta since removal of it seems to be the only effective way of reversing the symptoms (Rey et al., 2009). PE is a highly heterogeneous disease associated with differential placental gene expression and disease biomarkers (Cox et al., 2011). Heparin is classically an anticoagulant drug, which has more recently been shown to have properties that are independent of its anticoagulant properties (Rey et al., 2009). Heparin treatment in pregnancy has been shown to result in 50% reduction in the recurrence rate for patients with prior history of severe PE (Rodger et al., 2013). The beneficial effects of heparin treatment with respect to reducing the rate of recurrence of severe PE are independent of this drug’s anticoagulant properties (Drewlo et al., 2010). As a result, heparin is thought to have other properties are suspected to be associated with its beneficial effects in improving the pregnancy outcome in humans. The mechanisms by which heparin improves the pregnancy outcome are currently unknown. Understanding the mechanisms by which heparin carries out its functions are of great importance since the use of heparin treatment in obstetrics is associated with potentially serious side effects, all of which are caused by the anticoagulation properties of heparin (Kingdom et al., 2011). Hence, shedding light on the anticoagulant independent properties of heparin responsible for reducing the rate of recurrence of severe PE would result in a better understanding of the underlying pathology of PE, and ultimately the development of novel therapeutic approaches for the treatment of this prevalent, and potentially fatal disorder of pregnancy.

The structure of the placenta from mothers with sPE is abnormal (Benirschke, 2006). During human pregnancy, the exchange region between the maternal and fetal blood is formed by a continuous layer of syncytiotrophoblast (SCT) cells. The SCT cell layer is rejuvenated by terminal differentiation of the underlying proliferative villous cytotrophoblast (VCT) cells (Kingdom et al., 2011). In PE, the SCT cell layer has structural and functional abnormalities and the underlying pool of VCT cells is suspected to be depleted (Macara et al., 1996). There are certain lines of evidence suggesting the potential role of heparin with respect to altering trophoblast cell proliferation (Kingdom and Drewlo, 2011). This thesis is aimed at examining the effects of heparin treatment with respect to trophoblast cell proliferation and differentiation in
established human trophoblast cell lines as an in vitro model, along with mice as an in vivo model.

Human cell lines were chosen in order to study the effects of heparin treatment on a homogeneous placental trophoblast cell lines in isolation without the contributions of other factors present in an in vivo model, which could potentially skew the results. On the other hand, mice were used for the second part of this study due to the similarities present between the mouse and the human placenta, and also in order to serve as a physiologically relevant in vivo model yet allowing experimental manipulation aimed at studying the effects of heparin treatment in pregnancy. Understanding how heparin carries out its functions with respect to trophoblast cell proliferation and differentiation in the placenta would lead the way to the development of novel therapeutic approaches for prevention, and treatment of PE.
1.1 Healthy pregnancy in humans and mice

Pregnancy is a non-permanent physiological state associated with a wide variety of changes and adaptations in both the mother and the fetus during which one or more embryos form and develop thanks to a transient organ called the placenta. Formation of the placenta, a transient organ of fetal origin, is essential for the survival of the fetus. The placenta is responsible for bringing the maternal and fetal blood compartments into close proximity to allow the exchange of oxygen and nutrients while transporting metabolic by-products away from fetal circulation. The placenta acts as an immunological barrier during pregnancy by protecting the fetus against the maternal immune system as well as providing the fetus with immunological privileges by supplying the fetus with maternal immunoglobulins (Malek, 2013). Moreover, the placenta acts as a substance barrier, and is responsible for production of various endocrine factors, which have wide effects on both the fetus and the mother and are essential for a successful pregnancy (Myatt, 2002). Proper placental function is necessary for normal maternal adaptation to pregnancy and normal fetal growth and development.

1.2 Hypertensive pregnancy disorders

Normal pregnancy is associated with increases of around 40% in the maternal cardiac output and blood volume. This elevation in blood volume and cardiac output does not result in elevated maternal blood pressure since systemic resistance goes down significantly starting from the first trimester (Abbas et al., 2005). The normal vasodilation present in the uteroplacental arteries is a major contributor to sufficient blood flow into the placenta to support the needs of the developing fetus in a healthy pregnancy (Abbas et al., 2005). Maternal hypertension in pregnancy can ensue and co-exist in certain cases where the hemodynamic adaptation processes are thought to be abnormal. Hypertensive disorders of pregnancy are gestational hypertension, chronic hypertension, PE, and concurrent chronic hypertension with PE (Chandiramani and Shennan, 2008).

1.2.1 Preeclampsia (PE)

1.2.1.1 Features and symptoms

Severe early onset PE (sPE) is clinically defined as having any of the following: hypertension (above 160/110 mmHg) measured at least 4 hours apart, platelet depletion
(thrombocytopenia), renal insufficiency, impaired liver function, pulmonary edema, or cerebral and visual disturbances. PE affects 5% of all pregnancies globally and is regarded as a major cause of fetal and maternal mortality and morbidity (Arulkumaran and Lightstone, 2013). sPE is a multi-organ disorder that affects the placenta, kidneys, liver and the brain. Placental pathology of sPE will be explored in detail later (1.4.4). In the kidneys, endothelial dysfunction associated with sPE results in abnormal glomerular function leading to presence of protein in the urine known as proteinuria (Franco et al., 2011). In the liver, underperfusion and ischemia is thought to be associated with elevated liver enzyme levels, hemolysis, and low platelets, which are collectively known as the HELLP syndrome (Mammaro et al., 2009). The most severe stage of PE is called eclampsia during which the mother experiences cerebral ischemia that can potentially give way to seizures, coma and death (Arulkumaran and Lightstone, 2013). Due to the severity of the maternal signs, preterm delivery is unpredictably necessary in many of the cases via Cesarean section, which in turn increases preterm related mortality and morbidity in the fetus (Sibai, 2011).

1.2.1.2 Interventions

The life-threatening signs, along with the high prevalence of this disease and the burden it puts on healthcare have long prompted wide investigation into therapeutic and preventative approaches. Unfortunately, the only effective method for reversing the disease symptoms in women suffering from the disease is removal of the placenta. Over two decades of interventions aimed at reducing the disease risk have been unsuccessful (Dodd et al., 2013). The majority of the attempted interventions are oral administration of supplements such as calcium, antioxidants, and fish oils due to the yet undetermined underlying pathology of this disease (Basaran et al., 2010; Bujold et al., 2010; Hofmeyr et al., 2006; Zhou et al., 2012). Administration of low-dose Aspirin (81 mg/day) has shown to be of benefit for reducing the risk of developing PE only if treatment is commenced before 16 weeks of gestation (Bujold et al., 2010). Nonetheless, aspirin has not been shown to improve perinatal mortality and morbidity associated with PE to this date (Dodd et al., 2013). Despite the previous clinical interventions that have largely been unsuccessful, treating women at risk of sPE with low molecular weight heparin (LMWH) has been shown to reduce the risk of recurrence of sPE by 50% (Rodger et al., 2013). The aforementioned meta-analysis carried out for six controlled trials where the incidence of sPE
recurrence in ~900 women at risk of recurrent pregnancy complications were observed to drop by half (Rodger et al., 2013).

1.3 Heparin

1.3.1 Structure and pharmacology

Heparin is a complex macromolecule with repeating sulphated saccharide units reaching molecular weights in excess of 15 KDa (Linhardt and Gunay, 1999). Heparin is isolated from animal mucosa, and the non-modified heparin has 200 to 300 saccharide units. Anti-thrombin (AT) binding sites present on this sulphated polysaccharide are responsible for the anti-coagulant properties of this drug. The AT binding sites on heparin bind to anti-thrombin and result in structural changes in anti-thrombin. Altered structure of anti-thrombin in turn prevents the activation of the intrinsic and extrinsic coagulation cascades (Drewlo et al., 2010). Non-modified unfractionated heparin (UFH) consists of polysaccharide chains ranging from 12-15 kDa whereas the enzymatically modified low molecular weight heparin (LMWH) contains polysaccharides with the molecular weight range of 3-6 kDa (Fig. 1) (Sasisekharan and Venkataraman, 2000).
Figure 1: Molecular structure of heparin. The picture shows the simplified molecular structure of heparin’s sulphated polysaccharide chain. The anti-thrombin binding site (AT-bs) is responsible for the anticoagulant actions of heparin. The AT-bs is a pentasaccharide repeating unit within the macro structure of heparin. The structure of the AT-bs can be altered in various ways by removing the sulphate groups responsible for the anti-coagulant properties of heparin, or removing the AT-bs altogether resulting in a species of heparin that lacks the anti-coagulant properties. Removal of the underlined residue results in species of heparin without anti-coagulant properties known as “glycol-split” heparin.

UFH is administered via IV infusion clinically due to the short half-life of this variant of heparin, as well as a less predictable clinical dose-response associated with it (Greer et al., 2014). LMWH has a half-life of 4-5 hours, along with ideal subcutaneous absorption which makes subcutaneous administration of this drug possible (Toyoda, 2013).

As a result of being a complex macromolecule the structure of heparin can be modified in various ways. An example of such modification is glycol-split heparin, which can be obtained by removing the AT binding sites from the original structure of heparin. By removing the AT binding sites from the polysaccharide structure, heparin loses its anti-coagulant ability and further mimics the structure heparan sulfates found on the extracellular matrix of cells (Kingdom and Drewlo, 2011; Page, 2013; Toyoda, 2013). A species of heparin without any anti-coagulant
activity would be ideal for studying the non-coagulative actions of this drug (Casu et al., 2008). Nonetheless, non-anticoagulant variations of heparin have not yet been verified or approved for clinical use, and their production is still a costly process.

1.3.2 Heparin in obstetrics

Heparin has been known to be an anticoagulant drug for a long time. Classically used as an anti-coagulant, heparin as a result has long been administered to pregnant women at risk for various pregnancy-related disorders (Bates et al., 2008; D'Ippolito et al., 2011; Hirsh J, 1970). Due to its anticoagulant properties, heparin has long been administered to women suffering from hypercoagulability disorders such as deep vein thrombosis (DVT), antiphospholipid (APS) syndrome, factor V Leiden mutation etc (D'Ippolito et al., 2011). However, during the past decade heparin has been shown to improve pregnancy outcome in women who do not suffer from coagulation disorders. Namely, with respect to PE heparin has been shown to reduce the rate of recurrence of sPE by 50% (Rodger et al., 2013). Moreover, LMWH treatment during pregnancy in clinical trials has also resulted in reduced perinatal mortality, preterm birth before 34 and 37 weeks, and infant birthweight below 10th centile for gestational age (Rodger et al., 2013). The aforementioned effects of heparin treatment are thought to be independent of the anticoagulant properties of this drug even though the underlying mechanisms by which heparin carries out its actions are yet not understood (Greer et al., 2014).

Use of LMWH is preferred over UFH in obstetrics because of higher bioavailability, longer half-life allowing daily subcutaneous injections as opposed to continuous IV infusions, and a predictable dose-response curve which allows for treatment based on body weight (Hirsh, 2001). Furthermore, use of heparin is favored over other anti-coagulants (such as Warfarin) during pregnancy since unlike Warfarin, LMWH does not pass the blood-placental barrier, and therefore does not pose any teratogenic risks on the fetus (Hirsh J, 1970).

Despite its beneficial actions in improving pregnancy outcome, use of heparin in obstetrics is associated with certain side effects all of which are caused by the anticoagulation properties of this drug. These side effects include accelerated bone loss which increases the risk of vertebral fractures, increased risk of heparin-induced low platelet count, as well as making the use of epidural pain relief possible only 24 hours after the last LMWH injection (Dodd et al., 2013). In women at risk of sPE, the patients who benefit from heparin treatment the most, the
side effects are compounded. Namely, they are at risk of postoperative hematoma after the usually indicated and unpredictable Caesarian section, and they have to undergo general anesthesia during the procedure since an epidural block cannot be used for this patient population (Dodd et al., 2013). PE is thought to originate from abnormal, and insufficient placental function and development and as a result studying the effects of heparin treatment on the organ of interest, namely placenta, will result in better understanding of the underlying mechanisms by which heparin improves the pregnancy outcome independent of its anticoagulation properties.

1.4 Placenta

1.4.1 Normal placental function in humans and mice

The placenta is a transient organ of fetal origin, which allows for the growth and nourishment of the fetus throughout gestation via interaction with the mother while keeping the mother healthy in both humans and mice. The dynamic exchanges and interactions between the mother and the fetus in the placenta occur through regions referred to as the maternal-fetal interfaces. The placenta, as a result, is responsible for the exchange of oxygen and active and passive transport of nutrients into the fetal circulation, while eliminating metabolic by-products from the fetal circulation (Evain-Brion and Malassine, 2003; Sibley et al., 1998). Placenta also has pivotal functions regarding evading the maternal immune response during pregnancy (Hemberger, 2013). Moreover, the placenta has an important role of acting as an endocrine organ during pregnancy by producing various hormones such as steroid hormones, placenta-like growth factor (PLGF), placental lactogens, and human chorionic gonadotropin (HCG) etc (Evain-Brion and Malassine, 2003). Understanding the structural features of the human placenta is necessary for proper interpretation of placental pathology present in PE. More detailed information regarding the structural, and functional features of the human and mouse placenta is as follows:

1.4.2 Human placenta

The human placenta can be divided into three distinct anatomical regions, each of which has a distinct physiological function associated with it.

Starting from the maternal side, the decidua basalis is formed via invasion of the fetal-derived trophoblast cells into the maternal endometrial tissue bordering the implantation region.
This establishment of both maternal and fetal cells, along with the immediate layer of myometrium (1/3 of it) adjacent to the decidua basalis is termed the placental bed in humans (Brosens et al., 1967). The basal plate borders the placental bed from the maternal side and the villous region on the other side, and (see below) it is formed from fetal-derived trophoblast cells. The placental bed contains channels that are formed by fetal trophoblast cells that direct the maternal blood towards the exchange region of the placenta where the maternal circulation comes into close proximity with fetal circulation (Brosens et al., 1967). The exchange region of the placenta is known as the villous region in humans (Georgiades et al., 2002). The exchange between the maternal and fetal circulations occurs in this region of the placenta by means of blood circulating though separate maternal blood channels outlined by fetal trophoblast cells, as well as the fetal blood channels outlined by endothelial cells. As a result of this setup, maternal and fetal blood compartments come into close proximity to one another but never mix. The umbilical cord (collection of arteries and veins via which the fetal blood is brought to, and leaves the placenta) connects to the fetal surface of the villous region. Maternal blood enters the villous region from the opposite side (maternal side) by means of utero placental circulation (Georgiades et al., 2002) (Fig. 2). The region of exchange within the placenta, namely the villous region, is though to be involved in the pathogenesis of PE due to the abnormal formation of this region, which is suspected to be the caused by abnormal spiral artery remodeling and shallow EVT invasion (Kingdom and Drewlo, 2011). The villous region is of interest for the purpose of this study.
Figure 2: Simple representation of mature mouse and human placentas. The analogous decidual regions of the mouse and human placenta are shown. The basal plate of the human placenta and the junctional zone in mice are similar in the sense that both contain conduits for the transfer of the maternal blood into and out of the exchange region. The exchange region of the placenta is known as the labyrinth and villous region in mice and humans respectively. (Rai and Cross, 2014).\(^1\)

\(^1\) Reprinted from Developmental Biology. Volume 387. Rai, A., and Cross, J.C."Development of the hemochorial maternal vascular spaces in the placenta through endothelial and vasculogenic mimicry". Pg.132. (2014), with permission from ACADEMIC PRESS.
1.4.2.1 Human placental trophoblast cell subtypes

Trophoblast cells are a key component of the placenta, and their maintenance and proper functioning is needed for normal placental physiology during gestation. There are two major trophoblast lineages in human placentation, and each has their own distinct functions and characteristics. These two lineages are extravillous trophoblast (EVT), and villous cytotrophoblast (VCT). EVT cells are responsible for invasion into the placental bed during human placentation. EVT cells are also responsible for the process of spiral artery remodeling, and altering the maternal immune response to the fetal-derived cells of the placenta (Benirschke, 2006). VCT cells are responsible for the formation of the exchange structures within the villous region namely the floating placental villi, which as the name suggests project into the intervillous space and come into direct contact with maternal blood in the villous region (Benirschke, 1998) (Fig. 2). The maternal and fetal blood compartments come into close proximity to each other at the regions of the placental villi known as terminal villi. More detailed description of each trophoblast cell lineage is as follows:

1.4.2.1.1 Extravillous trophoblast (EVT) lineage

EVT cell lineage is derived from the anchoring villi (column of placental villi that mechanically stabilize the maternal-fetal interface), and they are themselves divided into two subtypes with respect to their phenotype and functions. The EVT cells derived from the basal side of the anchoring villi columns display a proliferative phenotype whereas the EVT cells located at the other end of the anchoring villi have an invasive phenotype (Chaddha et al., 2004). As a result, EVT cells invade the decidual and myometrial region in a tightly controlled manner while maintaining their numbers to establish the connection of the villous region with the maternal side. Thereafter EVT cells modulate many critical processes of pregnancy such as immunological processes in the placental micro and macro environment, remodeling of the spiral arteries allowing undisrupted and ample blood supply to the placenta hence the fetus, and maintenance of pregnancy (Kadyrov et al., 2013).

1.4.2.1.2 Villous cytotrophoblast (VCT) lineage

The VCT cell lineage is responsible for establishing the exchange region of the placenta within the villous region. Maternal blood in the villous region comes into direct contact with a continuous layer of syncytiotrophoblast (SCT) cells that arise from the VCT lineage throughout
pregnancy in the human placenta. VCT cells are situated underneath the continuous layer of SCT cells on a basal lamina surface that separates them from the stromal compartment of the placental villi. The VCT cells play a pivotal role in the formation, and maintenance of the trophoblast compartment in the exchange region of the placenta. The VCT cell population undergoes two major physiological processes during gestation (Benirschke, 2006). Early in placentation, VCT cells mostly undergo symmetrical rounds of mitosis in order to expand the population of the VCT cells (Baczyk et al., 2006). Later on in pregnancy and for the rest of human gestation, the VCT cells switch from symmetrical proliferation into a bimodal state of asymmetrical proliferation. Namely, the VCT cells undergo terminal differentiation and fusion in order to form the outer continuous SCT cell layer while a subset of VCT cells retain their progenitor-like state of proliferation in order to accommodate the growing villous exchange surface area, as well as rejuvenating the existent layer of SCT cells throughout pregnancy (Baczyk et al., 2006; Benirschke, 2006).

In the human placenta, the biomodal asymmetric phase of VCT cells is regulated by the trophoblast transcription factor glial cell missing-1 (GCM1) (Baczyk et al., 2006). Acting as the master regulator of the asymmetric proliferation state of VCT cells, GCM1 in turn promotes the transcription of syncytin-1 (SYN1). Syncytin-1 is a fusogenic protein, which is responsible for the terminal fusion and formation of the SCT cell layer (Frendo et al., 2003). Once terminal differentiation and fusion has taken place, the beta subunit of human chorionic gonadotropin (β-HCG) within the placenta is expressed solely by the terminally differentiated and fused SCT cell layer. Moreover, the level of released β-HCG is dependent upon the level of syncytial differentiation since β-HCG is secreted after trophoblastic fusion (Benirschke, 2006; Hoshina et al., 1984).

Overall, the VCT cells can go through a series of events, which are proliferation, differentiation, apoptosis and shedding. The aforementioned series of events are collectively known as trophoblast turnover.

1.4.3 Mouse placenta

Similar to the human placenta, the mouse placenta can also be viewed as being comprised of three distinct regions with respect to anatomy and physiological function. First, starting from the maternal side the mouse decidua basalis is located adjacent to the maternal myometrium.
Similar to the human placenta, fetal trophoblast cells invade this region yet unlike humans trophoblast invasion does not continue into the myometrium as seen in the human placental bed (Brosens et al., 1967). Decidual region of the mouse is considered to be analogous to the human decidual region with respect to its relative location (Muntener and Hsu, 1977). Second, the placental junctional zone is situated between the decidual and the exchange region of the placenta in mice (Muntener and Hsu, 1977). Similar to the basal plate of the human placenta, the junctional zone does not contain any fetal blood. Instead the junctional zone contains arteries and veins derived from the fetal-derived trophoblast cells that are continuous with the channels found in the decidual region of the placenta that transport maternal blood in and out of the exchange region (Cross, 2000; Georgiades et al., 2002). Third and last, the exchange region in the mouse placenta is known as the labyrinth, which is equivalent to the villous region in humans.

