DISSERTATION

Effects of the diabetic environment on the feto-placental vasculature

M.Sc. Luciana Lassance Gomes

For the academic degree of
Doctor of Philosophy
(PhD)

at the
Medical University of Graz
Clinics of Genecology and Obstetrics

under supervision of
Assoc. Prof. Dr. Gernot Desoye

2012

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Please note that parts of this thesis are already accepted for publication in Lassance L, Miedl H, Konya V, Heinemann A, Ebner B, Hackl H, Desoye G, Hiden U **Differential response of arterial and venous endothelial cells to extracellular matrix is modulated by oxygen.** Histochemistry and Cell Biology, 2012.

Luciana Lassance Gomes

Graz, January 2012.

To my father, that worked hard for giving me the opportunity of being WHERE I am.
To my mom, that worked hard for giving me the opportunity of being WHO I am.

To my supervisor, Gernot Desoye, that much more than a supervisor is an example of motivation, good character and keeps friendship into his team.

To my co-supervisor and friend, Ursula Hiden, that was always 24 hours available for my questions, doubts and thoughts.

To all from the Forschungslabor group, those accepted me with open arms and are always ready for helping who ever needs.

To Heidi Miedl, that is my beloved best friend since my first week in the lab.

"To steal ideas from one person is plagiarism. To steal from many is research."

Contents

Abstract

In a pregnancy complicated by maternal diabetes, insulin and IGF2 levels are elevated in the fetal circulation. As the insulin/IGF system regulates fetal and placental growth and development, dysregulation of insulin and IGF2 may have profound effects on the placenta. At term of gestation placental receptors for insulin and IGF2 are mainly located on the feto-placental vasculature, but insulin and IGF2 effects on placental endothelium have not yet been understood. Placentas of diabetic pregnancies show several alterations including increased weight and size and hypervascularization. Here I hypothesized that insulin and IGF2 can regulate placental endothelial cell (EC) processes such as gene expression and intracellular signaling, that may contribute to the alterations observed in placentas from diabetic pregnancies. As thickening of the placental basement membrane is also a feature of placentas from diabetic pregnancies, I investigated the effects of different basement membrane proteins on placental EC proliferation, survival and behavior.

Global gene expression profiling of arterial (AEC) and venous (VEC) EC revealed that insulin and IGF2 mainly regulate genes involved in lipid metabolism and cell adhesion/junction, and AEC are more susceptible than VEC herein. Results also showed that insulin increases *in vitro* angiogenesis and mediates actin assembly in AEC via the Akt/eNOS/Rac1 signaling pathway. By using different basement membrane (ECM) proteins to investigate their role in EC proliferation, survival and behavior, VEC were more susceptible to changes in matrix composition than AEC. These effects were mediated by focal adhesion kinase (Fak) and modulated by oxygen. Collectively, these data suggest that altered insulin/IGF2 levels and basement membrane constitution may alter placental vascular morphology and function in diabetic pregnancies.

Zusammenfassung

Schwangerschaftsdiabetes ist mit einem erhöhten Spiegel an Insulin und IGF2 in der fetalen Zirkulation assoziiert. Da das Insulin/IGF System Wachstum und Entwicklung der Plazenta reguliert, könnten veränderte Konzentrationen von Insulin und IGF2 die Plazenta entscheidend beeinflussen. Am Ende der Schwangerschaft sind die Rezeptoren, an die Insulin und IGF2 in der Plazenta binden, hauptsächlich an der fetalen Vaskulatur exprimiert, jedoch ist über die Effekte, die Insulin und IGF2 auf dieses Gewebe haben, noch nicht viel bekannt.

Schwangerschaftsdiabetes führt zu Veränderungen in der Plazenta, wie zum Beispiel einem erhöhten Gewicht sowie einer Hypervaskularisierung. Deswegen war meine Hypothese, dass Insulin und IGF2 die Endothelzellen der Plazenta beeinflussen, Prozesse wie Signaltransduktion und Genexpression anschalten und regulieren und so zu den Veränderungen beitragen, die bei Plazenten von Gestationsdiabetikerinnen auftreten. Da die Verdickung von Basalmembranen der Plazenta auch charakteristisch für Schwangerschaftsdiabetes ist, wurde der Effekt von unterschiedlicher Zusammensetzung der Basalmembran auf Proliferation, Survival und Verhalten der Endothelzellen untersucht.

Die globale Genexpression von arteriellen und venösen Plazenta-Endothelzellen zeigte, dass Insulin und IGF2 hauptsächlich Gene regulieren, die in Lipid Metabolismus und Zelladhäsion involviert sind. Außerdem zeigten sind die arteriellen Endothelzellen hier sensitiver als die venösen Endothelzellen. Insulin stimulierte *in vitro* Angiogenese und Aktin-Zusammenbau in arteriellen Endothelzellen über den Akt/eNOS/Rac1 Signatransduktionsweg. Bei der Untersuchung der Auswirkung der Zusammensetzung der Basalmembran auf Proliferation, Survival und Verhalten zeigte sich, dass venöse Endothelzellen darauf sensitiver reagierten als die arteriellen. Diese Effekte wurden durch die focal adhesion kinase (Fak) mediiert und durch Sauerstoff beeinflusst.

Zusammenfassend weisen diese Daten darauf hin, dass veränderte Insulin und IGF2 Konzentrationen sowie Zusammensetzung der Basalmembran die Morphologie und Funktion der Plazentavaskulatur bei Schwangerschaftsdiabetes modifiziert.

Introduction

1- Gestational Diabetes Mellitus (GDM)

Pregnancy is a glucose-intolerant state due to a transient insulin resistance which leads to postprandial hyperglycemia among other metabolic changes (Carpenter, 2007). Dysglycemia in normal pregnancy can increase blood pressure resulting in an enhanced risk for vascular dysfunction (Banerjee and Cruickshank, 2006). During the course of pregnancy the increasing insulin resistance augments insulin demand in order to keep a balance between demand and supply. If insulin resistance overcomes insulin production, the women become hyperglycemic and GDM is established (Catalano et al., 1993).

According to the American College of Obstetricians and Gynecologists (*Gestational Diabetes*, Practice bulletin no 30) GDM is defined as a carbohydrate intolerance that starts or is first recognized during pregnancy. Similar to type 2 diabetes (T2D), and as mentioned before, GDM is associated with both impaired insulin secretion and insulin resistance and presents the same risk factors as T2D (Kuhl, 1991) such as family history of diabetes. However, for GDM, high maternal age, weight, and parity, and previous delivery of a macrosomic infant are also other risk factors often reported (Ben-Haroush et al., 2004). Usually, short after delivery, glucose levels are restored to non-pregnancy levels. Woman whose pregnancy was affected by GDM, the risk to develop T2D later in life is elevated. Early GDM diagnosis, in the first half of pregnancy, is also a high risk factor for future T2D (Bartha et al., 2000). Nevertheless, pharmacological interventions or even changes in diet and lifestyle, such as physical exercises, could delay or prevent the onset of T2D in GDM affected women (Bellamy et al., 2009).

Besides insulin resistance, pregnant women with GDM possess β-cell dysfunction, higher body mass index (BMI), central obesity and hyperlipidemia, which make GDM also very similar to metabolic syndrome. Dyslipidemia and obesity are frequently associated with oxidative stress, endothelial dysfunction and inflammation. All together, they can be a predictor for gestational hypertension and vascular disease (Carpenter, 2007). Endothelial dysfunction and impaired prostaglandin-dependent relaxation were observed in the vessels of women with GDM as compared to a non diabetic pregnant group (Knock et al., 1997). Postpartum consequences, such as large arterial stiffness, impaired response of microcirculation

to hyperemia and impaired endothelium-dependent vaso dilatation were also shown to be more frequent in women with history of GDM (Hu et al., 1998, Hannemann et al., 2002, Anastasiou et al., 1998). The association of GDM and hypertension is still not well understood even though hypertension occurs in about 28% of GDM patients (Kvetny and Poulsen, 2003). A study made by Barden et al. showed that in a cohort of 184 women with GDM, the presence of high blood pressure, high BMI, fasting glucose, insulin, uric acid and C-reactive protein predicted pre-eclampsia (Barden et al., 2004).

Maternal diabetes can bring adverse consequences not only for the mother but as well an unfavorable environment for embryonic and feto-placental development, resulting in an increased risk for congenital malformations, placental abnormalities and changes in the intrauterine programming (Vambergue and Fajardy, 2011). GDM also increases the risk of perinatal morbidity and mortality, often associated with fetal macrosomia. In 1952 Pedersen proposed that maternal hyperglycemia drives increased transplacental glucose transfer and, as a consequence, fetal hyperinsulinemia as a compensatory effect. This hyperinsulinemia will then induce fetal growth (Pedersen, 1952). Moreover, in pregnancies affected by GDM the fetuses are exposed to metabolic abnormalities and relative fetal hypoxia with lower oxygen levels being documented for both, umbilical artery and vein (Taricco et al., 2009). This lower oxygenation would then be reflected in an altered placental exchange and metabolism.

For the offspring of diabetic women the risk for developing hypertension and other cardiovascular diseases as well as developing diabetes at young age is increased (Vambergue and Fajardy, 2011). The association between GDM and the risk for congenital malformations is still not clear. Some studies failed to find an association of GDM and major malformations (Bartha et al., 2000, Kalter, 1998) whereas others demonstrated that GDM is a significant risk factor for holoprosencephaly, spine/rib anomalies and renal and urinary system disorders (Martinez-Frias et al., 1998). Because of increased fetal weight and larger shoulder diameter, shoulder dystorcia is also often associated with GDM (Kim, 2010).

Fetal consequences of GDM have been widely investigated and are clearly recognized. Developmental and morphological alterations in the placenta have also been reported. However, the role of the diabetic placenta on these fetal outcomes or

whether altered processes in the fetuses from diabetic mothers will have an effect on the placenta resulting in its changes is not yet well understood.

2- Function and morphology of placentas from normal x diabetic pregnancies

The placenta is a fetal organ located at the interface between the maternal and fetal circulation which executes endocrine, metabolic and nutritional activities essential for fetal growth and development, and for maintenance of pregnancy. Most central is the supply of nutrients and oxygen to the fetus and the production of a range of hormones and growth factors that, when released, may affect mother or fetus, or both. Reciprocally, maternal and fetal hormones and growth factors present in both circulations may substantially regulate placental development (Fig 1).

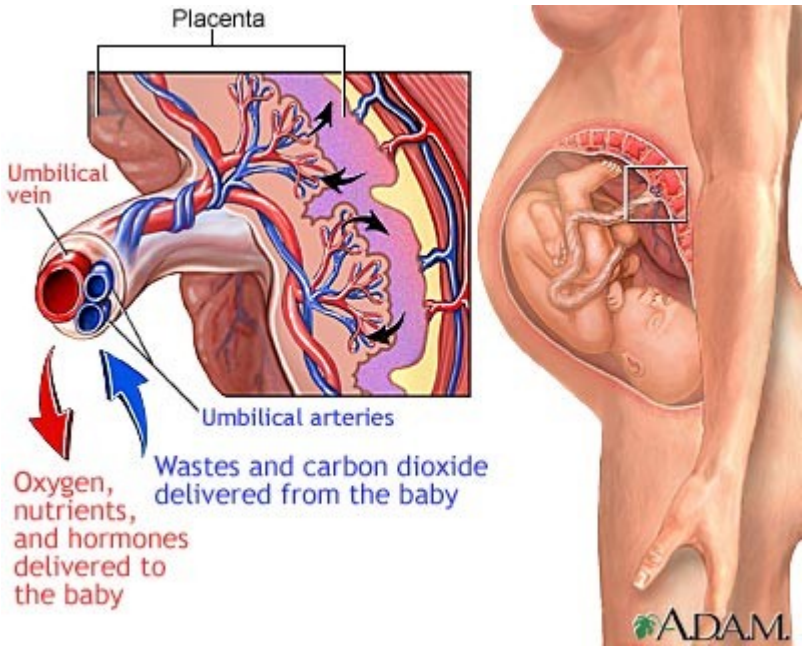


Figure 1: Schematic representation of the human placenta. The human placenta is a fetal organ located between the mother and fetus and is responsible for maintenance of the pregnancy and for sustaining fetus development by supplying gases and nutrients, exerting endocrine functions and delivering fetal waste and carbon dioxide back to the maternal circulation. (Source: http://www.medhelp.org/medical-information/show/2752/Placenta)

The chorionic villi are composed of an outerlayer of syncythiotrophoblast, an inner layer of cytotrophoblasts, stroma, and the fetal vessels. These villi bath in maternal blood coming from the uterine spiral arteries (Fig 2). The maternal blood is in contact with the syncytiotrophoblast, the outermost surface of the placental villous trees, and the fetal blood circulates in fetal vessels and capillaries, which are lined by endothelial cells (Fig 3).

This architecture allows exchanges between both circulations without mixing of them. The feto-placental vasculature is continuous with the fetal circulation making the later vulnerable to changes that may occur in the feto-placental vasculature and vice-versa (Leach et al., 2009).

2.1- Maternal surface of the placenta

The levels of growth factors such as insulin-like growth factor 1 and 2 (IGF1 and IGF2), platelet-derived growth factor (PDGF), fibroblast growth factors (FGF2 and FGF4), epidermal growth factor (EGF) and members of transforming growth factor (TGF)-β superfamily, are increased in the maternal circulation throughout gestation, indicating that they play a crucial role in promoting growth and development of the fetus and the placenta (Forbes and Westwood, 2010). Additionally, insulin, IGF1 and IGF2 are also expressed by the fetus and the placenta, therefore dysregulation of these growth factors may have direct effects on both. Placental receptors for insulin, IGF1 and IGF2 are expressed on distinct surfaces (Hill et al., 1993, Thomsen et al., 1997, Birnbacher et al., 1998, Han and Carter, 2000, Dalcik et al., 2001).

Placentas of diabetic pregnancies show several alterations including increased weight and size (Winick and Noble, 1967, Naeye, 1987, Desoye and Shafrir, 1996, Molteni et al., 1978), known as placentomegaly, and abnormal placental weight / fetal weight ratio (Lao et al., 1997). Thus, this ratio (Molteni et al., 1978, Heinonen et al., 2001) is used as an indicator of fetal health during intrauterine life, since it is altered in pathological conditions such as maternal hypertension, diabetes and fetal intrauterine growth restriction (Zorn et al., 2011). Although placentomegaly is correlated with fetal macrosomia it is still not possible to determine whether placental overweight is cause or consequence of fetal overweight. Alterations in placental transport and metabolism have also been reported. The expression of the glucose

transporter GLUT1 was shown to be increased at the basal plasma membrane of the syncytiotrophoblast of term insulin-dependent GDM placenta (Gaither et al., 1999, Jansson et al., 1999). As mentioned before, changes in blood oxygenation in the placental vasculature as a consequence of fetal hypoxia have also been demonstrated (Taricco et al., 2009).

Placentomegaly is associated with an altered placental histology and cellularity and has been reported in both humans (Diamant, 1991) and rats (Gewolb et al., 1986, Padmanabhan and al-Zuhair, 1990, Giachini et al., 2008). Theses alterations include villous immaturity, which could alter feto-maternal exchanges, and increased branching, that could be a compensatory effect (Bartha et al., 2000). Other alterations such as fibrinoid necrosis, villous edema, villous fibrosis, cytotrophoblast hyperplasia, thickening of the trophoblast basal membrane and thickening of the villous capillary vessels have been described (al-Okail and al-Attas, 1994, Wasserman et al., 1980). Increased glycogen content is also a characteristic of GDM placentas (Robinson et al., 1988, Aerts et al., 1990).

2.2- Effects of diabetes on the placental vasculature

The human placenta is a highly vascularised organ in order to exert its functions of feto-maternal exchange of solutes and gases, functioning as a key selective barrier between the mother and the fetus. Vasculogenesis or formation of new feto-placental vessels occurs in the first 4 weeks of pregnancy while formation of the microvascular bed (angiogenesis) occurs later in pregnancy. Feto-placental vessels are positioned in the chorionic villi.

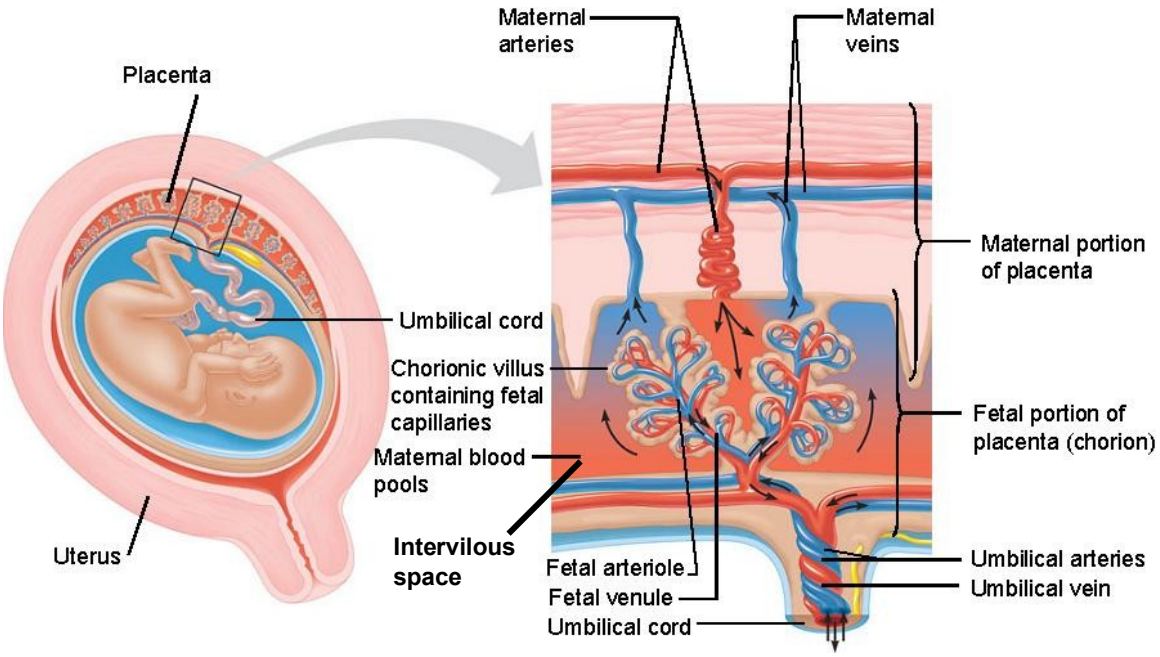


Figure 2: Simplified morphology of a placental villus showing the maternal and the fetal portions of the placenta. The chorionic villous trees project from the chorionic plate into the intervillous space and bathe in maternal blood coming from maternal spiral arteries.
Source: http://bio1152.nicerweb.com/Locked/media/ch46/placenta.html

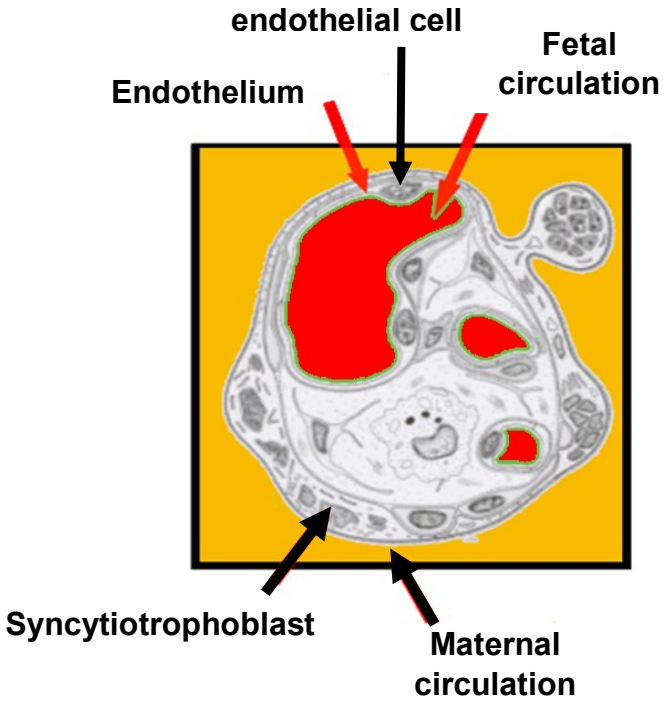


Figure 3: Schematic representation of a cross section of a terminal villus demonstrating the outermost layer i.e. syncytiotrophoblast which is in contact with the maternal circulation, and fetal capillaries lined by endothelial cells.

These feto-placental vessels are sensitive to growth factors, glucose, oxygen, nutrients and vasoactive molecules in the fetal blood which can modulate angiogenesis and villous branching (Leach, 2011). Concentration changes in the fetal blood would then have direct effect on the fetal endothelium. On the other hand, vasoactive agents produced by the feto-placental vessels will enter the fetal circulation and affect fetal development. Altered conditions, such as GDM, may lead to impaired vasculogenesis and/or angiogenesis.

Indeed, several pieces of evidence show that maternal diabetes can lead to an increase in placental angiogenesis and capillary proliferation (Sherer and Divon, 1996, Jirkovska et al., 2002, Leach et al., 2004). Hyperglycaemia per se has shown to act as vasoconstrictor, pro-coagulatory and pro-angiogenic agent as well as to increase permeability. This high glucose would either act via PKC or via the polyol pathway that will end up in decreasing NO generation and increasing VEGF production, both being key molecules in the pro-angiogenic and pro-permeability aspects of hyperglycaemia (Leach et al., 2009). In the second and third trimesters of pregnancy fetal pancreas starts insulin secretion. Concomitantly longitudinal growth of fetal capillaries occurs. This coincides with the switch of insulin receptor expression from the syncytiotrophoblast to the endothelial surface of the feto-placental vessels (Desoye et al., 1994, Hiden et al., 2009a) and suggests that fetal insulin can contribute to placental angiogenesis and control of vascular permeability. During periods of maternal hyperglycaemia, transplacental glucose transport leads to fetal hyperglyceamia that results in increased fetal insulin production (Nold and Georgieff, 2004). Fetal hyperinsulinemia would have effects on both fetal and feto-placental endothelium and may thus contribute to the hypervascularization of diabetic placentas. In diabetic retinopathy, it has been demonstrated that insulin can increase VEGF expression and production via PI3K and MAPK pathways and thus stimulate angiogenesis (Poulaki et al., 2002). And it can increase NO production via endothelial nitric oxide synthase (eNOS) activation resulting in vasodilation (Kuboki et al., 2000). The hypervascularization observed in GDM placentas can then lead to an increased surface for fetal-maternal exchanges that could compensate, for instance, the low oxygen supply for the fetus.

Changes can also extend to molecular phenotypes such as vascular endothelial cadherin (VE-cadherin) and beta-catenin expression in feto-placental vessels. These changes may cause impaired barrier integrity with an increase in

endothelial cell proliferation and angiogenesis, and consequently, augmentation in capillary length (Leach et al., 2004). Vascular permeability of the endothelium can be also be compromised (Bazzoni and Dejana, 2004). In human placenta, effects of hyperinsulinemia are still not well understood. It has been shown that perfusion of the fetal circulation of term placentas with high insulin concentration results in increased VEGF expression, junctional disruption and consequently vascular leakage (Leach et al., 2009). Increased expression VEGF receptors VEGFR-2 (KDR) and VEGFR-1 (Flt1) (Helske et al., 2001) was also observed. Besides that, Wright et al. demonstrated that VEGF is able to induce junctional molecule reorganization and angiogenesis in human umbilical venous endothelial cells (HUVECs) (Wright et al., 2002). Junctional adhesion molecules are key players in regulating endothelial monolayer integrity and vascular permeability. It has been demonstrated that higher levels of VEGF in GDM leads to phosphorylation and loss of vascular endothelial cadherins (VE-cadherins) and beta catenin (b-catenin) from junctional domains. Disruption of cell–cell junctions is one of the first steps in angiogenesis, leading to increased paracelular permeability and endothelial cell migration (Babawale et al., 2000). Moreover, once phosphorylated, b-catenin can also act as a signaling molecule participating in cell proliferation and growth regulating pathways (Conacci-Sorrell et al., 2002) as well as in F-actin stress fiber organization (Millan et al., 2010).

Another feature of diabetic placentas is the thickening, or hypertrophy, of the basement membrane (Burstein et al., 1957). Some authors found an increase of the collagen networks (Pietryga et al., 2004), while others found increase in fibronectin deposition in rat placentas (Giachini et al., 2008, Forsberg et al., 1998). In fact, thickening of the basement membrane is an ultrastructural hallmark in diabetic patients. Accumulation of basement membrane components, such as collagens, fibronectin, elastin among others, in type 2 and type 1 diabetic patients, was found in the endothelial capillary beds of the renal, retinal, neuronal units, myocardial and skeletal muscle, and in the arterial vasculature itself. This accumulation of basement membrane proteins represents the most important cause of diabetic complications such as cardiovascular and microvascular diseases (Hayden et al., 2005).

3- Insulin/IGFs axis in the human placenta

The insulin/IGF system regulates fetal and placental growth and development. Hence, dysregulation of insulin and IGFs may have profound effects on the fetus and

on the placenta. These growth factors are normally present in the maternal circulation during pregnancy but can also be produced by the fetus. Moreover signaling receptors for insulin, IGF1 and IGF2 (insulin receptor and IGF1 receptor) are expressed on the placenta at different locations in a spatio-temporal manner. In the first trimester of pregnancy, insulin receptors are mainly expressed on the microvillous membrane of the syncytiotrophoblast (Fig 4), being directly in contact with the maternal blood (Hiden et al., 2008). At term of gestation, insulin receptor expression shifts to the placental endothelium (Fig 4) which is in contact with fetal blood, which consequently becomes a target of fetal insulin. Hence, at term, placental arterial and venous endothelial cells are targets for insulin effects (Desoye et al., 1994, Desoye et al., 1997, Hiden et al., 2006), suggesting a regulatory role of fetal insulin on placental development. The IGF1 receptor (IGF1R) also shifts its expression pattern along pregnancy which however differs from IR expression pattern. In the first trimester, IGF1R expression was found in the proliferative cytotrophoblasts (Fig 5), which allows IGF1 to stimulate cytotrophoblast proliferation. At term, IGF1R is mainly expressed on the basal membrane of the syncytiotrophoblast and villous cytotrophoblasts and to a lesser extent on the endothelial cells (Fig 5). Both, IR and IGF1R are also expressed on tissue resident macrophages in the placenta (Hiden et al., 2009a).

In GDM, there is an increase in insulin and IGF2 levels in both, maternal and fetal circulation (Desoye and Shafrir, 1996, Desoye and Hauguel-de Mouzon, 2007). Therefore, in a pregnancy complicated by maternal diabetes, altered insulin/IGFs levels will affect the placenta. Hiden and Desoye proposed that elevated insulin and IGF2 as a consequence of maternal diabetes may promote proliferation, hypervascularization and placental growth (Hiden and Desoye, 2010).

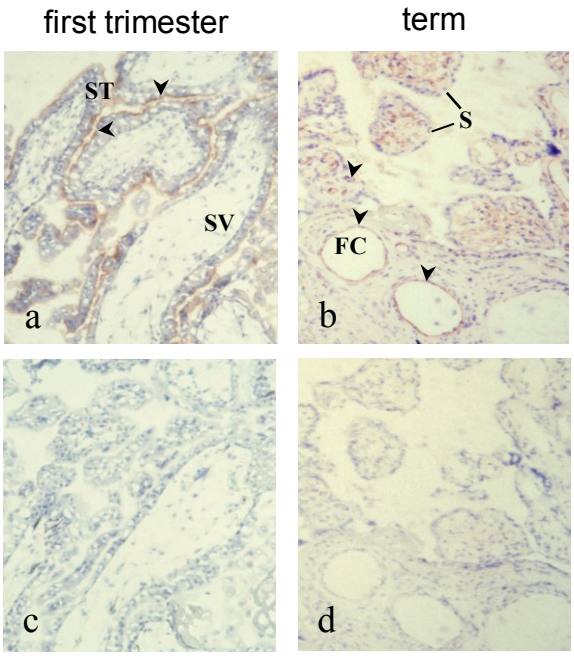


Figure 4: Immunohistochemical staining of insulin receptor in first trimester (a) and term placental tissue (b). Negative controls (c, d). The arrows mark the predominantly stained compartment (i.e. syncytiotrophoblast in first trimester and endothelium at term). *ST*, syncytiotrophoblast; *SV*, stem villus; *FC*, fetal capillary. Original magnification x100. Images kindly provided by Gernot Desoye.