### 1.4.3.1 Placental labyrinth

Placental labyrinth in the mouse starts forming roughly halfway into gestation at E9.0. The events leading to the formation of the labyrinth region become pronounced starting at 7.5 days into gestation where small clusters of cells located on the chorion start expressing Gcm1 (mouse analogue of human GCM1) (Basyuk et al., 1999). By E9.0 after an elaborate series of events Gcm1 is exclusively expressed in the cells that are located at the tip of the growing trophoblast cell column. Gcm1 is thought to be the driver in a process known as branching morphogenesis, which marks the formation of the placental labyrinth (Cross, 2000). Starting from the maternal side of the mature mouse placenta, maternal blood in the placental labyrinth region of the mouse comes into contact with mononuclear sinusoidal trophoblast giant cells (S-TGC) present in the maternal sinuses, as well as layer I of the syncytial bi-layer. Syncytial layer I (SynT-I) in the mouse labyrinth is underlined by a second ultrastructurally distinct layer of continuous syncytium known as syncytial layer II (SynT-II) (Simmons et al., 2008a). The two syncytial layers constitute a continuous exchange surface above the fetal endothelium in the mouse placental labyrinth, and one basement membrane is situated between SynT-II and fetal endothelium in the mouse placenta (Georgiades et al., 2002). As a result, the exchange region in the mouse placenta is different from the humans in the sense that the barrier to exchange is formed by two layer of syncytium in the mouse as opposed to the single layer seen in humans. Moreover, mice have an additional cell type, namely the S-TGC cells, in direct contact with...
maternal blood (Fig. 3). The trophoblast cell subtypes of the labyrinth region will be discussed in more detail next.

Figure 3: Structural representations of the mouse placenta at different times during gestation. The exchange region within the mouse placenta. The trilaminar structure of the labyrinth region at the microscopic level is shown. The structure consists of sinusoidal trophoblast giant cells (S-TGCs) which are in contact with maternal blood (m), two layers of syncytium (SynTI, SynT-II), and finally fetal endothelium (Endo) containing fetal red blood cells (Frbc). Reproduced with permission from the Journal of Embryology and Experimental Morphology (Simmons et al., 2008a).

1.4.3.1.1 Syncytial layers I and II

In the mature mouse placental labyrinth, Gcm1 is only expressed in the SynT-II layer and it directs the expression of syncytin-b (Synb), and CCAAT/ enhancer binding protein alpha (Cebpa) in that layer (Simmons et al., 2008a). The expression of those three aforementioned genes is tightly restricted to the SynT-II layer since none of them are seen in SynT-I layer. Syncytin-a (Syna) expression is restricted to the SynT-I layer and it is not yet established as to what factors direct the expression of Syna in the SynT-I cell layer of the mouse placenta. Nevertheless, recent literature points towards the involvement of EPCAM positive epithelial progenitor-like cells in the formation of the SynT-I layer which will be discussed in more detail later (1.4.3.1.3) (Ueno et al., 2013).
1.4.3.1.2 Sinusoidal trophoblast giant cells (S-TGC)

S-TGCs are the only subtype of trophoblast giant cells found in the labyrinth area of the mouse placenta. They are situated inside the maternal blood lacunae and hence in direct contact with maternal blood. S-TGCs can be distinguished from other trophoblast cell types in the labyrinth by means of expressing cathepsin-q (Ctsq), and prolactin-II (Prl3b1) (Simmons and Cross, 2005). Starting at E11.5, Ctsq is expressed by the S-TGC cells for the rest of gestation. Prl3b1 expression starts at E12.5 in the S-TGC cell population, and continues to be expressed for the rest of gestation like Ctsq (Simmons et al., 2007; Simmons et al., 2008b).

1.4.3.1.3 Trophoblast progenitor cells

In the mouse placenta, the trophoblast stem (TS) cell population is epithelial in nature and it completely disappears by E8.5, a day after Gcm1 is first detected on the chorion (Uy et al., 2002). Caudal type homeobox 2 (Cdx2), eomesodermin homobox (Eomes), and estrogen related receptor beta (Esrrb) are expressed by the TS cells that give rise to the chorionic region which in turn are thought to later give rise to the labyrinthine trophoblast subtypes (Simmons and Cross, 2005). The TS cell population in the mouse placenta is fibroblast growth factor 4 (Fgf4)-dependent, and is maintained through activation of Erk1/2 MAP kinases downstream of the FGF4 receptor (FGFR2 IIIc) (Ornitz and Itoh, 2001; Tanaka et al., 1998; Xu et al., 1998). However, the identity of the precursors giving rise to the growing population of labyrinthine trophoblast cells once the labyrinth starts forming around E9.0 remain unidentified. Unlike in humans where the population of trophoblast progenitor cells is maintained for the duration of gestation, mice experience a considerable loss in the number of those proliferative trophoblast cells by 14.5 days into gestation (Fig. 4) (Chen et al., 2004; Iguchi et al., 1993).
Figure 4: Percentage of proliferative mouse placental cells during gestation. MMD; Decidual cells on the mesometrial side. AMMD; decidual cells located on the antimesometrial side. D; placental decidual cells. EPC; ectoplacental cells. L; labyrinthine cells. ST; spongiotrophoblasts. The mitotic index of labyrinthine cells decreases by more than 80% from E10.5 to E14.5. Reprinted with permission from the Society for the Study of Reproduction (Iguchi et al., 1993).

Structurally speaking, there are clusters of cells scattered throughout the placental labyrinth area that do not belong to the aforementioned well-characterized three trophoblast cell types (Simmons and Cross, 2005). Moreover Ehomox, another TS cell marker seen at E6.5, as well Eomes are seen as being expressed in cell clusters in the labyrinth region in the mature mouse placenta (Wu et al., 2003) (Jackson et al., 2003). Interestingly, Cdx2 expression re-appears in the placental labyrinth region around E12.5 after it disappears after the depletion of TS cells at E8.5 (Beck et al., 1995; Tanaka et al., 1998). The most recent endeavours to characterize the trophoblast progenitor cells point toward the involvement of hepatocyte growth factor (Hgf)-dependent subset of EPCAM positive cells that are maintained through C-Met signaling and can give rise to all the three trophoblast cell subtypes in the labyrinth (Ueno et al., 2013). It is very important to mention that none of the recent findings are inconsistent with, or contradict the involvement of other suspected factors (Eomes, Ehomox, Cdx2, Sca1) in those trophoblast progenitor cells.
1.4.4  Placental pathology in preeclampsia

1.4.4.1  Gross and histological abnormalities

There are certain differences in the gross, and histological features of the placenta from mothers with PE when compared to their healthy counterparts. The placentas from preeclamptic patients are generally smaller for gestational age with almost 70% of the placentas from mothers with PE, and/or IUGR fall below the 10\textsuperscript{th} centile for weight (Kingdom et al., 2011; Walker et al., 2012). Moreover, 65% of PE placentas have numerous sites of intervillous infarcts, which are defined as necrosis of all the cells involved in the region (trophoblast, stroma, blood vessels etc) secondary to the obstruction of blood supply to the region (Benirschke, 2006).

Histologically speaking, roughly half of placentas from mothers suffering from sPE have shallow invasion of EVT cells into the maternal decidual and outermost third of the myometrium seen microscopically as spiral artery smooth muscle hypertrophy. Shallow invasion, and lack of normal spiral artery remodeling processes is thought to be responsible for abnormal remodeling of the spiral arteries leading to decidual, and possibly whole placental pathology seen in PE (Viero et al., 2004). When it comes to the exchange region of the placenta, the villous structure of PE placentas has very evident abnormalities. Namely, there are sites of excessive accumulation of syncytial cells known as syncytial knots (Kingdom and Drewlo, 2011; Kingdom et al., 2011). This abnormal aggregation of cells is associated with excessive tissue apoptosis and protein shedding into maternal blood by these syncytial knots as necrotic tissue. Moreover, these knots are transcriptionally active and are thought to be responsible for the secretion of various proteins involved in the pathogenesis of PE, which will be discussed later (1.4.5). Furthermore, there seems to be depletion of VCT cells underlying the abnormally formed syncytial knots in placentas affected with PE (Fig. 5) (Macara et al., 1996).
Figure 5: Abnormal fetal maternal interface as seen in preeclampsia. There is a shallow and unsuccessful invasion of extravillous trophoblast cells into maternal decidua seen in placentas with preeclampsia (lower panel) compared to healthy pregnancy (upper panel). Shallow invasion of the extravillous trophoblast cells results in lack of proper spiral artery remodeling which is thought to be responsible for decreased placental perfusion and other features of preeclampsia. Reprinted by permission from Macmillan Publishers Ltd: [Placenta] (Everett and Lees, 2012).

The shallow EVT cell invasion seen in PE (Benirschke, 2006), coupled with abnormal remodeling of the maternal arteries responsible for placental blood supply are thought to result in hypoxia-reperfusion injury in the placenta. Preeclamptic placental tissue collected at term have molecular evidence of hypoxia (Soleymanlou et al., 2005). Moreover, exposure of human
placental tissue to hypoxic conditions results in expression of anti-angiogenic cytokines and factors thought to be involved in the pathogenesis of PE (Nevo et al., 2006).

1.4.4.2 Gene expression abnormalities

The observed structural and pathological features observed in PE placentas are associated with differential regulation of genes important for normal trophoblast turnover processes. Placental tissue from pregnancies affected with PE have upregulated expression of hypoxia-inducible factor 1 (Hif1α) in the SCT cell layer (Caniggia and Winter, 2002). The upregulated expression of Hif1α in the preeclamptic placentas is thought to be responsible, at least partly, for the altered trophoblast turnover processes observed in pregnancies affected with PE (Caniggia and Winter, 2002). Placental samples isolated from pregnancies affected with PE also have reduced expression of syncytial markers GCM1 and SYN1 (Chen et al., 2004; Vargas et al., 2011). Primary VCT cells isolated from women with PE have an accelerated rate of apoptosis, coupled with reduced terminal differentiation and fusion as assessed by decreased SYN1 expression, as well as reduced β-HCG secretion compared to healthy controls (Langbein et al., 2008).

1.4.5 Anti-angiogenic factors in preeclampsia

Maternal hypertension being the foremost clinical feature of PE raises the question regarding the levels of circulating angiogenic factors affecting endothelial function in maternal blood for pregnancies affected with PE. The hypoxic stress imposed on the placental villi, coupled with the altered trophoblast turnover processes in the placentas affected with PE are thought to result in upregulation of expression and secretion of soluble fms-like tyrosine kinase1 (sFLT1). sFLT1 is a soluble spliced variant of vascular endothelial growth factor receptor (VEGFR1) generated by means of alternative splicing of FLTI gene and it can bind to all of the isoforms of VEGF-A and PLGF and hence impair their respective downstream signaling pathways. Disruption of VEGF and PLGF signaling leads to disrupted maternal endothelial function and most likely contribute to the maternal endovascular dysfunction observed in PE (Maynard et al., 2003) (Ahmad et al., 2011). sFLT1 levels are elevated above the non pregnant levels even in normal pregnancy (Rosenberg et al., 2011). Nevertheless, maternal serum levels of sFLT1 can be as much as five times higher in pregnancies affected with PE compared to their healthy counterparts (Maynard et al., 2003). Moreover, the rise in circulating levels of sFLT1
precedes the onset of symptoms in pregnancies affected with PE by as much as 4 weeks (Diab et al., 2008). In animal models for PE, induced expression of sFLT1 in both rats and mice results in PE-like signs by late gestation (Bergmann et al., 2010; Murphy et al., 2010).

PLGF, a member of the VEGF family, is a pro-angiogenic factor. The circulating levels of PLGF are reduced in pregnancies affected with PE when levels are compared to gestational matched controls(Ahmad and Ahmed, 2004; Moore Simas et al., 2007). In fact, in a subset of women the reduction in maternal PLGF levels are indicative of PE pathology as early as 16 weeks in gestation when sFLT1 levels are still not elevated (Chappell et al., 2013; Powers et al., 2012).

1.5 Heparin and preeclampsia

Heparin is classically known for its anticoagulant properties, and as a result it has been administered to pregnant women at risk for PE, particularly in thrombophilia screen positive women with recurrent venous thromboembolism (Bates et al., 2008; Hirsh J, 1970). Use of LMWH in women considered to be at a high risk for developing PE is justified by the observations that those women affected with PE are at a higher risk of pro-thrombotic complications after delivery regardless of prior blood coagulation disorders (Franco et al., 2011). Moreover as it was discussed previously, placentas from pregnancies affected with PE have numerous sites of placental villous infarction, which were originally thought to be caused by blood hypercoagulability in those women (de Maat and de Groot, 2011; Moldenhauer, 2003).

Nonetheless, the notion that heparin treatment was improving pregnancy outcome in women at risk for developing PE solely via its anticoagulant properties took a turn in 2009. The novel aspects of heparin treatment came about after the clinical trial carried out by the Rey group in Montreal, which showed that LMWH also reduces the incidence of recurrent PE in thrombophilia screen negative women without any history of thromboembolism to an equal extent when compared to women with coagulation disorders (Rey et al., 2009). In fact, the human placenta even intrinsically prevents the formation of blood clots via an intrinsic self-anticoagulation mechanisms (Kingdom and Drewlo, 2011). Furthermore, placental infarcts are now known to be strongly associated with, and most likely caused by altered trophoblast turnover processes manifesting itself into abnormal placental villous formation as opposed to maternal hypercoagulability (Franco et al., 2011). Taken together, the aforementioned evidence
suggested that heparin is carrying out its beneficial actions at least partly via coagulation-independent pathways in the placenta to improve pregnancy outcomes.

1.6 Heparin-sFLT1 paradox

Interestingly, treatment with LMWH results in an increase in the maternal circulating sFLT1 levels beyond the levels seen in normal pregnancy, namely by as much as 3-4 fold similar to the levels seen in women with PE (Rosenberg et al., 2011). In vitro evidence using human placental explants shows increased release, as well as increased synthesis of sFLT1 after LMWH treatment (Drewlo et al., 2011). The same trend is seen in male CD-1 wildtype mice where heparin treatment results in increased plasma levels of sFLT1 (Searle et al., 2011). As a result, there exists a paradox between the beneficial actions of heparin in decreasing the rate of recurrent sPE despite causing an elevation in circulating sFLT1 levels.

1.7 Rationale for the study

Heparin treatment in women at risk for developing sPE is associated with a 50% reduction in the rate of recurrence in those women (Rodger et al., 2013). The decrease in the recurrence of sPE is independent of heparin’s anticoagulant properties since women without hypercoagulability disorders benefit equally from heparin treatment, and also the pathological features of placentas from sPE pregnancies are not caused by coagulation disorders (Rey et al., 2009). Heparin is a highly negatively charged macromolecule, and it is known to interact with various receptors and proteins on the cell surface yet the way heparin affects downstream signaling and cellular events is still largely unknown (Kingdom and Drewlo, 2011). The processes of proliferation and differentiation appear to be altered in the VCT cells in placentas of preeclamptic women (Benirschke, 2006). Various lines of evidence suggest that heparin might be involved in altering the balance of cell proliferation, and differentiation in both human and mouse trophoblast cells. First, heparin acts as a cofactor in FGF4 signaling by facilitating receptor dimerization and as a result augments signaling through FGF4R2 IIIc (Tanaka et al., 1998). Second, VCT cells in the explanted human placental villi proliferate more as a response to heparin and FGF4 treatment (Baczyk et al., 2006). Third, heparin is used to maintain mouse trophoblast stem cells in vitro and withdrawal of heparin results in differentiation of the
trophoblast stem cell population (Tanaka et al., 1998). Heparin treatment also paradoxically causes an elevation in the maternal circulation levels of sFLT1 (Rosenberg et al., 2011), which is an anti-angiogenic protein thought to be responsible at least partly for the endothelial dysfunction and hypertension seen in PE despite improving the pregnancy outcome in women at risk.

Taken together, we suspect that heparin can be reducing the rate of recurrence of sPE by restoring the altered balance of VCT cell in PE placentas which overrides the potentially harmful effects brought about by elevated sFLT1 levels. Hence, we decided to study the effects of heparin treatment with respect to trophoblast cell proliferation and differentiation using an in vitro, and an in vivo model.

1.8 Models used for the study

Two different cell lines were used for the in vitro part of the study in order to assess the effects of heparin treatment on trophoblast cell proliferation and terminal differentiation. HTR8/SVneo cells were used as an EVT-like cell line (Graham et al., 1993) in order to study the effects of heparin treatment with respect to trophoblast cell proliferation only. BeWo cell line was used as a model for the VCT lineage (Orendi et al., 2010) to study the effects of heparin treatment with respect to proliferation and terminal differentiation and fusion in this cell line.

Wild type mice were used for the in vivo part of the study in order to study the effects of heparin treatment on the trophoblast cells in the exchange region of mouse placenta. Mice were used to study the changes in mRNA expression of selected trophoblast cell subtype markers within the exchange region of the placenta and placental morphometry along with maternal circulating levels of angiogenic factors after heparin treatment.

1.9 Hypotheses and aims

1.9.1 Hypotheses

1) We hypothesized that heparin treatment will increase cell proliferation in the human trophoblast cell lines without directly altering syncytial differentiation.
2) We hypothesized that heparin treatment in mice will increase trophoblast cell proliferation without directly altering differentiation collectively resulting in remodeling of the placenta, as well as placental and fetal overgrowth.

1.9.2 Aims:

The following aims were carried out to test our hypotheses:

1.9.2.1 Human cell lines

- Analysis of proliferation in HTR8/SVneo cells after being treated with different doses and types of heparin

- Analysis of proliferation and syncytial differentiation in BeWo cells after being treated with different doses and types of heparin.

1.9.2.2 Mice

- Analysis of gene expression for trophoblast cell subtypes found in the placental labyrinth region of the placenta after heparin treatment.

- Analysis of gene expression of proliferation markers, as well as angiogenic factors in the labyrinth region of the placenta after heparin treatment.

- Analysis of maternal circulating factors of angiogenic factors after heparin treatment.

- Analysis of placental gross measures and morphometry after heparin treatment
Chapter 2 Human placental cell lines
2.1 Overview and hypothesis

This section of the project was aimed at determining the effects of heparin treatment on proliferation and differentiation of established human placental cell lines.

We hypothesized that heparin treatment will increase cell proliferation in the human trophoblast cell lines without directly altering syncytial differentiation.

The effects were studied using both unfractionated (LEO® Pharma), and the more clinically prevalent and potent low molecular weight heparin (LMWH) (Enoxaparin®). HTR8/SVneo cells were used to study the effects of heparin treatment on an extravillous trophoblast-like cell line incapable of differentiation, whereas BeWo cells were used as a model for studying the effects of heparin treatment on a fusogenic choriocarcinoma cell line. The BeWo cell line more closely mimics the bi-potential behavior of the cytotrophoblast cell population in the human placenta due to its proliferative and differentiating potential. Proliferation of HTR8/SVneo cells was assessed via direct cell counting. Proliferation of BeWo cells was studied using indirect proliferation assays because their fusion is a confounding factor, as well as by assessing proliferation marker mRNA expression. Terminal differentiation and fusion in the BeWo cells was assessed by measuring the gene expression of differentiation factors at the mRNA level, as well as measuring the protein content of differentiated trophoblast markers in the cell media under different treatment conditions.

2.2 Rationale

HTR8/SVneo cells are immortalized first trimester human trophoblast cells capable of proliferation and invasion, but not fusion (Graham et al., 1993). On the other hand, BeWo cells are a human choriocarcinoma cell line capable of proliferation, as well as fusion (Orendi et al., 2010). There are various lines of evidence suggesting that heparin influences trophoblast cell proliferation. Moreover, heparin is a cofactor of FGF4 signaling through FGFR2 (Tanaka et al., 1998). Therefore, the aim of this study was to assess the effects of heparin treatment in vitro on
two trophoblast-only cell lines without the contribution of any exogenous factors contributed by the neighbouring cells, or other cells present in *in vivo* models. Human trophoblast cell lines were used for this part of the thesis in order to have a homogeneous population of trophoblast-only cells to study the trophoblast-specific effects of heparin treatment in the absence of other physiological factors involved in an *in vivo* model. The cells were treated with three different doses of LMWH and UFH to model the prophylactic equivalent (0.25 IU/ml) and the therapeutic equivalent (2.5 IU/ml) doses of heparin in the cell culture media (both of which are measured in the plasma of women treated with heparin) (Kingdom et al., 2011), along with a higher pharmacological (10 IU/ml) dose, not used clinically in humans. The higher dose was studied in order to see if the effects of heparin treatment follow a pharmacological drug-response curve.