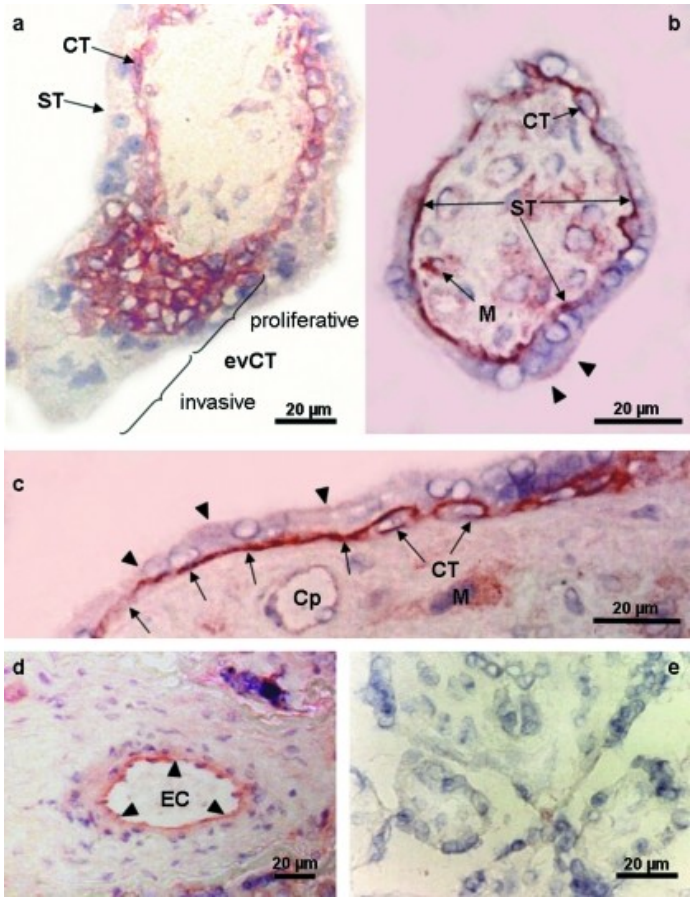


Figure 5: Immunohistochemical localization of IGF1R in first trimester human placenta (a) and at term of gestation (b–d). (a) In the first trimester the plasma membrane of the villous cytotrophoblast (CT) was strongly stained as was the basal membrane of the syncytiotrophoblast (ST). Among the extravillous cytotrophoblast (evCT) only three to five proximal cellular layers of the cell columns, representing the proliferative phenotype, were strongly labeled. (b–d) Cross-sections of a mature intermediate villus (b) and stem villus (c,d). The basal plasma membrane of the ST and the villous CT are strongly and continuously stained. The microvillous ST membrane is weakly and discontinuously stained (arrowheads). Tissue macrophages (M) are also strongly labelled. Endothelial cells in capillaries (Cp in c) are weakly and discontinuously labelled, whereas in larger calibre vessels they are moderately and continuously stained (d). (e) Negative control by omission of primary antibody. Magnification bars represent 20 µm. To allow easier identification of key structures the surrounding villi were erased in (a–c) using photoshop software. (Hiden et al., 2009a)

4- Insulin and IGF2 signaling

Insulin and IGFs are involved in regulating key steps for metabolism, growth and survival in mammalian cells (Nakae et al., 2001). They play their roles by binding to specific receptors present on the surface of target cells. Even though they can have different effects, they share the same signaling pathways. There are at least 3 receptors that can interact with insulin and IGFs. The insulin receptor (IR), the insulin-

like growth factor 1 receptor (IGF1R) and the insulin-like growth factor 2 receptor (IGF2R)(Krywicki and Yee, 1992). IR and IGF1R are the main receptors for driving intracellular signaling responsible for metabolic or mitogenic effects whereas IGF2R (IGF2/cation-independent mannose-6-phophate receptor) mainly regulates IGF2 clearance by trafficking excess ligand to lysosomes for degradation but can also mediate IGF2 signaling (Harris et al., 2011, Sferruzzi-Perri et al., 2008).

The IR exists in two isoforms that differ by presence (IR-B) or absence (IR-A) of 12 amino acids residues positioned at the carboxyl end of the α-subunit, as a result of alternative splicing of the exon 11 of its gene (Kosaki and Webster, 1993, Seino and Bell, 1989, Mosthaf et al., 1990). Alternative splicing of exon 11 is regulated developmentally (Kosaki and Webster, 1993), tissue specifically and hormonally (e. g. by insulin and glucocorticoids, (Sell et al., 1994, Kosaki et al., 1998). The molecular mechanisms involved are not understood although the regions of intron 10 and exon 11 participating of the alternative splicing have been identified (Kosaki et al. 1998). Both isoforms are co-expressed but their proportion will depend on the stage of development and tissue origin (Kosaki and Webster, 1993). IR-A is more expressed in fetal and cancer cells whereas IR-B is h expressed in differentiated insulin target cells like muscle, liver and fat (Frasca et al., 1999, Sacco et al., 2009). Insulin binds with two fold more affinity to isoform A but isoform B possesses a greater kinase activity (Yamaguchi et al., 1991, Kellerer et al., 1992, Kosaki et al., 1995). IGF2 can bind to IR-A and to IGF1R with same affinity, and to a lesser extent to IR-B, and trigger, in both cases, pro-mitogenic and anti-apoptotic effects (Frasca et al., 1999, Krywicki and Yee, 1992). Insulin binding to IR-B signals more efficiently to metabolic pathways. Although IGF1 and IGF2 are highly homologous, IGF1 binds with high affinity to IGF1R, whereas its binding affinity to IR-A is 10-fold lower than IGF2 to IR-A. Several studies raise the idea that IGF2 may exert its biological effects via IR-A rather than via IGF1R. It has been demonstrated that mouse fibroblasts expressing only IR-A and not IGF1R were more susceptible to the mitogenic effects of IGF2 than of insulin (Frasca et al., 1999). IGF2 was also more potent than insulin in stimulating cell chemotaxis in rhabdomyosarcoma cells lacking functional IGF1R (Sciacca et al., 2002)

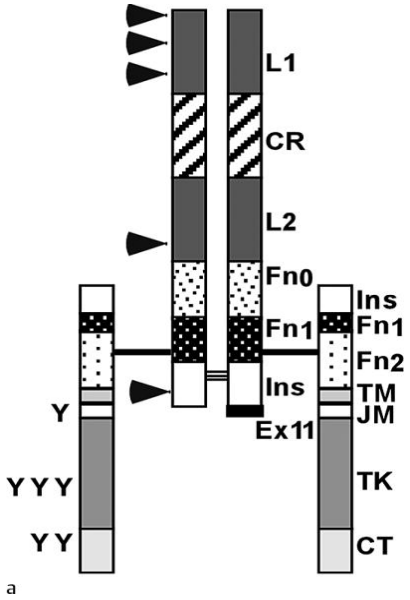


Figure 6: Schematic representation of the insulin receptor (IR) and its domains. L1 and L2; large domains 1 and 2 (leucine-rich repeats); *CR*, cysteine rich domain; *Fn_0, Fn_1, Fn_2*, fibronectin type III domains; *Ins*, insert domain; *Ex11*, exon 11 encoded peptide marked with an arrow; *TM*, transmembrane domain; *JM*, juxtamembrane domain; *TK*, tyrosine-kinase domain; *CT*, carboxy terminal tail. Solid arrows represent the major receptor sites involved in insulin binding. Y indicates potential phosphorylation sites (Denley et al., 2003).

Ligand binding to the extracellular alpha subunits of the receptors will trigger conformational changes in the beta subunits activating the receptor tyrosine kinase activity that in turn activates several intracellular signaling pathways. The best established pathways triggered by IR and IGF1R activation are the Akt/PKB (Akt is also called protein kinase B – PKB) and MEK (MAP kinase kinase) kinase pathways, and they will regulate cell metabolism and gene expression. The first and crucial step for activating these pathways is the auto-phosphorylation of the receptors followed by tyrosine phosphorylation of IR substrates for instance IRS1 and IRS2 or Shc (Src homology 2 domain-containing), that continue with phosphorylation and activation of PI3K (Phosphoinositide 3-kinase) and/or the small G-protein Ras. There is evidence that IRS1 triggers glucose metabolism whereas IRS2 regulates lipid metabolism, but the mechanism determining such specificity is still not understood. Fig 7 illustrates the best studied insulin/IGF2 pathways.

PI3K phosphorylation leads to activation of protein serine kinase cascades by phosphorylation of PDK1 (phosphoinisitide-dependent kinase 1) and its substrate

kinase Akt/PKB and the atypical protein kinase Cs (aPKCs). Akt/PKB activation will trigger different downstream pathways, depending on the cell type. The best established Akt/PKB downstream signals include GSK3, that stimulates glycogen synthesis, PKC, that regulates glucose transport via GLUT4 glucose transporter, mTOR/eIF4 that stimulates protein synthesis, and activation of fox transcription factors that will regulate gene expression. Translocation of GLUT4 to the membrane can also be driven by activation of Rho family of GTPases, for instance Rac1, which is involved in actin remodeling. Insulin can furthermore regulate adipose tissue lipolysis in an Akt dependent or independent manner (Siddle, 2011).

As discussed above, IR/IGF1R can trigger not only metabolic changes, but also cell growth via Ras/MAP kinase pathways. In this case, the recruitment of the adaptor guanine nucleotide exchange factor complex Grb2 to the phosphorylated Shc is the initial step followed by Ras/MAP kinase activation (Fig 7). In some cases, instead of activation of the substrate Shc, IRS-dependent activation can also occur (Takahashi et al., 1997). Activated Ras will activate the MEK/MAPK/ERK cascade that will mostly regulate gene expression and modulate growth and proliferation (Siddle, 2011). Shc and IRS substrates appear to compete for IR/IGF1R binding and this would then signal for metabolic vs mitogenic effects (Sasaoka et al., 2001).

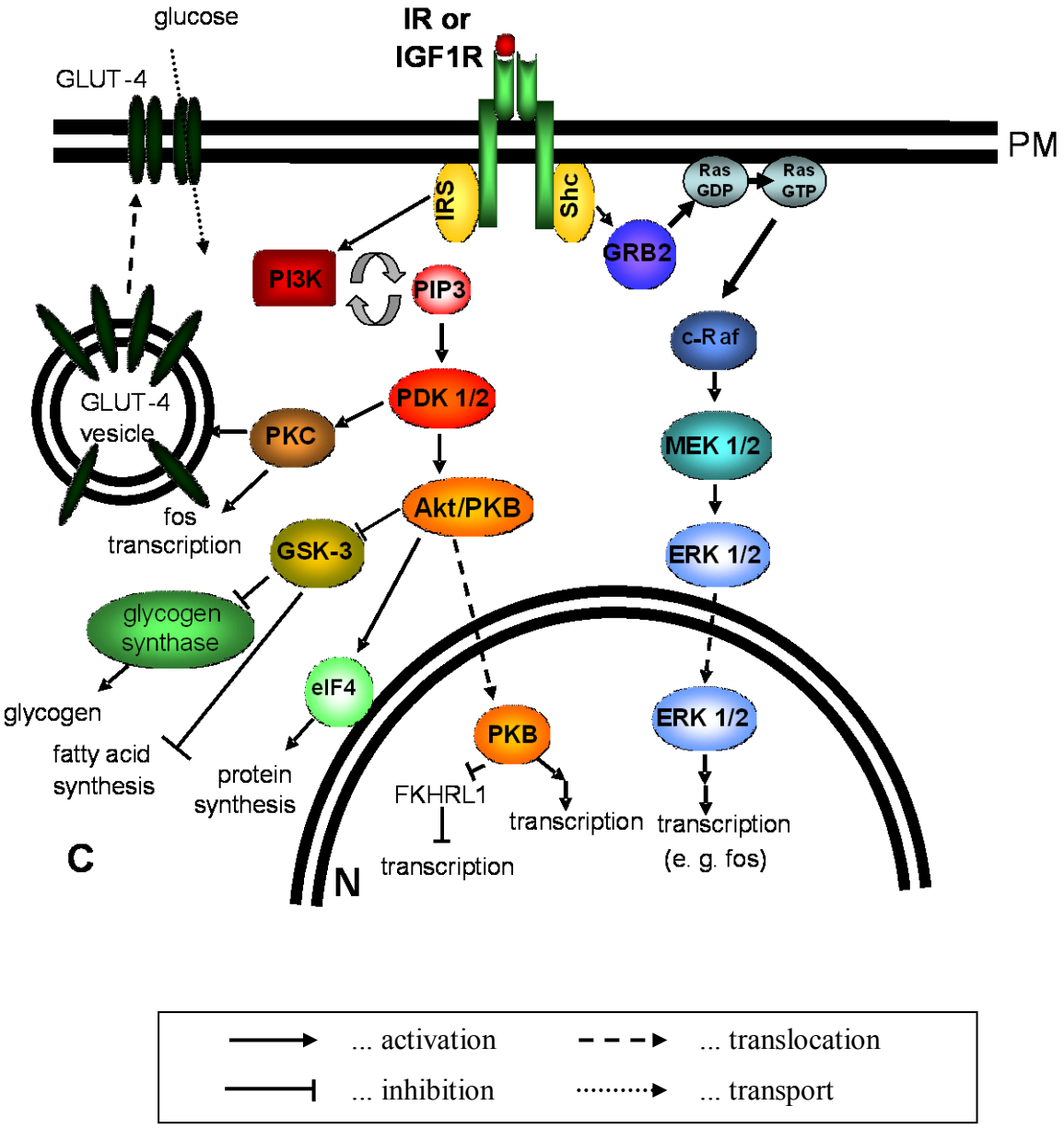


Figure 7: Insulin and IGF2 signalling. The two main insulin signalling pathways represent the PI3-kinase pathway on the left and the ERK1/2 MAPK pathway on the right side. The three MAPK pathways (ERK1/2, JNK1/2, p38; shown on the right side) affect transcription, proliferation, differentiation and apoptosis. *C*, cytoplasm; *N*, nucleus; *PM*, plasma membrane; *PIP3*, phosphatidylinositol-3,4,5-trisphosphate.

Additional pathways triggered by IR/IGF1R activation have been described. One of them indicates that IR/IGF1R activation leads to generation of reactive oxygen, such as superoxide and hydrogen peroxide, via Nox4 (NADPH oxidase 4) and Rac1 activation (Meng et al., 2008, Goldstein et al., 2005)Meng et al. 2008) Insulin resistance and several diabetic complications can be also a consequence of chronic oxidative stress (Giacco and Brownlee, 2010).

The pathways and processes regulated by insulin and IGF2 have been exhaustively studied, but the distinct and overlapping regulation of the IR and IGF1R receptors by these 2 growth factors remain inconclusive.

5- Insulin/IGF2 and angiogenesis

Angiogenesis is the development of new blood vessels from pre-existing ones and is a key process for tissue development and wound healing. The circulatory system is essential for the proper supply of oxygen and nutrients to the tissues and removal of by-products of metabolism (Folkman and Shing, 1992). In adults, angiogenesis rarely occurs, except during wound healing and in a number of angiogenesis dependent diseases such as solid tumors, proliferative retinopathies and rheumatoid arthritis (Folkman, 1987, Folkman, 1985, Folkman and Klagsbrun, 1987). In the placenta, angiogenesis is crucial for placental growth and development and to maintain its functions of sustaining the pregnancy and exchange between the mother and the fetus. Abnormal vascular development of the placenta can be associated with pathologies i.e. miscarriage, intra-uterine growth restriction and pre-eclampsia (Herr et al., 2010). As mentioned above, GDM is associated with placental hypervascularization.

Development of new blood vessels requires endothelial cell proliferation, migration and differentiation. All of these steps will depend on the interaction of endothelial cells with the surrounding environment, which includes numerous growth-factors, angiogenic factors, extracellular matrix and metalloproteinases that will degrade and remodel the basement membrane. Several soluble pro-angiogenic anti-angiogenic factors have been identified, including the members of the vascular endothelial growth factor (VEGF) family, transforming growth factors (TGF), angiopoietins, platelet-derived growth factor, tumor necrosis factor-α, interleukins and members of the fibroblast growth factor (FGF) family (Otrock et al., 2007). In

endothelial cells, VEGF-A is the best characterized and interacts with the transmembrane tyrosine kinase receptor VEGFR-1 and VEGFR-2.

In the 1960's, insulin started being used for purposes other than treating diabetic patients. Insulin was used to improve wound healing in rats (Gregory, 1965), later to improve incision or burn wounds of the skin (Rosenthal, 1968, Liu et al., 2004) and cutaneous ulcerations in diabetic and non-diabetic mice (Hanam et al., 1983). Insulin also showed vascular-protecting effects on endothelial cells by activating the endothelial nitric synthase (eNOS) and stimulating nitric oxide (NO) release (Montagnani et al., 2001). NO is a potent modulator of vascular permeability (Persson et al., 2003), inhibits vascular dysfunction caused by oxidative stress (Rath et al., 2006) and reduces reperfusion-induced cardiac injury (Wang et al., 2005). Additionally, mice lacking IR on vascular endothelial cells exhibit reduced retina-neovascularization. Similarly, mice lacking IGF1R had the same effects, although weaker (Kondo et al., 2003). Recently, Liu et al. demonstrated that subcutaneous injections of insulin into the dermis of mice stimulated the development of longer vessels with more branching. In culture, they verified that these effects were PI3K-Akt signaling mediated, leading to Rac1 activation (Liu et al., 2009). IGF2 was shown to stimulate *in vitro* angiogenesis in human umbilical venous endothelial cells (HUVECs) and is highly expressed in a series of tumors (Lee et al., 2000). IGF1 stimulated angiogenesis in ECV304 (human umbilical endothelial cells) in combination with hyperglycemia (Shigematsu et al., 1999). All together, these date raised the question whether insulin and IGFs could also act as pro-angiogenic factors in the placenta.

6- Human placental endothelial cells

The vascular endothelium is derived from the mesoderm and its inner surface is lined by a monolayer of endothelial cells (EC). It participates in several processes including control of vascular tone, developing and remodeling of the vasculature and blood flow and trafficking of nutrient and gases. Because of its position between the blood and tissue, it functions as a semi-permeable physical barrier. Arteries and veins differ in their morphology and function. Veins are usually larger in diameter than arteries with thinner walls. The walls are mainly composed by collagens and elastic fibers that provide the ability for the vessels to stretch, and smooth muscle cells that provide support. The inner surface of the endothelium is composed by a monolayer

of EC, with flat shape, ranging from 0.2µm in height at the periphery to 3µm at the nucleus. EC are heterogeneous concerning their morphology and function and will differ according to their origin. Arterial EC are usually thicker than venous EC and are long and narrow, probably as a consequence of their alignment in the direction of the undisturbed blood flow. Venous EC are short and wide, due to the lower blood flow in the venous as compared to the arterial circulation. In general in the body, arteries transport oxygenated blood to the tissues, whereas veins transport low oxygenated blood. The exceptions are the pulmonary and the placental vascular system (dela Paz and D'Amore, 2009). The semi-permeable character of the endothelium is a result of the intercellular junctions that mediate intercellular adhesion and communication. There are 3 types of intercellular junctions: the tight junctions or zona occludens, the adherens junctions, or zona adhaerens that mediate adhesion, and the gap junctions that mediate cell communication (Bazzoni and Dejana, 2004).

Our laboratory successfully established a method for the isolation of human placental arterial and venous cells. They maintain their endothelial cell characteristics in culture as expression of endothelial cell markers, Von-willebrand factor, and are characterized by LDL uptake (Lang et al., 2008). Human placental arterial and venous endothelial cells can also be distinguished by their morphology and growing patterns. Arterial endothelial cells (AEC) have a polygonal cell shape with a smooth surface and grow in a characteristic endothelial cobble-stone pattern whereas the venous endothelial cells (VEC) have a spindle-shaped appearance growing closely apposed to each other, reaching in confluence a fibroblastoid form (Fig 8). *In vitro* proliferation and generation times are also different between placental AEC and VEC. VEGF induced higher proliferative response in AEC whereas placental growth factors (PlGFs) only had an effect on VEC (Lang et al., 2008).

Human placental arterial endothelial cells express artery-related genes, such as *hey-2*, *connexin40* and *depp*, and venous endothelial cells have higher expression levels of development-associated genes, such as *gremlin*, *mensenchym homoebox2* and *DSC54*. Furthermore, these cells have distinct functions, as they respond to different growth factors (dela Paz and D'Amore, 2009, Lang et al., 2008).

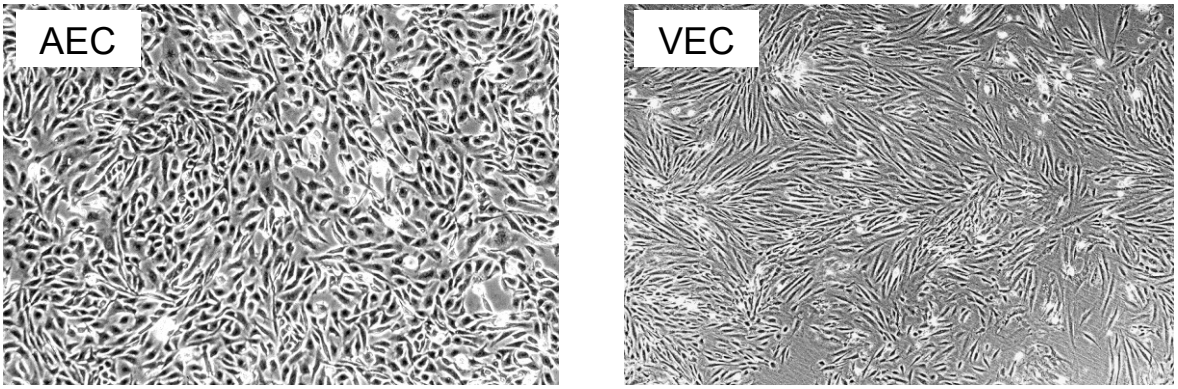


Figure 8: Morphology of AEC and VEC. Arterial endothelial cells have a polygonal cell shape with a smooth surface, whereas venous endothelial cells are spindle-shaped.

Although endothelial cells originate from the same embryonic precursor cells (hemangioblasts), they present different morphology, behaviour and function depending on their vascular bed, being a highly dynamic and adaptable system (Mehta, 2006, Simionescu, 2000). Recently, the placenta came into the focus as a reservoir of cells for regenerative medicine. Moreover, because of its high degree of vascularisation and its availability, as it is discarded post-partum, the placenta is an excellent source of fetal endothelial cells (Lang et al., 2008).

7- The role of endothelial basement membrane

EC represent the innermost continuous layer of arterial and venous blood vessels and thus are directly involved in vascular homeostasis. The endothelial cell has a luminal surface making contact with the blood, and an abluminal surface in close interaction with the subendothelial basement membrane. Consequently, the

extracellular-matrix (ECM) is one of the most important factors determining the endothelial cell properties *in vivo* and *in vitro* (Madri and Pratt, 1986, Kuehn K, 1982, Adachi et al., 2010). Oxygen tension varies within the vasculature (Read et al., 1995, Adachi et al., 2010) and thus can also affect a broad range of biological functions i.e. cell proliferation, apoptosis, inflammation and angiogenesis (Weidemann and Johnson, 2008, Simon and Keith, 2008).

In general, collagens, laminins, entactins, heparin sulfate proteoglycans, matrix metalloproteinases, osteonectin, fibulins, thrombospondins and fibronectin are components of vascular basement membranes. The dynamics of the basement membrane surrounding the endothelium will then co-modulate its functionality. In pathologies such as diabetes, hypertension and atherosclerosis, ECM remodeling can affect EC phenotype and function (Williamson et al., 1988, Skjot-Arkil et al., 2010, Loppnow et al., 2011, Schiffrin, 2001). Laminins, collagens and fibronectin play pivotal roles in adhesion and migration of endothelial cells, and laminin is considered to be the main biologically active basement membrane component (Sanz and Alvarez-Vallina, 2003, Arnaoutova et al., 2009). In addition to providing structural support, ECM components can also induce signals to stimulate proliferation, differentiation, survival and determine cell shape and morphology in various cell types from different tissues (Loeffler et al., 2011b, Larsen et al., 2006, Hynes, 2009, Kojima et al., 1998). These signals are relayed through integrin-ECM interaction which will lead to a series of signal transduction events downstream of the initial focal adhesion kinase (Fak) activation (Parsons et al., 1994, Pichard et al., 2001, Honore et al., 2000, Legate et al., 2009).

In the human placenta, collagens, including collagen I and IV, laminins and fibronectin are present in the stroma of chorionic villi. These are the structural and functional units of the human placenta in which the feto-placental vessels are embedded (Sati et al., 2008, Korhonen and Virtanen, 1997, Amenta et al., 1986, Yamada et al., 1987). The placenta, with its complex vascular system, is an easily available human source of venous and arterial EC (VEC and AEC, respectively) providing enough material for comparative studies of EC from different vascular origins, but from the same organ. Placental EC show genotypic and phenotypic heterogeneity depending on their vascular bed of origin i.e. vein or artery (Hiden et al., 2009a, Lang et al., 2003, Lang et al., 2008). So far, few studies compared EC derived from arteries or veins and these were usually performed with human

umbilical VEC and AEC (Annas et al., 2000, Van Rijen et al., 1997). Effects of ECM on EC from different vascular beds and same organ, however, have not yet been investigated.

Human feto-placental vessels (arteries and veins) are surrounded by a fibrilar matrix containing collagen I and IV, laminins and fibronectin (Sati et al., 2008, Korhonen and Virtanen, 1997, Amenta et al., 1986, Yamada et al., 1987, Huppertz et al., 1996).

As in other organs, proliferation and differentiation of EC are essential steps for a successful development of the placental vascular system, and will depend on key players such as growth factors and cytokines, ECM components, oxygen and shear stress (Herr et al., 2010). ECM proteins can also modulate angiogenesis through interaction with these chemokines and growth factors facilitating their binding to their specific receptors. The further signaling events will modulate EC migration, invasion, proliferation and survival. Binding of ECM to their specific integrins will regulate cytoskeleton reorganization. All together, these processes will drive vascular morphogenesis by which new EC will organize into new vessels (Davis and Senger, 2005)

As in the lungs and in contrast to all other vascular beds, in the human placenta AEC are exposed to lower oxygen tension, i.e. blood coming from the fetal heart, whereas their venous counterpart (VEC) is exposed to blood enriched in oxygen, which will be transported to the fetus (Nodwell et al., 2005). Changes in oxygen conditions could therefore govern placental vascular development. As already described above, GDM is associated with alterations of normal placental oxidative stress (Lappas et al., 2010) and thickening of placental basement membrane, laregely due to an increase in collagen production, and with an increase in endothelial cell proliferation (Pietryga et al., 2004).

Despite the importance of ECM for cellular function, information is sparse about how endothelial cells from different vascular beds in the same tissue respond to ECM signals present in the surrounding environment induced through their interaction with integrins.

Hypothesis and aims

Insulin and IGF2 are central growth factors for the placenta and the fetus with elevated fetal concentrations in GDM. Their receptors are expressed on the placental endothelium, which is in contact with the fetal circulation, but their cellular effects on the feto-placental vasculature have remained elusive. ECM composition is also altered in placentas from pregnancies complicated with GDM which are bigger and more vascularized than placentas from normal pregnancies. We hypothesized that insulin and IGF2 activate different signaling pathways and regulate different cellular effects in AEC and VEC. These effects may also depend on oxygen levels. Among these effects, proliferation and angiogenesis are candidate processes, involving also gene expression, and they may also be altered by the composition of the extracellular matrix (ECM).

Therefore, the aims of the project are:

- To analyse whether insulin and IGF2 can modulate gene expression in AEC and VEC
- Identify placental processes regulated by fetal insulin and IGF2
- To delineate the major signalling pathways induced by insulin regulating these processes
- To investigate whether insulin has an effect on angiogenesis of placental EC and delineate the pathways that could be involved
- To study potential effects of ECM on EC proliferation, survival, and behaviour
- To determine whether oxygen plays a role on the insulin/IGF2 and ECM effects on EC

The thesis is organized in three chapters:

- Chapter 1: **Differential effect of insulin and IGF2 on global gene expression of arterial vs venous human placental endothelial cells**
- Chapter 2: **Insulin stimulates *in vitro* angiogenesis in human placental arterial endothelial cells via IRS/PI3K/Akt and endothelial nitric oxide synthase activation**
- Chapter 3: **Differential response of arterial and venous endothelial cells to extracellular matrix is modulated by oxygen**

Materials and methods

Isolation and culture of third trimester human placental endothelial cells

Primary VEC and AEC were isolated from third trimester human placentas, after uncomplicated vaginal delivery as described previously (Lang et al., 2003). The amnion was removed and the apical surface of the chorionic plate was cleaned with an antiseptic solution (Betaisadona, Mundipharma, Vienna, Austria). Placental macrovascular arteries and veins were cut out in a size of 1.5 – 2 cm and washed with HBSS. A cannula was inserted into the vessel and fixed with a yarn. Following insertion of the syringe containing 0.05% (w/v) collagenase/dispase solution (Roche, Germany) in HBSS pre-warmed to 37°C, the enzyme solution was slowly rinsed through the vessel. The released cells were collected in a 50mL falcon containing 5mL FCS. The obtained cell suspension was centrifuged, resuspended in Endothelial Basal Medium (EBM, Clonetics™, Lonza, Walkersville, MD, USA) supplemented with the EGM™-MV BulletKit (Clonetics™, Lonza) containing gentamicin/amphotericin, hydrocortisone, recombinant-human epidermal growth factor, bovine brain extract and 5% pregnant woman serum and cultured in 12 well culture plates that had been pre-coated with 1% (v/v) gelatin (Sigma) in HBSS for 1 h at 37°C. After reaching confluence on the well, cells were transferred to 25cm^2 flasks and again, after confluence, transferred and cultured in 75cm^2 flasks. At this point cells were subjected to rigorous immunocytochemical characterization for identity and purity (Blaschitz et al., 2000, Cervar et al., 1999, Lang et al., 2003) as described below.

Characterization and culture of human primary placental endothelial cells

For the immunocytochemical characterization of VEC and AEC a panel of monoclonal antibodies was used. The sets of antibodies were chosen in order to achieve positive as well as negative verification. Antibodies and target molecules are listed in table 1. For immunocytochemistry, cells were grown on chamber slides (Lab-Tek®, Naperville, IL) for 48 hours and then fixed in acetone (Merck) for 3 min. Cells were then washed with HBSS 1x and the side panels removed. After removal of the side panels, the chamber slides can be used as optical slides. The cell-covered area was bordered with a DakoCytomation Pen (Dako) to avoid flow

out of solutions which were subsequently pipetted onto the cells. Cells were washed in TBS (tris buffered saline) pH 7.5 with 0.05% Tween 20 (Sigma) for 3 min. All further incubation steps were performed in a humid chamber. Primary antibodies (diluted in Thermo Scientific antibody diluent) were applied to the cells followed by 30 minutes incubation at room temperature. After 4 washings in TBS/Tween, a primary antibody enhancer (Thermo Scientific) was applied and slides were incubated for 10 minutes at room temperature. Slides were once again 4 times washed in TBS/Tween and slides incubated with an HRP polymer for 15 minutes in the dark. Following further 5 minutes washing, the slides were incubated with the ready-to-use peroxidase-compatible chromogen (Thermo Scientific) for 5 min and washed 4 times with distilled water. Unstained cells were visualized using haemalaun (Sigma) solution which developed a blue colour when immersed in tab water. Slides incubated with mouse, unspecific immunoglobulin fractions (Dako) in the same concentration as the primary antibody served as negative controls. The slides were mounted with cover slips and observed with bright field microscopy in a Zeiss AxioPlan microscope, AxioCam camera, and analyzed with the Zeiss AxioVison 4.8 software.

Table 1: Antibodies used for characterisation of endothelial cells.

Antibodies for characterisation of endothelial cells		**Company**	**Dilution**
Anti-von Willebrand factor	specific marker for endothelial cells	Dako	1:2500
Anti-Human CD90	specific marker for fibroblasts	Dianova	1:100
Anti-Human Desmin	stains muscle cells	Dako	1:100
Anti-Human Smooth Muscle Actin	stains muscle cells	Dako	1:200
Anti-Human Vimentin	stains intermediate filaments (IF)	Dako	1:200
Anti-mouse IgG1	Negative control	Dako	1:250

Unless stated otherwise cells were cultured on 1% (v/v) gelatin-coated plates using Endothelial Basal Medium (EBM, Clonetics™, Lonza, Walkersville, MD, USA) supplemented with the EGM™-MV BulletKit (Clonetics™, Lonza)

containing gentamicin/amphotericin, hydrocortisone, recombinant-human epidermal growth factor, bovine brain extract and 5% FCS. To mimic the oxygen variation in placental vascular beds AEC were expanded at 12% oxygen and 37^{o}C and VEC at 21% oxygen and 37^{o}C respectively.

Cell counting

VEC and AEC were harvested with trypsin/EDTA (Clonetics^{TM}, Lonza) and resuspended in 10 ml fully supplemented EBM. The number of viable and dead cells was counted in the cell counter and analyser system CASY 1 (Schärfe System, Reutlingen, Germany) using a 150 µm capillary. This system detects changes in conductivity along an aperture during the flow of a cell-containing liquid and gives a size-distribution curve of the cells (Falkenhain A, 2002), in which cell debris, dead and viable cells can be identified by their distinct diameters. The size distribution for viable and dead placental AEC and VEC was previously determined in our laboratory by mixing equal proportion of dead (after ethanol treatment) and viable cells prior of measurement. The size ranges thus determined allowed counting the viable and dead cells in the samples separately but in one measurement run. This method has been used to demonstrate the effects of hyperglycaemia on choriocarcinoma cells (Weiss et al., 2001).

Insulin and IGF2 treatment of primary placental AEC and VEC for microarray anaylsis

VEC and AEC pairs isolated from 6 different placentas were seeded ($8x10^5$ cells) in 1% (v/v) gelatin-coated flasks ($75cm^2$) and cultured in EBM medium containing 2% FCS without supplements for 24 hours at 21% oxygen and 37^{o}C. Cells were then treated either with 10nM insulin (Calbiochem Merck, Darmstadt, Germany*),* 10nM IGF2 (R&D systems, Minneapolis, MN, USA) or vehicle (HBSS 1x, Gibco, Paisley, UK) for 24 hours. After treatment cells in each flask were washed with cold HBSS 1x and RNA was isolated.

RNA isolation and microarray analysis of primary placental AEC and VEC

Total RNA was isolated with RNeasy mini kit (QIAGEN, Dusseldorf, Germany) and scrutinized for quality on the BioAnalyzer BA2100 (Agilent, Santa Clara, CA, USA) with the RNA 6000 Nano LabChip Kit (Agilent, Cat No 5067-1511). The RIN (RNA Integrity Number) of the samples ranged between 8.7 and 10. Total RNA was labeled using Affymetrix GeneChip® Whole Transcript (WT) Sense Target Labeling Kit (Affymetrix, Santa Clara, CA, USA; Cat No. 900652) and then prepared for hybridization. For expression analysis cRNA was hybridized against GeneChip® Human 1.0 ST arrays (Affymetrix, Cat No. 901087) according to the manufacturer's instructions. Labeling controls and hybridization controls were evaluated with Expression Console EC 1.1. Hybridizations and first analysis were carried out at the Division Core Facility for Molecular Biology at the Centre of Medical Research at the Medical University of Graz, with collaboration of Birgit Ebner. Microarray data were analysed with Partek Genomic Suite v6.4 software (Partek Inc, St Louis, MO, USA). The import process of the CEL files contained RMA normalization (robust multi-chip average) including background correction, quantile normalization across all arrays and median polished summarization based on log transformed expression values. For comparison of global expression profiles of different samples principal component analysis (PCA) was performed applying *prcomp* from the R package *stat* on normalized data using all probesets and the 30% upper quantile of probesets with highest interquartile range, respectively. Based on xy-plot of the first two principal components, one sample (49) were identified as outlier and removed from further analysis resulting in 5 biological replicates for each of the six conditions (AEC-HBSS, AEC-INS, AEC-IGF2, VEC-HBSS, VEC-INS, VEC-IGF2). Only probesets annotated with Refseq (NM_...) were considered for further analysis using current annotation file from NetAffx center (Affymetrix) for Human Gene 1.0 ST arrays. Probesets were filtered for genes with low variability across all samples (interquantile range < 1). To test probesets for statistical significant differential gene expression between groups, moderated (paired) t-test using R/Bioconductor package *limma* was used (Smyth, 2004) p-values were subjected to multiple testing correction by the Benjamini-Hochberg method based on the false discovery rate (FDR). Genes/probesets with >1.3 fold change and $p<0.05$ were considered significantly differentially expressed. Log2-fold changes for each group against the control group (treated with HBSS)

for in at least one group differentially expressed genes were visualized as heatmap using Genesis (Sturn et al., 2002). Overrepresented gene ontology terms for up- and downregulated genes separately were analysed based on gene symbols using DAVID Bioinformatice Resource (Huang da et al., 2009). Analyses of microarray data were done in collaboration with Hubert Hackl, Division for Bioinformatics, Biocenter, Innsbruck Medical University.

Validation of the microarray experiment

To validate the results of the microarray experiment, randomly selected insulin and IGF2 regulated genes and AEC x VEC differentially expressed integrins were verified by real-time PCR. One µg total RNA in 10 µl water was mixed with the random hexamer primers (200 ng) and 1 µl dNTPs (each 10 mM) and heated to 65°C for 5 min. Then, 5x first strand buffer and 0.1 M DTT were added. After 2 min at 25°C, 1 µl of Supersript II was added and gently mixed. For cDNA synthesis, the reactions were further incubated for 10 min at 25°C and 50 min for 42°C in a thermocycler. Thereafter, the cDNA of each reaction was subjected to quantitative real time PCR using carboxy-fluorescein-dye (FAM) labeled TaqMan Gene Expression Assays (table 2) and the TaqMan Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ, USA). Components were mixed according to the manufacturer's instructions and amplified in 20 µl total volume/well (96 well plates, Roche, Mannheim, Germany) using a ABI7900 (Applied Biosystems) real-time cycler. Ct values were automatically calculated by the software (SDS2.2) and relative gene expression was quantified by standard $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the expression of the ribosomal protein L30 as reference. Statistical analysis used the ΔCt values. Reactions were run in technical duplicates.

Table 2. Assay-on-Demand™ IDs used for real-time PCR.

Gene symbol	Assay-on-Demand™ ID
PTCH2	Hs00184804_m1
OCLN	Hs00170162_m1
PLA2G4	Hs00233352_m1
VAV3	Hs00196125_m1
KCNJ6	Hs00158423_m1
PIGF	Hs00601696_m1
EFCAB4B	Hs00261932_m1
RPL30	Hs00265497_m1
ITGAV	Hs00233808_m1
ITGB3	Hs01001469_m1
ITGB8	Hs01110394_m1
HPRT1	Hs01003267_m1
CDC42	Hs00377831_m1
KRT6B	Hs00749101_s1

Insulin stimulation of AEC for signal transduction

AEC were seeded (180,000 cells/well) in 1% gelatin coated 6 well plates and cultured for 12 hours in supplemented EBM medium containing 5% FCS, at 21% and 12% oxygen and 37°C. Cells were then serum starved in non-supplemented EBM medium for 6 hours. After starvation cells were stimulated either with 10nM insulin, 25ng/mL VEGF (654 pM) or vehicle (HBSS) for 2, 5 and 15 minutes at 12% and 21% oxygen at 37°C. After stimulation, plates were placed on ice, medium was immediately removed and cells were washed with cold cell wash buffer (Bio-Plex cell lysis kit, BioRad, CA, USA). Total protein was isolated with cell lysis buffer (Bio-Plex cell lysis kit, BioRad) following manufacturer instructions. Protein concentration was determined by DC protein assay (BioRad).

Phosphorylation status of the insulin receptor isoform B (IRb), insulin receptor substate 1 (IRS1) and of signaling kinases ERK 1/2, PKB/Akt, and GSK-3 after insulin and IGF2 treatment was determined using the Bio-Plex Phosphoprotein Detection kit (BioRad) following manufacturer instructions. This system allows a multiplexing analysis of up to 9 phosphoproteins in one single well in a 96 well format. Analysis was done in triplicates of 3 independent experiments with 3 different cell isolations. The phosphorylation values were normalized by total protein concentration.

Insulin stimulation of cell cycle in AEC

AEC (300,000/75cm^2 flasks) were cultured in non-supplemented EBM containing 5% FCS and incubated for 24 hours at 21% oxygen and 37^{o}C. Insulin was then added to a final concentration of 10nM and cells were incubated for another 24 hours under the same conditions. VEGF (650pM) and HBSS 1x were used as positive and negative controls, respectively. After 24 hours incubation, BrdU was added to a final concentration of 10µM and cells again incubated for 2 hours. Cells were then harvested with trypsin/EDTA (Clonetics^{TM}, Lonza), centrifuged at 1000 RPM for 6 minutes and supernatant was discarded. The cells were fixed, permeabilized and processed following the FITC BrDU Flow kit (BD Bioscineces) instructions. Samples were analyzed using a BD FACSCaliburTM system (BD Biosciences, Bedford, MA, USA) with BD CellQuest Pro software (BD Biosciences) for data analysis.

Insulin stimulation of actin reorganization and F-actin immunofluorescence staining in AEC

AEC (50,000 cells/well) were seeded in 1% gelatin coated chamber slides in 5% FCS supplemented EBM medium and incubated at 21% oxygen and 37^{o}C. After 12 hours, medium was replaced to a non supplemented medium containing 0.5% FCS and incubated in the same conditions for 24 hours. Medium was once again replaced and cells were serum starved in a non supplemented medium for another 24 hours. Insulin was added to a final concentration of 10nM and cells were incubated for 30 minutes at 21% oxygen and 37^{o}C. Non-starved cells and

starved cells treated with EGF 20ng/mL for 30 minutes were used as positive controls for F-actin distribution on AEC. Monolayers were washed with HBSS 1x and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. After washing 3 times with PBS the cells were permeabilized with 0.1% Triton X-100 in PBS for 25 min at room temperature. The slides were again 3 times washed with PBS and then blocked with 1% BSA in PBS for 25 min at room temperature. After blocking, slides were washed once with PBS and incubated with 1 U/200µl methanolic phalloidin-Texas Red (Molecular Probes, Invitrogen) for 20 minutes in the dark at room temperature for F-actin staining. Stained cells were washed 3 times with PBS and slides mounted with Dako fluorescent mounting medium (Dako) with DAPI (1:2000) for nuclei observation. After overnight drying, actin organization was observed in a Zeiss Axioplan fluorescence microscope with 200x magnification using the AxioVision software (Carl Zeiss Imaging Solutions GmbH).

Pathways analysis of insulin effects on actin assembly by using pharmacological inhibitors

AEC were seeded and starved as described above. Previous to insulin treatment cells were pre-treated for 1 hour with the following inhibitors: NSC 200µM (Rac1 inhibitor), Wortmannin 100nM (PI3K inhibitor), UO126 10µM (MAPK inhibitor), L-NAME 400µM (e-NOS inhibitor) and DMSO (vehicle). Insulin was then added to a final concentration of 10nM and cells were incubated for more 15 minutes at 21% oxygen and 37^{o}C. Non-starved cells and starved cells treated with EGF 20ng/mL for 15 minutes were used as positive controls for F-actin distribution. Cells treated with inhibitors alone were used as control for inhibitor effects. After the treatments, monolayers were washed and actin staining was performed as previously described.

Insulin stimulation of angiogenesis in AEC

Angiogenesis was analyzed by 2-D matrigel network formation assay. AEC were harvested with trypsin/EDTA (Clonetics^{TM}, Lonza) from 75cm^2 flasks, centrifuged and resuspended in non supplemented EBM containing 2% FCS. Insulin or VEGF were added to a final concentration of 10nM and 25ng/mL

(645pM), respectively and HBSS 1x was used as control (vehicle). Cells were then seeded (8,000/well) in triplicates on 96 well plates pre-coated with 50µL of growth factor reduced matrigel (BD Biosciences, Bedford, MA, USA). Coating procedure was according to manufacturer instructions. Cells were incubated at 21% oxygen and 37^{o}C for 24 hours and real time monitored in a Zeiss Cell Observer microscope with an AxioCam HRm camera and an A-Plan 5x/0.12 Ph0 objective using the software AxioVision (Carl Zeiss Imaging Solutions GmbH). Pictures of the 2-D networks were taken every 1 hour and the quantitative analysis of total tube length and number of branching points was done using the AngioJ-Matrigel assay plugin for the ImageJ software (NIH). The plugin was developed and kindly provided by Diego Guidolin (Department of Human Anatomy and Physiology, Section of Anatomy, University of Padova, Italy).

Insulin-induced angiogenesis pathways analysis

AEC were harvested with trypsin/EDTA (Clonetics^{TM}, Lonza) from 75cm^2 flasks, centrifuged and resuspended in non supplemented EBM containing 2% FCS. Previous to insulin treatment cells were pre-treated for 1 hour with the following inhibitors: NSC 200µM (Rac1 inhibitor), Wortmannin 100nM (PI3K inhibitor), UO126 10µM (MAPK inhibitor), L-NAME 400µM (e-NOS inhibitor) and DMSO (vehicle). Insulin or VEGF were then added to a final concentration of 10nM and 25ng/mL (645pM), respectively. Cells were then seeded (8,000/well) in triplicates on 96 well plates pre-coated with 50µL of growth factor reduced matrigel (BD Biosciences) and the experiment followed as described above, with network formation being real time monitored.

Culture of VEC and AEC on different extracellular matrices (ECM)

Six-well plates (plastic) were coated for 1 hour at 37^{o}C either with 3 µg/cm^2 of rat collagen I (Cultrex, R&D systems, Minneapolis, MN, USA), 3 µg/cm^2 of collagen IV (Cultrex, R&D systems) or 1% gelatine (Sigma, St. Louis, MO, USA), 30 min at 37^{o}C with 1 µg/cm^2 of bovine fibronectin (Cultrex, R&D systems) and overnight at 37^{o}C with 1 µg/cm^2 mouse laminin I (Cultrex, R&D systems). Prior to plating of cells, the coated wells were washed with PBS. VEC and AEC grown to

subconfluency (40%) in gelatine coated flasks were harvested with trypsin/EDTA (Clonetics™, Lonza) and resuspended in 10ml fully supplemented EBM. Cell number was determined as described above. Cells (90,000 cells/well) were then seeded on matrix-coated wells in EBM (Clonetics™, Lonza) supplemented with the EGM™-MV BulletKit (Clonetics™, Lonza). After 48 hours incubation, cells were harvested either for determination of cell number and viability or cell cycle and apoptosis analysis. For Fak inhibition assays, Fak inhibitor 14 (Tocris, Bristol, UK) was added to the culture medium to a final concentration of 25µM, prior to seeding. When assays required 48 hours incubation, medium containing Fak inhibitor was changed after 24 hours.

Proliferation of VEC and AEC on different ECM

Proliferation was measured by a direct cell counting method in the cell counter and analyser system CASY 1 (Schärfe System). Cells were cultured under the conditions defined above for the different ECM coating. After 48 hours, cells were detached by trypsin/EDTA (Clonetics™, Lonza) and the number of viable and dead cells was counted.

Flow cytometric analysis of cell cycle and apoptosis

After growing in the different ECM for 48 hours as described above, cells were harvested with trypsin/EDTA (Clonetics™, Lonza), resuspended in EBM (Clonetics™, Lonza) supplemented with the EGM™-MV BulletKit (Clonetics™, Lonza). Cell suspensions were then centrifuged at 1000 rpm for 4 min at 4°C and resuspended in 500µl PBS. The distribution of cells in the various phases of the cell cycle was measured by FACS after propidium iodide (PI) staining. Cells were fixed by adding 5ml of ice-cold ethanol (70%) and 10 min at 4°C incubation. After fixation, the samples were washed twice with PBS and the pellet resuspended in 250µl PI-staining buffer (50µg/ml PI and 200µg/ml RNAseA in PBS, Beckman Coulter, Miami, USA). Samples were incubated for 15 min at 37°C and PI incorporation was measured.

Apoptosis was determined by FACS analysis using the antibody against the large fragment of cleaved caspase 3 (Cell Signaling Technology, Boston, MA,

USA). The cells were fixed by adding 500 µl of formaldehyde (2%) to 500 µl cell suspension in PBS and by subsequent incubation for 10 min at 37°C and for 1 min on ice. After fixation cells were permeabilized by adding 9ml methanol and incubated for 30 min at 4°C. Samples were washed with 2ml of PBS containing 0.5% FCS and the pellet resuspended in 90µl PBS. After 10 min at room temperature, 2µl antibody (0.4µg/ml) was added and the samples incubated in the dark for 1 hour. Then, cells were centrifuged, resuspended in 300 µl PBS and the antibody conjugates analysed by FACS. Both PI and caspase 3 measurements were made using a BD FACSCaliburTM system (BD Biosciences, Bedford, MA, USA) with BD CellQuest Pro software (BD Biosciences) for data analysis.

Endothelial integrin expression

Placental endothelial cells were grown in 48-well plates. The cells were detached by using a detachment buffer (25mM HEPES, 10mM EDTA in PBS). The endothelial cells were then stained with fluorescently-labeled CD49e (APC), CD61 (FITC) and VE-Cadherin (PE) or the corresponding isotype control antibodies for 30 minutes at 4°C in dark. The antibodies were diluted (1:25) in Antibody diluent. The ITGB8 integrin expression was defined using a primary mouse anti-ITGB8 antibody or a corresponding isotype control antibody (10µg/mL) and a goat anti-mouse secondary antibody labeled with Alexa Fluor 488 (4µg/mL). Endothelial integrin molecule expression was determined by means of flow cytometry (FACSCalibur, BD Biosciences) by analysis of the fluorescence intensity of 5,000 cells in each sample; data are shown in histograms (Gao et al., 2000). Flow cytomtric analyses were done in collaboration with Viktoria Konya and Akos Heinemann, from the Institute of Experimental and Clinical Pharmacology, Medical University of Graz. CD49e-APC, CD61-FITC antibodies and isotype control IgG1,κ-FITC, IgG2b, κ-PE and IgG2b, κ-APC antibodies were purchased from Biolegend (San Diego, CA).VE-Cadherin-PE antibody is from BD (Vienna, Austria). The ITGB8 primary mouse antibody is from antibodies-online Inc. (Atlanta, GA) and the goat anti-mouse Alexa Fluor 488-labeled secondary antibody is from Invitrogen (Carlsbad, CA). Antibody diluent is from Dako Österreich GmbH (Vienna, Austria).

F-actin and vinculin immunofluorescence staining and laser scanning microscopy (LSM)

VEC and AEC (75,000 cells/well) were seeded in matrix-coated chamber slides (as described above). After 24 and 48 hours the monolayers were washed with HBSS 1x and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. After washing 3 times with PBS the cells were permeabilized with 0.1% Triton X-100 in PBS for 25 min at room temperature. The slides were again 3 times washed with PBS and then blocked with 1% BSA in PBS for 25 min at room temperature. After blocking, slides were washed once with PBS and then overnight stained with vinculin antibody (Thermo Scientific, CA, USA, 1:100). Slides were once again washed 3 times with PBS and incubated with cy2-conjugated goat anti-mouse secondary antibody (Jackson immunoresearch, PA, USA) and with 1 U/200µl methanolic phalloidin-Texas Red (Molecular Probes, Invitrogen) for 1 hour in the dark at room temperature. Stained cells were washed 3 times with PBS and slides mounted with Dako fluorescent mounting medium (Dako) with DAPI (1:2000). After overnight drying, actin organization and focal adhesions were observed in a Zeiss LSM 510 Meta microscope, objective Plan-Apochromat 63x/1.4 Oil DIC, lasers 405nm, 488nm and 543nm and LSM Image Browser software.

Immunobloting for detection of Fak phosphorylation

VEC and AEC (75,000 cells/well) were seeded in matrix-coated 12 well-plates (as described above). After 4 hours, when the cells were attached to the matrices, they were washed with ice-cold HBSS 1x (Gibco, Paisley, UK), lysis buffer was added [0.01M Tris pH 7.4, 1% SDS, 1mM Na_3VO_4, 25mM NaF, 10mM NaPP and 1 tablet of Complete protease inhibitor cocktail (Roche) dissolved in 1ml distilled water to a final volume of 25ml] and monolayers were scratched. The lysates were then boiled for 5 min. Samples were subjected to SDS-PAGE on 10% precise protein gels (Pierce Protein Research, Rockford, IL, USA) using HEPES buffer, and electrophoretically transferred onto PVDF membranes in Tris-glycine-methanol buffer. The membranes were blocked for 1 hour at room temperature with blocking solution [5% BSA and 0.1% (v/v) Tween-20 (Sigma) in 0.14M Tris-buffered saline, pH 7.2-7.4] and hybridized overnight at 4^{o}C with the primary

antibody against pTyr397-Fak (1:500 dilution, Cell Signaling) or GAPDH (1:20,000; NOVUS Biologicals, Littleton, CO, USA). After overnight incubation membranes were washed in 0.1% (v/v) Tween-20 (Sigma) in 0.14 M Tris-buffered saline, pH 7.2-7.4, and incubated with the secondary antibodies goat anti-rabbit 1:1000 and goat anti-mouse 1:2000 for pTyr397-Fak and GAPDH, respectively (BIORAD, Hercules, CA, USA). The bands were detected by an immunochemiluminescence method and membranes exposed to high performance chemiluminiscence film (Hyperfilm ECL, Amersham, Buckinghamshire, UK). The optical density of specific bands was quantified by Bio-Rad Quantity One software.

Statistical Analysis

The data are expressed as mean values ± SD. The raw data of viability, cell cycle and apoptosis were statistically tested by Wilcoxon signed rank test. Student's t-test was applied for western blot and FACS analysis after testing for normal distribution (Kolmogorov–Smirnov test). For the time course experiment, Two way ANOVA was used with Holm-Sidak method as post-hoc. Significance was accepted when $p<0.05$.

Chapter 1

Differential effect of insulin and IGF2 on global gene expression of arterial vs venous human placental endothelial cells

The placental endothelium is lined by fetal endothelial cells and is in contact with the fetal blood (Leach, 2011). In GDM, altered insulin and IGF2 levels in the fetal blood would then have a direct effect on the fetal endothelium which is rich in IR and IGF1R (Hiden et al., 2009a). Global gene expression profiling tools have been extensively used to understand the molecular basis for pre-eclampsia (Hoegh et al., 2010, Reimer et al., 2002, Sitras et al., 2009, Nishizawa et al., 2007), however, only 3 studies focused on GDM effects on placental gene expression (Enquobahrie et al., 2009, Radaelli et al., 2009, Radaelli et al., 2003). All of these studies were performed using whole placental tissue, thus providing only an overview of the differential gene expression caused by GDM in the entire organ. Here I focused on the effects of GDM-associated altered fetal insulin and IGF2 levels on only one compartment of the placenta, the feto-placental endothelium.