### 2.3 Methods summary

In order to study proliferation in BeWo and HTR8/SVneo cells, as well as differentiation in BeWo cells, both cell lines were plated at a low confluency to allow longitudinal study of cells for up to 72 hours. Both cell lines were treated with different doses of unfractionated (UFH), as well as low molecular weight heparin (LMWH). HTR8/SVneo cells were only used for measurements of cell proliferation in this project. HTR8/SVneo were detached from the plate by means of trypsinization since these cells do not fuse or form clumps, and cell numbers were obtained directly by counting them with a cell counter (CASY Model TTC cell counter, Roche, Switzerland). BeWo cells undergo fusion and as a result, direct counting of the number of cells does not result in an accurate assessment of cell numbers. Instead, Fluorescent cell viability assay (Promega®) was used to obtain cell numbers under different treatment conditions. Cell number under all treatments was expressed relative to the media control (no heparin added). mRNA levels of *PCNA*, a proliferation marker, were measured in BeWo cells to verify the results obtained by the assay. Western blotting was used to study the levels of FGF4 protein in the BeWo cell media 24 hours after treatment in order to verify the possibility of FGF signaling being responsible for altered proliferative response in the BeWo cell line.

Terminal differentiation and fusion of BeWo cells was assessed via measuring the mRNA levels of differentiation markers *GCM-1* and *SYN1* after 24 hours of being exposed to different
treatment conditions, followed by the assessment of secreted free β–HCG, a marker of fused syncytium, after 48 hours of treatment using ELISA.

A protein phosphorylation array was carried out to study the changes in intracellular signaling protein phosphorylation in BeWo cells after heparin treatment. Protein from BeWo cells under different treatment conditions was extracted at 3 and 24 hours post-treatment. The extracted protein samples were used in standardized quantities suggested by the manufacturer as substrates for the Pathscan® intracellular signaling phospho-antibody array kit in order to examine the phosphorylation state of suspected intracellular proteins and signaling nodes. Moreover, conditioned media from BeWo cells collected 24 hours following treatment was concentrated using the TCA/Acetone method and later used for screening for the presence of FGF4 in the media using standard Western blotting procedures to assess the possibility of FGF4 mediated FGFR2 being responsible for changes in proliferation after heparin treatment in the BeWo cell line.

The detailed methodology used is as follows:

2.3.1 HTR8/SVneo cell line

The HTR8/SVneo cell line was only used for direct cell counting for measurement of cell proliferation after heparin treatment in this study. This cell line, a gift from Dr. Graham (Queen’s university, Kingston, Ontario), mimics the characteristics of extravillous trophoblast cells of the placenta (Graham et al., 1993). These cells have a rapid proliferation rate and are incapable of undergoing terminal differentiation and fusion. They were adopted as a model for studying the effects of heparin treatment on trophoblast cell proliferation due to their highly-proliferative nature and also since they lack the syncytial differentiation axis. No other measurements rather than cell proliferation were done in HTR8/SVneo cells since they are an extra-villous trophoblast model rather than a more suitable cytotrophoblast model for this study. Hence, they were used as a trophoblast cell line to study the effects of heparin on trophoblast cell proliferation. Altogether, HTR8/SVneo cells were initially used to see if heparin affects proliferation differentially in trophoblast cells of different origin and function in the human placenta.
2.3.2 BeWo cell line

The BeWo cell line was used to study both cell proliferation, as well as terminal differentiation, after heparin treatment. The BeWo cell line is an established choriocarcinoma cell line capable of differentiation and terminal fusion. Hence, the physiology of BeWo cells is similar to the villous cytrophoblast cells of the human placenta, which makes them a great model for studying the processes affecting trophoblast turnover due to the bi-potential nature of this cell line. The cells were fingerprinted at the Centre for Applied Genomics (SickKids, Toronto, ON, Canada) for verification purposes before initiating the experiments. The cells were cultured in F12K medium at 37°C at 20% O2 (Wiset Inc, QC, Canada) supplemented with 10% Fetal Bovine Serum (Heat-inactivated, Invitrogen®). Moreover, the cell media was supplemented with 100 µg/mL Streptomycin and 100 U/mL Penicillin along with L-glutamine at a final concentration of 2 nM. The cells were followed longitudinally as opposed to making the measurements at a specific time point since fusion happens more readily in this cell line after the cells have grown into a denser layer on the plate. We were interested in examining the effects of heparin with respect to both proliferation and differentiation, and hence followed the cells over a 72-hour period.

2.4 Proliferation assays

2.4.1 Cell counting for HTR8/Svneo cells

HTR8/SVneo cells were plated at the density of $10^5$ cells per well (~30% confluence) in 24-well plates to allow for further growth over 72 hours. Cells were either treated with heparin at the time of seeding to rule out the potential effects of heparin with respect to cell attachment, or left overnight to attach and treated the next day with varying doses of UFH and LMWH to study the effects of heparin on cell proliferation once the cells have attached to the plate and have started their normal growth. Cell numbers were measured directly by detaching the cells via trypsinization of the cells from the wells, followed by counting the suspended intact cells directly using the cell counting instrument as suggested by the manufacturer (CASY Technology, Switzerland). Cell numbers for each well were measured every 24 hours for a total duration of 3 days (72 hours) in order to study the effects of heparin treatment on cell proliferation in the
HTR8/SVneo cell line. Longitudinal measurements of cell numbers were taken by means of culturing cells in equal numbers in three separate plates, and measuring cell numbers of each plate every 24 hours. Each plate used for cell measurements were then discarded and hence the cells were not plated again after trypsination, and cell number measurements. The aforementioned interventions were taken in order to allow for longitudinal comparison with the BeWo cell line.

2.4.2 Relative cell numbers for cultured BeWo cells

BeWo cells were used as a model of villous cytotrophoblasts in order to study the effects of heparin treatment on proliferation of this trophoblast cell subtype. The BeWo cells were plated at a density of $10^4$ cells per well (~40% confluence) in a 96-well plate (to be compatible with plate fluorescent plate readers), and left to attach to the plate overnight before being treated with different doses and types of heparin (LMWH and UFH) the following day. A relatively low confluence level was chosen initially to allow for growth of the cells over 72 hours and minimize premature terminal fusion and cell death. Cell number was then indirectly assessed every 24 hours by means of measuring intracellular enzymatic activity (CellTiter-Fluor™ Cell Viability Assay, Promega, Madison, USA) following the manufacturer’s protocol. Measurements were taken after 24, 48 and 72 hours of heparin treatment. The assay does not cause detachment, or death of the cells on the plate after each measurement. Fresh media corresponding to each treatment condition was added to the wells after each measurement. Cell numbers were compared to vehicle control media (no added heparin) in order to allow for normalization of cell proliferation since this assay does not yield absolute cell numbers. Other controls included are as follows: “media only (no cell) control” to account for the background signal from the assay, and ”lysed cell control” to serve as a negative control for the assay.

2.5 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

RNA extraction was performed for the BeWo cells to study mRNA levels of GCM-1, SYN1 and Ki67 using the RNeasy Plus Mini Kit (Qiagen®, Toronto, Canada) following the
user’s manual. Cells were cultured in 24-well plates and after being washed with ice-cold PBS, they were collected using a 10% solution of β-mercaptoethanol (Fischer Scientific, ON, Canada) in the supplied RLT Plus buffer. RNA was isolated using 70% ethanol after removing genomic DNA and the final RNA was eluted in 30 µl of RNase-free water.

After measuring the RNA content using NanoDrop 1000 instrument (Thermo Scientific, ON, Canada), one microgram of RNA from each sample was reverse-transcribed using the iScript™ Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada) by means of incubating the mixed ingredients at 25°C for 5 minutes followed by 42°C for 30 minutes before a final incubation time of 5 minutes at 85°C.

Reverse transcribed cDNA was diluted to 10 ng/µl and 1 µl of cDNA was used per qPCR reaction. Gene expression was then measured on the CFX384 Real-Time PCR Detection System (Bio-Rad, ON, Canada). LuminoCt® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich, ON, Canada) was used as the fluorophore for all the qPCR reactions for the thermal cycler. Reagents were activated for 5 minutes at 95°C then followed by 40 cycles of denaturation at 95°C while having an annealing temperature of 60°C for a 20-second duration. A melting curve was also performed for each new primer (0.5°C increments increase from 65°C to 95°C).

Primer design

Table 1: Primers used for qPCR experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’ to 3’</th>
<th>Reverse 3’ to 5’</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA</td>
<td>CCA TCC TCA AGA AGG TGT TGG</td>
<td>GTG TCC CAT ATC CGC AAT TTT</td>
</tr>
<tr>
<td>GCM1</td>
<td>ATG GCA CCT CTA GCC CCT ACA</td>
<td>GCT CTT CTT GCC TCA GCT TCT AA</td>
</tr>
<tr>
<td>SYN1</td>
<td>ATG GAG CCC AAG ATG CAG</td>
<td>AGA TCG TGG GCT AGC AG</td>
</tr>
<tr>
<td>TBP</td>
<td>TGC ACA GGA GCC AAG AGT GAA</td>
<td>CAC ATC ACA GCT CCC CAC CA</td>
</tr>
</tbody>
</table>

2.6 ELISA for β-HCG and LDH

Lactate dehydrogenase (LDH) enzyme levels in the cell media signify cell wall disruption, and hence cell death (Chan et al., 2013). Enzyme-linked immunosorbent assays
(ELISA) were used to quantify the presence of LDH, and beta-human chorionic gonadotropin (β-HCG) in the conditioned media of BeWo cells under different treatment conditions. Cell-conditioned media was collected from 24-well plates and centrifuged at room temperature for 5 minutes at 400g in order to remove cell debris from the media. The media was then frozen in aliquots for further analysis.

β-HCG is secreted by differentiated and fused syncytiotrophoblast cells. Terminal cell differentiation at the protein level in BeWo cells was studied by measuring β-HCG in the media of BeWo cells after 72 hours of treatment. β-HCG protein ELISA Kit (Phoenix Pharmaceuticals, CA, USA) was used for the measurements. The assay procedure was followed as suggested by the manufacturer and the final results were expressed compared to the vehicle control conditioned media (no drug treatment).

Cell death and cell cytotoxicity after treatment was only assessed in BeWo cells by means of measuring LDH activity in the conditioned media from BeWo cells 48 hours after treatment. Pierce™ LDH Cytotoxicity Assay Kit (Thermo Scientific®) was used to measure conditioned media LDH activity levels and the results were then normalized to the vehicle control conditioned media (not treated with heparin) to allow for comparison between heparin treated and non-treated cells.

### 2.7 Western blotting for FGF4

Western blotting was used in order to determine the presence of FGF4 protein in BeWo cell media. Presence of FGF4 media in BeWo cell media is required for allowing FGFR2-mediated signaling in BeWo cells. Conditioned media from BeWo cells was collected after 24 hours of treatment under various conditions to study the levels of this protein in the media when proliferation is happening at a fast pace and before it plateaus. The collected media was studied for presence of FGF4 protein by concentrating the collected media using the TCA/Acetone method (Appendix A) for western blot analysis. 35 μg of protein diluted in protein lysis buffer (RIPA, Thermo Scientific, Canada) and 4X Loading Dye (Invitrogen) containing 10% β-mercaptoethanol were electrophoresed at 100 volts in TG-SDS buffer (Winset Inc) using precast 20% Mini-PROTEAN TGX™ Gels (Bio-Rad, Canada). The proteins were then transferred to a
PVDF membrane (0.2 µm) using the Trans-Blot Turbo™ Transfer Pack (Bio-Rad). Blocking of the membrane was followed after the transfer using 5% milk in TBS-T buffer solution for the duration of 1 hour at room temperature. A 5% milk solution was used to dilute the primary human FGF4 antibody and the membrane was incubated with the primary antibody overnight at 4°C. The membrane was washed three times (20 minutes each) in TBS-T solution before being incubated with the secondary antibody (1:3,000 in 5% milk solution) for 1 hour at room temperature (Table. 2). The membrane was then developed using Western Lightning Plus-ECL (Thermo Scientific®) on autoradiography film (Denville Scientific®) to visualize the bands.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Retrieval</th>
<th>Supplier</th>
<th>Cat#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (Western)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF4</td>
<td>1/400</td>
<td>N/A</td>
<td>SantaCruz</td>
<td>sc-1363</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1/500</td>
<td>N/A</td>
<td>AbCam</td>
<td>ab2113</td>
</tr>
</tbody>
</table>

2.8 Phospho-Protein array

Differential phosphorylation of the proteins involved in intracellular signaling was used to study the signaling pathways affected by heparin drug treatment. Phospho-protein antibody array was done in the treated BeWo cells to study intracellular protein phosphorylation. BeWo cells were plated in 10 ml flasks to yield sufficient cell numbers, and allowed to reach 70% confluence. The cells were not allowed to grow past 70% confluence in order to minimize cell death and shedding and excessive terminal fusion. Thereafter, the cells were treated with UFH or LMWH using the previously indicated 3 doses of each. The cells were then harvested after either 3, or 24 hours of being treated using the provided lysis buffer. PathScan® Intracellular Signaling Chemiluminescent Array Kit (Cat#7323) was then used to assess the phosphorylation status of intracellular proteins and signaling nodes.

Protein was isolated from the cells after the media was aspirated from the flasks in order to carry out the protein phosphorylation array. The cells were washed once with ice-cold PBS to minimize further intracellular changes and kept on ice in the cold room at 4°C for the duration of
sample isolation. After removing the PBS, 1 mL of the manufacturer’s provided ice-cold lysis buffer was added to each 10 ml flask of cells, and the flasks were incubated on ice at 4 °C for 5 minutes. The cells were then scraped and sonicated in a test tube before being centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was then aliquoted and frozen at -80°C. Measurement of total protein content from the isolated samples, as well as normalization of the samples with respect to their total protein content was achieved using the Pierce BCA Protein Kit (Thermo Scientific®) according to the user’s manual. The lysates were then diluted to a concentration of 0.6 mg/ml (using the provided cell lysis buffer). 75 μl of the diluted lysate was added to each well and the rest of the procedure was followed according to the manual. The array slides were then developed on autoradiography film to visualize the signals.

2.9 Statistical analysis

GraphPad Prism 5.2 Software was used for statistical analysis (San Diego, USA). The tests used were unpaired student t-test, as well as One-way ANOVA for multiple group comparison using a Dunnett’s post-hoc test. Two-way ANOVA with Bonferroni correction was performed for determining the interaction between variables, as well as contribution from each P values of less than 0.05 were defined as being statistically significant. Data was represented as mean ± SEM. “n” represents biological (experimental) replicates.

2.10 Results

2.10.1 Effects of heparin treatment on proliferation in the HTR8/SVneo cell line

In the first experiment, UFH and LMWH appeared to decrease cell proliferation in the HTR8/SVneo cell line in a dose-dependent manner (Fig. 6). However, data showed very high variability in biological replicates in numerous attempts to reproduce the results (e.g. Fig. 7). Due to the non-reproducibility of the measurements, it was suspected that heparin might be affecting cell attachment to the wells during the daily wash, and daily media-change routine. In order to examine the effects of heparin treatment on the attachment of the HTR8/SVneo cells to
the bottom of the well, different concentrations of heparin were added to the freshly-seeded cells at the time of plating instead of adding the heparin after attachment has taken place. Cell numbers were then measured after 24 hours, and cell numbers in heparin treated groups were significantly reduced when compared to vehicle control at 24 hours (Fig. 8). The interaction between heparin type (UFH vs LMWH) has a significant effect of measured HTR8/SVneo cell numbers after 24 hours (Fig. 8). The data suggest that even though there is a reduction in HTR8/SVneo cell numbers, the results would be hard to interpret since heparin is likely affecting the attachment of the cells to the wells. In conclusion, there is a lot of variability associated with measuring cell proliferation in HTR8/SVneo cells, which can be at least partly attributed to heparin affecting the attachment of the cells to the plate wells.

Figure 6: Initial experimental round showing growth curves showing HTR8/SVneo cell number under different UFH concentrations. Measurements are taken every 24 hours in triplicets. IU, International units. U, Unfractionated heparin. Concentrations: 0.25 units/ml (U1), 2.5 units/ml (U2), 10 units/ml (U3). Data represented as mean for each time point. n=1.
Figure 7: An example of subsequent experiments showing growth curve showing HTR8/SVneo cell number under different Unfractionated heparin concentrations. Measurements are taken every 24 hours in triplicates. The cells demonstrated high variability with respect to cell numbers under different treatment conditions in the subsequent experiments and this graph is shown as an example. IU, International units. U, Unfractionated heparin. Concentrations: 0.25 units/ml (U1), 2.5 units/ml (U2), 10 units/ml (U3). Data represented as mean for each time point. n=1.
Figure 8: HTR8/SVneo cell numbers after 24 hours of treatment after heparin was added at the time of seeding. Heparin was added to the culture medium at the time of seeding and cell numbers were measured after 24 hours. IU, International Units. U, Unfracionated heparin. L, low molecular weight heparin. Concentrations: 0.25 units/ml (U1, L1), 2.5 units/ml (U2, L2), 10 units/ml (U3, L3). Data represented as mean ± SEM. One-way ANOVA followed by Dunnett’s Multiple Comparion Test showed significance indicated by **(P<0.01) and *** (p<0.001) relative to vehicle control (n=3). There is an interaction between heparin type, UFH vs LMWH, and heparin Dose (P<0.0001) shown by two-way ANOVA.
2.10.2 Effects of heparin treatment on proliferation and differentiation in the BeWo cell line

2.10.2.1 BeWo cell proliferation

BeWo cell proliferation was measured following treatment with the same three doses of LMWH and UFH in order to study the effects of heparin treatment on proliferation for these cells during the time of rapid proliferation (24 to 48 hours), as well as when proliferation approaches a plateau (72 hours). The cells showed a trend of increased proliferative response after 72 hours of being treated with 10 units/ml of LMWH (supra-therapeutic dose); nonetheless the changes were not statistically significant (n=3, p>0.05) (Fig. 9). Complimentary experiments measuring the mRNA levels of PCNA (marker of proliferation) in BeWo cells after heparin treatment were performed. PCNA expression showed a significant increase only after only 24 hours of treatment with the highest doses of UFH and LMWH (10 units/ml) (n=4, p<0.01) (Fig. 10). In order to measure cell death and cytotoxicity, the LDH assay was carried out on the BeWo cell culture medium after 72 hours of heparin treatment as a direct measure to assess the effects of heparin treatment on cell death. There was no significant difference between the vehicle control conditioned media (no heparin added), and treatment groups containing heparin (n=3) (Fig. 11) suggesting that none of the treatment conditions used were toxic to the cells. Collectively the data suggest that heparin treatment results in an augmented proliferative response in BeWo cells without accompanying cytotoxicity.
BeWo Cell Number-24 hours

Vehicle Control  U1  U2  U3  L1  L2  L3
0.0  0.5  1.0  1.5
BeWo Cell Number-48 hours

Vehicle Control  U1  U2  U3  L1  L2  L3
0.0  0.5  1.0  1.5

Relative Cell Proliferation

Treatment

Relative Cell Proliferation

Treatment
Figure 9: Cell proliferation in BeWo cells. The figure shows cell numbers relative to the vehicle control for 3 different doses of UFH and LMWH. The measurements were taken every 24 hours for a total of three days. U, unfractionated heparin. L, low molecular weight heparin. Concentrations: 0.25 units/ml (U1, L1), 2.5 units/ml (U2, L2), 10 units/ml (U3, L3). Data represented as mean ± SEM. One-way ANOVA. No significance. n=3.
Figure 10: Normalized PCNA mRNA expression after 24 hours of treatment. Gene expression of PCNA was measured after 24 hours of treatment then normalized to the housekeeping genes and vehicle control. U, unfractionated heparin. L, low molecular weight heparin. IU, International units. Concentrations: 0.25 international units/ml (U1, L1), 2.5 international units/ml (U2, L2), 10 international units/ml (U3, L3). Data represented as mean±SEM. One-way ANOVA followed by Dunnett's Multiple Comparison Test showed significance indicated by ** (p<0.01, n=4).
Figure 11: Normalized Lactose Dehydrogenase (LDH) assay for BeWo cells. Cytotoxicity was measured in BeWo cells after 72 hours of treatment. U, unfractionated heparin. L, low molecular weight heparin. IU, International units. Concentrations: 0.25 international units/ml (U1, L1), 2.5 international units/ml (U2, L2), 10 international units/ml (U3, L3). Data represented as mean±SEM. One-way ANOVA showed no significance, n=4.

2.10.2.2 BeWo cell terminal differentiation and fusion

In order to assess the process of syncytialization in the BeWo cells, GCM-1 and SYN1 mRNA levels were measured once the cells were exposed to 24 hours of different heparin treatments. There were no statistically significant differences between the treatment groups with respect to gene expression for the aforementioned two genes (n=4) (Fig. 12). Secreted free β–HCG was measured in order to assess the extent of terminal differentiation on the protein level. The protein levels of free β–HCG secreted from BeWo cells into the media after 48 hours of
treatment were significantly decreased under all conditions when compared to the vehicle control conditioned media. Nonetheless, there was no dose-dependent response observed, and the effect appeared to have resulted from heparin treatment irrespective of heparin type or dose (Fig. 13) (n=3, p<0.05).