Hypothesis

I hypothesized that elevated fetal insulin and IGF2 levels caused by GDM would have long term effects and affect the gene expression profile of AEC and VEC isolated from the feto-placental vasculature which is in contact with the fetal blood. These effects may also be different in AEC versus VEC.

Results

1- Global analysis (microarray) of insulin and IGF2 effects on gene expression in human placental endothelial cells

The insulin/IGF system can regulate fetal and placental development and growth. As their levels are elevated in GDM in the mother and the fetus, this can have profound effects on the placenta. In order to investigate these effects on

gene expression on the placental endothelium, which is in contact with fetal blood, a microarray experiment was performed. To this end, both AEC and VEC were isolated from six different placentas. The use of AEC and VEC pairs from the same donor placenta allowed their isogenetic analysis. Once isolated, the six AEC and VEC pairs were treated with 10nM insulin or IGF2 and incubated for 24 hours in EBM medium containing only 2% FCS. After 24 hours the RNA was isolated, quantified and used for cRDNA synthesis which was hybridized against the microarray Affymetrix GeneChip Human Gene 1.0 ST Array.

The initial data processing revealed that there were larger differences between AEC and VEC than caused by the treatments. Figure 9 shows the number of genes differentially expressed between AEC and VEC ($p<0.05$) and provide an overview of the number of genes regulated by insulin and IGF2, and. To achieve a larger number of insulin and IGF2 regulated genes, the fold change cut off was set to 1.3, while for differentially expressed genes between AEC vs VEC, the threshold of fold change >2 was kept .

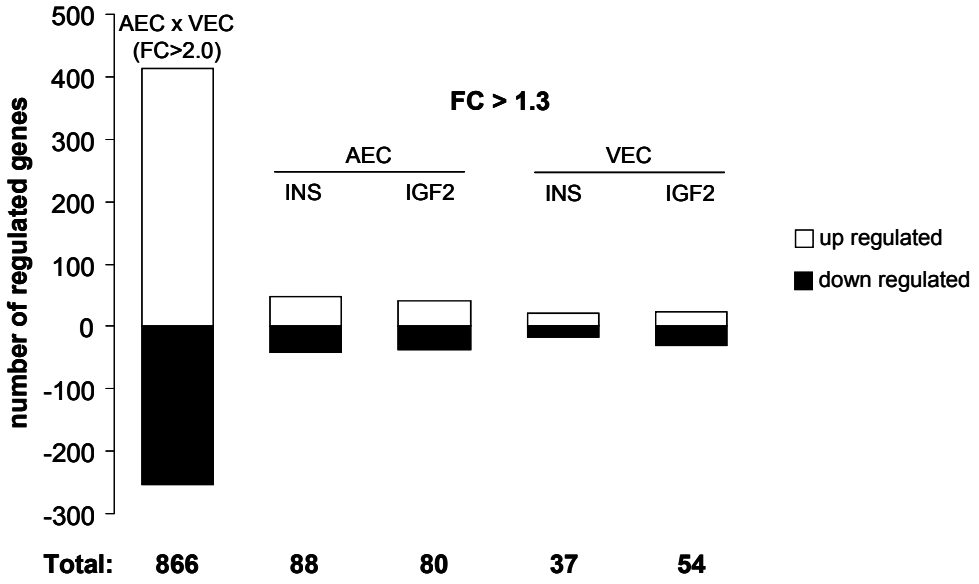


Figure 9: Number of differentially expressed genes identified by microarray analysis. AEC and VEC pairs isolated from 6 different placentas were seeded (800,000 cells/flask) and cultured in supplemented EBM containing 2% FCS, at 21% oxygen and 37°C. Cell were then treated either with with 10nM insulin, 10nM IGF2 or vehicle for 24 hours. After treatment RNA was isolated and cRNA was hybridized against GeneChip® Human 1.0 ST arrays. Data processing revealed more differentially expressed genes among the cell types, with fold changes >2.0, than by the treatments, for which fold changes >1.3 are shown.

The number of differentially expressed genes between AEC and VEC was 866, with 413 higher and 253 lower expressed in AEC as in VEC. Treatment with insulin regulated 88 genes in AEC and 37 in VEC, while treatment with IGF2 revealed 80 genes being regulated in AEC and 54 in VEC. In fact, preliminary unpublished immunocytochemistry and semi-quantitative RT-PCR data in our laboratory demonstrated that AEC express more IR than VEC (personal communication by Ursula Hiden, data not shown) and this would partially explain the differences found in the microarray.

Figure 10 shows a heat map and Venn diagrams summarizing the distribution of the differentially regulated genes in AEC and VEC by insulin and IGF2. From the 88 insulin regulated genes in AEC, 14 were equally regulated by IGF2, which in total regulated 80 genes in AEC. In VEC, the number of regulated genes was smaller than in AEC. Here, 37 and 54 genes were regulated by insulin and IGF2 respectively. In VEC, 4 were equally regulated by both growth factors. There were only two overlapping insulin-regulated genes in AEC and VEC, but they were up regulated in AEC while down regulated in VEC. A similar observation was made by the IGF2 regulated overlapping genes in AEC and VEC. The list of all insulin and IGF2 regulated genes is presented in the appendix of the thesis (appendix 1).

The genes up-regulated by insulin in AEC include MAP2 (microtubule-associated protein 2), TUBB2C (tubulin 2C), OCLN (occludin), and RND1 (Rho family GTPase 1), all of them being involved in cytoskeleton organization and cell contact, important steps for EC proliferation and angiogenesis. PLA2G4A (phospholipase A2) was also up-regulated by insulin and is involved in prostaglandin production, also participating in the angiogenic process (Wendum et al., 2005). IGF2 up-regulated expression of CLDN6 (claudin 6), a component of tight junctions, and also of MAP2, which stabilizes microtubules against depolymerisation (Kamath et al., 2010). Furthermore, IGF2 regulated a variety of zinc fingers transcription factors genes (appendix 1). Among the insulin down-regulated genes, HBG1 and HBG2 (haemoglobin gamma 1 and 2) are involved in oxygen transport, had the largest fold change. Other genes down-regulated by insulin in AEC include AKR1C1 (aldo-keto reductase family 1, member C1), involved in progesterone metabolism, CASP1 (caspase 1), and genes associated with inflammatory response such as the gene for HP (haptoglobin) that has also

been linked to diabetic nephropathy and the incidence of coronary artery disease in type 1 diabetes (Simpson et al., 2011). IGF2 down-regulated genes include G0S2 (G0/G1 switch 2), that encodes an anti-apoptotic protein (Welch et al., 2009), LIPN (lipase, family member N) and VAV3 (vav 3 guanine nucleotide exchange factor), involved in RhoA and RhoG activation.

The overlapping genes up-regulated by insulin and IGF2 in AEC comprise GSTM3 (glutathione S-transferase mu 3) that participates in detoxification of oxidative stress products, PTCH2 (patched homolog 2), KRT6B (keratin 6B) and MAP2. From the down regulated overlapping genes, the ones with highest fold changes are HSD11B1 (hydroxysteroid 11-beta dehydrogenase 1), PRDM1 (PR domain containing 1, with ZNF domain), a transcriptional repressor, GPR116 (G protein-coupled receptor 116) and PPARG (peroxisome proliferator-activated receptor gamma).

In VEC, the number of genes regulated by insulin and IGF2 was smaller. The genes up-regulated by insulin with higher fold change comprise ZNF257, a zinc finger transcription factor, EFCAB4B (EF-hand calcium binding domain 4B) and TPTE2 (transmembrane phosphoinositide 3-phosphatase and tensin homolog 2), TUBB2A (tubulin beta 2A), CALCA (calcitonin-related polypeptide alpha) and CDC42 (cell division cycle 42 (GTP binding protein, 25kDa). The most down-regulated genes were IVL (involucrin), FKBP6 (FK506 binding protein 6), that plays a role in immunoregulation and NHEDC1 (Na+/H+ exchanger domain containing 1). In VEC, IGF2 increased the expression of ADAM21 (ADAM metallopeptidase domain 21), several genes for zinc finger transcription factors, GRIN1 (glutamate receptor, ionotropic, N-methyl D-aspartate 1), GJA5 (gap junction protein, alpha 5, 40kDa), among others. Moreover, IGF2 down-regulated expression of GLUD1 (glutamate dehydrogenase 1), APOM (apolipoprotein M), S100A1 (S100 calcium binding protein A1) and others, listed in the appendix 1.

Interestingly, the two overlapping insulin-regulated genes in AEC and VEC were regulated in opposite direction: PDE10A (phosphodiesterase 10A) and GAGE12B (G antigen 12B) were up-regulated by insulin in AEC, while down-regulated in VEC. Similarly, the 3 overlapping IGF2-regulated genes had also opposing regulation in AEC and VEC. KIR2DL3 (killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3), CD209 and the unknown C3orf79

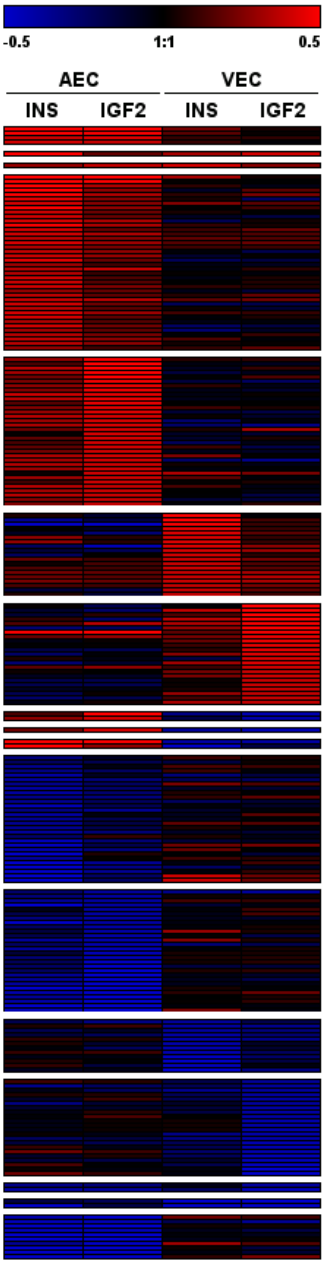
(chromosome 3 open reading frame 79) were up-regulated by insulin and down-regulated by IGF2.

In the next step gene ontology analysis was performed by using DAVID_GO (DAVID Bioinformatics Resources 6.7) software, in collaboration with Hubert Hackl, from the Division for Bioinformatics, Biocenter, Innsbruck Medical University, to identify biological processes being influenced by elevated insulin and IGF2 levels. All genes regulated by insulin and IGF2 in AEC and VEC with fold change higher than 1.3 and $p<0.05$ were used. Most of the processes found up-regulated in AEC by insulin treatment were related to development and cell differentiation, while the down-regulated processes involve oxygen transport and steroid dehydrogenase activity. Many of the processes regulated by IGF2 are involved in lipid metabolism (Table 3). For VEC, insulin also seems to up-regulate developmental processes, while IGF2 showed up-regulation of transmembrane transport processes (Table 4).

Biological processes usually involve a wide variety of pathways that contribute to their success. In order to delineate which pathways are regulated by insulin and IGF2 in AEC and VEC, the software Pathway Explorer from the Pathway Mapping Center Graz (https://pathwayexplorer.genome.tugraz.at) was used. Again lists containing insulin and IGF2 regulated genes in AEC and VEC, with fold change higher than 1.3 and $p<0.05$, were used. In AEC, pathways such as p38 MAPK, cell adhesion, cell junction, prostaglandin metabolism and actin cytoskeleton were up-regulated by insulin, while the pathways for visceral fat deposit, PPARG, lipid metabolism and steroid hormone metabolism were down-regulated (Table 5). IGF2 up-regulates pathways for lipid degradation, prostaglandin production and also cell junction, cell adhesion and VEGF signalling pathways. Similar to insulin, IGF2 also down-regulates visceral fat deposit, lipid metabolism and PPARG pathways, but also immunoregulatory pathways (Table 6).

As a result of the insulin-induced up-regulation of CDC42 in VEC, which is involved in several cellular processes (Teramoto et al., 1996, Coso et al., 1995, Popoff and Geny, 2009, Nobes and Hall, 1995, Olson et al., 1995), a variety of pathways involving this gene appeared to be up-regulated. These include Ras and Rho signalling pathways, cadherin-mediated cell adhesion, cell growth, tight junction and VEGF signalling pathway, among others listed in Table 7. Lipid and

purine metabolism, as well as anion transport are down-regulated by insulin in VEC (Table 7). In VEC IGF2 up-regulates pathways such as cell junction, regulation of cytoskeleton and cell proliferation, and down-regulates amino acid synthesis pathways, androgen and strogen metabolism pathways and immuno-related pathways (Table 8).



p<0.05; FC>1.3

AEC

Insulin IGF2

74 14 66

Gene symbol	FC INS	FC IGF2
GSTM3	1.42	1.46
PTCH2	1.47	1.40
KRT6B	1.39	1.35
MAP2	1.35	1.30
CCL20	-1.31	-1.43
DKKL1	-1.38	-1.38
ETV1	-1.35	-1.42
DUSP4	-1.41	-1.38
LYZL2	-1.41	-1.46
PPARG	-1.45	-1.44
ZNF280A	-1.36	-1.57
GPR116	-1.40	-1.83
PRDM1	-1.74	-1.56
HSD11B1	-1.96	-1.69

VEC

Insulin IGF2

33 4 50

Gene symbol	FC INS	FC IGF2
KIR2DL3	-1.30	-1.37
PDE10A	-1.33	-1.33
CES1	-1.53	-1.64
GOLGA6A	-1.69	-1.59

Insulin

AEC VEC

86 2 35

Gene symbol	FC AEC	FC VEC
PDE10A	1.54	-1.33
GAGE12B	1.61	-1.40

IGF2

AEC VEC

77 3 51

Gene symbol	FC AEC	FC VEC
CD209	1.60	-1.36
C3orf79	1.34	-1.35
KIR2DL3	1.34	-1.37

Figure. 10: Heat map illustration and Venn diagrams summary of insulin and IGF2 regulated genes in AEC and VEC. (collaboration with Huber Hackl, Division for Bioinformatics, Biocenter, Innsbruck Medical University)

Table 3: DAVID gene ontology of the insulin and IGF2 differentially regulated genes in AEC

Category	%	PValue	Genes
AEC INS UP			
cell development	13.33	2.0E-02	GSTM3, RND1, SOX1, FSCN2, MAP2, KCNE1
system development	26.67	2.2E-02	MYF6, GSTM3, PLA2G4A, RND1, KRT6B, SOX1, FSCN2, MAP2, KCNE1, PTCH2, LHB, TLL1
tissue development	13.33	2.3E-02	MYF6, GSTM3, PLA2G4A, KRT6B, KCNE1, PTCH2
anatomical structure development	26.67	3.7E-02	MYF6, GSTM3, PLA2G4A, RND1, KRT6B, SOX1, FSCN2, MAP2, KCNE1, PTCH2, LHB, TLL1
multicellular organismal development	28.89	3.8E-02	MYF6, KRT6B, FSCN2, SOX1, THEG, PLA2G4A, GSTM3, RND1, MAP2, KCNE1, PTCH2, LHB, TLL1
multicellular organismal process	37.78	3.9E-02	MYF6, KRT6B, SOX1, FSCN2, THEG, CALCB, PLA2G4A, GSTM3, RND1, SLC1A6, MAP2, KCNE1, PTCH2
cell differentiation	20.00	4.3E-02	MYF6, GSTM3, RND1, SOX1, FSCN2, MAP2, KCNE1, THEG, TLL1
epithelial cell development	4.44	5.1E-02	GSTM3, KCNE1
neuron development	8.89	5.1E-02	RND1, SOX1, FSCN2, MAP2
cellular developmental process	20.00	5.3E-02	MYF6, GSTM3, RND1, SOX1, FSCN2, MAP2, KCNE1, THEG, TLL1
cellular amino acid and derivative metabolic process	8.89	5.6E-02	PLA2G4A, SNCAIP, OCLN, ASRGL1
organ development	20.00	5.9E-02	MYF6, GSTM3, PLA2G4A, KRT6B, SOX1, FSCN2, KCNE1, PTCH2, LHB
cellular amino acid derivative metabolic process	6.67	6.4E-02	PLA2G4A, SNCAIP, OCLN
developmental process	28.89	7.1E-02	MYF6, KRT6B, FSCN2, SOX1, THEG, PLA2G4A, GSTM3, RND1, MAP2, KCNE1, PTCH2, LHB, TLL1
neuron differentiation	8.89	9.4E-02	RND1, SOX1, FSCN2, MAP2
cell fraction	17.78	1.3E-02	CALCB, TMEM59L, CYP4X1, GSTM3, PLA2G4A, SLC1A6, MAP2, LHB
structural molecule activity	11.11	6.8E-02	KRT6B, OCLN, TUBB2C, MAP2, KRTAP1-1
AEC INS DOWN			
oxygen binding and transport	4.88	2.8E-02	HBG1, HBG2
gas transport	4.88	3.9E-02	HBG1, HBG2
hemoglobin complex	4.88	2.5E-02	HBG1, HBG2
extracellular region	21.95	5.7E-02	LYZL2, GKN2, PSG8, CCL20, ENAM, HP, CASP1, DKKL1, HSN2
steroid dehydrogenase activity	4.88	6.2E-02	HSD11B1, AKR1C1
AEC IGF2 UP			
ectoderm development	1.02	6.6E-02	FRAS1, KRT6B, PTCH2
plasma membrane	4.76	4.2E-02	FRAS1, OR6F1, CFB, CLDN6, TMPRSS6, OR1D5, FOLH1, TMEM47, OR10G7, CD209, PTCH2, KIR2DL3, I
intermediate filament cytoskeleton	1.02	6.0E-02	KRT6B, KRTAP24-1, KRT33A
serine-type endopeptidase activity	1.02	4.4E-02	CFB, KLK1, TMPRSS6
serine hydrolase activity	1.02	5.8E-02	CFB, KLK1, TMPRSS6
receptor activity	2.72	9.7E-02	OR10G7, OR6F1, CD209, LAIR2, PTCH2, KIR2DL3, OR1D5, HTR3D
AEC IGF2 DOWN			
locomotion	13.16	1.7E-02	VAV3, CCL20, CXCR4, TGFBR3, XCL1
regulation of lipid metabolic process	7.89	2.8E-02	CAB39L, VAV3, PPARG
response to lipid	5.26	4.1E-02	PPARG, TGFBR3
chemotaxis	7.89	5.4E-02	CCL20, CXCR4, XCL1
cell surface receptor linked signal transduction	23.68	6.0E-02	VAV3, HEY1, CCL20, CXCR4, GPR45, TGFBR3, OR4B1, BRS3, GPR116
glutathione metabolic process	5.26	6.3E-02	GSTA1, GGT5
regulation of fatty acid oxidation	5.26	6.3E-02	CAB39L, PPARG
immune response	13.16	7.5E-02	CCL20, CXCR4, PPARG, TGFBR3, XCL1
negative regulation of specific transcription from RNA polymerase II promoter	5.26	9.2E-02	HEY1, PPARG
behavior	10.53	9.6E-02	CCL20, CXCR4, BRS3, XCL1
extracellular region	21.05	8.8E-02	LYZL2, CCL20, RNASET2, LIPN, TGFBR3, XCL1, SERPINI1, DKKL1
G-protein coupled receptor activity	15.79	3.9E-02	CXCR4, GPR45, PPARG, OR4B1, BRS3, GPR116
coreceptor activity	5.26	4.3E-02	CXCR4, TGFBR3
transmembrane receptor activity	18.42	6.0E-02	CXCR4, GPR45, PPARG, TGFBR3, OR4B1, BRS3, GPR116
chemokine activity	5.26	9.6E-02	CCL20, XCL1

The processes showed here presented fold enrichment score >1.0 and FDR % >10.0.

Table 4: DAVID gene ontology of the insulin and IGF2 differentially regulated genes in VEC

Category	%	PValue	Genes
VEC INS UP			
cognition	27.78	1.0E-02	CALCA, OR6B2, OR2H2, KIT, RD3
regulation of cell projection assembly	11.11	1.8E-02	CDC42, KIT
positive regulation of kinase activity	16.67	2.1E-02	CALCA, CDC42, KIT
positive regulation of transferase activity	16.67	2.3E-02	CALCA, CDC42, KIT
neurological system process	27.78	2.7E-02	CALCA, OR6B2, OR2H2, KIT, RD3
myeloid leukocyte differentiation	11.11	3.3E-02	CDC42, KIT
multicellular organismal process	50.00	3.3E-02	CALCA, CDC42, OR6B2, OR2H2, TUBB2A, KIT, CSRP2, TIMM8A, RD3
sensory perception	22.22	4.3E-02	CALCA, OR6B2, OR2H2, RD3
regulation of kinase activity	16.67	4.7E-02	CALCA, CDC42, KIT
regulation of transferase activity	16.67	5.1E-02	CALCA, CDC42, KIT
system process	27.78	5.4E-02	CALCA, OR6B2, OR2H2, KIT, RD3
system development	33.33	6.7E-02	CALCA, CDC42, TUBB2A, KIT, CSRP2, TIMM8A
cell proliferation	16.67	6.8E-02	CALCA, KIT, CSRP2
regulation of phosphorylation	16.67	7.6E-02	CALCA, CDC42, KIT
nervous system development	22.22	8.8E-02	CDC42, TUBB2A, KIT, TIMM8A
anatomical structure development	33.33	8.9E-02	CALCA, CDC42, TUBB2A, KIT, CSRP2, TIMM8A
positive regulation of catalytic activity	16.67	9.2E-02	CALCA, CDC42, KIT
GTP binding	16.67	3.9E-02	CDC42, TUBB2A, EFCAB4B
VEC INS DOWN			
response to stimulus	41.18	3.3E-02	OR4C13, UCN3, CES1, SLC22A8, KIR2DL3, IVL, GAGE12B
response to toxin	11.76	4.7E-02	CES1, SLC22A8
solute:solute antiporter activity	11.76	4.5E-02	NHEDC1, SLC22A8
VEC IGF2 UP			
transmembrane transporter activity	18.18	6.7E-02	GRIN1, AQP7, TIMM23, GJA5
channel activity	13.64	6.9E-02	GRIN1, AQP7, GJA5
passive transmembrane transporter activity	13.64	6.9E-02	GRIN1, AQP7, GJA5
VEC IGF2 DOWN			
extracellular region	25.00	5.0E-02	GPC2, PSG8, CD209, FAM24A, OTOR, APOM, NPFF
antigen binding	7.14	5.8E-02	CD209, KIR2DL3

The processes showed here presented fold enrichment score >1.0 and FDR % >10.0.

From the insulin and IGF2 regulated genes in AEC, 4 different insulin (PTCH2, OCLN, PLA2G4A and KRT6B) and 5 different IGF2 (PLA2G5, VAV3, KCNJ6, PIGF and KRT6B) regulated genes were chosen for validation by real time RT-PCR. For VEC, 2 genes were chosen for confirmation of insulin regulation by real-time PCR (EFCAB4B and CDC42) and 1 gene regulated by IGF2 (PDE10A). These genes were selected based on their appearance in the pathway analysis. The real-time PCR confirmed the microarray analysis. Table 9 shows the confirmation of the microarray expression data by real time PCR with the respective fold changes for each method.

Table 5: Pathway analysis of the insulin regulated genes in AEC

Insulin UP-regulated Pathways in AEC

Subsection	Pathway	pathway % of genes	Genes
Immunology	Neutrophil and Its Surface Molecules	8.33	e-selectin
Neuroscience	Role of Parkin in the Ubiquitin-Proteasomal Pathway	6.25	SNCAIP
Metabolism	Eicosanoid Metabolism	5.88	PTGIS, PLA2G4A
Adhesion	Adhesion Molecules on Lymphocyte	5.56	e-selectin
Immunology	Monocyte and its Surface Molecules	5.00	e-selectin
Lipid Metabolism	Prostaglandin and leukotriene metabolism	4.69	PTGIS, PLA2G4A, AKR1C1
Cell Cycle Regulation	Protein Kinase A at the Centrosome	4.35	MAP2
Lipid Metabolism	Phospholipid degradation	3.45	PLA2G4A
Biodegradation of Xenobiotics	gamma-Hexachlorocyclohexane degradation	3.39	ALPI, CYP4X1
Physiological Process	Circadian	2.78	MYF6, GSTM3
Cell Signalling	Fc Epsilon Receptor I Signaling in Mast Cells	2.44	PLA2G4A
Metabolism of Other Amino Acids	Glutathione metabolism	1.75	GSTM3
Ligand-Receptor Interaction	Cell adhesion molecules (CAMs)	1.71	OCLN, NCAM2, e-selectin
Cell Signalling	p38 MAPK Signaling Pathway	1.69	PLA2G4A
Cellular Component	cell junction	1.56	RND1, OCLN
Neurodegenreative Disorders	Parkinson's disease	1.52	SNCAIP
Biological Process	anion transport	1.42	SLC1A6
Lipid Metabolism	Glycerolipid metabolism	1.31	PLA2G4A, ALPI
Amino Acid Metabolism	Tryptophan metabolism	0.79	CYP4X1
Signal Transduction	VEGF signaling pathway	0.75	PLA2G4A
Immune System	Natural killer cell mediated cytotoxicity	0.55	ULBP2
Molecular Funktion	chaperone activity	0.52	TUBB2C
Development	Axon guidance	0.48	RND1
Molecular Funktion	catalytic activity	0.45	PDE10A
Cellular Component	actin cytoskeleton	0.33	FSCN2

Insulin DOWN-regulated Pathways in AEC

Subsection	Pathway	pathway % of genes	Genes
Metabolism	Visceral Fat Deposits and the Metabolic Syndrome	11.11	PPARG, HSD11B1
Cytokines/Chemokines	IL 18 Signaling Pathway	11.11	caspase1
Cell Signalling	Basic mechanism of action of PPARa, PPARb(d) and PPARg and effects on gene expression	8.33	PPARG
Lipid Metabolism	C21-Steroid hormone metabolism	7.69	HSD11B1
Expression	Role of PPAR-gamma Coactivators in Obesity and Thermogenesis	7.14	PPARG
Cell Signalling	Regulation of MAP Kinase Pathways Through Dual Specificity Phosphatases	5.88	DUSP1
Adhesion	D4-GDI Signaling Pathway	3.23	caspase1
1.3 Lipid Metabolism	Androgen and estrogen metabolism	2.33	HSD11B1
Adhesion	Caspase Cascade in Apoptosis	1.89	caspase1
Cell Signalling	Nuclear Receptors in Lipid Metabolism and Toxicity	1.75	PPARG
Neurodegenreative Disorders	Huntington's disease	1.43	caspase1
Molecular Function	GPCRs, Other	1.28	GPCR116
Biological Process	lipid metabolism	1.13	PPARG, PTGIS, PLA2G4A
Endocrine System	PPAR signaling pathway	0.98	PPARG
Cell Growth and Death	p53 signaling pathway	0.79	SESN3
Biological Process	innate immune response	0.74	CCL20, e-selectin
Signal Transduction	MAPK signaling pathway	0.66	DUSP1, PLA2G4A, caspase1

Table 6: Pathway analysis of the IGF2 regulated genes in AEC

IGF2 UP-regulated Pathways in AEC

Subsection	Pathway	pathway % of genes	Genes
Immunology	Alternative Complement Pathway	11.11	CFB (complement factor B)
Cell Cycle Regulation	Protein Kinase A at the Centrosome	4.35	MAP2
Physiological Process	Circadian	4.17	GSTM3, PIGF, G0S2
Metabolism of Other Amino Acids	Glutathione metabolism	3.51	GSTM3, GSTA1
Lipid Metabolism	Phospholipid degradation	3.45	PLA2G5
Lipid Metabolism	Glycerophospholipid metabolism	1.89	GPD1, PLA2G5
Lipid Metabolism	Prostaglandin and leukotriene metabolism	1.56	PLA2G5
Immune System	Natural killer cell mediated cytotoxicity	1.09	KIR2DL3, VAV3
Immune System	Antigen processing and presentation	0.85	KIR2DL3
Cellular Component	cell junction	0.78	CLDN6
Signal Transduction	VEGF signaling pathway	0.75	PLA2G5
Cell communication	Tight junction	0.6	CLDN6
Ligand-Receptor Interaction	Cell adhesion molecules (CAMs)	0.57	CLDN6

IGF2 DOWN-regulated Pathways in AEC

Subsection	Pathway	pathway % of genes	Genes
Metabolism	Visceral Fat Deposits and the Metabolic Syndrome	11.11	HSD11B1, PPARG
Cell Signalling	Basic mechanism of action of PPARa, PPARb(d) and PPARg and effects on gene expression	8.33	PPARG
Lipid Metabolism	C21-Steroid hormone metabolism	7.69	HSD11B1
Expression	Role of PPAR-gamma Coactivators in Obesity and Thermogenesis	7.14	PPARG
Molecular Function	GPCRs, Class A Rhodopsin-like 2	6.25	GPR45
Immunology	Role of Tob in T-cell activation	6.25	TGFBR3
Cell Signalling	Regulation of MAP Kinase Pathways Through Dual Specificity Phosphatases	5.88	DUSP4
Hematopoeisis	Pertussis toxin-insensitive CCR5 Signaling in Macrophage	3.7	CXCR4
Developmental Biology	CTCF: First Multivalent Nuclear Factor	3.33	TGFBR3
Cytokines/Chemokines	CXCR4 Signaling Pathway	2.86	CXCR4
Metabolism of Complex Carbohydrates	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	2.44	PIGF
Molecular Function	Peptide GPCRs	2.41	BRS3, CXCR4
Metabolism of Complex Carbohydrates	O-Glycans biosynthesis	2.38	GALNT3
Lipid Metabolism	Androgen and estrogen metabolism	2.33	HSD11B1
Cell Signalling	ALK in cardiac myocytes	2.08	TGFBR3
Biological Process	chemotaxis	1.84	CXCR4, CCL20, XCL1
Cell Signalling	Nuclear Receptors in Lipid Metabolism and Toxicity	1.75	PPARG
Immune System	Leukocyte transendothelial migration	1.5	VAV3, CXCR4
Molecular Function	Nuclear Receptors	1.47	PPARG
Cellular Process	TGF Beta Signaling Pathway	1.33	TGFBR3
Biological Process	humoral immune response	1.28	CCL20, XCL1, CBF
Molecular Function	GPCRs, Other	1.28	GPR116
Biological Process	innate immune response	1.11	CCL20, CXCR4, ORM1
Immune System	B cell receptor signaling pathway	0.97	VAV3
Molecular Function	GPCRs, Class A Rhodopsin-like	0.87	BRS3, CXCR4
Ligand-Receptor Interaction	Cytokine-cytokine receptor interaction	0.77	CXCR4, CCL20, XCL1
Cell communication	Integrin-mediated cell adhesion	0.7	VAV3
Molecular Funktion	cytokine activity	0.66	CCL20, XCL1
Development	Axon guidance	0.48	CXCR4
Molecular Funktion	lipid binding	0.48	VAV3
Signal Transduction	MAPK signaling pathway	0.44	DUSP4, TGFBR3
Cell Motility	Focal adhesion and Regulation of actin cytoskeleton	0.31	VAV3

Table 7: Pathway analysis of the insulin regulated genes in VEC

Insulin UP-regulated Pathways in VEC

Subsection	Pathway	pathway % of genes	Genes
Adhesion	Role of PI3K subunit p85 in regulation of Actin Organization and Cell Migration	7.14	CDC42
Cell Signalling	Ras and Rho Signaling Pathways	3.45	CDC42
Apoptosis	Regulation of BAD phosphorylation	3.03	KIT
Cellular Process	G13 Signaling Pathway	2.27	CDC42
Cell Signalling	p38 MAPK Signaling Pathway	1.69	CDC42
Cell communication	Cadherin-mediated cell adhesion	1.1	CDC42
Cell communication	Adherens junction	0.95	CDC42
Signal Transduction	VEGF signaling pathway	0.75	CDC42
Immune System	Leukocyte transendothelial migration	0.75	CDC42
Immune System	Hematopoietic cell lineage	0.7	KIT
Cell communication	Integrin-mediated cell adhesion	0.7	CDC42
Cell communication	Tight junction	0.6	CDC42
Molecular Funktion	chaperone activity	0.52	DNAJC28
Biological Process	cell growth	0.52	CRSP2
Development	Axon guidance	0.48	CDC42
Physiological Process	Myometrial Relaxation and Contraction Pathways	0.47	CALCA
Cell Motility	Focal adhesion and Regulation of actin cytoskeleton	0.31	CDC42

Insulin DOWN-regulated Pathways in VEC

Subsection	Pathway	pathway % of genes	Genes
Physiological Process	Irinotecan Pathway	4.76	CES1
Cell Signalling	Nuclear Receptors in Lipid Metabolism and Toxicity	1.75	RARB
Immune System	Antigen processing and presentation	0.85	KIR2DL3
Biological Process	anion transport	0.71	SLC22A8
Immune System	Natural killer cell mediated cytotoxicity	0.55	KIR2DL3
Molecular Funktion	catalytic activity	0.45	PDE10A
Cellular Component	cytosol	0.45	IVL
Nucleotide Metabolism	Purine metabolism	0.41	PDE10A
Ligand-Receptor Interaction	Neuroactive ligand-receptor interaction	0.24	UCN3

Table 8: Pathway analysis of the IGF2 regulated genes in VEC

IGF2 UP-regulated Pathways in VEC

Subsection	Pathway	pathway % of genes	Genes
Endocrine System	PPAR signaling pathway	0.98	AQP7
Cellular Component	cell junction	0.78	GJA5
Molecular Funktion	chaperone activity	0.52	TUBB2C
Biological Process	humoral immune response	0.43	CLEC2D
Cell Motility	Regulation of actin cytoskeleton	0.31	C3orf10
Biological Process	cell proliferation	0.28	MTCP1

IGF2 DOWN-regulated Pathways in VEC

Subsection	Pathway	pathway % of genes	Genes
Metabolism of Other Amino Acids	D-Glutamine and D-glutamate metabolism	20	GLUD1
Neuroscience	Erythropoietin mediated neuroprotection through NF-kB	5.26	GRIN1
Physiological Process	Irinotecan Pathway	4.76	CES1
Neurodegenreative Disorders	Amyotrophic lateral sclerosis (ALS)	3.85	SLC1A2
Carbohydrate Metabolism	Pentose and glucuronate interconversions	3.7	UGT2B17
Metabolism of Cofactors and Vitamins	Porphyrin and chlorophyll metabolism	2.5	UGT2B17
Lipid Metabolism	Androgen and estrogen metabolism	2.33	UGT2B17
Nervous System	Long-term potentiation	1.98	GRIN1, CALML6
Biological Process	cell surface receptor linked signal transduction	0.97	SIGLEC9, CLEC2D
Immune System	Antigen processing and presentation	0.85	KIR2DL3
Biological Process	anion transport	0.71	SLC1A2
Carbohydrate Metabolism	Starch and sucrose metabolism	0.64	UGT2B17
Immune System	Natural killer cell mediated cytotoxicity	0.55	KIR2DL3
Ligand-Receptor Interaction	Neuroactive ligand-receptor interaction	0.49	NPFF, GRIN1
Molecular Funktion	catalytic activity	0.45	PDE10A
Nucleotide Metabolism	Purine metabolism	0.41	PDE10A
Cellular Component	extracellular matrix	0.27	GPC2

Table 9: Validation of microarray results for insulin- and IGF2 regulated genes in placental human primary AEC and VEC by real-time PCR

	Insulin regulated genes		
	Gene symbol	**Microarray**	**Real time PCR**
AEC	PTCH2	1.48	1.25
	OCLN	1.80	1.32
	PLA2G4A	1.48	1.47
	KRT6B	1.39	1.54
VEC	EFCAB4B	1.48	1.35
	CDC42	1.30	2.87
	IGF2 regulated genes		
	Gene symbol	**Microarray**	**Real time PCR**
AEC	PLA2G5	1.40	10.14
	VAV3	-1.59	-1.16
	KCNJ6	-1.56	-1.66
	PIGF	-1.52	-1.37
	KRT6B	1.35	3.35
VEC	PDE10A	-1.33	-2.04

Results are expressed as fold-change compared to the expression of untreated cells. Data are given as mean of expression levels of 6 biological replicates. The highlighted gene shows one of the overlapping regulations of insulin and IGF2

Discussion

I tested the hypothesis that elevated levels of insulin and IGF2 found in the fetal circulation associated with GDM affect the gene expression profile of AEC and VEC isolated from the feto-placental vasculature. Because AEC and VEC originate from different vascular beds, I furthermore hypothesized that these effects would also be different in AEC versus VEC. The hypothesis was also based on unpublished observation (U. Hiden) that AEC express higher levels of receptors for insulin and IGF2. Lately, global expression profiling tools, such as microarray analysis, are broadly used in order to gain insight into the molecular basis of pathologies (Mufson et al., 2006, Bauer et al., 2009, Espinosa et al., 2011, Shai, 2006). Gene expression studies in placentas from pregnancies with adverse outcomes can also be a powerful tool to delineate how pathologies can directly affect the fetus and the placenta itself, and vice versa. Most placental global expression studies were done with placentas derived from pre-eclamptic pregnancies, or compared SGA and pre-eclampsia as both pathologies share many aspects (Hoegh et al., 2010, Reimer et al., 2002, Sitras et al., 2009, Nishizawa et al., 2007). Few whole-genome microarray studies were also done with placentas derived from GDM or pre-existing maternal diabetes (Enquobahrie et al., 2009, Radaelli et al., 2009, Toft et al., 2008, Radaelli et al., 2003). All of them used whole placenta tissues, but studying gene expression in individual placental compartments could give a better insight of how these disorders can affect particular compartments and provide data for developing new hypothesis.

The present study is the first to analyze the effects of elevated insulin and IGF2 levels present in the fetal circulation during GDM exclusively on the feto-placental endothelial gene expression. Insulin and IGF2 treatment of human primary AEC and VEC isolated from normal term placentas revealed that among more than 28,000 transcribed genes evaluated in the microarray, insulin regulated expression of 88 genes in AEC and 37 in VEC while IGF2 regulated 80 genes in AEC and 54 in VEC.

The main findings of this study were: 1) By using gene ontology tools, insulin and IGF2 regulate different biological processes in AEC and VEC; 2) Insulin and IGF2 share regulation of more similar biological processes in AEC than in VEC; 3) Pathway analysis confirmed the differential effect of insulin and IGF2 on AEC and VEC and 4) In general, cell adhesion and junction, lipid metabolism, cell

growth, angiogenesis and immuno-related pathways are the main targets of insulin and IGF2 on feto-placental EC.

The limitation of the study was the small number of regulated genes by the growth factors, especially in VEC, and the small fold-changes. Radaelli et al. 2009 and Enquobahrie et al. 2009 also obtained only a small number of regulated genes comparing normal vs diabetic placentas.

Other microarrays studies comparing GDM and normal placental samples identified several genes involved in transport and trafficking, inflammatory responses, endothelial differentiation, signal transduction and lipid and glucose metabolism (Enquobahrie et al., 2009, Radaelli et al., 2009). Both studies verified that the leptin pathway, already known to be involved in the development of GDM (Miehle et al., 2012, Gauster et al., 2011b) was up-regulated. In the present study however, regulation of genes involved in leptin signaling was not verified. This would suggest that leptin signaling related genes are deranged in GDM mostly in non-endothelial cells in the placenta. Rather, pathway analysis revealed that VEGF signaling pathway is up-regulated by insulin in both, AEC and VEC, but only in AEC by IGF2. In every case, different genes involved in this pathway were regulated. Insulin and IGF2 up-regulated PLA2G4A and PLA2G5 (phospholipase A2) respectively in AEC, involved in prostaglandin production (Nomura et al., 2011), and CDC42 in VEC, involved in various cell communication and lumen formation pathways (Leszczynska et al., 2011, Sacharidou et al., 2011, Stengel and Zheng, 2011, Qi et al., 2011). Insulin also regulates many genes in AEC involved in lipid metabolism. This process was also found regulated by GDM in the study of Radaelli et al. 2009. However, we identified different genes not identified in other GDM and placental expression studies, such as PTGIS, PLA2G4A, AKR1C1, PPARG and HSD11B1. In common with Radaelli et al. 2009 we found PLA2G5, and in common with Enquobahrie et al. 2009 we identified one of the claudins (CLDN7 in our study).

Because lipid metabolism, cell adhesion/junction and architecture were the main processes regulated by insulin and IGF2 in the present work, I will focus on these processes. Among the genes involved in lipid metabolism identified in this study the ones which appeared in the gene ontology and pathway analysis often seemed to be associated with diabetes, metabolic syndrome, obesity, cardiovascular disease and atherosclerosis. Insulin up-regulates PTGIS,

PLA2G4A and ALPI (table 10). PTGIS (prostaglandin I2 (prostacyclin) synthase) catalyzes the conversion of prostglandin H2 to prostacyclin (prostaglandin I2), a potent vasodilator and inhibitor of platelet aggregation, and is also involved in the synthesis of cholesterol, steroids and other lipids. Imbalance of prostacyclin can contribute to myocardial infarction, stroke and atherosclerosis (Nakayama, 2010, He et al., 2010, Xie et al., 2009). PLA2G4A, a phospholipase from the A2 familiy is related to the hydrolysis of the platelet-activating factor and oxidized phospholipids and also often associated with atherosclerosis and cardiac dysfunction (Silva et al., 2011, Zalewski and Macphee, 2005, Sharma et al., 2011). The insulin down-regulated genes associated with lipid metabolism are AKR1C1, PPARG and HSD11B1 (table 10). AKRC1 encodes a member of the aldo/keto reductase superfamily and converts progesterone to its inactive form, 20-alpha-dihydroxyprogesterone (20-alpha-OHP). Progesterone is a steroid hormone required for the support of gestation and embryogenesis (Spencer and Bazer, 2002). PPAR-gamma is a regulator of adipocyte differentiation and is associated with several pathologies including obesity, diabetes, metabolic syndrome and atherosclerosis. Agonists of PPAR-gamma reduce the risk for atherosclerosis and cardiovascular disease (Bego et al., 2011, Bassaganya-Riera et al., 2011, Kuusisto et al., 2007). The protein encoded by HSD11B1 is an enzyme that catalyzes the conversion of cortisol to the inactive metabolite cortisone and can also catalize the reverse reaction, the conversion of cortisone to cortisol. Cortisol excess is associated with obesity and metabolic syndrome (Gambineri et al., 2011). Reduced activity of HSD11B1 was shown to have a protective effect in subjects with type 2 diabetes (Jang et al., 2007, Mlinar et al., 2011).

IGF2 also regulated lipid metabolism-related genes in AEC, such as PLA2G5, GPD1, that were up-regulated, and - overlapping with insulin - down regulates PPARG and HSD11B1 (table 10). GPD1 (glycerol-3-phosphate dehydrogenase 1) catalyzes the reversible reaction of dihydroxyacetone phosphate (DHAP) and NADH to sn-glycerol-3-phosphate (G3P) and NAD+. It plays a role in the synthesis of triacylglycerol and in the transport of reducing equivalents from the cytosol to the mitochondria and its down-regulation is associated with weight loss-induced improvements in insulin action in human skeletal muscle of obese patients (Park et al., 2006). PLA2G5 (group V phospholipase A2) is involved in prostaglandin production (Nomura et al., 2011)

and its blockade inhibited EC barrier dysfunction in pulmonary EC (Dudek et al., 2011). Furthermore, PLA2G5 increases the atherogenic potential of LDL in mice (Boyanovsky et al., 2009). Insulin and IGF2 altered expression of genes involved in lipid metabolism associated with diabetes, metabolic syndrome and obesity and thus it seems that these growth factors may have both negative as well as protective effects in AEC.

Adhesion molecules and genes encoding for structural proteins were also targets of insulin and IGF2 gene expression regulation (Table 11). Here we found that insulin up-regulates the following genes in AEC: SELE (e-selectin), OCLN (occludin), RND1 (Rho family GTPase 1), TUBB2C (tubulin beta 2C), MAP2 (microtubule-associated protein 2) and FSCN2 (fascin homolog 2, actin-bundling protein, retinal), and down-regulates NCAM2 (neural cell adhesion molecule 2) (Table 11). In AEC IGF2 up-regulates TMEM47 (transmembrane protein 47), FRAS1 (Fraser syndrome 1) and, overlapping with insulin, MAP2 (Table 11). In VEC, insulin up-regulates TUBB2A (tubulin beta 2A) and CDC42, involved in actin organization, and IGF2 up-regulates GJA5 (gap junction protein, alpha 5, 40kDa) and c3orf10 that codes for a protein involved in regulation of actin and microtubule organization (Table 11).

SELE is an endothelial selectin, member of the cellular adhesion proteins (CAMs) that attach to the cytoskeleton and activate intracellular signaling cascades involved in cell proliferation, differentiation, motility, trafficking, and apoptosis. It participates in the interaction between leukocytes and endothelium, it is associated with the pathogenesis of atherosclerosis (Oh et al., 2007, Davies et al., 1993, Guray et al., 2004) and is elevated in GDM women (Bo et al., 2007). SELE may also have a role in capillary morphogenesis (Guray et al., 2004, Kumar et al., 2003, Oh et al., 2007, Oishi et al., 2000). Occludins and claudins play a role in the formation and regulation of the tight junction (TJ) paracellular permeability barrier. Lower levels of OCLN are associated with an increase in vascular permeability in diabetic rats (Antonetti et al., 1998). It was also shown that occludin is down-regulated in large vessels of placentas in pregnancies complicated by GDM (Babawale et al., 2000). RND1 is a member of the Rho GTPase family and regulates the organization of the actin cytoskeleton in response to extracellular growth factors (Nobes et al., 1998) but is also involved in junction assembly and barrier regulation (Schneeberger and Lynch, 2004). Cytoskeletal organization and

dynamics furthermore depend on protein self-associations and interactions with regulatory elements such as microtubule-associated proteins (MAPs) and microtubule (Maccioni and Cambiazo, 1995). I found insulin up-regulation of TUBB2C, a constituent of microtubule, and MAP2, a microtubule-associated protein. Fascins are also part of the cytoskeleton organization, crosslinking with actin into filamentous bundles within dynamic cell extensions. Overall, the microarray findings strongly suggest that insulin is regulating processes including architecture, intracellular transport, modulation of surface receptors, mitosis, cell motility, and differentiation. All of these processes participate in endothelial cell function and alterations could lead to vascular dysfunction (Leach et al., 2009). Lately, insulin is more and more considered to be one of the factors causing placental vascular dysfunction. For instance, perfusion of the fetal circulation of term placentas with high insulin resulted in increased VEGF expression, junctional disruption and increased vascular leak suggesting that fetal insulin directly affects the placental vascular system (Leach et al., 2009), probably via regulation of adhesion molecules and cell architecture.

In VEC, insulin regulates TUBB2A and CDC42. CDC42 is involved in several processes and aberrant CDC42 activation was shown to be associated with the pathogenesis of pathologies such as cardiovascular disease and diabetes (Sinha and Yang, 2008, Schmidt and Hall, 2002).

Among the IGF2-regulated adhesion molecules in AEC, TMEM47 encodes a member of the PMP22/EMP/claudin protein family (Christophe-Hobertus et al., 2001), FRAS1, that encodes an extracellular matrix protein involved in the regulation of epidermal-basement membrane adhesion and organogenesis during development (Short et al., 2007), MAP2 and claudin 6. The possible role of MAP2 and claudin in GDM was already discussed above. In VEC, IGF2 up-regulates GJA5 and C3orf10. The protein encoded by GJA5 is a component of gap junctions providing a route for the diffusion of low molecular weight materials from cell to cell. Mutations in this gene are associated with atrial fibrillation (Parvez and Darbar, 2011). C3orf10, also called BRICK1, SCAR/WAVE actin-nucleating complex subunit, is involved in regulation of actin and microtubule organization.

In summary, IGF2, although to a lesser extent than insulin, regulates cell adhesion, cell junction and cell architecture genes in endothelial cells from human term placentas.

For the confirmatory real-time PCR studies, genes were chosen according to their distribution in the pathway analysis and their fold changes in the microarray. Thus 12 genes regulated by insulin and IGF2 (6 insulin-regulated and 6 IGF2-regulated, respectively) were confirmed in AEC and VEC. Discordances in fold change when comparing the two methods, i.e. microarray and real-time PCR, could be due to different location of target sequences for the microarray and real-time probes.

The differences found in insulin and IGF2 regulated gene expression in AEC versus VEC in the microarray experiment can be explained not only by the fact that AEC and VEC differentially express IR and IGF1R, with higher expression of both in AEC (personal communication by Ursula Hiden, data not shown). Moreover AEC and VEC diverge in many aspects including cell morphology, response to VEGF and basal gene expression (Lang et al., 2008). The study about insulin and IGF2 regulated gene expression in placental AEC and VEC also provides strong evidence that both growth factors may regulate placental EC function in normal and GDM pregnancies by influencing key processes in vascular function such as cell adhesion, VEGF signaling but also lipid metabolism,

Table 10: Insulin and IGF2-regulated genes in AEC and VEC associated with Lipid Metabolism. Red and green arrows represent up and down regulation, respectively.

	AEC		**VEC**	
Gene	**Insulin**	**IGF2**	**Insulin**	**IGF2**
PTGIS	↑	-	-	-
PLA2G4A	↑	-	-	-
ALPI	↑	-	-	-
AKR1C1	↓	-	-	-
PPARG	↓	↓	-	-
HSD11B1	↓	↓	-	-
PLA2G5	-	↑	-	-
GPD1	-	↑	-	-

Table 11: Insulin and IGF2-regulated genes in AEC and VEC associated with Cell adhesion/junction/architecture. Red and green arrows represent up and down regulation, respectively.

	AEC		VEC	
Gene	**Insulin**	**IGF2**	**Insulin**	**IGF2**
SELE	↑	-	-	-
OCLN	↑	-	-	-
RND1	↑	-	-	-
TUBB2C	↑	-	-	-
MAP2	↑	-	-	-
FSCN2	↑	-	-	-
NCAM2	↓	-	-	-
TMEM47	-	↑	-	-
FRAS1	-	↑	-	-
MAP2	↑	↑	-	-
TUBB2A	-	-	↑	-
CDC42	-	-	↑	-
GJA5	-	-	-	↑
C3orf10	-	-	-	↑

Chapter 2

Insulin stimulates *in vitro* angiogenesis in human placental arterial endothelial cells via IRS/PI3K/Akt and endothelial nitric synthase activation

The insulin/IGF system regulates fetal and placental growth and development. Hence, dysregulation of insulin and IGFs may have profound effects on the fetus and on the placenta. During GDM, there is an increase in insulin and IGF2 levels in both maternal and fetal circulation (Desoye and Shafrir, 1996, Desoye and Hauguel-de Mouzon, 2007). Therefore, in a pregnancy complicated by maternal diabetes, altered insulin/IGFs levels in both maternal and fetal circulation will affect the placenta. Hiden et al. proposed that insulin and IGF2 elevated in the fetal circulation as a consequence of maternal diabetes may promote proliferation, hypervascularization and placental growth (Hiden and Desoye, 2010). IR and IGF1R are expressed on the placental endothelium, which is in contact with the fetal circulation, but their cellular effects on the feto-placental vasculature have remained elusive. Placental hypervascularization observed in GDM is a consequence of an increase in angiogenesis of the pacental vessels. Development of new blood vessels requires endothelial cell proliferation, migration and differentiation. All of these steps will depend on the interaction of endothelial cells with the surrounding environment, including growth-factors.

Hypothesis

Against this background, in this part of the thesis I tested the hypothesis that insulin stimulates angiogenesis in placental AEC and this effect would be signaled via PI3K-Akt-eNOS pathway. The effect of insulin on actin-dependent cell remodeling was also investigated. Because AEC expresse more IR and IGF1 receptors than VEC (Hiden, unpublished) and, as demonstrated in the previous chapter, insulin and IGF2 regulated more genes related to structure and adhesions/junctions in AEC than in VEC, AEC were chosen for the experiments performed in this chapter.

Results

1. Insulin stimulates angiogenesis in human placental AEC

The development of new blood vessels is a complex process that depends on endothelial cell proliferation, migration and cell differentiation which in turn result in the interaction with the surrounding environment, such as growth and vascular factors, and basement membrane (Hoeben et al., 2004, Risau, 1997). Recently, it has been demonstrated that insulin was able to stimulate *in vivo* and *in vitro* angiogenesis in mice skin injected with insulin and in human microvascular endothelial cells (HMEC), respectively (Liu et al., 2009). Insulin action on HUVEC has been shown to be predominantly via IRS/PI3K/Akt activation ultimately leading to eNOS activation and VEGF production (Zeng et al., 2000). As placental hypervascularization is one of the features of the diabetic placentas (Sherer and Divon, 1996, Jirkovska et al., 2002, Leach et al., 2004, Gauster et al., 2011a) and placental EC express IR (Hiden et al., 2009a), I investigated whether insulin stimulate angiogenesis of primary placental AEC isolated from term placentas. To test this hypothesis, a 2D-*in vitro* network formation assay on growth factor reduced matrigel was performed. In this assay, AEC were treated either with insulin or VEGF as a positive control and seeded in 3 different conditioned media: 1) EBM without FCS and without supplements, 2) EBM with 2% FCS but without supplements and 3) EBM with 5% FCS and supplements. Figure 11A shows AEC immediately and 6 hours after seeding on matrigel. The parameters chosen to measure insulin effects on network formation were the number of branching points and the total tube length (Figure 11A). When cells were treated in the presence of fully supplemented medium (FCS + supplements), no insulin or VEGF effect on total tube length of the network was observed. When EBM deprived of FCS and supplements was added, only VEGF had a significant effect in increasing total tube length. However, if FCS concentration was reduced to 2% but no supplements were added to the medium, I observed that insulin increased the total tube length of the network to similar levels as VEGF, used as positive control (Fig 11B). Similar effects were found when the branching point number was counted (Fig 11C). The observation that insulin stimulates *in vitro* angiogenesis only in the absence of supplements and reduced FCS concentration can be due to the fact that the supplement bullet kit contains growth factors necessary for EC growth,

and also FCS itself contains soluble growth factors. In the absence of FCS and supplements, network formation was extremely reduced and insulin had no effect. A plausible explanation could be that insulin is increasing angiogenesis probably in cooperation with other growth factors present in the FCS.