![Graphs showing mRNA expression of GCM1 and SYN1](image)

**Figure 12:** Normalized mRNA expression of syncytial markers. mRNA levels of *GCM-1* and *SYN1* were measured in BeWo cells after 24 hours of being treated with heparin then normalized to the house keeping genes and vehicle control. U, unfractionated heparin. L, low molecular weight heparin. IU, International units per ml; Concentrations: 0.25 units/ml (U1, L1), 2.5 units/ml (U2, L2), 10 units/ml (U3, L3). Data represented as Mean±SEM, One-way ANOVA showed no significance, n=4.
Figure 13: Relative secreted free $\beta$-HCG protein levels in BeWo conditioned medium after 48 hours of treatment. The levels of free $\beta$-HCG protein were measured in the cell culture medium after 48 hours of treatment and the results were normalized to Vehicle Control. U, unfractionated heparin. L, low molecular weight heparin. IU, International units. Concentrations: 0.25 units/ml (U1, L1), 2.5 units/ml (U2, L2), 10 units/ml (U3, L3). Data represented as Mean±SEM, One-way ANOVA, Dunnett's Multiple Comparison Test *P<0.05, **P<0.01 relative to vehicle control, n=3.

BeWo cell intracellular signaling protein phosphorylation.

The Phospho-array was performed to study the effects of treatment on intracellular signaling protein phosphorylation after 3 and 24 hours of treatment with different doses of UFH, and LMWH. The results of the phospho-array showed subtle qualitative changes in the phosphorylation status of intracellular proteins such as ERK1/2 (Fig. 14). The changes in intracellular protein phosphorylation were less pronounced after 24 hours of treatment. This
antibody array is meant for qualitative observations but quantitative analysis of the array can be carried out using western blotting. Nonetheless, none of the changes were deemed large enough to warrant quantitative analysis.

Figure 14: Intracellular protein phosphorylation in BeWo cells after 3 and 24 hours of treatment. BeWo cells were treated for 3 hours (top 4 rows), or 24 hours (bottom 4 rows) with three different concentrations of LMWH (left column) (top: 0.25 IU/ml, middle: 2.5 IU/ml, bottom: 10 IU/ml), or UFH (right column) with the same concentrations as LMWH. U, unfractionated heparin. L, low molecular weight heparin. IU, International units. n=1.
2.10.3 **BeWo cell media FGF4 protein content**

The presence of FGF4 protein was assessed in BeWo conditioned media after 24 hours of treatment to determine whether or not FGF4 is present in the BeWo media to allow for FGFR2 mediated signaling. The levels of FGF4 were measured after 24 hours of treatment to assess the presence of FGF4 when the cells are at a low confluence percentage resulting in proliferation happening at a fast pace. FGF4 protein was detected in the media isolated from BeWo cells in trace quantities after 24 hours of treatment (Fig. 15). Nonetheless, FGF4 was nearly absent from the media isolated from the cells treated with the highest dose (10 IU/ml) of LMWH. It is important to mention that the evidence is not conclusive since the experiments were not replicated further (n=1) and future rounds of experiments could potentially have altered results.

![Western blotting for FGF4 in BeWo cell conditioned media](image)

**Figure 15**: Western blotting for FGF4 in BeWo cell conditioned media. BeWo cells media was collected 24 hours after treatment and used for measuring the amounts of FGF4 protein normalized to media fibronectin levels in the concentrated media. IU, International units. UFH, unfractionated heparin. LMWH, low molecular weight heparin. n=1.

2.11 **Discussion**
2.11.1 Effects of heparin treatment on proliferation in the HTR8/SVneo cell line

The HTR8/SVneo cell line was used for this part of the project to study the effects of heparin on trophoblast cell proliferation in an EVT-like cell line incapable of differentiation and fusion. The initial work in HTR8/SVneo cells suggested that heparin treatment increases cell proliferation. Nonetheless, further rounds of experiments did not yield the same results for HTR8/SVneo cells. Namely, measuring proliferation by direct cell counting was not reproducible in these cells, and even the opposite trends were observed between two consecutive rounds of experiments (Figures. 6,7). Since during the daily media change for the HTR8/SVneo cells the wells are washed, it was suspected that heparin could potentially influence the attachment of the cells to the bottom of the wells, which in turn can cause inconsistency in the results. The inconsistency was suspected to be caused by means of the cells being washed off after the daily media change due to detachment from the plate. Heparin treatment indeed influenced the way cells attach to the bottom of the plate by means of decreasing attachment since cell numbers were significantly reduced after 24 hours when heparin was added to the cell culture media before the cells were added to the wells (Fig. 8). Conclusive interpretation of the results would be challenging since even thought the number of cells measured decreased after 24 hours of adding heparin at the time of seeding, the lack of proper attachment to the plate wells coupled with potential changes in cell number are both contributing to the decreased cell numbers measured at 24 hours. Namely, the high variability observed when cells were treated after attachment complemented by the observations once heparin was added at the time of seeding suggest that heparin might be affecting both the proliferation and cell attachment and hence confounding the results. The significant interaction between the type of heparin, and the dose of treatment when heparin was added at the time of seeding further point towards multifactorial effects associated with heparin treatment in HTR8/SVneo cells. Other possible contributors to the non-reproducibility of the results can be the very rapid growth rate of this cell line, which can contribute to the inconsistency of results observed. Moreover, once HTR8/SVneo cells reach full confluency, they appear to grow further on top of the confluent cell layer as seen with certain other EVT-like cell lines (Graham et al., 1993). Hence, cell numbers do not seem to plateau in a consistent manner as expected from cells in a dish unless cells are starved of nutrients, which would be an undesirable deviation from the optimal physiological environment for the cells.
2.11.2 Effects of heparin treatment on proliferation and differentiation in the BeWo cell line

BeWo cells were used as a model for human villous trophoblast cells since BeWo cells are capable of both proliferation and differentiation. BeWo cells were treated with different doses and types of heparin and screened for proliferation and differentiation markers over a 72-hour time period.

BeWo cells treated with the highest dose of LMWH displayed a nonsignificant trend towards an increase in proliferation after 72 hours of treatment (Fig 9). The observed trend towards maintained proliferation under the highest dose of LMWH is of great value since it suggests that heparin treatment can be responsible for maintaining the proliferative state of BeWo cells after 72 hours when the cells would normally have the tendency to reach a plateau with respect to proliferation and terminally differentiate. Following the aforementioned observation of maintained proliferation trend, mRNA was isolated from BeWo cells under different treatment conditions to study the gene expression of a proliferation marker, proliferating cell nuclear antigen (PCNA) after 24 hours of treatment when the cells are in a rapid proliferation phase in order to see if heparin treatment is associated with increased proliferative response in the BeWo cell line. Quantitative PCR results for PCNA showed an increase in gene expression for PCNA after only 24 hours of heparin treatment for the highest doses of both UFH and LMWH (10 IU/ml) in support of our hypothesis (Fig. 10).

We hypothesized that there would be an upregulation of proliferation without affecting differentiation and syncytial fusion in the BeWo cells. mRNA levels of differentiation markers (GCM-1 and SYN1) appeared to show a trend of slight increase after heparin treatment, although none of the changes were statistically significant (Fig. 11). On the other hand, free β-HCG protein levels in the conditioned medium were decreased after 48 hours of treatment, which corresponds to a potentially lower level of terminally differentiated cells. It is noteworthy to mention that despite a significant decrease in the release of free β-hCG following heparin treatment, the physiological significance of this finding is questionable. In a physiological setting as seen in the human placenta, fold changes are needed to infer physiologically relevant alteration in the rate and extent of fusion and differentiation for these cells when free β-HCG
levels are used as a differentiation marker (Baczyk et al., 2013). Another plausible factor contributing to the lowered free β-HCG levels after heparin treatment is this drug’s potential in reducing global protein secretion. Heparin treatment was previously seen to be associated with decreased protein shedding from placental explants into media (Drewlo et al., 2011). As pertains to the reduced β-HCG levels after heparin treatment in this study, downregulation of secreted free β-HCG was not dose-dependent which raises the question that perhaps the decreased levels of free β-HCG is an artifact of heparin treatment by means of the very presence of heparin in the cell media preventing protein secretion from the cells, hence lowering secreted free β-HCG levels in the conditioned media. Thus differentiation is likely not affected by heparin treatment since the decrease in β-HCG levels do not reach physiologically significant levels.

2.11.3 BeWo cell intracellular signaling protein phosphorylation.

Altered phosphorylation state of intracellular proteins involved in signaling pathways was studied to detect signaling pathways affected by heparin treatment in the BeWo cell line. The study was carried out by means of measuring differential signaling protein phosphorylation under different doses of UFH and LMWH. The results of the array did not show striking differences between different treatment arms regardless of whether BeWo cells were harvested 3 or 24 hours after initiation of treatment. Nonetheless, there appeared to be an upregulation of phosphorylated ERK1/2 levels, a downstream node of FGF signaling, in the cells treated with the highest dose of LMWH (10 IU/ml (Fig. 14). It is important to mention that this array is not meant for quantitative analysis, and instead is meant to serve as a hypothesis-generating measure for narrowing down on potential intracellular targets of interest. The suspected changes observed in the protein phosphorylation after heparin treatment (e.g ERK1/2) can be followed up by means of performing western blotting analysis for those proteins.

2.11.4 BeWo cell media FGF4 protein content

Presence of FGF4 is essential for the activation of the FGFR2 signaling pathway and the downstream Erk1/2 as the effectors of the FGFR2. We suspect FGF4-mediated signaling to be at
least partly responsible for the altered mitogenic response of the BeWo cells to heparin treatment due to heparin binding capacity of FGF4 and heparin acting as a cofactor for FGFR2 (Powers et al., 2000). Western blotting was carried out in order to assess the presence of FGF4 protein in the conditioned media of BeWo cells under different treatment conditions after 24 hours of treatment when proliferation is yet favored over terminal differentiation and fusion. FGF4 was observed to be present in trace amounts in the isolated media except when the cells were treated with the highest dose of LMWH (10 IU/ml) (Fig. 15).

FGF4 was detected in conditioned media from BeWo cells but whether it originated from the cell line is not known. A lowered FGF4 level in the media under the highest dose of LMWH was rather unexpected and suggest that the effect is dependent on the type, and dose of heparin used. Interestingly, heparin reduces protein shedding and secretion from human placental explants (Kingdom and Drewlo, 2011). That being said, it is plausible to suspect that treating cells with such supra-therapeutic dose of the more potent LMWH results in dampened global protein secretion independent of protein synthesis. This could apply to FGF4 secretion the same way as for β-HCG secretion into the media after heparin treatment described above. The presence of FGF4 in the conditioned media suggests there could be a contribution of FGF4-dependent FGFR2 signaling pathway to BeWo cell proliferation. It is noteworthy to mention that no conclusive statements can be made after one round of experiment since further replicates might not show the presence of FGF4 in the BeWo conditioned media.

It is unknown as to whether the FGF4 detected had been secreted by the BeWo cells, or was originally present in the media, or was added in media supplements (e.g fetal bovine serum). Despite the well-defined nature of human trophoblast cell lines for studying effects of drug treatment, unknown exogenous factors are added to the medium. For example, the fetal bovine serum routinely added to the BeWo medium contains various potentially mitogenic factors which can in turn alter the response of these cells to drug treatment (Rauch et al., 2011). Therefore if the presence of FGF4 in the conditioned media is truly valid, it can be concluded that FGF4 is contributing to the augmented proliferative response seen in BeWo cells after heparin treatment. Moreover, activation of ERK1/2 (section 2.11.3), a downstream effector of FGF4-dependent signaling, further suggests that heparin’s mitogenic effects in BeWo cells are FGF4-dependent.
2.11.5 Conclusions

Overall, heparin treatment in human trophoblast cell lines was associated with variable proliferative responses in HTR8/SVneo cells (EVT model) due to confounding effects with respect to cell attachment and proliferation for HTR8/SVneo cells. Furthermore, heparin treatment caused an augmented proliferative response in the BeWo cell line (villous cytotrophoblast model) without changing the syncytial differentiation and fusion pathway in a physiologically significant manner in BeWo cells. Collectively, HTR8/SVneo cells were determined to be less than ideal for measuring trophoblast cell proliferation due to their unreproducible and rapid proliferation rate, and BeWo cells were deemed the more feasible model for our study.

Although apparently small responses, the trend towards increased cell proliferation and the upregulation of proliferation markers in BeWo cells could have large cumulative effects over the duration of gestation. This section of the project suggests that heparin may reduce the rate of recurrence of severe PE by means of re-establishing the disrupted balance of cytotrophoblast cell proliferation vs. terminal differentiation (trophoblast turnover) in the human placenta by means of increasing, and maintaining cytotrophoblast cell proliferation.
Chapter 3 Heparin treatment in mice
3.1 Rationale and hypothesis

This part of the study was carried out in order to examine the effects of heparin treatment on mouse pregnancy and placentation using mice as an in vivo model of placentation. Mice were used in order to study the effects of heparin treatment in a more clinically relevant physiological setting. As mentioned in Section 1.7, there are various lines of evidence suggesting the involvement of heparin with trophoblast cell proliferation.

We hypothesized that heparin treatment in mice will increase trophoblast cell proliferation without directly altering differentiation collectively resulting in remodeling of the placenta, as well as placental and fetal overgrowth.

We suspected that signaling through the FGFR2 could potentially be affected given the role of heparin as a cofactor for the FGFR2. The labyrinth region in the mouse starts forming around E9 after the chorioallantoic attachment takes place 8.5 days into gestation (Cross, 2000). FGF4 signaling plays an important role in the development of the mouse placenta since all forms of Fgf4, and Fgfr2 mutations result in embryonic lethality due to abnormal formation of the placenta (Feldman et al., 1995; Goldin and Papaioannou, 2003; Xu et al., 1998). Specifically, heterozygous mice with a knockout mutation of the FGF4 receptor either fail to undergo chorioallantoic fusion (1/3 of the cases), or they do not survive past E12.5 due to a striking abnormality in the formation of the labyrinth region of the placenta (Xu et al., 1998). Moreover, in vitro studies have demonstrated that FGF signaling is necessary to maintain trophoblast stem cells in their proliferative state until they disappear at E8.5 (Simmons and Cross, 2005) (Kaufman and Bard, 1999). Furthermore, FGFRII is still expressed in the labyrinthine region of the placenta later on at E12.5 (Fig. 40). Trophoblast cell proliferation is at its maximum at E10.5 in the labyrinth, but is significantly decreased by E14.5 (Fig. 4) (Iguchi et al., 1993). Therefore, we hypothesized that in vivo treatment of mice with heparin will result in a longer retention of the proliferative trophoblast progenitor cells. Heparin is known to interact with the FGF pathway but in addition interacts with various proteins involved in a wide range of physiological processes. The list includes, but is not limited to, interactions with the TGF-β family and its binding proteins, placental growth factors (PLGF), and vascular endothelial factors (VEGF) (Drewlo et al., 2010).
This part of the project was aimed at studying the morphological changes caused by heparin treatment in mice. Furthermore, expression levels of various trophoblast cell type markers involved with placentation were studied in this part of the project. It is currently not known how heparin administration in an in vivo model of placentation affects the main signaling pathways in the mouse placenta. Hence we carried out hypothesis-generating experiments using the Qiagen® Mouse Transcription Factors PCR array to screen for 84 genes responsible for controlling the pattern of gene expression. These hypothesis-generating experiments were carried out to better understand the underlying effects associated with the morphological changes observed in mice treated with heparin.

3.2 Overview

Mice were chosen for the in vivo model due to similarities to humans with respect to placental morphometry and physiology (Georgiades et al., 2002), and other maternal responses to pregnancy involving angiogenesis related factors (e.g. PLGF, sFLT1) (Saad et al., 2014). Moreover, mice have a short gestation time (18.5 days) allowing for convenient and practical experimental procedures.

All the experimental procedures involving mice were carried out using CD-1 (ICR) mice purchased from, and kept at the Toronto Centre for Phenogenomics (TCP). ICR mice were originally established into a line by Dr. Hauschka (Institute for Cancer Research, Philadelphia). All the mice were housed and handled according to standard procedures set by the Canadian Council for Animal Care. All the experimental procedures were approved by the Animal Care Committee of the Toronto Centre for Phenogenomics (Toronto, ON, Canada). Virgin female mice of 8-10 weeks of age were put into the mating cages overnight and the noon of the day at which the vaginal plug was detected was labeled as day 0.5 (E0.5) of pregnancy. Only the first pregnancy of the mice was used for our studies.

In this study, markers of proliferative trophoblast progenitor cells were measured in the mouse labyrinth across different experimental groups and gestational ages. Moreover, expression levels of different trophoblast cell markers in the labyrinth region of the placentas were measured. Localization, and expression pattern of a subset of markers were assessed by regular
immunohistochemistry (IHC), and fluorescent immunohistochemistry (FIHC). Moreover, placental morphology measurements were carried out to examine the effects of heparin treatment on placental and fetal weight, fetal and maternal blood space, and labyrinth area and perimeter. The density of BrdU positive nuclei within the labyrinthine area was assessed in a subset of mice to quantify the effects of heparin on global cell proliferation in the collected placental samples.

3.3 Methodology

Three separate series of mouse experiments were performed for this project. For the first two series, ICR mice were injected subcutaneously with LMWH every 12 hours commencing 9.5 days into gestation. Treatment was continued until E12.5 in Series 1 and to E14.5 in Series 2. In vivo BrdU labeling for the aforementioned two series was carried out by intraperitoneal injection five hours prior to euthanizing the animals and collections were carried out in the same manner for all the mouse series. In Series 3, the ICR mice were implanted with drug-eluting microosmotic pumps starting at 5.5 days into gestation until euthanizing them at E12.5. Tissue collection and processing was carried out the same way for all three series.

3.3.1 Intra-peritoneal injection of BrdU

BrdU was administered by intraperitoneal injection to mice as follows: Intraperitoneal injections were carried out according the standard operating procedures at the Toronto Centre for Phenogenomics. Pregnant mice were weighed five hours prior to being euthanized, and BrdU Labeling Reagent (Invitrogen, Cat#00-0103) was drawn into the syringe (1 ml syringe) at dose of 10 µl per gram of body weight as directed by the users manual for the reagent. The mice were then restrained by pinching the skin behind their neck. The mice were then tilted head down at a 45 degree angle with the abdomen facing up. Injections were then made at the lower right abdominal region using a 25-gauge needle. The needle was inserted 1 cm into the abdominal cavity and aspirated before injection to ensure no misplacement of the needle. The BrdU solution was then injected as a bolus five hours prior to euthanasia.
3.3.2 **Modes of heparin treatment**

3.3.2.1 **Subcutaneous injection**

Mice in the first two series of experiments were injected subcutaneously every 12 hours (8 a.m. and 8 p.m.) by pinching the skin behind the neck to make a tent into which the subcutaneous injection was given from E9.5 to E12.5 (Series 1) or E14.5 (Series 2). Low Dose mice were injected with 5 IU of LMWH for every injection and the High Dose group were injected with 35 IU every time corresponding to 10 IU/day and 70 IU/day respectively. The Vehicle Control mice were injected with PBS only (PBS was used as the vehicle for diluting the drug).

Dose adjustments for LMWH to were done by means of normalizing the clinical dose by body weight as follows: clinically, women are given 5,000 IU/day regardless of their bodyweight and adjusting that dose for an average body weight of 70 kg and normalizing that to a 30g mouse at midgestation would translate to 2.5 IU per bolus. Accurate scaling of a clinical drug for a smaller animal species requires empirical and experimental adjustments based on previous studies (Sharma and McNeill, 2009), which were not available in our case. The mice were injected twice per day with double the weight-adjusted clinical bolus dosage for heparin administration to be somewhat similar to the clinical setting of once per day injection. The aforementioned modifications were made in the mice in an effort to mimic the clinical setting, while accounting for the much shorter gestational time, and hence much faster pregnancy progression rate, and the much faster metabolic rate, which results in a higher dose requirement than that based on weight-adjusted dosing alone (Sharma and McNeill, 2009).

3.3.2.2 **Drug-eluting subcutaneous pumps**

Drug eluting microosmotic pumps were used to infuse mice with heparin in the third series of mouse experiments in order to achieve a stable plasma heparin levels, and to avoid peaks and troughs associated with twice daily injections. Mice in the third series of experiments were surgically implanted with the pumps at E5.5. The pumps were loaded and primed following the manufacturer’s guidelines (Alzet® 1007D) to deliver a dose similar to the dosage administered via subcutaneous injections for the first two mouse series. The pumps were set to
infuse the mice with 10, and 70 international units of LMWH per 24 hours for the low dose and high dose groups respectively, which corresponds to the dosage administered per 24 hours for the mice injected twice daily in the first two series.