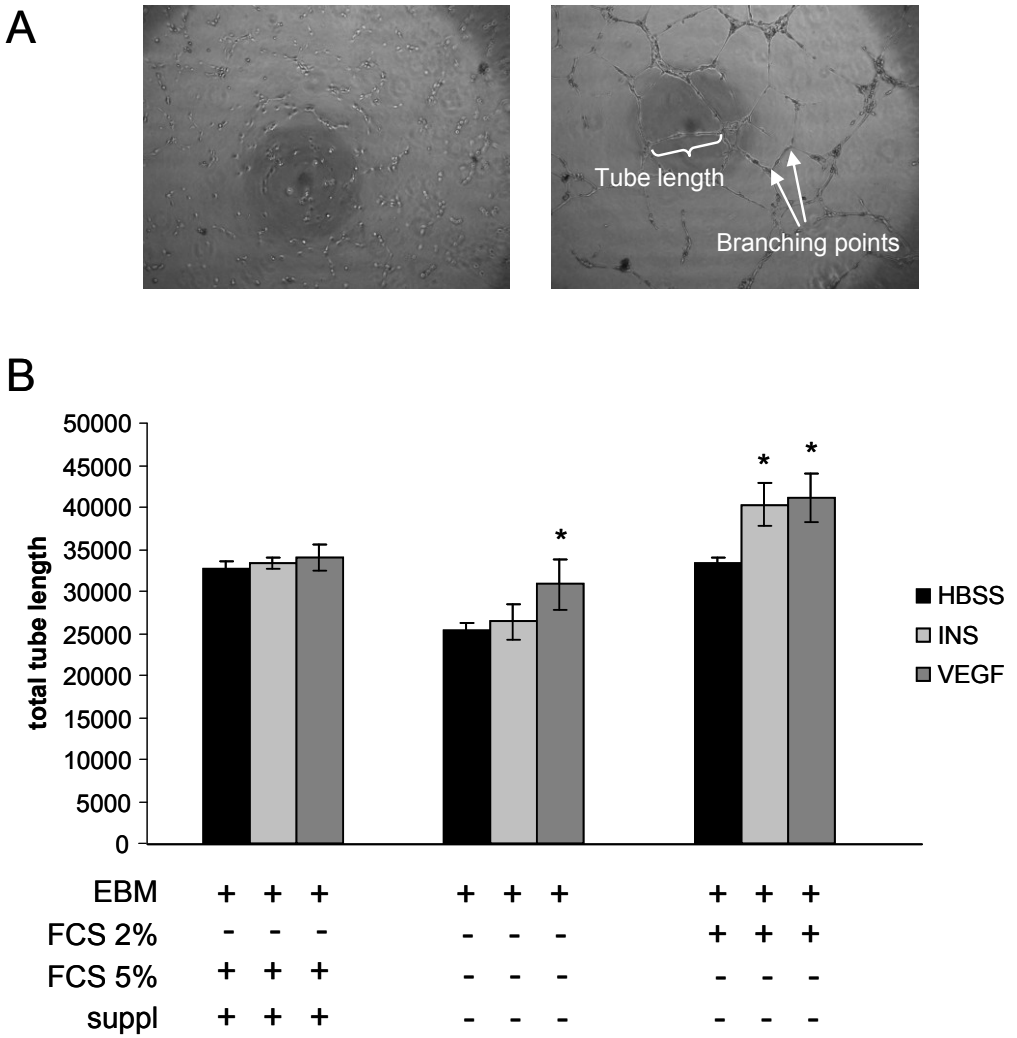


Figure 11: Insulin effect on *in vitro* angiogenesis assay. A) AEC immediately (left image) and 6 hours (right image) after seeding in growth factor reduced matrigel. The parameters tube length and branching points used for the quantitative analysis are indicated. B) AEC (8,000/well) were treated with insulin (10nM) or VEGF (650 pM) as positive control and seeded in growth factor reduced matrigel in three different conditioning medium: 1) fully supplememted EBM, containing 5% FCS and supplements; 2) EBM without FCS and without supplements and 3) in EBM without supplements but with only 2% FCS. When medium was fulley supplemented, no growth factor effect was observed. In poor medium conditions, only VEGF gad an effect. Insulin effect was observed when FCS concentration was reduced to 2% and no supplements were added to the medium. C) Insulin effects on total tube length and number of branching points in the condition of no supplements and 2% FCS. VEGF was used as a positive control. As by treatment with VEGF, insulin increased the total length and number of branching points of the network. Quantitative analysis was done six hours after seeding the cells using the AngioJ-Matrigel assay plugin for the ImageJ software (NIH). Data are means ± SD of 3 different cell isolations each measured in triplicates. * $p<0.05$

2. Insulin effect on cell cycle progression of AEC

Cell proliferation is one of the first steps for angiogenesis to occur (Senger and Davis, 2011) and it has been extensively shown that insulin can enhance proliferation and viability of endothelial cells (Shrader et al., 2009, Qiao et al., 2005, Zhang et al., 2008). Thus, the next step was to investigate whether insulin is capable of increasing proliferation of human placental AEC. Cells were treated with insulin or, as a positive control with VEGF, and incubated at 21% oxygen and 37^{o}C for 24 hours. Using the BrdU incorporation assay combined with flow cytometry it was possible to determine the proportion of cells in G0/G1, S phase and G2/M phase of the cell cycle. Analysis by flow cytometry revealed that insulin was able to significantly increase the proportion of cells in S-phase by around 24% as compared to the untreated cells (Fig 12). This increase was not as pronounced as with VEGF treatment. Intriguingly, the proportion of cells in G2/M phase was not altered by insulin treatment, meaning that even though insulin stimulates progression from G0/G1 to S-phase, cell cycle progression is then put on hold (Fig 12). These results are in line with proliferation measurements (CASY automatic counting) in which insulin did not increase the number of viable AEC (data not shown) up to 48 hours. However, VEGF treatment increased the proportion of cells in G2/M phase as compared to insulin or untreated cells (Fig 12), confirming its role as a potent mitogenic factor in endothelial cells.

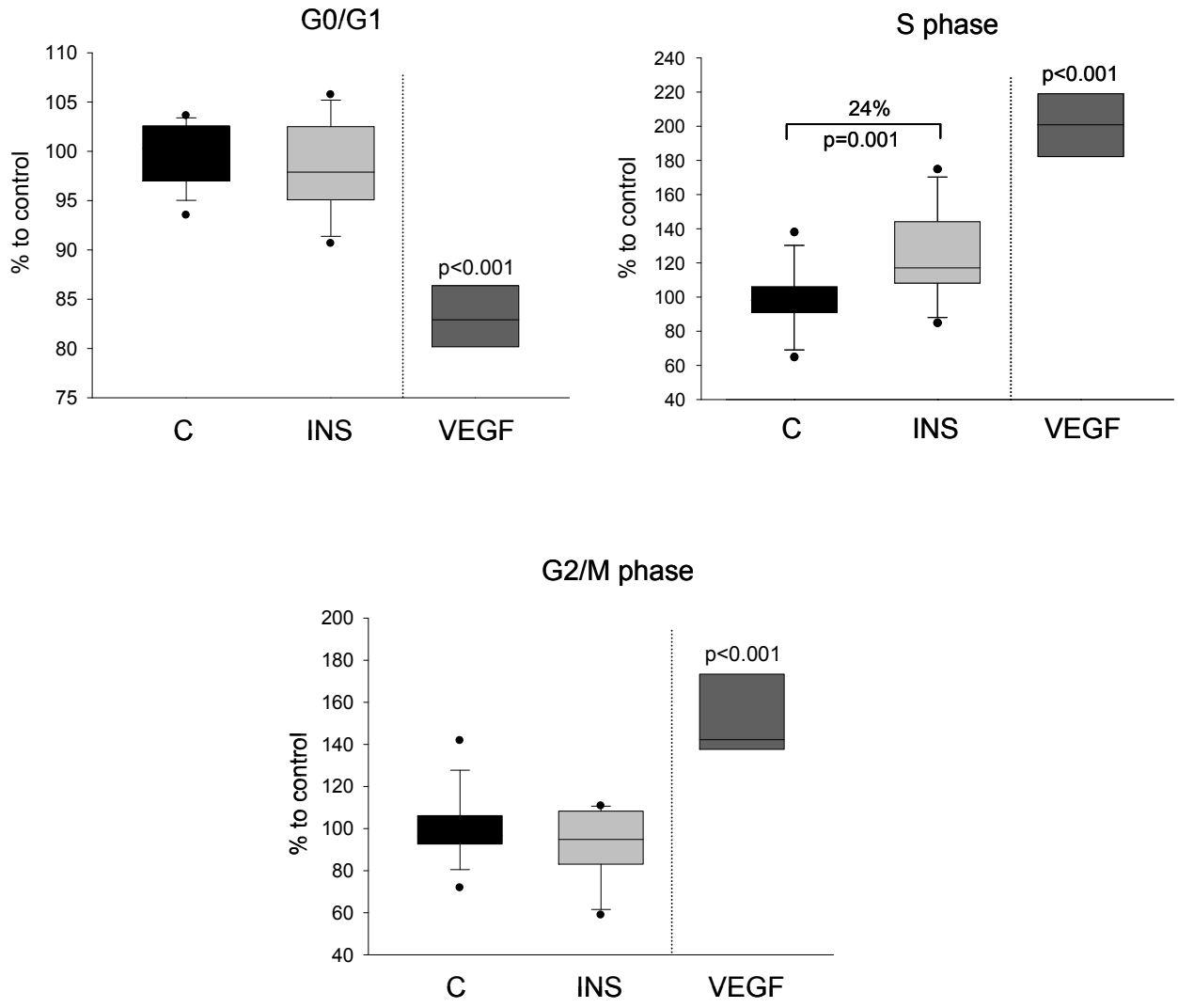


Figure 12: Insulin effect on AEC cell cycle progression. AEC (300,000/75cm^2 flasks) were cultured in non-supplemented EBM containing 5% FCS and incubated for 24 hours at 21% oxygen and 37^{o}C. Insulin (10nM) was added and cells were incubated for another 24 hours, BrdU was then added followed by 2 hours of incubation. Flow cytometry analysis revealed an increase of 24 % in the proportion of AEC in S phase after insulin treatment. The proportion of G0/G1 G2/M phase cells was not altered by insulin treatment as compared to the untreated cells. VEGF (650pM) was used as positive control. Data are means ± SD of 3 different cell isolations each measured in triplicates.

3. Insulin activates IRS/PI3K/Akt/eNOS and IRS/PI3K/Akt/GSK3β signaling pathways in AEC but not MAPK pathway.

As shown above insulin increases angiogenesis and stimulates progression of AEC from G0/G1 to S phase, but not from S phase to G2/M phase. The next aim was then to delineate the signaling pathways involved in these insulin effects in AEC. Others have reported that insulin action on EC from different origins is predominantly mediated via IRS/PI3K/Akt activation, that will lead to phosphorylation of endothelial nitric oxide synthase (eNOS) and stimulation of VEGF production (Salt et al., 2003, Zeng et al., 2000, Kim et al., 2001, Ritchie et al., 2010, Kohlhaas et al., 2011). However, insulin can also stimulate proliferation via the MAPK pathway (Isenovic et al., 2009, Montagnani et al., 2002). Recently, the glycogen-synthase kinase beta 3 (GSK3β) has been found to play an important role in angiogenesis by controlling EC migration and differentiation via β-catenin stabilization (Skurk et al., 2005) or via stimulating cell cycle progression (Liang and Slingerland, 2003). In fact, PI3K/Akt/GSK3, together with MAPK pathways often cooperate in regulating many aspects of cell cycle progression, such as Cyclin D, c-Myc, p27and p21 among others (Liang and Slingerland, 2003).

In order to investigate which signaling pathways are mediating the observed insulin effects, AEC were treated for 2, 5 and 10 minutes with 10nM of insulin, or 650pM of VEGF as a positive control, and incubated at 21% oxygen and 37^{o}C. A phospho-multiplex assay, which allows the detection of multiple phospho-signaling proteins in a 96-well format, was used to investigate insulin signaling transduction. Time course analysis showed that 5 minutes after stimulation highest phosphorylation levels were reached (data not shown). Thus, this was subsequently used as time point for further experiments, Insulin treatment increased insulin receptor B autophosphorylation of Tyr 1446 residue by about 300% and, consequently, stimulated IRS1 activation (Fig 13A and B). Downstream IRS1, the main signaling pathways that could be triggered are the Akt/PKB kinase or MAPK pathways. Insulin increased Akt/PKB phosphorylation by about 60% (Fig 13C), but surprisingly, had no effect on Erk1/2 phosphorylation (Fig 13D). VEGF, however, already well established as a potent MAPK pathway activator (Narasimhan et al., 2009), and thus again used as a positive control, increased Erk1/2 phosphorylation (Fig 13D). When investigating further downstream moleculaes of the Akt/PKB pathway, I also verified that insulin treatment increased

GSK3β phosphorylation by about 60% as compared to the untreated cells (Fig 13E). This data suggest that insulin stimulates the PI3K/Akt/GSK3β but not the MAPK pathway. In endothelial cells, stimulation of IRS1/PI3K/Akt pathway not only can activate the downstream GSK3β, but can also lead to phosphorylation of eNOS. This may contribute to the potential insulin regulation of vascular function, including NO production, increased vasodilation and angiogenesis (Maeno et al., 2011). Based on this, I further investigated whether insulin can also lead to eNOS activation in AEC. A time course experiment was performed and after immunoblot analysis an increase in eNOS phosphorylation by insulin stimulation was observed. There is an increase in eNOS phophorylation over the time, with significance reached at 15 minutes after insulin treatment (Fig 13F). This data suggest that, in placental AEC, insulin is activating the PI3K/Akt/GSK3β and the PI3K/Akt/eNOS pathways. This could explain the insulin-induced angiogenesis and the stimulation of G0/G1 to S phase progression. As cell cycle progression is governed by a cooperation of different intracellular signaling events, the inability of insulin to activate MAPK pathways could explain that, upon insulin stimulation, AEC progressed to S phase, but did not enter M phase.

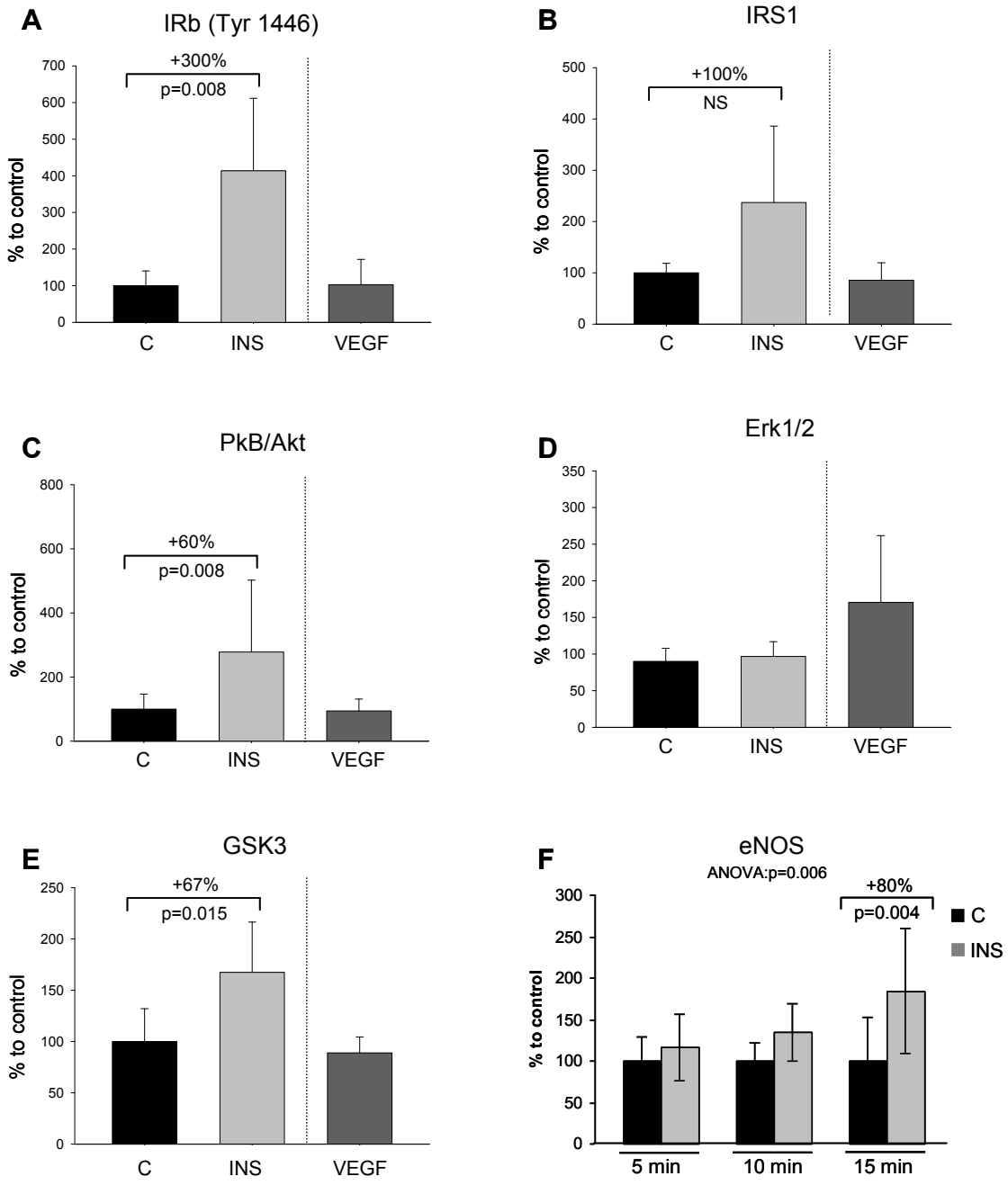


Figure 13: Insulin signaling in placental AEC. After 12 hour starvation, AEC (180,000 cells/well) were stimulated either with 10nM insulin, 25ng/mL VEGF (654 pM) or vehicle (HBSS) for 5 minutes (A-E) and only with insulin for 2, 5, 10 and 45 minutes (F). A-E: phosphorylated forms of IRB (Tyr 1446), IRS1, Akt, Erk1/2 and GSK3β were measured in AEC lysates after insulin and VEGF treatment using a phospho-multiplex assay (BioRad). Insulin stimulation increased phosphorylation of IRB (Tyr 1446), IRS1, Akt and GSK3β but not of Erk1/2, which was activated only by VEGF. F: Time course for insulin effects on eNOS phosphorylation. Insulin increased, even though not significantly, eNOS phosphorylation up to 10 minutes after stimulation. eNOS phosphorylation droped down to control levels after 45 minutes. Data are means ± SD of 3 different cell isolations each measured in triplicates. A-E: Students t-test, F: Two way ANOVA with Holm-Sidak method as post hoc.

4. Insulin stimulates angiogenesis via eNOS activation

Participation of eNOS in the development of vascular trees by promoting angiogenesis has been demonstrated in experiments where animals presenting eNOS deficiency showed defective development of lung vascular tree (Han and Stewart, 2006). Besides that, some pro-angiogenic mediators, such as VEGF and angiopoietin, depend on NO production to modulate angiogenesis (Ahmad et al., 2006). So far I demonstrated that insulin increases angiogenesis in placental AEC most likely via the PI3K/Akt/eNOS signaling pathways. To confirm this finding, new *in vitro* network formation assays were performed, this time in the presence of pharmacological inhibitors of the key molecules involved in this pathway. AEC were pre-treated for 1 hour either with wortmannin, a PI3K inhibitor, with UO126, a MAPK inhibitor or with L-NAME, an eNOS inhibitor. Angiogenesis comprises several processes that will depend also on cytoskeleton rearrangements. These are mostly dependent on activation of a member of the small GTPases family, Rac1 (Van Aelst and D'Souza-Schorey, 1997), (Tan et al., 2008). To verify whether Rac1 also plays a role in insulin-induced angiogenesis, NSC23766, a inhibitor of this GTPase, was also used. After pre-treatment with the inhibitors, cells were seeded on growth factor reduced matrigel and treated with insulin. *In vitro* angiogenesis was monitored in a cell observer and quantitative analysis of total tube length and number of branching points was done six hours after seeding the cells. VEGF was used as a positive control. Pre-treatment with wortmannin, UO126, and NSC23766 alone reduced total tube length and the number of branching points of the network to lower levels than in the control (Fig 14 and 15), which confirms the important role of PI3K, MAPK and Rac1 signaling pathways in placental angiogenesis. Because these inhibitors reduced angiogenesis below control levels, we could not confirm that insulin is mediating angiogenesis in placental AEC via these pathways, even though insulin alone increased angiogenesis. Nevertheless, when cells were pre-treated with the eNOS inhibitor L-NAME, basal levels of total tube length and number of branching points were not reduced (Fig 14 and 15). However, pre-treatment with L-NAME reduced insulin-induced angiogenesis to the same levels as in the control. These findings confirm that insulin-induced angiogenesis in placental AEC is mediated via eNOS. The finding that wortmannin, UO126 and NSC23766 reduced basal angiogenesis in AEC without insulin treatment could be due to the fact that, even though growth

factor reduced matrigel was used, it still contains small amounts of growth factors and basement membrane proteins. Besides that, 2% FCS was added to the media, which also contains soluble growth factors. They can also trigger angiogenesis via these pathways, but probably not involving eNOS, which will only be activated by insulin to contribute to insulin-stimulated angiogenesis.

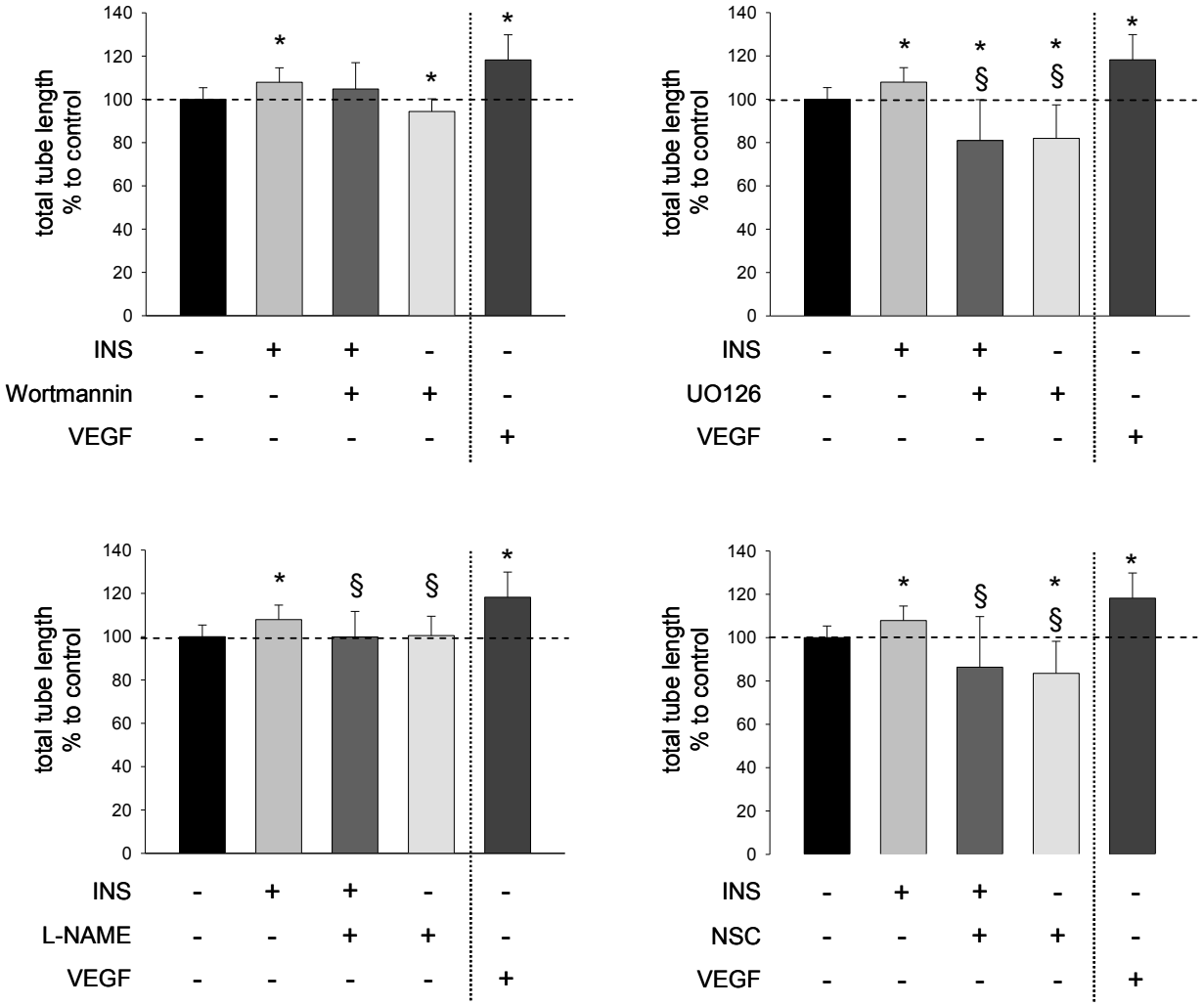


Figure 14: Use of pharmacological inhibitors to delineate the signaling pathways involved in insulin-induced angiogenesis by measuring total tube length of the network. AEC (8,000 cells) were pre-treated for 1 hour either with wortmannin (100nM), a PI3K inhibitor, or with UO126 (10µM), a MAPK inhibitor, or with L-NAME (400µM), an eNOS inhibitor or with NSC23766 (200µM), a Rac1 inhibitor. After pre-treatment with the inhibitors cells were seeded in growth factor reduced matrigel and treated with insulin (10nM) or VEGF (650 pM) as positive control. Network formation was monitored in a cell observer and quantitative analysis of total tube length was done six hours after seeding the cells using the AngioJ-Matrigel assay plugin for the ImageJ software (NIH). Data are means ± SD of 3 different cell isolations each measured in triplicates. * $p<0.05$ vs control, § $p<0.05$ vs insulin

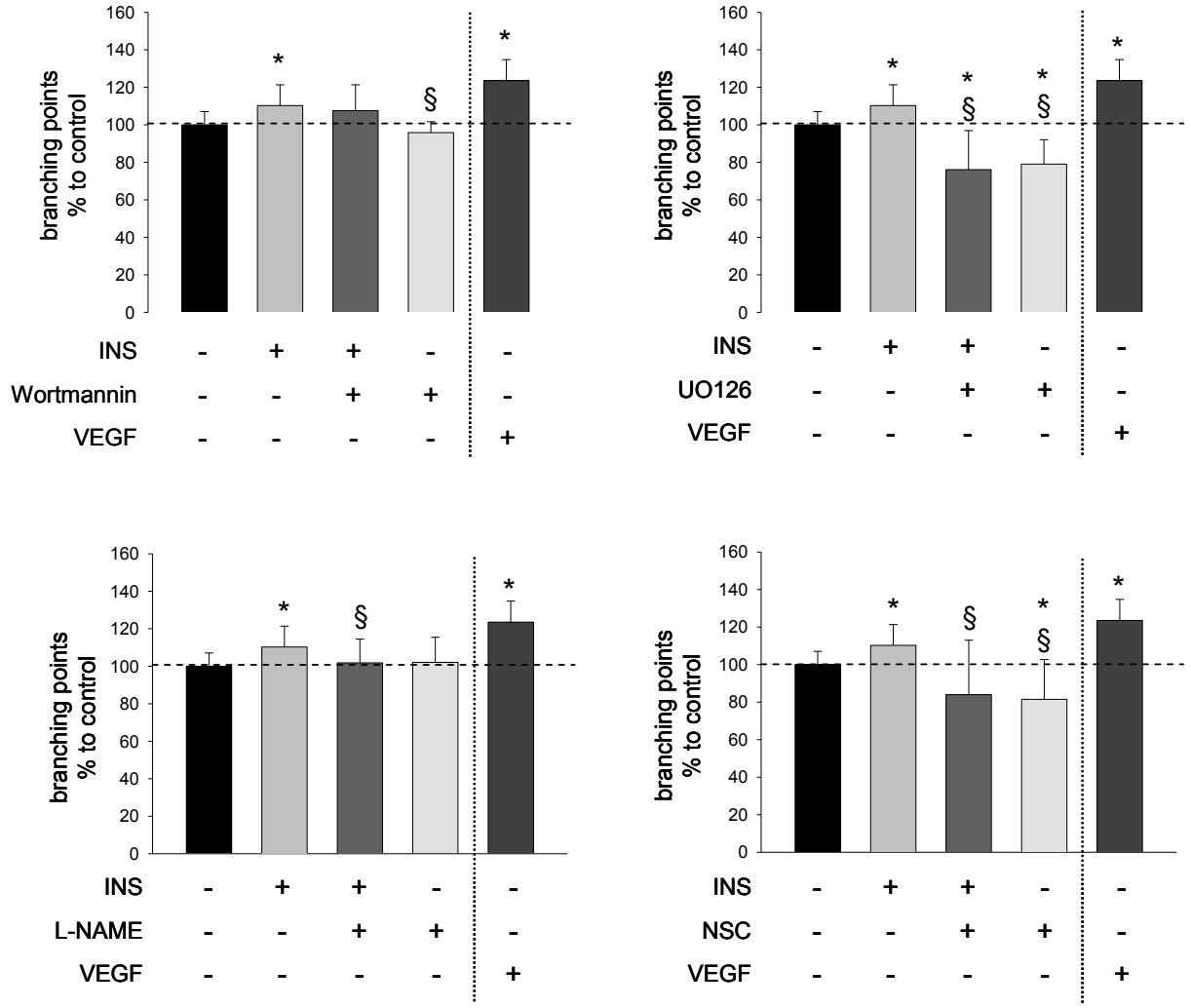


Figure 15: Use of pharmacological inhibitors to delineate the signaling pathways involved in insulin-induced angiogenesis by measuring the number of branching points of the network. AEC (8,000 cells) were pre-treated for 1 hour either with wortmannin (100nM), a PI3K inhibitor, or with UO126 (10µM), a MAPK inhibitor, or with L-NAME (400µM), an eNOS inhibitor or with NSC23766 (200µM), a Rac1 inhibitor. After pre-treatment with the inhibitors cells were seeded in growth factor reduced matrigel and treated with insulin (10nM) or VEGF (650 pM) as positive control. Network formation was monitored in a cell observer and quantitative analysis the number of branching points was done six hours after seeding the cells using the AngioJ-Matrigel assay plugin for the ImageJ software (NIH). Data are means ± SD of 3 different cell isolations each measured in triplicates. * $p<0.05$ vs control, § $p<0.05$ vs insulin

5. Insulin effect on actin organization and cell shape is eNOS and Rac1 mediated

As a dynamic process, angiogenesis comprises a series of events that include cell motility, cell shape, cell adhesion and differentiation. All of them depend on cytoskeleton rearrangements that are mostly driven by activation of several members of the RhoGTPases family (Van Aelst and D'Souza-Schorey, 1997) including Rac1, which plays an essential role in EC (Tan et al., 2008). Like other members of RhoGTPases, Rac1 cycles between the inactive GDP-bound to the active GTP-bound forms which will then activate downstream signaling effectors for actin reorganization. Guanine exchange factors (GEFs) control RhoGTPases activity by exchanging GDP to GTP and thereby make them active, and by GTPase-activating proteins (GAPs) which will hydrolyze GTP into GDP making them inactive (Van Aelst and D'Souza-Schorey, 1997). Rac1 activation can be a response to growth factor activation or to cell-ECM interactions. When active Rac1 mediates actin polymerization and lamellipodia formation (Bosco et al., 2009). Recently, it has been shown that insulin can stabilize microvascular endothelial barrier function via PI3K/Akt and NO/cGMP-induced Rac1 activation (Gunduz et al., 2010).