Mice were prepared for aseptic surgery under a laminar flow cabinet as follows: Anesthesia was induced using 5% isofluorane and maintenance was achieved by a facemask delivering 2% isofluorane while the mice were put on a sterile underpad. A small area behind the neck was shaved and cleaned with 70% isopropyl alcohol and antiseptic solution (Betadine). A small incision (1 cm) was made using clippers with a #40 scalpel in order to insert the primed pump under the skin of the mouse behind the shoulders, and into a pocket slightly larger than the pump created by inserting a sterile hemostat into the incision site. The incision was then closed after inserting the pump with wound clips and the animals were allowed to recover. The animals were housed individually in separate cages until the day they were euthanized.

3.3.3  **Tissue and blood collection**

3.3.3.1  **Saphenous vein blood collection**

Saphenous vein blood collection was performed in order to collect EDTA plasma for measuring maternal circulating sFLT1 and PLGF levels. The collection was done 30 minutes prior to being euthanized. The E12.5 pregnant mice were constrained in an uncapped 50 ml falcon tube with holes dilled at the end to allow for air exchange. One of the legs was secured by pinching the skin between the tail and the thigh region to expose the saphenous vein. The region was shaved and wiped with 70% ethanol before the vein was punctured at a 45-degree angle using a 25-gauge needle. 400 µl of blood was collected using an EDTA coated capillary tube (MICROCAPS cat#1-000-2000-E). The blood was centrifuged immediately for 20 minutes at 4°C at the speed of 2,000 g and the plasma (supernatant) was aliquoted (20 µl per tube), and frozen at -20°C.

3.3.3.2  **Ultrasound-guided cardiac puncture**

Citrate plasma was needed to measure plasma heparin concentrations. Citrate plasma was obtained from blood collected by ultrasound-guided cardiac puncture while mice were anesthetized using the following procedure. Pregnant mice were weighed on a scale then anaesthesia was induced by 5% isofluorane and 1000ml/min of oxygen in a closed container. 2%
isofluorane was then used for maintenance via a secured facemask. The thoracic and abdominal area was then shaved and pre-warmed gel was used as the ultrasound coupling medium for imaging the mice transectaneously. The 30-MHz transducer was operated at 30 frames per second (Model Vevo 770, Visual Sonics, Toronto, ON, Canada). The probe was secured at a longitudinal section of the left ventricle and blood was collected from the left ventricle using a 3 ml syringe containing sodium citrate under ultrasound. 1.5 ml of blood was collected to give a final content of 3.2% Sodium Citrate in the solution. The blood was centrifuged for 20 minutes at 4°C at 2,000g. The plasma was then frozen as five aliquots of 100 μl each at -20°C.

3.3.4 Tissue collection for immunohistochemistry

Placental samples from the mothers were collected for immunohistochemistry right after the mice were euthanized at E12.5. The umbilical cord, and the yolk sac were removed on the fetal side along with the fetus. The myometrium was left attached to the maternal side of the placenta. Placentas were then weighed and immediately fixed in 20 ml of 4% paraformaldehyde (PFA) and left on a shaker overnight at 4°C. Specimens were washed in PBS three times (10 minutes each) then dehydrated in a series of increasing methanol concentrations (25%, 50%, 75%, 100%), half an hour each. The dehydrated specimens were then embedded in paraffin.

3.3.5 Tissue collection for mRNA and protein analysis

In order to collect placentas for mRNA and protein analysis, uterine horns were removed from the euthanized mice and immediately put into ice cold PBS. All the dissection was performed inside a biosafely cabinet cleaned thoroughly, and wiped using RNAseZap® RNase Decontamination Solution (Cat# AM9780). While keeping the specimen on ice, a subset of the samples labelled the “labyrinth samples” were microdissected under the surgical microscope to enrich for the labyrinth region of the placenta only. Meanwhile, another subset of samples labelled the “whole placenta” samples were flash frozen in liquid nitrogen after removal of only the myometrium, the umbilical cord, and the fetus while leaving the decidua and yolk sac attached. All the flash frozen samples were then transferred to the -80°C freezer for long-term storage.
3.3.6 **Histology and immunohistochemistry**

IHC was carried out on 4 μm sections that were cut from the embedded samples using a microtome (LEICA RM2255). IHC for BrdU and CD34 was done by the Pathology core of the Centre for Modeling Human Disease (CMHD, Toronto, ON, Canada).

3.3.6.1 **Non-fluorescent immunohistochemistry**

The slides were stored until required. They were then rehydrated as follows. The slides were incubated at 60°C for 10 minutes followed by three steps of 5-minute incubation in xylene. Then the slides were rehydrated down from 100% ethanol to 95% ethanol for 2 minutes each before being soaked in 0.6% H₂O₂ (in methanol) for 30 minutes to quench exogenous peroxidase activity. Rehydration was continued by soaking the slides in 90%, 80%, and 70% ethanol respectively for one minute each before transferring the slides into PBS.

Antigen retrieval was performed by boiling the slides in sodium citrate (10mM, PH6.0) for 5 minutes and 3 minutes respectively while having a 15-minute gap between each step. The slides were then incubated with blocking reagent (Code X0909, DAKO). The slides were then incubated with the antibodies overnight at 4°C diluted in serum-free blocking solution (DAKO) (Table 3). The slides were washed in PBS three times in the morning after being incubated with the primary antibody overnight. The washed slides were then incubated with the secondary antibody (diluted 1:300 in PBS) for one hour at room temperature. Another set of PBS washes was performed before the slides were incubated with Streptavidin with Horseradish Peroxidase (HRP) (Invitrogen, Cat#SA100-01) for one hour at room temperature. The slides were then washed three times in PBS before starting the DAB/AEC (conjugate for HRP) colour reaction. The colour reaction was terminated using tap water and the slides were then treated with undiluted haematoxylin for 20 seconds before being dehydrated a stepwise ethanol gradient. The slides were then incubated in xylene for 2 minutes before coverslipping. IHC for BrdU was done on the embedded blocks by the CMHD Pathology Lab (Toronto Centre for Phenogenomics) for which the protocol is attached (Appendix B).
3.3.6.2 Fluorescent immunohistochemistry

Only freshly sectioned slides (within a week of being sectioned) were used for FIHC. The slides were rehydrated as for IHC except that the hydrogen peroxide step was skipped. Incubation of the primary antibodies which were diluted in serum-free blocking solution (DAKO) was performed overnight at 4°C (Table 3). Antigen retrieval was performed like IHC but the slides were then treated with Sudan Black B for 10 seconds to quench auto-fluorescence before being blocked with the serum-free blocking solution (DAKO) as described in the IHC section. The slides were then washed three times in PBS before being incubated with the corresponding secondary antibodies for one hour at room temperature. The slides were then washed and coverslipped with FIHC coverslip reagent (Thermo Fischer, Cat #9990402)

Table 3: Antibodies used for immunohistochemistry in mice along with their corresponding dilutions.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Retrieval</th>
<th>Supplier</th>
<th>Cat#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epcam</td>
<td>1/150</td>
<td>Sodium Citrate</td>
<td>BD Pharmingen</td>
<td>552370</td>
</tr>
<tr>
<td>FGFRII</td>
<td>1/100</td>
<td>Proteinase K</td>
<td>R&amp;D systems</td>
<td>MAB716</td>
</tr>
<tr>
<td>Sca1</td>
<td>1/100</td>
<td>Tween-20</td>
<td>AbCam</td>
<td>ab51317</td>
</tr>
<tr>
<td>CD34</td>
<td>1/200</td>
<td>Sodium Citrate</td>
<td>AbCam</td>
<td>8158</td>
</tr>
<tr>
<td>Cyt18</td>
<td>1/100</td>
<td>Sodium Citrate</td>
<td>Fitzgerald</td>
<td>10R-C161a</td>
</tr>
<tr>
<td>BrdU</td>
<td>1/1000</td>
<td>Sodium Citrate</td>
<td>Abcam</td>
<td>Ab6326</td>
</tr>
<tr>
<td>PHH3</td>
<td>1/100</td>
<td>Sodium Citrate</td>
<td>Millipore</td>
<td>06-570</td>
</tr>
</tbody>
</table>

3.3.7 Placental histomorphometry

Histomorphometry was performed on slides stained with CD34 to visualize the fetal capillaries. The slides were visualized using a digital camera (BX-UCB, Olympus) mounted on light microscope (BX61, Olympus). Morphometry software (newCAST, Visiopharm) was used for quantitative analysis of the placental sections. Detailed instructions for the point counting method using the software are attached (Appendix C).

Placental regions were quantified on placental midline cross-sections under 10x magnification. Labyrinthine area was calculated by outlining the CD34 positive region bordering the junctional zone and chorionic plate (Fig. 16). The percentage of labyrinth within
the whole placenta was obtained by comparing the measured labyrinth area to the total placental area. Total placental area was obtained by outlining the whole placenta including decidua and chorionic plate. CD34 stained slides were also used to calculate maternal and fetal blood space measures (Fig. 16). 100% of the labyrinth was covered via random field images at 20x magnification. Each of the field images contained 12 equally spaced crosshairs, which were labeled according to their location within each random image (Fetal, Maternal, Other).

Figure 16: CD34 staining and point counting method for 3rd series of mice. Fetal capillaries stained brown by CD34 and other nuclei stained blue (hematoxylin). Labyrinth area is outlined in green and crosshairs (yellow) were used to determine maternal and fetal space areas, as well as area occupied by tissue in the labyrinth. Left: 2x magnification, Right: 20% magnification.
The density of BrdU-positive nuclei was calculated via outlining the labyrinthine area, then covering 60% of the outlined area via random field images at 20X magnification (Fig. 36). The number of BrdU positive cells within the 4 boxes in each image (each comprising 10% of the image area), were then counted. Results were expressed as BrdU-positive cells per labyrinth area. Results were then compared between different treatment arms.

3.3.8 qRT-PCR

3.3.8.1 RNA extraction

RNA isolation from the flash frozen labyrinth-enriched, and whole placenta samples were achieved by removing all the layers except for the chorionic plate and the labyrinth for the labyrinth enriched samples. For the whole placental samples the uterus, yolk sac, and the decidua were removed and the remaining tissue was flash frozen. RNA isolation was carried out using the TRIzol method for RNA isolation following the users manual (Invitrogen, ON, Canada). First of all the genomic DNA content was eliminated using a DNase treatment solution (Qiagen®, ON, Canada). Mini-elute mRNA cleanup columns were then used to purify and concentrate the RNA content (Qiagen®). RNA concentration and quality was assessed using NanoDrop 1000 (Thermo Scientific, ON, Canada).

3.3.8.2 Reverse transcription

After measuring the RNA content, one microgram of RNA from each sample was reverse transcribed using the iScript™ Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada) by incubating the mixed ingredients at 25°C for five minutes followed by 42°C for half an hours and a final incubation of 5 minutes at 85°C.

3.3.8.3 Real-Time PCR

Reverse transcribed cDNA was diluted to 5 ng/µl and 1 µl of cDNA was used per reaction. Gene expression was then measured on the CFX384 Real-Time PCR Detection System (Bio-Rad, ON, Canada). LuminoCt® SYBR® Green qPCR ReadyMix™ (Sigma- Aldrich, ON, Canada) was used as the fluorophore for all the qPCR reactions for the thermal cycler. Reagents were activated for 5 minutes at 95°C then followed by 40 cycles of denaturation at 95°C while
having an annealing temperature of 60°C for a 20 second duration. A melting curve was also performed for each primer (0.5°C increments increase from 65°C to 95°C).

Primer design was carried out using Primer-Blast (NCBI). The list of primers used for the genes analyzed in this study are shown in Table 4.

### 3.3.8.4 Primer validation and gene expression normalization

All the primers were validated using the CFX96 Real-Time PCR Detection System (Bio-Rad, ON, Canada) using the aforementioned reagents. Pooled placental cDNA from different gestational ages in the mouse was used as the standard for primer verification purposes. cDNA was used at the starting concentration of 5x10^-8 ng/µl and diluted 1:4 down the dilution series. qPCR reactions were performed to verify each primer.

qPCR data was normalized with respect to the geometric mean of the housekeeping genes (Tbp, Actin, Hprt, Gapdh) and the results were expressed as mRNA expression fold change.

**Table 4:** Primers used for qRT-PCR, and regular PCR experiments in mice.

<table>
<thead>
<tr>
<th>Host</th>
<th>Gene (or isoform)</th>
<th>Forward 5’ to 3’</th>
<th>Reverse 3’ to 5’</th>
</tr>
</thead>
<tbody>
<tr>
<td>qRT-PCR Mouse</td>
<td>Fgfr2</td>
<td>ACG TAG AGA GGT CCA TCC TG</td>
<td>GAT GCC ACA GAG AAG GAC CT</td>
</tr>
<tr>
<td></td>
<td>Ki67</td>
<td>GGT TCA GGC TGT CAC TGG A</td>
<td>ACT CCA AGA GTC TTT CAC CAA</td>
</tr>
<tr>
<td></td>
<td>Hif1a</td>
<td>TGG ATG CCG GTG GTC TAG A</td>
<td>ACC ATG TCG CCA TCA TCT GT</td>
</tr>
<tr>
<td></td>
<td>Eomes</td>
<td>CCT GGT GGT GTG TTG TTG TG</td>
<td>AAT CCA GCA CCT TGA ACG AC</td>
</tr>
<tr>
<td></td>
<td>Sca1</td>
<td>GCA GAA AGA GCT CAG GGA</td>
<td>CAC AAT AAC TGC TGC CTC CT</td>
</tr>
<tr>
<td></td>
<td>Cdx2</td>
<td>TCC TGC TGA CTG CCT TCT GA</td>
<td>CCC TTC CTT ATT TGT GGA GA</td>
</tr>
<tr>
<td></td>
<td>Rhox4b</td>
<td>GAG CCA GTT CTC AGG GAT G</td>
<td>GCC AGA TGT CTT CTT TCC TC</td>
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<td></td>
<td>Epcam</td>
<td>CGG GGA TTG TTG TCC TGG TTA T</td>
<td>CCA TCT CCT TTA CCT CAG CCT TCT</td>
</tr>
<tr>
<td></td>
<td>Igf2 (α isoform)</td>
<td>CTT CCA GCC TTG TTC TGT CTT C</td>
<td>AAA GAG ATG AGA AGC ACC AAC AT</td>
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<td></td>
<td>Gcm1</td>
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<td>GAT CAT GCT CGC CTT TGG</td>
</tr>
<tr>
<td></td>
<td>Synb</td>
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</tr>
<tr>
<td></td>
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<td>ATC TGG TCC ACG TGT CCT TCG</td>
</tr>
<tr>
<td></td>
<td>Ctsq</td>
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<td>GTA CTT CTT CCT CCG GAC TGT ATA</td>
</tr>
<tr>
<td></td>
<td>Prl3b1</td>
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<tr>
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<td>Plgf</td>
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<td>GGA CAC AGG AGC GAC TGA AT</td>
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<tr>
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<td>sflt1</td>
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<td>GCA GTG CTC ACC TCT AAC GA</td>
</tr>
<tr>
<td></td>
<td>Actin</td>
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<td>GAA CCG CTC GTT GCC AAT A</td>
</tr>
<tr>
<td></td>
<td>Tbp</td>
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</tr>
<tr>
<td>PCR</td>
<td>Hprt</td>
<td>TCT TTG CTG ACC TGC TGG ATT</td>
<td>TAT GTC CCC CGT TGA CTG ATC</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------</td>
<td>----------------------------</td>
<td>-----------------------------</td>
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<tr>
<td></td>
<td>Gapdh</td>
<td>AGG AGT AAG AAA CC TGG AC</td>
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<tr>
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<tr>
<td></td>
<td>Jarid1c-rev2</td>
<td>TGA GTT GGT ACG ACG AAG CTG CAG</td>
<td></td>
</tr>
</tbody>
</table>

### 3.3.9 qPCR array

Placental labyrinth samples collected from the second series of mice injected from E9.5 to E12.5 were used to examine the effects of heparin treatment with respect to altering the gene expression of common transcription factors at the exchange region of the mouse placenta. For each mouse the mRNA from one male placental labyrinth was combined with one female placental labyrinth collected from the second mouse series in order to eliminate sex bias. The aforementioned arrangement resulted in obtaining one pooled sample per mouse, and there were five mice per treatment group (N=5 for each treatment group). 10 ng of cDNA was then used from each pooled sample per reaction for the array as specified by the manufacturer (Mouse Transcription Factor PCR Array, Qiagen, cat#PAMM-075Z). The results of the array were analyzed using the provided software (RT² Profiler PCR Array Data Analysis software V4.0, SAbiosciences). Gene expression was normalized to the housekeeping genes provided on the array. Unpaired student t-test was used by the software and p<0.05 was considered significant. No false discovery rate correction was carried out by the software.

### 3.3.10 ELISA for sFLT1 and PLGF

Maternal circulating levels of sFLT1 and PLGF proteins were measured using commercial ELISA kits. The procedures were performed on EDTA-treated plasma samples so no protein extraction method was required before starting the assays.

The sFLT1 ELISA kit was used to measure maternal circulating levels of sFLT1. The kit was purchased from R&D systems (Cat# MVR100) which was used to quantify maternal circulating mouse sFLT1 content using plasma collected from the saphenous vein of mice half an hour before being euthanized (Section 2.2.3.1). The assay procedure was carried out as suggested
in the user’s manual for the kit to obtain final concentration values of sFLT1 in pg/ml units of plasma.

Maternal circulating mouse PLGF-2 was measured using the PLGF-2 Quantikine ELISA kit purchased from Cedarlane Laboratories (Cat#MP200). The plasma samples collected from the saphenous vein were used to measure the maternal circulating PLGF levels at the time of being euthanized. The assay procedure was followed directly from the manufacturer’s manual.

3.3.11 Anti-Xa assay

The concentration of LMWH heparin in maternal plasma was measured in 3.2% citrated plasma collected via ultrasound cardiac puncture using the standard anti-Xa assay (Vandiver and Vondracek, 2013). The Coatest LMW Heparin Kit (Chromogenix, Cat#82-1363-63) was used to perform the assay according to the user’s manual. Numerous dilutions of the collected plasma samples were carried out. The dilution resulting in a reading within the detection range of the assay (0.2 to 0.7 IU) was used. The final results were standardized and expressed in terms of International Units (IU) of activity per ml of undiluted maternal plasma.

3.3.12 Fetal and placental weights

Fetal and placental weights were measured for three arbitrarily chosen conceptuses in each litter. Choosing the conceptuses was done arbitrarily amongst both horns. The decidua and myometrium were removed from the placentas and the yolk sac was separated and discarded before weighing the placentas. The samples were weighed on a dry petri dish. The placental and fetal weights were recorded along with the litter size, and the maternal weight for each mouse.

3.3.13 Sexing the embryos

Fetal sex was determined for the placental labyrinth samples from the second mouse series used in the Qiagen qPCR array in order to allow for eliminating potential sex-related changes in gene expression. Fetal DNA was purified using Qiagen DNA Purification Mini Kit (Cat#12943). PCR was carried out using primers for the different *Jarid* genes (Table. 4) as originally described by Clapcote et al (Clapcote and Roder, 2005). The PCR products were then run on a 2% agarose gel as described by Clapcote et al and two bands were obtained for males and a single band for females (Fig. 42).
3.3.14 Statistical analysis

Software was used for statistical analysis (Graphpad Prism 5.2, San Diego, USA). The tests used were unpaired student t-test as well as one-way ANOVA for multiple group comparison using a Dunnet’s post-hoc test. One-way ANOVA with a Dunnett’s post hoc test was used to compare the different treatment groups to vehicle control. Newman Keul’s post hoc test was used to compared each of the treatment groups together. P values of less than 0.05 were defined as being statistically significant. Data were represented as mean ± SEM. Uppercase “N” was used to signify the number of dams and lower case “n” was used to signify the number of placentas or fetuses included in the data shown.

3.4 Results

3.4.1 Anti Xa assay

The anti-Xa assay was performed to determine plasma levels of heparin at the time of euthanasia in the 2nd series of mice injected from E9.5 until E12.5, or series 3 mice implanted with the pump from E5.5 to E12.5. Citrated plasma was collected using ultrasound guided cardiac puncture at the time of euthanasia for the assay. Mice in both series 2 and 3 were euthanized at 1 pm, which is 5 hours after the last bolus of LMWH in the second series. Plasma LMWH concentrations were increased by approximately 2 and 2.5 international units in mice treated with low dose, and high dose of heparin respectively for the series 2 group undergoing subcutaneous injection from E9.5 to E12.5 (p<0.0001, N=5). The same general trend was observed for series 3 mice infused with the drug eluting pumps from E5.5 to E12.5 but the low dose group seemed to have a lower plasma heparin concentration compared to the series 2 mice that were injected. In contrast, the high dose mice that were infused with heparin seemed to have a higher plasma heparin concentration at the time of euthanasia compared to the high dose mice from the twice-daily injection series 2 mice (N=6) (Fig. 17). Nonetheless none of the differences present between the different series of mice treated with the same dose of heparin, but with a different treatment method were significant.
Figure 17: Maternal plasma LMWH concentrations at the time of euthanasia. Left: Series 2 mice (twice-daily injections from E9.5 to E12.5). Right: Series 3 mice (infused from E5.5 to E12.5). Levels of plasma LMWH were determined using the anti-Xa functional activity assay. Vehicle control, saline; Low dose, 10 IU/day; High Dose, 70 IU/day; One-way ANOVA followed by Newman-Keuls post test. *P<0.05, **P<0.01, ****P<0.0001. N=5 for series 2 (left). N=6 for series 3 (right).

3.4.2 Fetal and placental growth

Placental weights were measured for mice euthanized at E12.5. Placental weight was significantly increased in the high dose mice compared to vehicle control when treatment was initiated at E5.5 (n = 18 from 6 dams). However, treatment starting at E9.5 did not result in significant changes for placental weight (n = 15 from 5 dams) (Fig. 18). The changes in the placental weight measures were not accompanied by changes in the fetal body weights. Namely, fetal body weight remained unchanged across different treatment arms independent of treatment initiation time, or administered dose of heparin (Fig. 19).
mRNA expression of the placental specific isoform of Insulin-like Growth Factor II, Igf2, a major modulator of placental growth (Constancia et al., 2002), was significantly upregulated at E12.5 in the whole placenta of high dose mice when treatment was started at E9.5. However, expression levels were not significantly different at E12.5 for mice treated from E5.5. Placental specific Igf2 gene expression was significantly reduced in the labyrinth-enriched samples of mice treated from E5.5 at the time of euthanasia (Fig. 20).

Figure 18: Placental weights. Mice were treated with vehicle or two different doses of LMWH in the 2nd series from E9.5 to E12.5 (left, N=5), or in the 3rd series from E5.5 to E12.5 (right, N=6). Three placentas were arbitrarily chosen and weighted from each litter. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-Way ANOVA. Dunnett’s Multiple Comparison Test. **p<0.01. n=15 for the 2nd series (left). n=18 for the 3rd series (right).
Figure 19: Fetal body weight. Mice were treated with vehicle or two different doses of LMWH in the 2nd series from E9.5 to E12.5 (left, N=5), or in the 3rd series from E5.5 to E12.5 (right, N=6). Three fetuses were arbitrarily chosen and weighted from each litter. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA, showed no significance. n=15 placetas for the 2nd series (left). n=18 placetas for the 3rd series (right).
3.4.3 **Placental mRNA, and maternal circulating levels of sFlt1 and Plgf**

Plasma sFLT1 levels in the mice euthanized at E12.5 were measured using EDTA plasma collected from the saphenous vein prior to euthanasia. There was a large variability observed between the mice even in the same treatment group, and no statistically significant difference was present between the treatment groups whether treated from E9.5 to E12.5 (N=5) or E5.5 to E12.5 (N=6) (Fig. 21). Maternal plasma PLGF protein concentrations showed a dose-dependent upregulation trend in heparin treated mice but the changes did not reach statistical significance (Fig. 23).

sFLT1 mRNA levels were measured in the labyrinth samples from series 2 and 3 mice. At the mRNA level, *sFlt1* expression was significantly increased (by 50%) in the placental labyrinth region of the high dose group treated from E5.5. Interestingly, this increase was paralleled by a significant 25% increase in gene expression of *Plgf* in the same labyrinth-enriched samples of this group (Fig. 22).
Figure 21: Maternal plasma sFLT1 concentrations 30 minutes prior to euthanasia at E12.5 for 2nd and 3rd series of mice. Left: 2nd series of mice (treated from E9.5 to E12.5). Right: 3rd series of mice (treated from E5.5 to E12.5). The concentration of sFLT1 was measured using blood collected from the saphenous vein 30 minutes prior to euthanasia. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA showed no significance. N=5 per group for 2nd series (left). N=6 per group for the 3rd series (right).
Figure 22: mRNA levels of sFlt1 and Plgf at E12.5 in the labyrinth. Labyrinth-enriched tissue was used for the measurements in the 3rd series of mice (treated from E5.5 to E12.5, N=6). Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA. Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01. n=12 per treatment group.

Figure 23: Maternal plasma PLGF concentrations 30 minutes prior to euthanasia at E12.5 for the 3rd series of mice (treated from E5.5 to E12.5). The concentration of PLGF was
measured using blood collected from the saphenous vein 30 minutes prior to euthanasia. Vehicle Control, saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA showed no significance. N=6 per treatment group.

### 3.4.4 Placental mRNA levels of the cell proliferation marker, *Ki67*

qRT-PCR analysis of E12.5 micro-dissected labyrinth samples were done to study mRNA levels of *Ki67* at the time of euthanasia. The results revealed an upregulation of *Ki67* expression in mice treated with high dose of LMWH compared to the vehicle control mice when treatment was initiated at E9.5. *Ki67* gene expression also showed an upregulation trend in the high dose mice when treatment was started earlier at E5.5 but the upregulatory trend was not statistically significant (Fig. 24).

![Figure 24: mRNA levels of *Ki67* at E12.5 in the labyrinth. Left: 2nd series of mice (treated from E9.5 to E12.5, N=5) Right: 3rd series of mice (treated from E5.5 to E12.5, N=6). mRNA levels of *Ki67* were measured in the placental labyrinth region. Vehicle control,](image-url)
Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA, Dunnett’s post test. *p<0.05. n=10 per group for the 2nd series (left). n=12 per group for the 3rd series (right).

3.4.5 Placental mRNA levels of suspected trophoblast progenitor markers

There was an upregulation in the expression of suspected trophoblast progenitor cell markers *Eomes* and *Sca1* in mice treated with heparin from E9.5 to E12.5 in the labyrinth region of the placenta at E12.5 (n=10 per treatment group). Moreover, the same results were observed when treatment was started earlier at E5.5 with respect to those markers (*Eomes, Sca1*) (n=12) (Fig. 25).

It was very recently published that a well established epithelial stem cell marker, namely *Epcam*, is involved in maintaining the trophoblast progenitor cells in the labyrinth via *Met* dependent hepatocyte growth factor (*Hgf*) signaling (Ueno et al., 2013). mRNA expression of *Epcam* was upregulated when whole placental tissue was looked at but surprisingly it was downregulated in the labyrinth region in mice treated with heparin from E5.5 to E12.5 despite placental overgrowth in those mice only (Fig. 26). *Epcam* gene expression was only measured in whole placental, and labyrinth enriched samples from the third mouse series only.

There was also a trend of mRNA expression upregulation for *Fgfr2* in the labyrinth-enriched samples of mice in the second series treated with heparin from E9.5 to E12.5 but the changes did not reach statistical significance (Fig. 27). *Fgfr2* gene expression was only measured in the labyrinth samples from the second mouse series.
Figure 25: mRNA levels of suspected trophoblast progenitor markers at E12.5 in the labyrinth region. Left: 2nd series of mice (treated from E9.5 to E12.5, N=5) Right: 3rd series of mice (treated from E5.5 to E12.5, N=6). The mRNA expression levels of suspected trophoblast progenitor markers were measured in the placental labyrinth region at E12.5. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA. Dunnett’s post test. *p<0.05, **p<0.01, ***p<0.001. n=10 per group (left column); n=12 per group (right column).

Figure 26: mRNA levels of Epcam at E12.5 for 3rd mouse series (treated from E5.5 to E12.5, N=6). Either whole placenta (left), or labyrinth only (right) tissue was used. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA, Dunnett’s Multiple Comparison Test (right). Left graph only significant by Unpaired t-test (not significant by one-way ANOVA). *p<0.05. n=12 per group.
Figure 27: mRNA expression of Fgfr2 at E12.5 for the 2nd series of mice (treated from E9.5 to E12.5, N=5). Labyrinth-enriched samples were isolated and gene expression of Fgfr2 was measured in the labyrinth enriched samples. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA showed no significance. n=10 per group.

3.4.6 Placental mRNA levels of syncytiotrophoblast markers

Micro-dissected E12.5 labyrinth samples were also used to measure mRNA levels of syncytiotrophoblast markers. Gcm1 expression was significantly decreased in mice treated with the low dose of LMWH as compared to the vehicle control when mice were injected from E9.5 to E12.5 (p<0.01, n=10). Starting treatment with continuous infusion of heparin earlier at E5.5 however, did not alter the pattern, and levels of Gcm1 expression. There were no significant changes observed in other syncytial markers measured in the labyrinth-enriched samples from mice in the second and third series (Fig. 28).
Figure 28: mRNA expression of syncytial markers in the labyrinth region at E12.5 for 2nd and 3rd series of mice. Left: 2nd series of mice (treated from E9.5 to E12.5, N=5). Right: 3rd series of mice (treated from E5.5 to E12.5, N=6). The mRNA levels of the syncytial markers were measured in placental labyrinth-enriched samples. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA showed no significance. n=10 per group (left); n=12 per group (right).
3.4.7 **Placental mRNA levels of sinusoidal trophoblast giant cell markers**

mRNA levels of the sinusoidal trophoblast giant cell (S-TGC) markers, placental lactogen 2 (*Prl3b1*) and cathepsin-q (*Ctsq*), were measured in the E12.5 placental labyrinth samples. Placental lactogen II expression was significantly upregulated in the high dose mice compared to controls when treatment was started earlier at E5.5. The same trend was observed when mice were treated starting later at E9.5, but the increase did not reach statistical significance in the latter group. *Ctsq* expression was decreased in both low dose, and high dose mice compared to the vehicle controls (p<0.05 and p<0.01 respectively) when mice were treated from E9.5 to E12.5. Nevertheless, starting treatment earlier at E5.5 resulted in the opposite trend for *Ctsq*: namely, there was a non-significant upregulatory trend in gene expression of *Ctsq* in the labyrinth region of mice treated with LMWH starting E5.5 (Fig. 29) (n=10 for treatment from E9.5 to E12.5), (n=12 for treatment from E5.5 to E12.5).
Figure 29: mRNA levels of sinusoidal trophoblast giant cell (S-TGC) markers at E12.5 for the 2\textsuperscript{nd} and 3\textsuperscript{rd} series of mice. Left: 2\textsuperscript{nd} series of mice (treated from E9.5 to E12.5, N=5); Right: 3\textsuperscript{rd} series of mice (treated from E5.5 to E12.5, N=6). Gene expression levels of the markers were measured in E12.5 placental labyrinth-enriched samples. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA. Dunnett’s Post Test. *p<0.05, **p<0.01. n=10 per group (left); n=12 per group (right).

3.4.8 Placental morphometry

There were no differences observed between the different treatment groups regarding the labyrinthine area and perimeter measured at placental mid-sections for samples collected at the
E12.5 or E14.5 time points corresponding to the 1\textsuperscript{st} and 2\textsuperscript{nd} series of mice respectively. (n=9 and n=15 respectively) (Fig. 30, 31). Since no change in the labyrinth area measurements were observed in the second series of mice, the placental samples from series 1 which were euthanized at E14.5 were examined to allow for further growth of the labyrinth section of the placenta but again there was no difference between the groups (Fig. 31). Starting treatment earlier at E5.5 for the 3\textsuperscript{rd} and last series of mice did not change the percentage labyrinth of the whole placental area across the different treatment groups at the time of euthanasia (E12.5) (Fig. 32). Similarly, starting heparin treatment at E5.5 via infusion did not result in any significant changes in the labyrinthine area despite the previously described increase in placental mass in the high dose group mice. (Fig. 32)

Fetal, and maternal blood space area measures were carried out using CD34-stained placental mid-sections of mice treated from E5.5 to E12.5. There was a significant increase in fetal blood space in the high dose mice compared to controls (Fig. 33). Moreover, there was a statistically significant, yet dose independent decrease in the maternal blood space area measurements in mice treated with heparin (both low dose and high dose) compared to vehicle controls (Fig. 34). There was no significant difference between the treatment groups with respect to the area of labyrinth other than maternal or fetal blood spaces (Fig. 35).

**Figure 30:** Perimeter, and area of the labyrinth portion of the placentas at E12.5 for 2\textsuperscript{nd} series of mice (N=5). The area, and perimeter of the labyrinth component of the placenta
was measured at E12.5 after commencing treatment at E9.5. Control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA showed no significance. n=15 per group.

Figure 31: Perimeter, and area of the labyrinth portion of the placentas at E14.5 for the 1st series of mice (N=5). The area, and perimeter of the labyrinth was measured using placental mid sections collected at E14.5 after commencing heparin treatment at E9.5. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA showed no significance. n=9 per group.
Figure 32: Total placental, and labyrinth area at E12.5 for the 3rd series of mice (N=6). The area of the whole placenta was measured, and the area of the labyrinth was normalized to the whole placental area using CD34 staining of the placental mid-sections. Mice were treated starting at E5.5. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA showed no significance. n=10 (vehicle control), n=7 (low dose), n=9 (high dose) per group.
Figure 33: Fetal blood space measurements at E12.5 for the 3rd series of mice (N=6). Fetal blood space area was computed via point counting using CD34 to stain the fetal endothelium in placental mid-sections. Mice were treated from E5.5. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One way ANOVA. Dunnett’s Post Test. *p<0.05. n=8 (vehicle), n=7 (low dose), n=10 (high dose) per group.

Figure 34: Maternal Blood space measurements at E12.5 for 3rd series of mice (treatment from E5.5 to E12.5, N=6). Maternal blood space area was computed via point counting using CD34 to stain the fetal endothelium in placental mid-sections. Mice were treated...
from E5.5. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA. Dunnett’s Post Test.**p<0.001. n=8 (vehicle control), n=7 (low dose), n=10 (high dose) per group.

Figure 35: Area of labyrinth not occupied by maternal and fetal blood spaces at E12.5 for the 3rd series mice (N=6). The area of the labyrinth not occupied by maternal or fetal blood spaces was computed via point counting using CD34 to stain the fetal endothelium in placental mid-sections. Mice were treated from E5.5. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA showed no significance. n=8 (vehicle control), n=7 (low dose), n=10 (high dose) per group.

3.4.9 Expression patterns of suspected progenitor markers

Placental samples collected at E14.5 were stained for BrdU. Measurement of the density of BrdU positive nuclei was carried out by counting BrdU-positive cells in the labyrinth of 1st series of mice injected with heparin from E9.5 to E14.5. There was no significant difference observed between the treatment groups with respect to the total number of proliferative cells (BrdU-positive) per area of placental labyrinth (Fig. 36, 37) (n=10).

Visualization of proliferative trophoblast cells was carried out by means of double fluorescent immunohistochemistry on placental samples from the E12.5 samples from the 2nd series mice via staining for cytokeratin-18 (trophoblast marker), and phospho-histone H3
(proliferative cell marker) (Fig. 38). Accurate pinpointing of double-stained cells, however, was not practical. Moreover, successful double staining FIHC procedures using the aforementioned two antibodies were not reproducible despite various subsequent attempts.

IHC staining for the suspected trophoblast progenitor markers Sca1, and FGFR2 IIIb,c were done in order to verify, and study the labyrinthine expression pattern of these markers which we hypothesized were involved with maintaining proliferative trophoblast progenitor cells. Both markers were expressed in cell clusters in the labyrinth region as previously hypothesized in the literature (Simmons and Cross, 2005; Ueno et al., 2013) (Fig. 39, 40).

E12.5 placentas were stained for EPCAM in order to determine whether this trophoblast progenitor marker was expressed within cell clusters. Similar to the expression pattern seen with Sca1 and FGFR II, and suggested by recent literature (Ueno et al., 2013), the expression pattern was seen as being localized in cell clusters scattered throughout the labyrinth region (Fig. 41).

Figure 36: BrdU staining of the mouse placenta at E14.5 for 1st series of mice (treated from E9.5 to E14.5). Cell nuclei are visualized with Hematoxylin (blue), BrdU-positive nuclei are stained brown. Left: 2x magnification, Right: 20x magnification.
Figure 37: Number of proliferative cells (BrdU-positive) per unit area of labyrinth for the 1st series of mice. Placental midsections stained for BrdU were used in mice treated from E9.5. Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-way ANOVA showed no significance. n=10, N=5 per group.
Figure 38: Double-stained PHH3 and Cyt18 placental samples at E12.5 from the 2\textsuperscript{nd} series of mice (treated from E9.5 to E12.5). Cell nuclei are visualized by DAPI (blue), Proliferating nuclei are visualized by PHH3 (green), and trophoblast cells are visualized by cytokeratin-18 (red). Left: 2x magnification, Right: 20x magnification.

Figure 39: SCA1 staining for the placental samples at E12.5 for the 3\textsuperscript{rd} series of mice (treated from E5.5 to E12.5). Sca1-positive membranes were stained brown and other cells stained blue (hematoxylin). Sca1 positive cells were seen to be localized in clusters in the labyrinth region of the placentas. Top left: 2x magnification, Others: 20x magnification.
Figure 40: FGFR2 IIIb staining for the placental samples at E12.5 for the 3rd series of mice (treated from E5.5 to E12.5). FGFR2 positive membranes were stained brown and other cells stained blue (hematoxylin). FGFR2 positive cells were seen as localized in clusters in the labyrinth region of the placenta. Staining for FGFR2 IIIc yielded a similar expression pattern (data not shown). Top: 2x magnification, bottom: 20x magnification.

Figure 41: EPCAM staining of the placental samples from the 3rd series of mice (treated from E5.5 to E12.5). EPCAM-positive membranes were stained brown and other cells stained blue (hematoxylin). EPCAM positive cells were observed to be localized in clusters in the labyrinth region of the placenta. Top left: 2x magnification, Others: 20x magnification.
3.4.10 Global transcription factor qPCR array

The fetuses corresponding to labyrinth-enriched samples collected for the E9.5 to E12.5 series 2 mice were sexed in order to eliminate sex-biased analysis of the qPCR array. Each sample was prepared by pooling the cDNA from one male, and one female labyrinth-enriched sample (Fig. 42). One pooled sample was used from each mouse resulting in 5 samples per treatment group. The pooled samples were run on a qPCR array containing major cell regulatory transcription factors (Qiagen, Mouse Transcription Factor Array).

The p-values are calculated using Student’s t-test (assuming equal variance and two-tail distribution) for each gene. No false discovery rate corrections were made and p-values of less than 0.05 were considered significant. Only two genes (Gtf2b and Nfkb) were upregulated in the low dose mice compared to the vehicle control mice (treatment from E9.5 to E12.5). The trend was different for the mice treated with high dose of LMWH (Table. 5). Namely, 12 genes were significantly downregulated in mice treated with high dose of LMWH heparin from E9.5 to E12.5 (Table. 5). Among the 12 genes significantly downregulated in the high dose group of mice, Hif1a and its heterodimerization partner Arnt were downregulated by 22, and 44 percent respectively in the high dose mice compared to vehicle controls (p=0.0003 and p=0.003 respectively). Other general transcription factors implicated in a variety of signaling pathways such as Fos, and Jun were also downregulated in the high dose mice compared to vehicle controls (Table. 6).

Further qPCR experiments were performed on placental samples from mice treated with the high dose of LMWH from E9.5 to E12.5 in order to verify the qPCR array results, which showed highly significant reduction in mRNA expression levels of Hif1a (Table. 6). Subsequent qPCR experiments did not show a reduction in Hif1a expression in the labyrinth-enriched samples of high dose mice as seen in the qPCR array. Interestingly nonetheless, there was a significant and consistent downregulation of Hif1a gene expression in mice treated with the low dose of heparin for both 2nd and 3rd series of mice (Fig. 43). Moreover, the effect was labyrinth-specific since mRNA levels did not change when mRNA levels of Hif1a were measured in the whole placental samples of the high dose mice in 2nd or 3rd mouse series (Fig. 43)
Figure 42: Sexing of fetuses at E12.5 for the 2\textsuperscript{nd} series of mice (treated from E9.5 to E12.5). Fetuses corresponding to the collected placentas were sexed using *jarid* gene isoforms. Males are represented by two bands, and females are visualized having a single band. Data not shown entirely.