As I found that insulin-induced angiogenesis is mediated via PI3K/Akt/eNOS pathway I decided to investigate whether insulin also has an effect on actin rearrangements of placental AEC downstream of NO-dependent Rac1 activation. Cells were either serum starved for 48 hours (24 hours with 0.5% FCS and additional 24 hours without FCS) for Rac1 inactivation and consequently analysis of insulin effects, or not serum starved as control for the pharmacological inhibitors. Starved cells were insulin treated to verify actin reorganization effects of insulin. Non-starved and starved cells were pre-treated for 1 hour either with wortmannin, UO126, L-NAME, or NSC23766 and then stimulated with insulin for 40 minutes to delineate the pathways activated by insulin. Actin organization under normal growing conditions (non-starved cells) can be observed by phalloidin staining (performed in collaboration with by Heidi Miedl, Department of Gynecology and Obstetrics, Medical University of Graz, Austria) as F-actin stress fibers arranged in parallel and well organized across the cell. There are no membrane ruffles occurring (Fig 16, arrows). Starved cells present less intense F-actin fibers staining, membrane ruffles are formed (Fig 16, filled arrows) and cells

lose their original shape acquiring a round, shrunken shape. Cell starvation leads to Rac1 inactivation (Kurokawa et al., 2004) thus resulting in actin disorganization and membrane ruffling. Insulin stimulation of starved cells restored actin distribution, the original cell shape and eliminated membrane ruffling, suggesting insulin participation in actin stabilization in placental AEC (Fig 16). Pre-treatment of starved cells with wortmannin, L-NAME and NSC23766 abolished insulin effects, consequently causing actin cytoskeleton disorganization with membrane ruffling formation (Fig 16, filled arrow). The MAPK inhibitor, UO126, only partially changed actin organization and did not abolish insulin effects, confirming the signaling data that showed no Erk activation after insulin stimulation. As Rac1 is the main mediator of actin organization, and the PI3K and eNOS inhibitors (wortmannin and L-NAME, respectively) abolished insulin effects, our data suggest that insulin mediates Rac 1 activation via the PI3K/Akt/eNOS pathway. Pre-treatment of the non-starved cells with the inhibitors was performed to verify their own inhibitory effects on actin organization and then, after insulin treatment, to confirm the data obtained for the starved cells. Treatment of non-starved cells with wortmannin, L-NAME and NSC 23766 reduced visible F-actin fibers and increased membrane ruffling (Fig 16) similar to the starved cells and abolished insulin effects, confirming that insulin contributes to actin organization in AEC via RAC1 activation through the PI3K/Akt/eNOS pathway. Again, pre-treatment with UO126 only partially disturbed actin organization and did not abolish insulin effects (Fig 16). The eNOS inhibitor abolished the insulin effect thus confirming the role of eNOS/NO in Rac1 activation and, consequently, actin cytoskeleton stabilization.

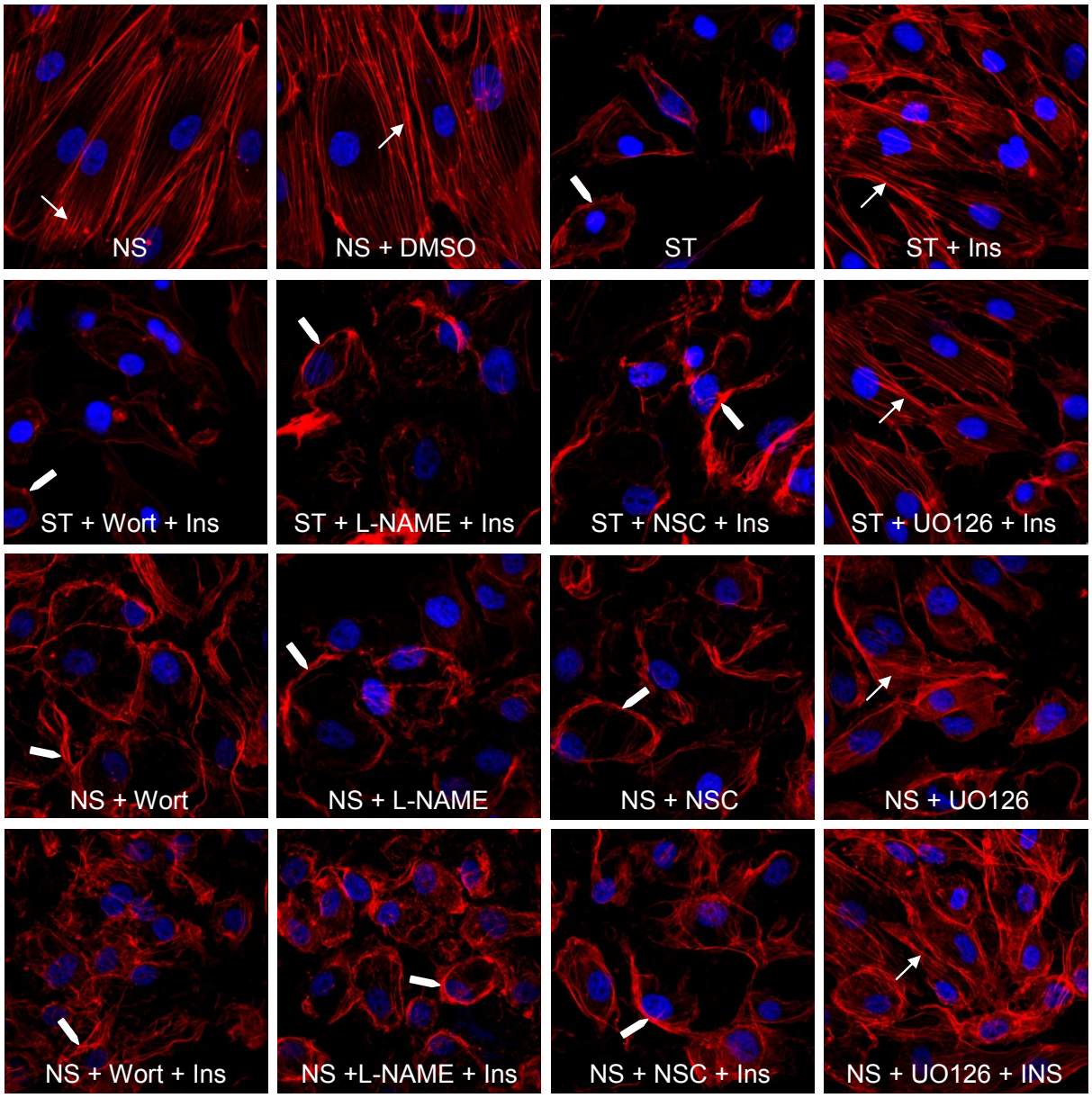


Figure 16: Effect of insulin in actin cytoskeleton of cultured human placental AEC. Cells (50,000/well) were seeded in gelatin coated glass chamber slides. Starved cells (ST) were cultured 24 hours in EBM containing 0.5% FCS and another 24 hours in EBM with no FCS and non-starved cells (NS) under normal conditions. Non-starved and starved cells were either pre-treated with wortmannin (100nM), L-NAME (400µM), NSC23766 (200µM) or UO126 (10µM) for 1 hour. After pre-treatment, cells were stimulated with insulin (10nM) for 40 minutes and stained with phalloidin texas-red. Non-starved cells show organized parallel F-actin stress fibers (arrows) with very intense phalloidin staining, while starved cells show membrane ruffles formation (filled arrows), less actin staining and cell shape is altered. After insulin stimulation, starved cells restore actin organization and the cell shape observed in the the non-starved cells. Pre-treatment of starved cells with wortmannin, L-NAME and NSC23766 abolished insulin effect of restoring F-actin fibers distribution and the cell shape. Treatment of non-starved cells with the inhibitors had the same effect of starvation and also abolishe insulin effect. UO126 did not have a strong effect and did no inhibit insulin stimulation (performed in collaboration with by Heidi Miedl, Department of Gynecology and Obstetrics, Medical University of Graz, Austria).

Discussion

Recent findings demonstrated insulin as vascular protective agent in endothelial cells promoting angiogenesis and wound healing (Liu et al., 2009). During embryogenesis, the insulin/IGF system is indispensible for proper fetal and placental growth and development (Hiden et al., 2009a, Hiden et al., 2006). During GDM, as a consequence of maternal hyperglycemia, there is an increase of insulin production by the fetus. Fetal circulation is in direct contact with placental vessels thus altered growth factor levels in fetal blood can have a direct effect on the feto-placental vasculature. (Desoye and Hauguel-de Mouzon, 2007, Hiden et al., 2009b). In fact, placentas from diabetic pregnancies are hypervascularized (Hiden and Desoye, 2010) and this is a consequence of increased angiogenesis, a process that requires endothelial cell proliferation, migration and differentiation (Hoeben et al., 2004). Here we hypothesized that insulin stimulates angiogenesis in placental AEC and this would partially explain hypervascularization observed on placentas from GDM. The main findings were 1) Insulin stimulates placental AEC *in vitro* angiogenesis but does not induce proliferation; 2) Insulin-induced angiogenesis in AEC signals via IRS1/PI3K/Akt signaling pathway and involves downstream eNOS and Rac1 activation and 3) insulin induces actin rearrangements in AEC via the eNOS/Rac1 signaling pathway. These results show for the first time the pro-angiogenic effect of insulin on placental endothelial cells and delineate the signaling pathways that could contribute to increased placental vascularization in GDM.

The formation of new vessels is a process that depends on the interaction of endothelial cells with the surrounding environment. The latter comprises a variety of soluble factors and their receptors, such as VEGF, FGF, PDGF, TGF-β, membrane-bound factors, such as integrins, adhesion molecules, matrix metalloproteinases and ECM (Otrock et al., 2007). Recently insulin has emerged as a potential pro-angiogenic factor and the data obtained here support this hypothesis. I verified that insulin is able to increase angiogenesis of placental AEC even though I was not able to find an effect on endothelial proliferation. Intriguingly, insulin stimulated AEC cell cycle progression from G0/G1 to S phase, but not from S to M phase, contrary to VEGF. Liu et al. also did not find a mitogenic effect of insulin in HMEC yet angiogenesis was increased (Liu et al., 2009). Most likely insulin requires cooperation with other growth factors, such as

VEGF, FGF or EGF, in order to promote proliferation of EC in the cell model used here.

Insulin activates the IRS1/PI3K/Akt signaling pathway leading to downstream GSK3β phosphorylation and consequently inactivation. It has been shown that the PI3K pathway is activated during G1/S transition and is indispensable for DNA synthesis in mouse fibroblasts, leukocytes and human mammary epithelial cells (HMEC) (Treinies et al., 1999, Brennan et al., 1997, Liang et al., 2002). Cyclin D1 and c-Myc are crucial regulators of G1/S progression and their proteolysis is regulated by the PI3K pathway (Treinies et al. 1999). Besides this, GS3Kβ is able to phosphorylate cyclin D1 leading to its ubiquitin-mediated proteolysis. However, inhibition of GS3Kβ by Akt-dependent phosphorylation stabilizes cyclin D1 and PI3K inhibitor accelerated cyclin D1 degradation (Diehl et al., 1998). PI3K pathway activation also reduces the levels of the cell cycle inhibitors p27 and p21 (Narita et al., 2002, Forti et al., 2002, Rossig et al., 2001, Zhou et al., 2001). It seems like c-Myc stabilization can be as well mediated by GS3Kβ inhibition via Akt-dependent phosphorylation but Ras and MAP kinases can also acivate c-Myc (Pelengaris et al., 2002). These pathways would then cooperate in cell cycle progression. It appears that in placental AEC, insulin would cooperate with other growth factors to promote EC proliferation. Since VEGF has shown here to increase the proportion of cells in the M phase, it would be a strong candidate to act in paralell or even in concert with insulin to stimulate placental EC proliferation.

eNOS is also one of the downstream targets of PI3K/Akt signaling pathway (Fulton et al., 1999). eNOS activation leads to NO production, a potent modulator of endothelial function, that regulates processes such as vasodilation, vascular remodeling, vascular permeability and angiogenesis (Sessa, 2009). Inhibition of NO production reduces angiogenesis and vascular permeability induced by VEGF (Ziche et al., 1997, Murohara et al., 1998). Here I found that insulin activates the IRS1/Akt pathway that leads to phosphorylation and activation of the downstream eNOS. This could mediate the increase in angiogenesis after insulin stimulation, a concept confirmed here by using L-NAME, a pharmacological eNOS inhibitor, which blocked the insulin effect of increasing angiogenesis on AEC. The use of wortmannin, a PI3K inhibitor, also reduced angiogenesis but to lower levels as in the control, reinforcing the central role of this pathway in angiogenesis. In fact,

PI3K/Akt modulates activation of several proteins that somehow participate in the different steps required for angiogenesis. The mammalian target of rapamycin (mTOR) is one of the targets of PI3K/Akt signaling and has been recently shown to mediate angiogenesis (Karar and Maity, 2011, Jung et al., 2011, He et al., 2011). GS3Kβ, that was also phosphorylated after insulin stimulation and is downstream of PI3K/Akt, promotes angiogenesis via regulation of β-catenin function (Skurk et al., 2005). Fukumoto et al. demonstrated that insulin signaling can modulate β-catenin activity via Akt/GSK3β signaling in different cell types (Fukumoto et al., 2001). All together these data can explain our finding that inhibition of PI3K signaling pathway with wortmaninn reduced angiogenesis of placental AEC to lower levels than in the control.

Inhibition of Rac1 by using the pharmacological inhibitor NSC23766 also led to a reduction of basal angiogenesis. Similar to PI3K, this reduction likely occurs because Rac1 regulates important steps in angiogenesis. Rac1, member of the Rho GTPases, mediates endothelial actin cytoskeleton rearrangements, polarization, permeability, and adhesion (Wojciak-Stothard and Ridley, 2003, Tzima, 2006). Recently, it has been shown in murine endothelial cells, that Rac1 can also stabilize and enhance eNOS activity by promoting endothelial uptake of L-arginine (Sawada et al., 2008), a precursor of NO synthesis (Moncada and Higgs, 1993). Down-regulation of Rac1 activity would then lead to impaired endothelial cell organization, adhesion and reduced NO production, resulting in decreased angiogenesis observed after Rac1 inhibitor treatment.

Given the role of Rac1 in crucial processes for angiogenesis, and because NSC23766 reduced AEC basal angiogenesis, I investigated whether insulin stimulation of AEC had an effect on actin cytoskeleton assembly and organization. Our results clearly showed that insulin activates endothelial cell actin reassembly via PI3K/Akt/eNOS pathway and Rac1 activation. This was confirmed by using the PI3K inhibitor wortmannin, the eNOS inhibitor L-NAME and the Rac1 inhibitor NSC23766. All of them abolished insulin effects in restoring and keeping actin organization and cell shape as demonstrated in the insulin-treated starved and non-starved cells after inhibitors pre-treatment. L-NAME had a high level of inhibition suggesting that NO production is essential for Rac1 activity and consequently actin assembly in AEC. To the best of my knowledge, the role of eNOS/NO in activating Rac1 in endothelial cells has been poorly investigated

(Gunduz et al., 2010, Wojciak-Stothard et al., 2009). Most published data were obtained in smooth muscle cells, fibroblasts and macrophages (Hou et al., 2004, Magazine et al., 2000, Zhou et al., 2009, Rolli-Derkinderen et al., 2010). NO production leads to an increase of cGMP which activates the cGMP-dependent protein kinases (cGKs) also known as protein kinase G (PKG) which in turn, activates Rac1 (Hou et al., 2004). This would explain why L-NAME inhibited the insulin effect on actin cytoskeleton reassembly, suggesting that Rac1 in placental AEC may be downstream of eNOS in insulin-mediated actin organization.

These data together reinforce the role of insulin as a pro-angiogenic factor in placental angiogenesis. Insulin-induced angiogenesis in placental AEC occurs through PI3K/Akt and eNOS/NO signaling pathways and downstream Rac1 activation (Fig 17). This would suggest that the hypervascularization observed in placentas from GDM is a consequence of fetal hyperinsulinemia as a response to increased maternal glucose transfer to the fetus.

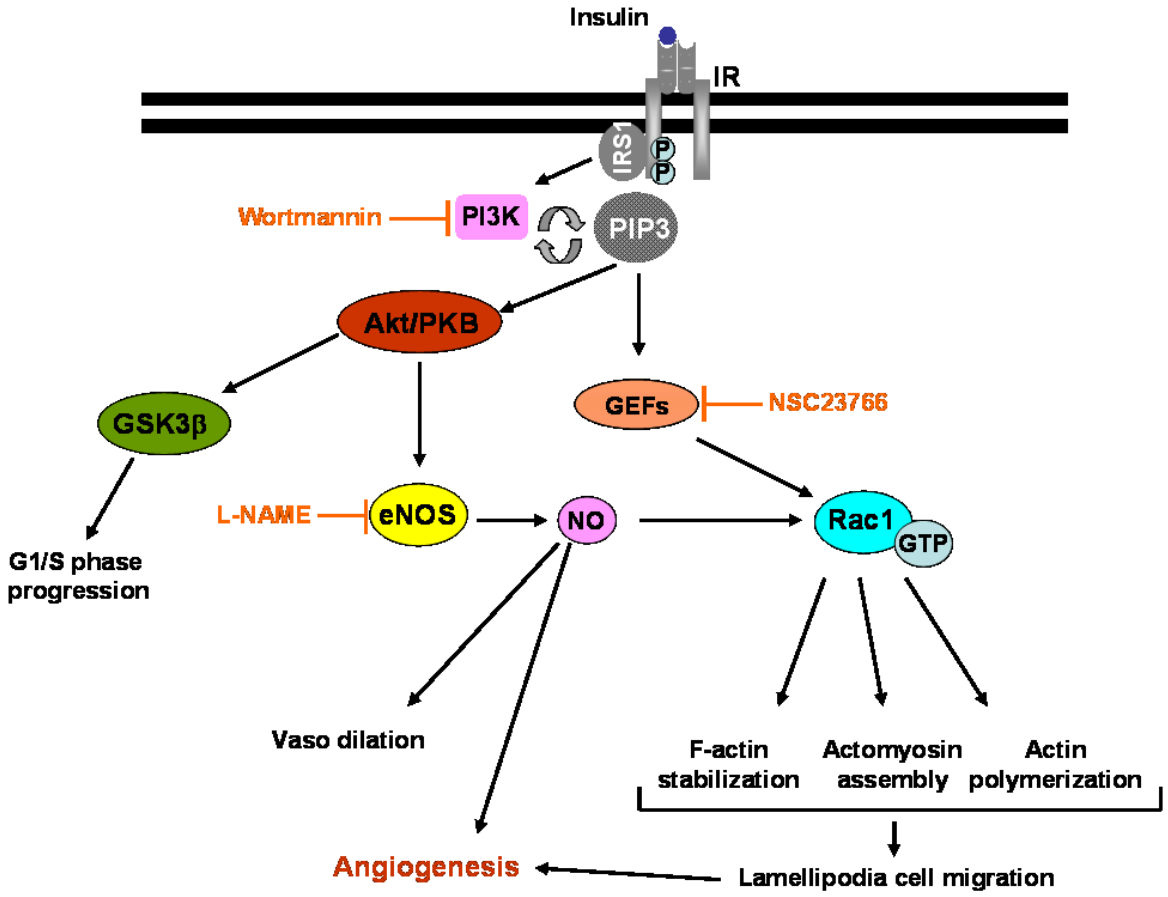


Figure 17. Insulin signaling in human placental AEC. Insulin binding to IR leads to its autophosphorylation and subsequent IRS1 phosphorylation and PI3K activation. Active PI3K phosphorylates Akt/PKB which in turn phosphorylates and consequently inactivates GSK3β, leading to cell cycle progression. Akt also phosphorylates eNOS leading to NO production and downstream Rac1 activation. NO generation and Rac1 activation will contribute to the insulin-induced angiogenesis in placental AEC. The use of the pharmacological inhibitors wortmannin, L-NAME and NSC23766 confirmed the role of insulin on actin reorganization and L-NAME confirmed insulin effect on increased angiogenesis.

Chapter 3

Differential response of arterial and venous endothelial cells to extracellular matrix is modulated by oxygen

As mentioned above, GDM is associated with thickening of the placental basement membrane, especially by an increase in collagen production, and an increase in endothelial cell proliferation (Pietryga et al., 2004). Besides that and despite the importance of ECM for cellular function, information is sparse about how endothelial cells from different vascular beds in the same tissue respond to ECM signals present in the surrounding environment induced through their interaction with integrins.

Hypothesis

In this part of the work I tested the hypothesis that AEC and VEC differ in their response to different basement membrane proteins and that this distinct response is modulated by oxygen. I have chosen anchorage-dependent processes as functional endpoints and measured proliferation, viability, survival and cell shape.

Results

1. Effects of ECM and oxygen on proliferation and viability of VEC and AEC

Most animal-derived cells depend on cell-cell and cell-ECM contacts for *in vitro* growth. Therefore, processes such as migration, proliferation, differentiation and survival are modulated by these interactions and, consequently, by the intracellular signaling generated by them (Stromblad and Cheresh, 1996, Loeffler et al., 2011a). In order to test whether VEC and AEC proliferation and viability are influenced by different ECM components, each cell type was grown for 48 hours on rat collagen I and IV, bovine fibronectin, mouse laminin I, gelatin, or uncoated plastic plates. To investigate whether oxygen modulates the ECM effect on cell viability, comparative experiments were performed at 12% and 21% oxygen.

Among the collagens present in the basement membrane surrounding the blood vessels, collagen I and IV are important constituents. In order to test whether collagen I and IV effects on VEC and AEC viability and proliferation would differ, we compared the number of viable cells grown on plates coated with both ECM proteins. As there was no difference in their effect in both VEC and AEC, neither at 12% nor 21% oxygen (Fig 18A), all of the subsequent experiments were carried out with collagen I.

In general VEC showed a stronger ECM dependence at 21% than at 12% oxygen, whereas AEC growth was not strongly influenced by ECM, if at all. At 12% oxygen, the number of viable VEC was higher in gelatin and fibronectin as compared to uncoated plates. In 21% oxygen, gelatin, collagen I, fibronectin and laminin increased the number of viable cells. Collagen I and fibronectin had the strongest effect (Fig 18B). In contrast, this effect was absent in AEC in both 12% and 21% oxygen. Only gelatin increased the number of viable cells in 21% as compared to the uncoated plates, but merely by around 40% (Fig 18B).

The stronger ECM effect on VEC as compared to AEC was also seen in the number of dead cells. At 21% oxygen, we found a significant reduction in the number of dead VEC on collagen I, fibronectin and laminin coated plates as compared to gelatin (Fig 18C). However, the number of dead cells in the uncoated plates as compared to the matrix-coated plates was substantially reduced by about 60%. Since there were also fewer viable VEC on the uncoated plates, plastic appears to keep the VEC in cell cycle arrest. In 12% oxygen, the different matrices also tended to decrease the number of dead cells as compared to gelatin but without reaching significance. The number of dead AEC was not influenced by the ECM at both 12% and 21% oxygen (Fig 18C). Only on laminin coated plates and at 12% oxygen, the number of dead cells was reduced. It seems that under this condition laminin stimulates viability rather than proliferation of AEC (Fig 18C).