Table 5: Normalized mRNA expression changes for measured genes of interest at E12.5 for low dose mice (10 IU/day of LMWH) in the 2\textsuperscript{nd} mouse series. The table includes the genes that were changed significantly (p<0.05) in the low dose mice compared to the vehicle controls in the qPCR array. Unpaired student t-test. n=5, N=5.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene</th>
<th>P-Value</th>
<th>Low Dose / Vehicle Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>General transcription factor IIB</td>
<td><em>Gtf2b</em></td>
<td>0.03</td>
<td>1.91</td>
</tr>
<tr>
<td>Nuclear factor NF kappa, p105</td>
<td><em>Nfkb1</em></td>
<td>0.019</td>
<td>2.29</td>
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</table>
Table 6: Normalized mRNA expression changes for the measured genes of interest at E12.5 for high dose mice (70 IU/day of LMWH) in the 2nd series of mice. The table includes the genes that were changed significantly (p<0.05) in the high dose mice compared to the vehicle controls in the Qiagen TF PCR array. Unpaired student t-test. n=5, N=5.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene</th>
<th>P-Value</th>
<th>High Dose / Vehicle Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
<td>Arnt</td>
<td>0.003</td>
<td>0.66</td>
</tr>
<tr>
<td>Activating transcription factor 3</td>
<td>Atf3</td>
<td>0.014</td>
<td>0.8</td>
</tr>
<tr>
<td>CAMP responsive element binding protein 1</td>
<td>Creb1</td>
<td>0.039</td>
<td>0.85</td>
</tr>
<tr>
<td>Down-regulator of transcription 1</td>
<td>Dr1</td>
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<td>0.83</td>
</tr>
<tr>
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<td>E2f1</td>
<td>0.004</td>
<td>0.86</td>
</tr>
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<td>Jun</td>
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<td>0.79</td>
</tr>
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<td>Fos</td>
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<td>0.78</td>
</tr>
<tr>
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<td>Gata3</td>
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<td>0.9</td>
</tr>
<tr>
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<td>0.78</td>
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<td>0.84</td>
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<tr>
<td>Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3</td>
<td>Nfate3</td>
<td>0.019</td>
<td>0.83</td>
</tr>
<tr>
<td>Trans-acting transcription factor 1</td>
<td>Sp1</td>
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<td>0.79</td>
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Figure 43: mRNA levels of *Hif1a* at E12.5 for the 2nd and 3rd series mice. Gene expression was measured in labyrinth-enriched samples (top row), as well as whole placental samples (bottom row). Left: 2nd series of mice (treated from E9.5 to E12.5, N=5). Right: 3rd series of mice (treated from E5.5 to E12.5, N=6). Vehicle control, Saline; Low dose, 10 IU/day; High Dose, 70 IU/day. One-Way ANOVA. Dunnett’s post test. *P<0.05. n=10 per group (left). n=12 per group (right).
3.5 Discussion

3.5.1 Overview

Altogether, only continuously infusing mice starting at E5.5 with a supra-therapeutic dose of heparin was shown to be associated with placental overgrowth without altering fetal weight. Moreover, heparin treatment increased the labyrinth expression of suspected trophoblast progenitor marker *Eomes* in a dose-dependent manner regardless of when treatment was started suggesting a higher retention of progenitor cells by heparin treatment. Heparin treatment did not significantly change the expression of syncytiotrophoblast markers *Gcm1*, *Syna*, and *Synb* suggesting that heparin treatment did not affect differentiation of the syncytial layer. Heparin treatment, nonetheless, altered the expression levels of S-TGC markers *Prl3b1* and *Ctsq* along with resulting in a reduced maternal blood space area in heparin treated mice suggesting altered S-TGC function. Moreover, heparin treatment was shown to result in increase fetal blood space area in mice treated from E5.5 with high dose of heparin.

3.5.2 Anti-Xa assay

Anti-Xa assay was performed to measure plasma levels of LMWH in the treated mice. Plasma LMWH levels were elevated in mice treated with heparin as expected regardless of the method of delivery (subcutaneous injection vs continuous infusion), or treatment initiation time (E5.5 or E9.5).

The measured plasma heparin concentration in the series 2 low dose mice (1.9 IU/ml) was near the expected concentration of around 3.75 IU/ml. The expected concentration was calculated based on a LMWH half-life of roughly five hours in humans (Hirsh, 2001). Moreover, drugs are pharmacologically considered to be cleared from circulation after five half-lives (Hirsh, 2001). Taking the aforementioned information into account, mice injected with heparin would have ~50% of the levels of the morning bolus, along with ~25% of the LMWH from the previous night in their circulation at the time of euthanasia. Therefore, the level of LMWH detected in low dose mice injected with 5 IU/bolus twice daily is expected to be 3.75 IU/ml. For
the high dose mice injected twice daily (35 IU/bolus), the plasma LMWH levels were significantly higher than the low dose group by +21% (2.3 IU/ml) but this was much less than the expected 7-fold increase based on the dosage difference (Fig. 17).

Continuously infusing mice with heparin resulted in plasma LMWH concentrations of 0.4 IU/ml, and 4.7 IU/ml at the time of euthanasia in low dose, and high dose mice respectively (Fig. 17). The plasma concentration in the high dose mice was ~12-fold higher than the low dose mice. However, the measured concentrations for both doses were 3-5 fold less than the expected plasma concentrations of 1.9 IU/ml and 13.5 IU/ml of LMWH at the time of euthanasia in the low dose, and high dose mice respectively. The expectations were based on the fact that continuously infused drugs reach a steady state concentration after 5 half lives given a constant infusion rate, and the plasma concentration of the drug is maintained until infusion is stopped (Hill, 2004). Therefore, the plasma levels of LMWH would reach a constant plateau after 25 hours (E6.5), and the levels will remain constant until the mice are euthanized at E12.5. The steady-state concentration can be relatively accurately approximated by treating every half-life of the drug (5 hours in this case) as a “bolus” at the end of each half-life (Hill, 2004). The low dose mice are infused with roughly 2 international units of LMWH per half-life (10 IU/day or 2 IU every five hours). Thus, the steady state plasma LMWH concentration for the low dose mice would be equal to the sum of the drug remaining from the previous five “boluses” (1+0.5+0.25+0.125+0.06 = 1.9). The expected levels for the continuously infused mice would be seven times the levels of low dose mice, which would be 13.3 IU/ml. The difference between the expectations and the much lower measured concentrations for both the low dose and high dose mice could be be attributed to the premature ceasing of infusion by the pumps as they reach the end of their reservoir. It is noteworthy to mention that the high variability in the high dose mice which were continuously infused is likely attributable to the low sensitivity of this assay (± 0.1 IU/ml). On the other hand, low variability observed within the high dose mice in the second series (injected twice daily) is likely due to the measurements having been close to the upper limit of assay detection, and hence the assay being saturated and giving a rather uniform reading for those high dose mice.

Detecting the levels of heparin in plasma has long been a challenge clinically (Vandiver and Vondracek, 2013). With the advent of LMWH, plasma heparin concentrations can be more accurately determined since LMWH has a more predictable level of activity, which can be in
turn measured by performing anti-Xa assays. Nonetheless, these assays generally have a very narrow range of detection and rather low sensitivity (Detection range: 0.2-0.7 IU/ml, Sensitivity: 0.1 IU/ml). Moreover, citrated plasma is highly unstable with respect to platelet activation, and unfortunately the only type of plasma suitable for coagulation assays since the activation, as well as blockage of the coagulation cascade needs to be avoided for these assays to be successfully carried out. The aforementioned factors are likely contributing to the variability of the results observed, coupled with the saturation of the assay seen for the high dose group in the 2nd series. Altogether, despite the weaknesses associated with anti-Xa assays, the results showed that the administered heparin makes it into the general circulation of the mice regardless of whether it is injected or infused. Moreover, the data suggest that the half-life of LMWH in mice roughly corresponds to the half-life of 5 hours seen in human pregnancies (Kingdom and Drewlo, 2011) because of the similarity between the measured and the expected concentrations calculated using this half-life for the series 2 low dose mice.

3.5.3 Fetal and placental weights, placental growth marker levels

We hypothesized that heparin treatment would result in longer retention of trophoblast progenitor cells ultimately causing placental overgrowth. Indeed, heparin treatment was seen to be associated with increased placental weight without an accompanying increase in the fetal weights in mice only when treatment was commenced early at E5.5. Commencing heparin treatment at E5.5 is during the peri-implantation period. Even though FGF4-dependent signaling was not looked at in this study, heparin may have resulted in placental overgrowth via altered FGF4-mediated FGFR2 signaling which plays a very important role in maintenance of trophoblast stem cells during that time of gestation. In order to look at gene expression level of a placental specific growth marker, mRNA levels of Igf2 were measured in placental samples collected from 2nd and 3rd series of mice. Interestingly, the mRNA levels of Igf2 were decreased in the labyrinth region in mice infused with heparin from E5.5. The labyrinthine-specific decrease of Igf2 gene expression in 3rd series of mice could suggest that the increased weight of the placentas in high dose mice might not be attributable to a larger labyrinth region per se.

The labyrinth region of the mouse placenta starts forming after the chorioallantoic attachment 8.5 days into gestation. Starting heparin treatment at E9.5 did not result in an
increased placental mass whereas starting heparin at E5.5 did, which points us towards suspecting that the increased placental mass in the E5.5 group is indeed not caused by a bigger labyrinth. Moreover, increased whole placental _Igf2_ gene expression in the high dose group mice treated from E9.5 was not paralleled by the high dose mice treated from E5.5 suggesting that the placental overgrowth in the E5.5 mice is likely attributable to the earlier growth phase of the placenta and independent of labyrinthine formation and growth. Alternatively, increased placental mass can be caused by changes in factors such as increased interhaemal membrane thickness, increased total cell numbers without a corresponding increase in area and volume, or simply tissue edema. It could also be possible that cell size in increased leading to increase in placental mass.

### 3.5.4 Placental mRNA, and maternal circulating levels of _sflt1_ and _Plgf_

Maternal plasma levels of sFLT1 were measured to examine the effects of heparin treatment on the levels of this anti-angiogenic protein in treated mice. Heparin is associated with increased circulating sFLT1 levels by ~4 fold in pregnant women (Rosenberg et al., 2011). Placental mRNA expression of _Flt1_ was elevated in the 3rd series of mice infused with high dose of heparin suggesting increased placental production of sFLT1 (Fig. 22). Surprisingly, plasma levels of sFLT1 were not statistically different in the mice treated with heparin compared to mice in the vehicle control group regardless of treatment method and treatment initiation times (Fig. 21). Searle et al (2011) found both an acute, and sustained upregulation in plasma sFLT1 protein levels in male mice after heparin treatment (10 minutes, and 3 days respectively) (Searle et al., 2011). The data show that CD-1 wild-type mice have a natural large variability with respect to circulating sFLT1 levels during normal pregnancy. However, the increase in sFLT1 levels after heparin treatment in male CD-1 mice reported by the Searle et al group is significantly less than the fold differences naturally present in normal pregnancy in CD-1 mice at E12.5. Therefore, the effects of heparin treatment with respect to plasma sFLT1 cannot be conclusively studied comparing different treatment groups to one another, since such comparison would not be plausible in pregnant CD-1 mice due to the heterogeneity of these mice with respect to circulating plasma sFLT1 levels.
At the mRNA level, Flt1 gene expression was significantly elevated in the labyrinthine region of the placenta of mice treated from E5.5 to E12.5 suggesting augmented de novo synthesis of sFLT1. The increase in Flt1 gene expression can be attributed to the trophoblast compartment, as well as mesenchyme and endothelium in the placental labyrinth region given the regional expression of this gene.

PLGF, a member of the VEGF family, is a pro-angiogenic factor and lowered circulating PLGF levels have recently been shown to be a very robust predictor of PE pathology in a subset of women independent of circulation sFLT1 levels as early as 16 weeks of gestation (Chappell et al., 2013) (Powers et al., 2012). The maternal levels of PLGF were measured in mice treated from E5.5 to examine the effects of heparin treatment with respect to this pro-angiogenic factor. There appeared to be a trend of dose-dependent increase in heparin treated mice but none of the changes reached statistical significance. Namely, there was a high level of variability observed for circulating PLGF in mice (COV of 23% for vehicle control) as seen with circulating sFLT1 levels (COV of 83% for vehicle control). Interestingly, there was an accompanying increase in gene expression of placental growth factor (Plgf) in the placental labyrinth region of heparin treated mice. Our observations suggest that the beneficial effects of heparin treatment in pregnancy are likely independent of circulating sFLT1 levels, or the detrimental effects brought about by elevated sFLT1 levels are overridden by a parallel elevation in PLGF gene expression and potentially by maternal circulatory levels of PLGF.

3.5.5 Placental mRNA levels of global proliferation marker (Ki67)

mRNA expression level of Ki67 was measured in the E12.5 labyrinth-enriched samples of mice treated with heparin starting at either E5.5, or E9.5 as a measure of global cell proliferation. In agreement with our hypothesis, heparin treatment was associated with increased mRNA expression of Ki67 in the labyrinth region of the placenta in the high dose group when mice were treated starting at E9.5, but the effects did not reach significance when treatment was started at E5.5. Coupled with reduced expression of Igf2 in the labyrinth region of heparin infused high dose mice (treated E5.5 to E12.5), unaltered expression of Ki67 in the labyrinth region of these mice further suggests that the placental overgrowth observed in the high dose group treated from E5.5 might not be attributable to a bigger labyrinth region. The IHC data
(section 3.4.8) further supports the previous statement since the area and perimeter of labyrinth was unaltered after heparin treatment.

It is important to mention that both $Ki67$ and $Igf2$ are not necessarily trophoblast-specific, and hence do not reflect the potential proliferative effects of heparin treatment in trophoblast progenitor cells in the labyrinth region of the placenta. Namely, the changes in placental morphometry and expression levels of the markers discussed so far do not necessarily nullify our hypothesis regarding heparin treatment causing a longer retention of proliferative trophoblast progenitor cells.

### 3.5.6 Placental mRNA levels, and expression patterns of suspected trophoblast progenitor markers

Signaling through FGFR2 is thought to be at least partly responsible for maintaining proliferative trophoblast progenitor cells. $Fgfr2$ mRNA was detected at E12.5 in the labyrinth-enriched samples of 2nd series of mice (Fig. 27), along with staining of E12.5 placentas from the 3rd mouse series (Fig. 40). The aforementioned pieces of evidence make possible the involvement of heparin in FGF4-mediated signaling in the labyrinth. Nonetheless we do not have evidence in this study to show that heparin caused changes in FGFR2 signaling in the labyrinth region of mice.

mRNA levels of the suspected trophoblast progenitor markers were measured in the labyrinth-enriched samples to examine the effects of heparin treatment by further pinpointing our cell subtype of interest. The increase in mRNA expression levels of $Eomes$ in the labyrinth region of 2nd and 3rd series mice suggests that heparin treatment may increase an $Eomes$-expressing subgroup of trophoblast progenitor cells. $Eomes$ is strongly expressed towards the chorionic side of the placenta where labyrinthine trophoblast stem cells are thought to reside. However, $Eomes$ is also expressed in the yolk sac (Kwon and Hadjantonakis, 2007; Wu et al., 2003) so it is possible that increased levels of $Eomes$ could be due to contamination from yolk sac tissue attached to the chorionic plate of the labyrinth-enriched samples. The use of laser capture microdissection in future studies could resolve this question by ensuring greater purity of labyrinthine specimens.
Also observed was an increase in mRNA expression of \textit{Sca1} in the 3\textsuperscript{rd} series of mice. It is noteworthy that \textit{SCA1} is also an established marker for hematopoietic stem cells (HSCs), which are extensively present in the mouse placenta at E12.5 (Wei et al., 2008). We do not have evidence in this study indicating that the increased expression of \textit{Sca1} was trophoblast-specific. Thus it is possible that heparin increased mRNA expression of \textit{Sca1} due to alterations in the HSC pool in the placenta. However, the levels of \textit{Hif1a} didn’t change suggesting no placental hypoxia hence the there would appear to be no reason for the HSC pool to change (Imanirad et al., 2014). Despite lack of evidence suggesting alteration of HSC population in this study, it might be the case that heparin indeed alters the HSC population in treated women and hence future studies are warranted.

Expression of neither \textit{Cd2}, nor \textit{Rhox4b} was altered by heparin treatment in disagreement with the hypothesis. As well as being suspected labyrinthine trophoblast progenitor markers (Wu et al., 2003), both the aforementioned markers are highly expressed in the spongiotrophoblast layer of the mouse placenta around E12.5 (Beck et al., 1995; Jackson et al., 2003). \textit{Eomes} on the other hand is strongly expressed towards the chorionic side of the placenta (Kwon and Hadjantonakis, 2007; Wu et al., 2003). Collectively, given our results it is plausible that heparin treatment is not altering the trophoblast progenitor cell population located in proximity of the spongiotrophoblast layer but may be augmenting trophoblast progenitor cell population in proximity to the chorionic plate.

Expression of \textit{Epcam}, a recently proposed marker for the labyrinthine trophoblast progenitor cell, was measured in both whole placenta and labyrinth-enriched samples from the 3\textsuperscript{rd} mouse series to see if gene expression levels of \textit{Epcam} were increased similar to the aforementioned markers after heparin treatment. At the mRNA level, \textit{Epcam} was upregulated in the whole placenta of the 3\textsuperscript{rd} series high dose mice. But because IHC staining of the 3\textsuperscript{rd} series placentas showed heavy staining for EPCAM in the decidual component of the placenta, gene expression data from the labyrinth-enriched samples pertains to our hypothesis more. Surprisingly, mRNA levels of \textit{Epcam} were downregulated in the labyrinth-enriched samples of 3\textsuperscript{rd} series mice (Fig. 26). EPCAM is a cell surface transmembrane marker and proteins of such class are well established to have a very low mRNA to protein expression correspondence. Therefore, assessment of EPCAM protein levels would provide a much more reliable measure for studying the effects of heparin on this epithelial marker. This would likely need to be done using semi-
quantitative IHC because EPCAM is expressed by only a very limited subset of cells within the labyrinthine area (Ueno et al., 2013). It is not known whether the same population of trophoblast progenitor cells expresses both Eomes and EpCAM or whether they are each expressed by different non-overlapping labyrinthine trophoblast progenitor cell types. Heparin, as a result, may selectively affect the Eomes-expressing subtype of trophoblast progenitor cells.

Collected placenta samples were then stained for suspected progenitor markers to examine the localization, and expression pattern of these markers. Initially, BrdU-positive cells were studied to measure the potential changes in global cell proliferation brought about by heparin treatment. Initial counting of the total number of proliferative cells (BrdU-positive) per area of labyrinth in 1st mouse series did not reveal significant differences between the treatment groups at E14.5 failing to support our hypothesis. In order to further pinpoint the hypothesized cell type of interest (namely proliferative trophoblast cells), Cytokeratin-18 and PHH3 fluorescent double staining (FIHC) has been carried out to label trophoblast, and proliferative cells respectively. Nonetheless, quantification was not possible since reliable quantification proved to be difficult due to the ambiguity of identifying double-labelled cells under the microscope. The ambiguity arose from the fact that the markers stain different cellular compartments. Namely cell membrane and cell nucleus for Cyt18 and PHH3 respectively hence making identification of double-labelled cells very difficult. Furthermore, the FIHC procedure for Cyt18 and PHH3 was not as successful in the subsequent rounds of experiments. Cyt18 was not labeling the trophoblast cells of the placenta despite various attempts to replicate the initial results.

IHC staining was then done for SCA1 and FGFR2 to study the expression pattern of these suspected proliferative trophoblast progenitor markers in the labyrinth area of the heparin treated mice. The results showed very selective staining of a few cell clusters in the labyrinth region. Expression of progenitor markers in cell clusters within the labyrinth area is consistent with the latest literature on mouse trophoblast progenitor cells, which shows a similar localization pattern for trophoblast progenitor cells (Ueno et al., 2013). It was later decided that double-labeling placental sections with EPCAM as a trophoblast progenitor marker, along with PHH3 would help us more accurately pinpoint the proliferative trophoblast progenitor cell population we hypothesized to be affected by heparin treatment. To do so, placentas collected from mice treated from E5.5 to E12.5 were successfully stained for EPCAM using regular IHC.
Nonetheless, fluorescent staining of placental sections with EPCAM was not successful. Staining the placentas with EPCAM and PHH3 would allow for very accurate visualization, and quantification of double-labeled proliferative trophoblast progenitor cells within the labyrinth region as a direct measure of examining the effects of heparin treatment on the numbers, and proliferative status of those cells.

3.5.7 **Placental mRNA levels of syncytiotrophoblast, and sinusoidal trophoblast giant cell markers**

mRNA levels of genes expressed by syncytial layers I and II were measured in order to examine the potential effects of heparin treatment on trophoblast cell differentiation. Since the levels of SynT markers were not changed by heparin treatment for the 2nd and 3rd series of mice, there is no evidence suggesting altered levels of syncytiotrophoblast differentiation in the high dose placentas in those series despite the aforementioned changes in the levels of a subset of trophoblast progenitor markers in the high dose mice. It can be concluded from our data that the syncytial compartment of the labyrinth region is likely not affected by heparin treatment as far as our data indicates. Altogether, heparin treatment does not directly affect syncytial differentiation as we hypothesized.

mRNA levels of S-TGC markers were measured to examine the effects of heparin treatment on this particular subtype of labyrinthine trophoblast giant cells, which are direct contact with maternal blood, and hence the administered heparin. The consistent upregulation of Prl3b1 expression observed in the labyrinth region of heparin treated mice could be pointing towards altered numbers/physiology of the S-TGC population in those mice. Since the expression of Ctsq by S-TGCs appears at E11.5, a day before Prl3b1, and both of the markers are thereafter expressed until term. The increased expression of Prl3b1 in the E12.5 placental samples treated with heparin suggests that heparin treatment is resulting in a more “mature” phenotype for S-TGC cells. Altogether, we can conclude that heparin treatment can be affecting the S-TGC population of the mouse placenta. Histological studies need to be carried out to confirm the changes.
3.5.8 Placental morphometry

Placental morphometry measures were carried out in order to examine whether heparin treatment altered structural features of the placenta. The study was started with measuring gross placental labyrinth measurements (area and perimeter) across the different treatment groups at both E12.5 and E14.5 end points when heparin was given starting 9.5 days into gestation. Labyrinthine area and perimeter remained unchanged between different treatment groups when heparin treatment was initiated at E9.5 regardless of when heparin treatment was stopped (E12.5 vs. E14.5). Labyrinthine area was similarly unchanged after heparin treatment when mice were given heparin starting at E5.5. Collectively, it would be plausible to infer that the change in placental weight in the high dose group was most likely not attributable to a grossly bigger labyrinth component in the placentas of high dose mice.

Since heparin treatment was seen to alter placental weight and expression of various trophoblast markers, maternal and fetal blood space area measurements were then carried out in the placental samples collected at E12.5 (treated from E5.5 to E12.5). For that purpose, embedded placental samples were stained using CD34 to visualize the fetal endothelium. Heparin treatment was associated with significantly reduced maternal blood space measures in both low dose and high dose groups, along with a statistically significant increase in fetal blood space area measures in the high dose group. It is noteworthy to mention that there is no alteration in fetal weight despite this decreased maternal space area in the treated mice so the effect does not seem to cause pathology in mice. In fact, the increased fetal blood space area seen in the high dose mice likely even counters the potentially detrimental effects associated with the reduced maternal blood space area seen in the high dose mice.

Sinusoidal trophoblast giant cells are in direct contact with the maternal blood by being situated inside the lacunae filled with maternal blood in the mouse placenta. Coupled with an upregulated expression of PL-II in the placentas of mice treated from E5.5, it would be plausible to suspect that the reduced maternal blood space in those mice could potentially be attributed to an increased area contribution by S-TGCs in the placentas of those mice. Alternatively, abnormal s-TGC function could be hampering normal formation of the MBS since S-TGCs play an important role in the formation of MBS.
3.5.9 Placental mRNA levels of global transcription factors

The qPCR array was carried out in order to examine the effects of heparin treatment with respect to gene expression levels of various key transcription factors involved in cellular processes. One of the most interesting findings of the PCR array were the decrease seen in Hif1a and Arnt mRNA expression levels in mice treated with high dose of LMWH compared to vehicle control mice when treatment was initiated at E9.5. Hif1a is the master regulator of the hypoxic response and it is significantly elevated in the syncytial compartment of the placenta in preeclamptic patients (Tal, 2012).

Separate qPCR experiments were run for the samples later to confirm downregulated Hif1a expression when treatment was started 9.5, as well as to assess mRNA levels of Hif1a in the labyrinth-enriched samples collected from mice that were treated starting 5.5 days into gestation. Surprisingly, the findings were not in agreement with the previous qPCR array results. Namely, Hif1a gene expression was only seen to be significantly downregulated in the low dose group independent of treatment time, and the changes in the high dose group were not statistically significant. It is noteworthy to mention that the downregulation of Hif1a gene expression in the labyrinthine-enriched samples was not seen when tissue from the whole placenta was examined (containing spongiotrophoblast and decidua layers. Taken together, decreased Hif1a expression in the syncytial layer of heparin treated mice can be partly attributed to the mechanism by which heparin improves the pregnancy outcome in humans if this effect of decreased Hif1a expression is also seen in human pregnancy after heparin treatment.

3.6 Summary

Overall, we have shown that starting heparin treatment during the peri-implantation period at E5.5 results in an increase in placental weight without altering fetal weight in mice treated with high dose of heparin. Depending on the way heparin is administered, heparin can upregulate the expression of certain proliferation markers and increase expression of certain suspected trophoblast progenitor markers Eomes and Sca1, along with changes in other trophoblast markers such as increased expression of a unique S-TGC marker Prl3b1, coupled with reduced levels of Ctsq, another S-TGC marker. Moreover, heparin treatment was shown to result in reduced maternal blood space area, and increased fetal blood space area in high dose mice when started at E5.5.
3.7 Conclusion

In conclusion, heparin treatment was shown to cause altered gene expression in the trophoblast cell types found in the exchange region of the mouse placenta. Even though the changes in the gene expression levels of some of the various trophoblast cell type markers studied were significant yet rather modest for the most part supporting the safety of giving heparin to pregnant women. Heparin was seen to result in a placental phenotype of placental overgrowth without altering fetal weight. Furthermore, those rather subtle changes in gene expression measured were shown to be associated with reduced maternal blood space area in heparin treated mice, coupled with increased fetal blood space area in mice treated with the high dose of heparin suggesting caution in terms of human pregnancy if such high doses (7x the current clinical dose) are ever to be administered in women at risk of developing sPE. As a result, heparin treatment in mouse pregnancy certainly results in altered trophoblast cell dynamics. Namely in agreement with our hypothesis, we have shown that heparin treatment can result in increased expression of certain suspected trophoblast progenitor markers, and can cause placental overgrowth in mice if continuously infused starting at E5.5.
Chapter 4 Overall conclusions of this thesis
Human trophoblast cell lines and mice were used in this project to study the effects of heparin treatment as complementary *in vitro* and *in vivo* models of placentation and pregnancy. Overall, our data show that heparin treatment in both human trophoblast cell lines and mice can result in an augmented proliferative response, and increased expression of progenitor markers without a significant effect on syncytial differentiation. We also had results that did not match the hypothesis since mRNA levels of *Cdx2, Rhox4b* did not seem to be affected by heparin treatment, and expression of *Epcam* in the labyrinth was reduced in mice. This means that with respect to trophoblast proliferation, heparin treatment resulted in augmented expression of some, but not all trophoblast stem cell markers. The aforementioned observation would be plausible given that trophoblast progenitor cells likely express some, but not all of the markers associated with trophoblast stem cells (Ueno et al., 2013). Altered expression of S-TGC markers *Ctsq* and *PL-II* suggest altered trophoblast differentiation towards the S-TGC type. However given the data presented in this study, it would not be possible to conclude whether or not the changes seen in the levels of those markers are a direct result of heparin treatment. Moreover, altered expression of S-TGC markers were different depending on heparin treatment initiation time further suggesting an indirect affect.

The effects of heparin treatment on trophoblast cell proliferation can be examined by comparing the effects of heparin on cell proliferation in both human cell lines and mice. We observed an increase in the expression of *PCNA*, a proliferation marker, after 24 hours in BeWo cells under high dose heparin treatment as our *in vitro* model. Expression of *Ki67*, another global proliferation marker, was also upregulated in the 2nd series of mice experiments. Nonetheless, it is important to keep in mind that unlike the homogeneous trophoblast-only BeWo cells, the labyrinth region of the mouse placenta contains endothelium, mesenchyme, blood cells etc as well. Hence, a direct comparison cannot be made by comparing expression levels of *PCNA* and *Ki67* in BeWo cells and mice. However, mice treated with heparin had increased expression levels of suspected trophoblast progenitor markers (*Eomes, Sca1*), which we predicted will be upregulated as a result of heparin treatment. The aforementioned observations collectively support our hypothesis, which was that heparin increases trophoblast cell proliferation.
The effects of heparin treatment with respect to syncytial differentiation can be elucidated by comparing the expression of syncytial markers for both of the models in this study. Neither the markers of syncytium in the human placenta measured in BeWo cells (GCM1, SYN1), nor the syncytial markers measured in the mice (Gcm1, Syna, Synb) changed significantly after heparin treatment. Lack of changes in the expression of syncytial markers in both of our models suggest that heparin treatment does not affect syncytial trophoblast cell differentiation significantly as we predicted.

Having used both trophoblast-only human cell lines along with mice in this study, we were able to demonstrate a similar effect associated with heparin treatment on trophoblast cells in an in vitro, and an in vivo setting. Combining these two models in order to come to a unifying conclusion was very informative since the two adopted in vivo and in vitro models in this study were complementary. We saw an augmented proliferative response in the BeWo cell line after high dose heparin treatment and upregulated levels of certain suspected trophoblast progenitor markers in mice treated with high dose of LMWH. Unfortunately direct assessment of trophoblast progenitor cells in mice proved to be quite challenging but other line of evidence presented in this study such as placental overgrowth, altered maternal and fetal blood space areas, as well as increased mRNA expression of suspected trophoblast progenitor markers all suggest higher numbers of trophoblast progenitor cells in the high dose mice treated with the supra-therapeutic dose of LMWH. The increased expression of proliferation markers and the trend towards increased cell numbers in BeWo cells after heparin treatment matches the data in mice, and further suggest that the effects observed are trophoblast specific. Our findings collectively suggest that heparin treatment improves the pregnancy outcome in women at risk of developing PE by means of rectifying the abnormally reduced proliferation seen in VCT cells seen in PE without affecting syncytial differentiation and fusion.
Chapter 5 Future directions
Is heparin degraded faster in cell culture medium of trophoblast cells compared to maternal circulation in human pregnancy?

In our studies, we added fresh media containing the desired concentrations of heparin to the cells every 24 hours. The adopted doses of prophylactic, or therapeutic heparin were chosen in correspondence to the clinical doses. Nonetheless, it is very important to take into account the fact that heparin is cleared from maternal circulation \textit{in vivo} with a half life of approximately 5 hours in human pregnancy, whereas the rate of elimination and breakdown of LMWH in the culture medium for BeWo cells is unknown, if it even occurs in the first place. The rate of heparin elimination from the cell culture medium can vary widely depending on the cell type studied (Mcduffie, 1979). I propose that heparin is broken down, and hence eliminated faster in the cell culture medium of BeWo cells compared to the \textit{in vivo} elimination rate seen in humans.

Knowing the elimination rate of LMWH in BeWo cell culture medium would be important since heparin treatment can in turn affect secretion and availability of FGF4 in circulation in an \textit{in vivo} setting. Since heparin is an avid binder of FGF4, when heparin is present in low and moderate amounts (Powers et al., 2000). Heparin-binding capacity of FGF4 can facilitate FGFR2 activation by means of making FGF4 available to the receptor dimers and hence directly amplify mitogenic FGFR2-mediated signaling. The elimination rate of LMWH would be very relevant both in an \textit{in vivo} setting, as well as in a pooled and enclosed environment experienced by the cells in an \textit{in vitro} plate well. In an \textit{in vitro} setting if no breakdown of LMWH occurs, the cells are exposed to the same concentrations of the drug due to the absence of systemic drug elimination means (e.g. renal or hepatic elimination). On the other hand, high concentrations of heparin can result in heparin acting as a “chelator”, which would bind and take the available FGF4 away from the receptor dimers and hence prevent presentation of FGF4 to its corresponding receptor. The expression levels, and activity of heparanase enzyme determines the levels of LMWH in the cell culture medium since renal and hepatic elimination pathways are not possible in an \textit{in vitro} cell culture model (Jingting et al., 2007). Heparanase is expressed \textit{in vivo} and in other choriocarcinoma cell lines and the level of expression, and activity of this enzyme would be implicated in metabolism of the LMWH added to the culture medium (Jingting et al., 2007). mRNA levels of \textit{HPSE} gene can be studied, followed by measuring the protein levels of this enzyme in the BeWo cells in order to study the expression levels of heparanase in BeWo cells. Thereafter, functional assays can be done using radio-labelled
substrates of heparanase to determine the activity of heparanase in BeWo cells (Pearson et al., 2011). Anti-Xa assays used in this project to measure plasma levels of LMWH in mice can be used to assess the concentration of heparin in cell culture media at regular time intervals after introducing heparin to the media at a known initial concentration in order to determine the half-life of LMWH in BeWo cell culture media.

If indeed the half-life of LMWH is shorter in the BeWo cell culture media compared to the in vivo setting of human pregnancy, the doses used for BeWo cells in this study would not be an accurate reflection of the levels seen by the placental trophoblast cells in vivo. Namely, higher doses and more frequent media changes would be necessary to accurately reproduce the in vivo circulating LMWH levels. Alternatively if LMWH has a longer half-life in the BeWo cell culture medium, the doses used in this study to treat BeWo cells would have be too high for heparin to act as a cofactor for FGF4. Instead, the high levels of LMWH could have potentially inhibited FGF4-dependent signaling through sequestering the available FGF4 away from the media through the aforementioned “chelation” effect.

Would replicating the mouse chapter of this study using a mouse model of preeclampsia result in more significant findings?

In this study we used wild type ICR mice as our in vivo model for examining the effects of heparin treatment on placental trophoblast cells. ICR mice are outbred heterogeneous mice readily used in a wide range of research fields (Aldinger et al., 2009). ICR mice are used as a healthy model for pregnancy studies due to their large litter size and normal fetal and placental development (Coan, 2004). I propose that replicating the experiments using the mouse model for PE established by Kalkunte et al (Kalkunte et al., 2010) where inbred C57BL/6 mice develop PE symptoms after a one time mid-gestation injection of human sPE would result in more significant changes in the measured parameters of our study.

There are various lines of evidence suggesting that replicating the experiments in the Kalkunte et al mouse model would be worthwhile. Firstly, the model is based on C57BL/6 mice, which are the most commonly used inbred mouse strain with placental abnormalities (Rennie et al., 2012). Similar to the reduced placental mass seen in women with PE (Section 1.4.4), placentas from C57BL/6 mice are 25% smaller if compared to placentas from ICR mice matched for gestational age (Plaks et al., 2011). C57BL/6 mice injected once midgestation with severe
preeclamptic human serum develop signs and symptoms of PE by late gestation (Kalkunte et al., 2010). Adopting this model would be beneficial for mainly two reasons. First, there is suspected to be much less variability associated with the measured outcomes in C57BL/6 mice due to their inbred nature compared to the outbred ICR mice. Second, C57BL/6 mice injected with sPE serum are directly influenced by the human pathology of sPE and hence serve as a model that more closely reflects the pathology of sPE since it shares more similarities to the human pathology when compared to wildtype healthy ICR mice used in this study.

If the proposition is correct, we expect to see a much larger increase in placental mass in C57BL/6 mice treated with high dose of heparin starting at E5.5 given our results in ICR mice (Section 3.4.2). Furthermore, the changes seen in trophoblast markers in ICR mice would potentially be more significant in C57BL/6 mice due to the homogeneous genetic background of the inbred C57BL/6 strain resulting in less natural variation. The lower natural variation seen in C57BL/6 mice could also potentially result in seeing significant changes in the maternal circulating levels of sFLT1 and PLGF after heparin treatment since these parameters were seen to be highly variable in wildtype ICR mice even without heparin treatment (Section 3.4.3) (COV 23%, and 83% for sFLT1 and PLGF measurements respectively).

**Does LMWH enter the syncytiotrophoblast cells in humans and mice?**

Regarding the pharmacology of LMWH, it is not yet very well known whether heparin is able to get into the trophoblast cells to affect signaling or whether it stays outside trophoblast cells to potentially activate/inhibit extracellular receptors, and alter the extracellular milieu. Heparin has been shown to enter other cell types such as endothelial cells (Page, 2013), yet heparin cannot be detected in fetal circulation since it does not cross the placental barrier (Greer et al., 2014), yet it remains a question as to whether heparin can cross or enter SynT, and VCT cells. It would be of great importance and value to know whether heparin can enter the placental syncytiotrophoblast layer and come in direct contact or enter the underlying VCT cells in order to directly influence the proliferation rate in VCT cells. In a physiological setting of the healthy human placenta the cytotrophoblast cells are not exposed directly to maternal blood since the multinucleated syncytiotrophoblast cells span across the placenta and tightly control the material “seen” by the cytotrophoblast cells. Nonetheless, there are “denuded” areas in the placenta and
they are seen more frequently in placentas from PE mothers, which constitute around 3-4% of the placental surface (Kingdom and Drewlo, 2011). The denuded surfaces are defined as areas where the syncytium is broken off resulting in direct exposure of the underlying cytotrophoblast cells to maternal circulation. Hence, it is possible that heparin is partly carrying out its beneficial effects in human pregnancy via altering VCT cell proliferation by coming into direct contact with the denuded cytotrophoblast cells. If heparin does not cross the syncytial layer, the contribution would likely be minor due to the low percentage of cytotrophoblast cells exposed.

It would be plausible to suspect that heparin can enter the syncytial layer of the placenta given it can enter endothelial cells since trophoblast cells share similarities with endothelium with respect to gene expression and function during pregnancy (Burrows et al., 1994; Kaufmann et al., 2003). Tracking heparin would be possible through fluorescent-tagging of certain residues within the polysaccharide structure (Babu and Kuberan, 2010). In order to test whether heparin can cross the syncytial layer, fluorescent-tagged heparin species can be administered to mice followed by fluorescent microscopy in order to study the localization of heparin in vivo. Furthermore, BeWo cells can serve as a great in vitro model to study the localization of fluorescent-tagged heparin species given the established nature of BeWo cells as a model for studying human trans-placental transport (Cartwright et al., 2012). It is important to mention that tagging heparin might alter its activity and affect the ability of heparin to enter the cell. As a result, tagging should preferentially be done on certain regions that are least likely to contribute to the activity, as well as ability of heparin to enter the cell.


Appendices

Appendix A:

Acetone/TCA method:

1. The media collected from the BeWo cells was mixed with 8 equivalent parts of ice-cold acetone, and one equivalent part of trichloroacetic acid (100% W/V)

2. The mixture was left at -20 °C for one hour to allow for a precipitate to form

3. The tubes were then centrifuged for 15 minutes at 18,000g at 4 °C

4. After discarding the supernatant, the pellet was resuspended in 0°C acetone and centrifuged again as described previously.

5. The re-suspension and centrifugation steps were repeated twice more in order to eliminate TCA from the pellet

6. After removing the supernatant (Acetone), the pellet was allowed to dry at room temperature.

7. The pellets were then stored at -80°C for later use.
Appendix B:

BrdU Immunohistochemistry

Deparafinization and rehydration of the sections was carried out as described in the IHC section. BrdU IHC was performed by the pathology core of the Centre for modeling Human Disease (Toronto Centre for Phenogenomics) as follows: Antigen retrieval was performed by incubating the slides in 98°C solution of 10nM sodium citrate for 20 minutes. The endogenous peroxidase activity was then blocked by incubating the slides in 3% hydrogen peroxide (in Methanol) for 30 minutes followed by 3 washes of five minutes each in PBST (PBS with 0.01% Tween-20). After being blocked for one hour using the DAKO blocking solution as described for other IHC procedures, the slides were incubated with the primary anti-BrdU antibody (Table. 3) overnight at 4°C. After being washed in PBS 3x, the slides were incubated for half an hour at room temperature with biotinylated secondary antibody (1:100) (Vector Labs®, cat# BA-4001). The slides were then washed three times before being incubated with Avidin-Biotin/HRP (1:50) for 1hr at room temperature (Vector Labs®, cat#PK-6100). DAB colour reaction was then performed on the slides as described for regular IHC.
Appendix C:

Visiopharm Software:

1. The newCAST platform was used for the measurement purposes.
2. Placental section of interest was selected using the selection tool at 10x magnification
   (For placental labyrinth area and perimeter measurements)
3. For measuring BrdU positive cells, MBS, FBS areas the selected area was then viewed
   under 20x magnification. 100% of the selected area (60% for BrdU) was then chosen for
   randomized meander sampling.
4. Every randomized frame had 12 equally spaced crosshairs (for MBS and FBS measures),
   or 4 boxes collectively covering 40% of each frame.
5. For maternal, and fetal blood space measures, the crosshairs were labeled as either MBS
   or FBS and the unlabeled crosshairs were considered as “other”. The results were then
   normalized to the total number of points per labyrinth
6. For BrdU positive measurements, BrdU-positive nuclei were labeled and the number of
   labeled nuclei, along with the number of boxes analyzed per placental labyrinth were
   used to compare between different treatment arms. The results were normalized per area
   of labyrinth.