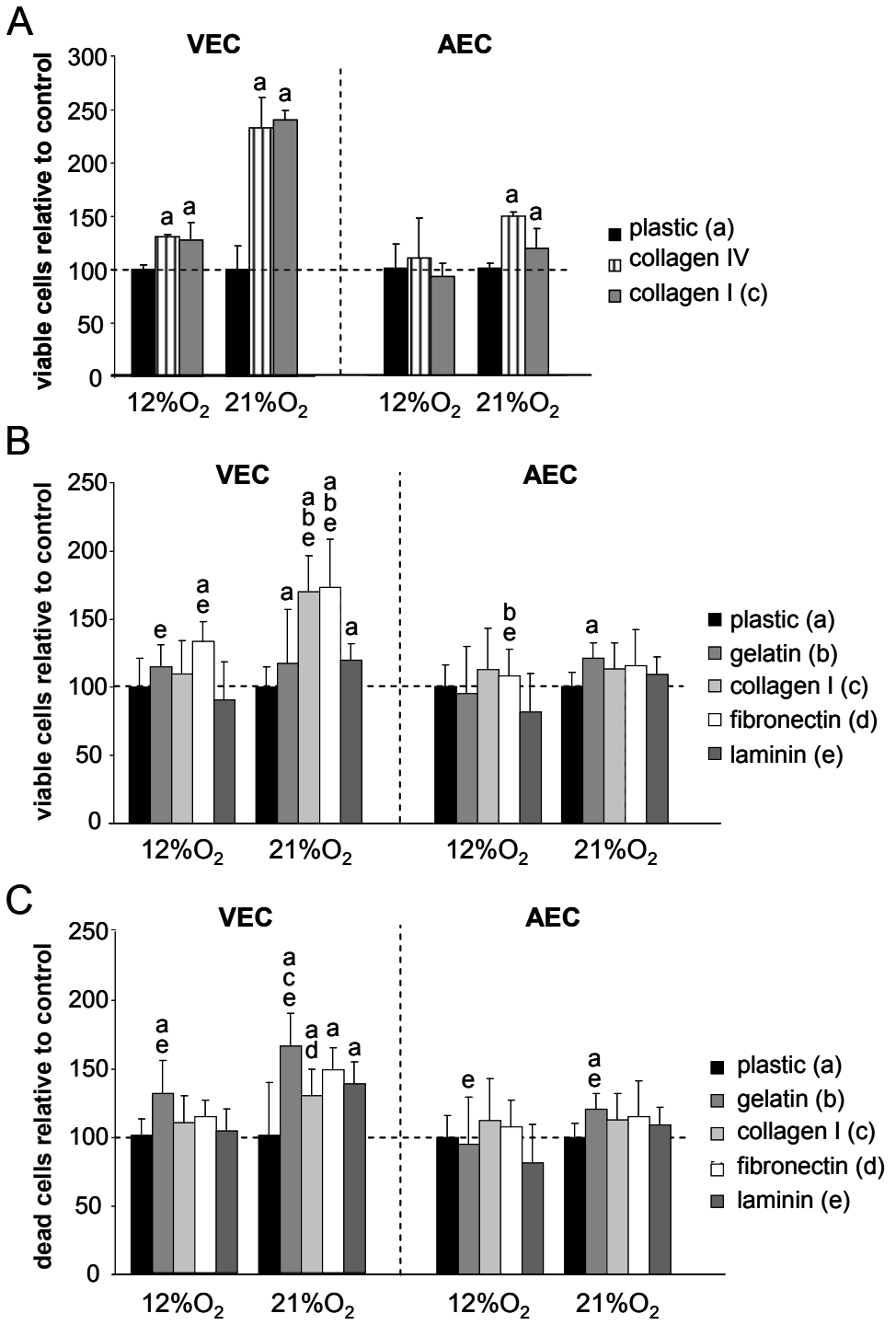


Figure 18: Viability of primary placental endothelial cells in different extracellular matrices and at different oxygen concentrations. VEC and AEC (90,000 cells/well) were plated on collagen I, fibronectin, laminin, gelatin and uncoated plates (plastic) and cultured for 48 hours either at 12% or 21% oxygen. Cells on uncoated plates were used as control (100%). A) The number of viable cells on collagen I and IV was compared. As there were no significant differences, the following experiments were performed in collagen I. The number of viable (B) and dead (C) cells in the different matrices was determined by automatic counting. Data are means ± SD of 3 different cell isolations each measured in triplicates. a, b, c, d and e denote $p<0.05$: a vs. plastic, b vs. gelatin, c vs. collagen, d vs. fibronectin, e vs. laminin

2. Effect of ECM and oxygen on cell cycle and apoptosis in VEC and AEC

ECM represents a survival factor for many cell types, rescuing them from apoptosis (Meredith et al., 1993, Priya and Sudhakaran, 2008). To determine whether the ECM effects on AEC and VEC proliferation are a result of cell cycle stimulation rather than increasing survival, cell cycle and apoptosis assays were performed. Each ECM tested increased significantly the proportion of AEC in S-phase when compared to the uncoated plates (Fig 19A). This effect was present at both 12% and 21% oxygen. Particularly collagen I at 12% oxygen showed a profound increase by about 75% in the proportion of cells in S-phase compared to the other matrices. For VEC, collagen I and fibronectin were more effective in inducing DNA duplication in both oxygen concentrations and again laminin had no effect on inducing cell cycle, which reinforces that laminin may act to maintain viability rather than stimulating proliferation (Fig 19A).

Caspase-3, which represents a key enzyme in the execution phase of apoptosis, when the process becomes irreversible, was measured here as a readout for apoptosis. Cells were treated with the apoptosis inducer staurosporin as a positive control. Uncoated plates were used not only as a control for the matrix coated surfaces, but also as a control for any potential ECM contamination present in the medium. The assay revealed that - when VEC were grown on different ECM - the proportion of apoptotic cells was reduced as compared to uncoated plates (Fig 19B). This was different from AEC which did not show a reduction in apoptosis when grown on ECM as compared to uncoated plates (Fig 19B). In general only about 5-7% of cells were apoptotic, a number which can be considered in the normal range of a vital cell culture. Thus, it seems that ECM is not only instrumental in stimulating proliferation of VEC, but also in rescuing them from apoptosis. This is at variance from AEC, for which ECM plays a role rather for proliferation than for survival. In contrast, oxygen does not affect apoptosis of both cell types

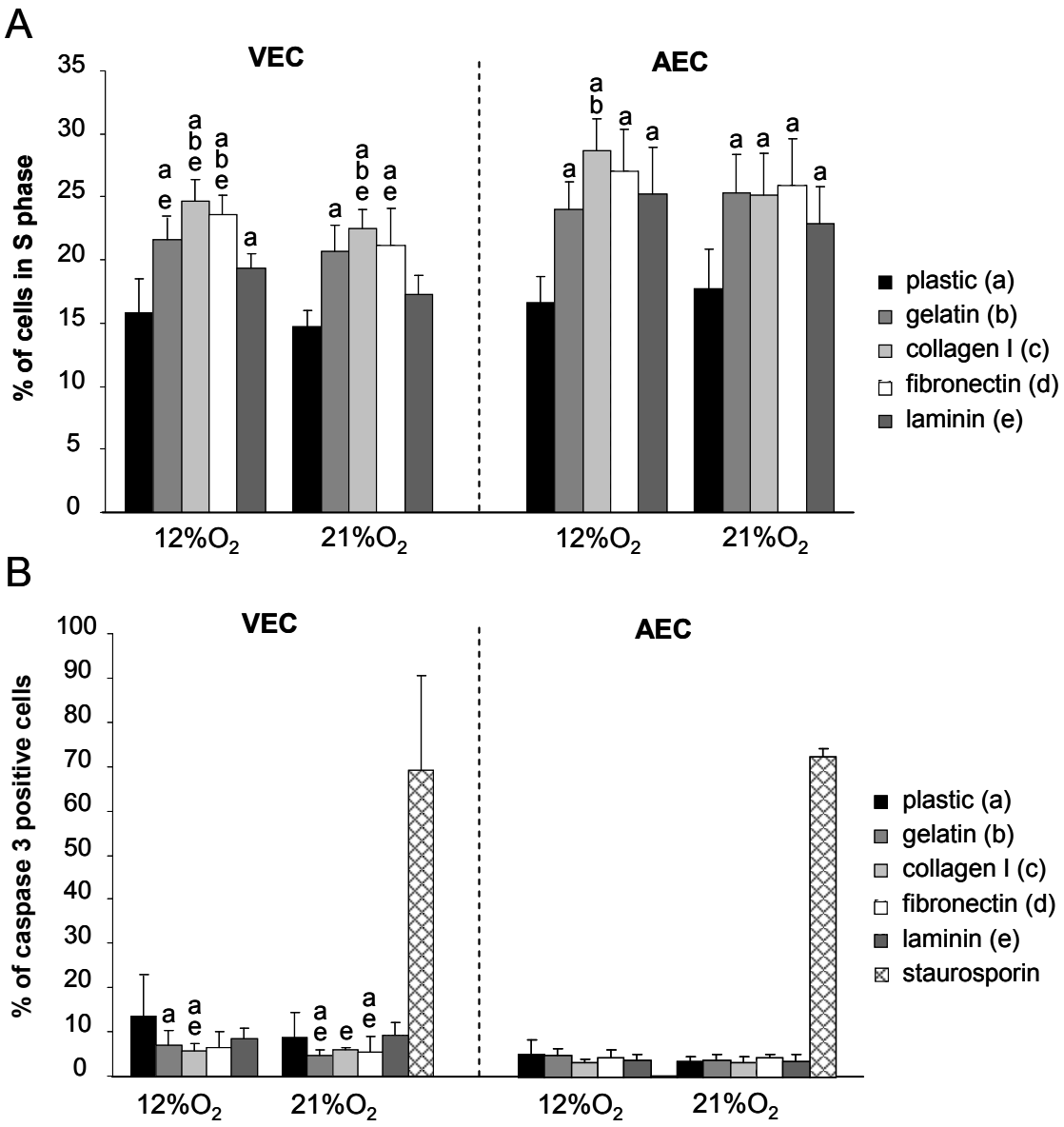


Figure 19: Proliferation and survival of primary placental endothelial cells in different extracellular matrices and at different oxygen concentrations. VEC and AEC (90,000 cells/well) were plated on collagen I, fibronectin, laminin, gelatin and uncoated plates (plastic) and cultured for 48 hours either at 12% or 21% oxygen. Cells on uncoated plates were used as control (100%). A) 48 hours after plating, cells were harvested, fixed, stained with propidium iodide (PI) and analyzed by flow cytometry. B) 48 hours after plating, cells were harvested, fixed, permeabilized and positive cells for cleaved caspase 3 were determined by flow cytometry. Data are means ± SD of 3 different cell isolations each measured in triplicates. a, b, c, d and e denote $p<0.05$: a vs. plastic, b vs. gelatin, c vs. collagen, d vs. fibronectin, e vs. laminin

3. Effect of ECM and oxygen on Fak phosphorylation in VEC and AEC

Integrin binding to extracellular components elicits many intracellular signals by activating a series of kinases. Among these, Fak is most upstream. The first step in its activation is autophosphorylation of Tyr397 residue. Therefore, Fak autophosphorylation was measured in VEC and AEC, which were seeded in plates coated with the different matrices and incubated at 12% and 21% oxygen. The cells attached to the plates 4-5 hours after seeding. They were harvested five hours after plating. This represents on average an about 30 minutes period between attachment and harvesting, i.e. activation period for FAK. In VEC at 21% oxygen, Fak phosphorylation was increased in response to collagen I and fibronectin, but not by gelatin and laminin (Fig 20). At 12% oxygen, however, only fibronectin increased significantly Fak phosphorylation. In AEC, there were no significant changes in Fak phosphorylation among the ECM tested (Fig 20), even though there was a small increase when cells were grown on gelatin, collagen and fibronectin at 21% oxygen (Fig 20). Laminin, however, decreased Fak phosphorylation in AEC at 21% oxygen. These results suggest that Fak phosphorylation after ECM binding is differentially activated in VEC and AEC. The ECM components themselves also had differential effect in activating Fak and this was modulated by oxygen.

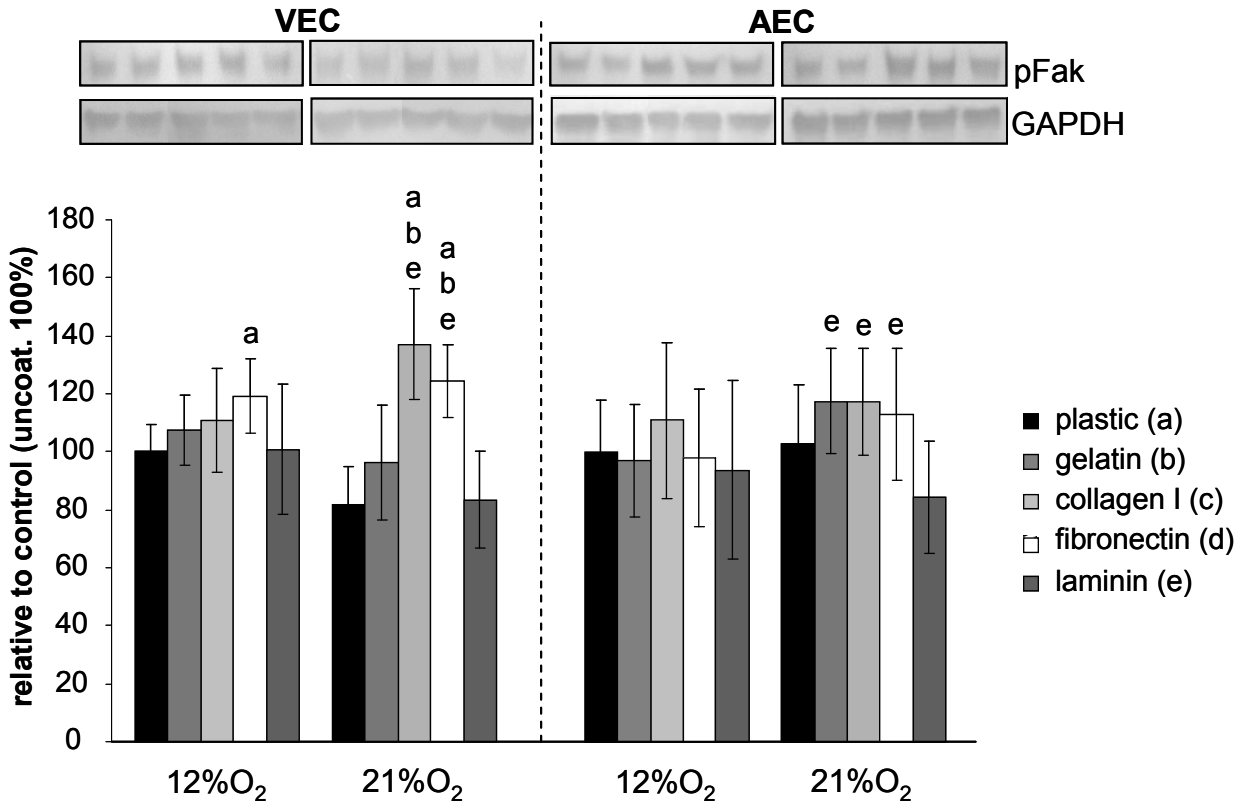


Figure 20: Immunoblot analysis of Fak tyrosine phophorylation (pTyr397) on different extracellular matrices at different oxygen concentrations. VEC and AEC were plated on collagen I, fibronectin, laminin, gelatin and uncoated plates (plastic) at 12 and 21% oxygen. Cells on uncoated plates were used as control. After 5 hours cells were harvested and lysates used for immunoblotting. The relative levels of phosphorylated Fak were determined by normalizing optical band densities of pFak to GAPDH. Data are means ± SD of 3 different cell isolations each measured in triplicates. a, b, c, d and e denote $p<0.05$: a vs. plastic, b vs. gelatin, c vs. collagen, d vs. fibronectin, e vs. laminin

4. Effect of Fak inhibitor on ECM induced proliferation and attachment of VEC and AEC

Fak is preferentially activated by cell-ECM interaction at 21% oxygen and this is paralleled by increased cell viability at this condition. In order to investigate whether Fak activation is mediating these ECM effects on cell viability and survival at 21% oxygen, Fak inhibitor 14, which blocks Fak autophosphorylation at Tyr397, was used. After performing a concentration-response pilot study in the range of 1-50 µM (Golubovskaya et al., 2008), the concentration of 25 µM was chosen for further experiments since it resulted in an acceptable level of 25-30% cell detachment (data not shown). Cells were grown for 48 hours at 21% oxygen on various ECM in the absence or presence of the inhibitor. Bright field microscopy revealed a higher number of viable VEC and AEC attached to the ECM as compared to the uncoated plates (Fig 21A). In the presence of the Fak inhibitor, approximately 30% of cells were not able to attach and remained floating in the medium (Fig 21A). Quantitative analysis using CASY measurements confirmed that there were more viable VEC and AEC cells attached to the ECM-coated as compared to the uncoated plates (Fig 21B). The Fak inhibitor decreased cell viability, thus confirming the visual impression from microscopy, probably as a consequence of a reduction in Fak activation. Similar observations were previously made in neuroblastoma cell lines, where Fak inhibition resulted in loss of cell attachment and, consequently, apoptosis of the non-attached cells (Beierle et al., 2010). Collectively, these data suggest that Fak mediates ECM-dependent cell viability and survival in primary placental VEC and AEC.

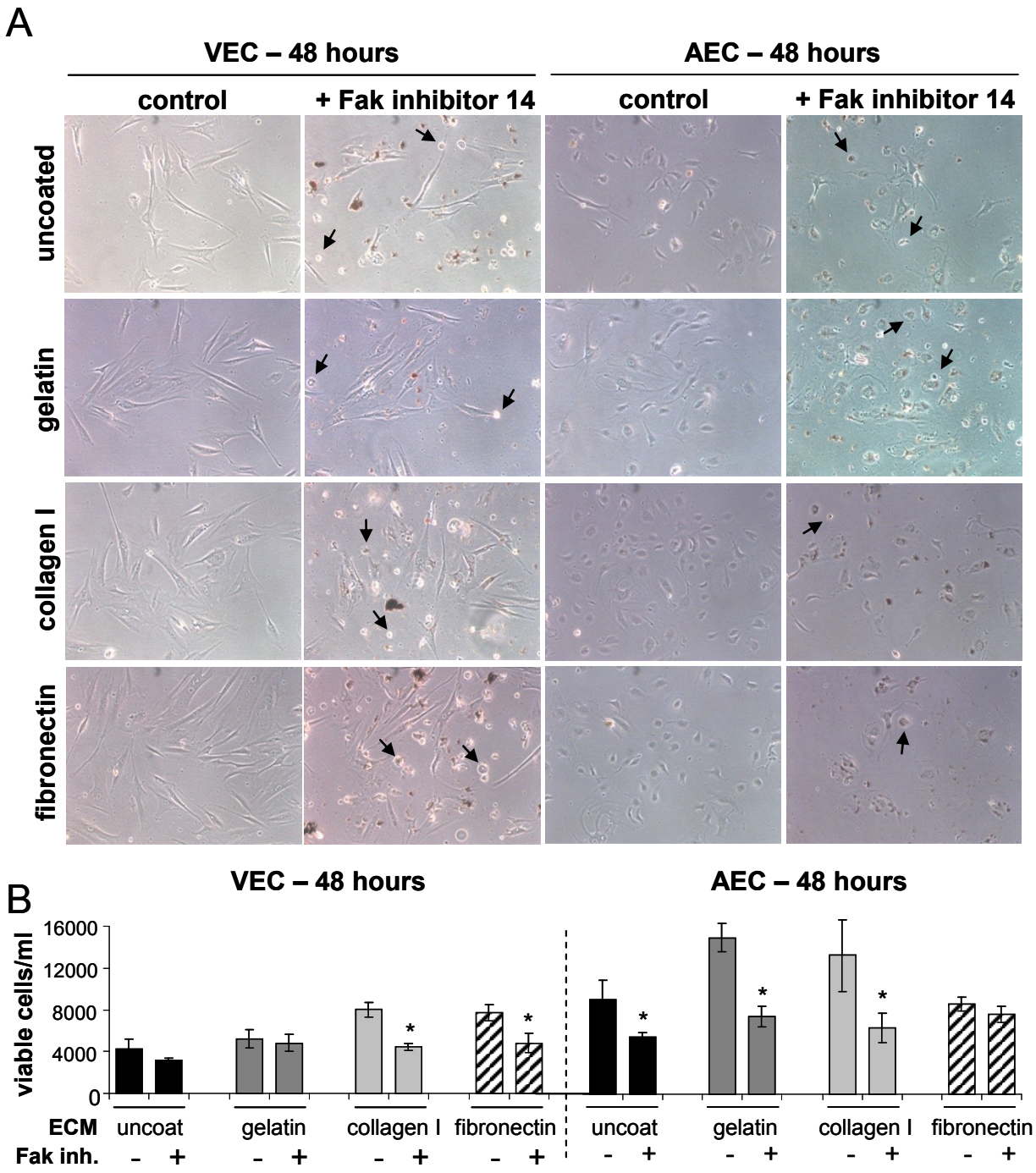


Figure 21: Fak inhibition and viability of primary placental VEC and AEC in different extracellular matrices at 21% oxygen. Cells were plated on ECM-coated and uncoated plates and cultured for 48 hours in the presence or absence of the Fak inhibitor 14 (25 µM). A) Cells were photographed in bright field (400x magnification). B) The number of viable cells was determined by automatic counting. Note that more cells are attached on the matrix-coated than on the uncoated plates. This difference is increased in the presence of Fak inhibitor, where a smaller number of attached and higher number of floating cells in the media can be observed (arrows). Data are means ± SD of 3 different cell isolations each measured in triplicates. * $p<0.05$ vs. absence of inhibitor. Scale bar: 200 µm

5. Differential integrin expression between arterial and venous endothelial cells

VEC and AEC show phenotypic differences, which result from anatomical and functional discrepancies between veins and arteries (Lang et al., 2008). Previous microarray data generated in our laboratory (Lassance, Desoye, Hiden, unpublished data) showed that VEC and AEC differentially express more than 3000 genes. In order to gain insight into potential heterogeneity in the integrin repertoire of VEC and AEC and their differential response to the ECM components, integrin expression was analyzed. Among the 18 α and 8 β integrin subunits described (Kim et al., 2011, Alam et al., 2007), AEC and VEC express 17 α and all of the 8 β subunits (Table 12). Among these, the integrins α5, αV, α4, β3, β4 and β8 are higher expressed in AEC than in VEC when they were grown on gelatine coated flasks at 21% oxygen (Table 12). Real-time PCR measurements and flow cytometric analysis for integrin αV (ITGAV), β3 (ITGB3) and β8 (ITGB8) confirmed the microarray results (performed in collaboration with Viktoria Konya and Akos Heinemann from Institute of Experimental and Clinical Pharmacology, Medical University of Graz). VE-cadherin, an endothelial cell marker, was used as positive control for flow cytometric analysis. Integrins αV, β3 and β8, key integrins for angiogenesis (Hood et al., 2003, Nisato et al., 2003, Zhu et al., 2002), are upregulated in AEC at both mRNA and protein level (Fig 22A,B). These findings confirm the differential expression pattern of αV, β3 and β8 in AEC and VEC, but do not explain their differential response to the ECM molecules.

Table 12 Human α and β integrin subunits expressed *in vitro* in primary placental arterial (AEC) and venous (VEC) endothelial cells as determined by microarray analysis.

		AEC	VEC	
Ligand binding properties	**integrins**	**expression**		**FC (p<0.05)**
Collagen receptors	α2	+++	+++	
(GFOGER)	α10	++	++	
	α11	++	++	
	β1	++++	++++	
RGD receptors	α5	++++	+++	1.25
(fibronectin, vitronectin and fibrinogen)	αV	++++	+++	1.39
	α8	+	+	
	αIIb	++	++	
	β3	+++	++	2.21
	β5	++	++	
	β6	+	+	
	β8	+++	++	6.15
Laminin receptors	α3	+++	+++	
	α6	+++	+++	
	α7	+	+	
	β4	+++	++	1.52
Leukocyte-specific receptors	α4	++++	+++	3.57
	α9	+	+	
	αE	+	+	
	αL	+	+	
	αM	++	++	
	αX	++	++	
	αD	++	++	
	β2	++	++	
	β7	++	++	

Differentially expressed integrins between AEC and VEC, with $p<0.05$ in ANOVA, are highlighted and the fold change (FC) indicated. The subunits are classified according to their ligand-binding properties and the individual expression levels represented by + (low expression) to ++++ (high expression). GFOGER and RGD sequences: peptides where single letter aminoacid nomenclature is used (O = hydroxyproline).

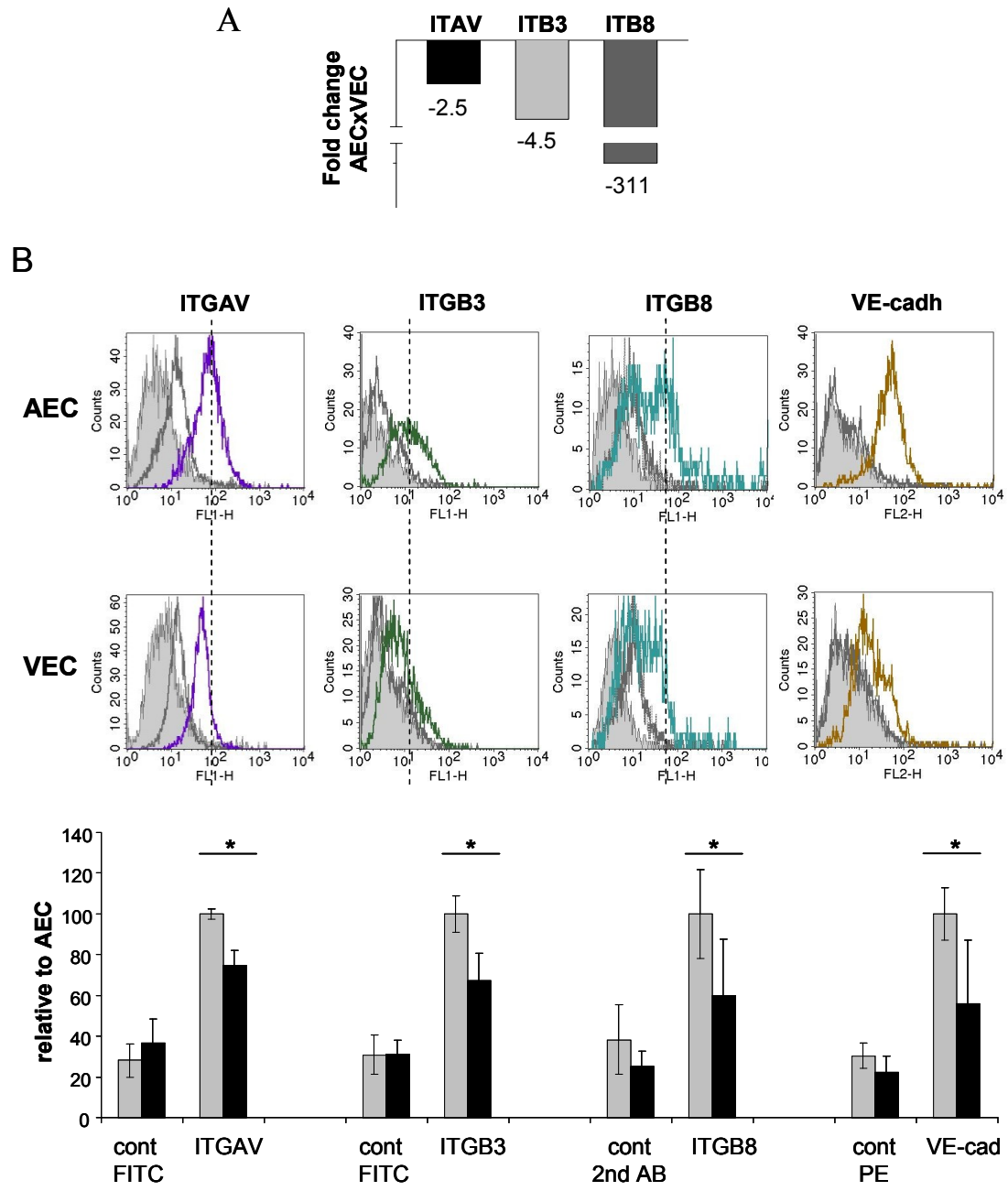


Figure 22: Validation of differential integrin expression between primary placental VEC and AEC. A) Real time PCR confirmed downregulation of the human integrin αV (ITGAV), β3 (ITGB3) and β8 (ITGB8) subunits in VEC compared to AEC. Data are presented as fold change with $p<0.02$ (*t*-test) from 6 VEC and AEC pairs, each of them in technical duplicates. B) Flow cytometry analysis of integrin subunits also confirmed the mRNA data (performed in collaboration with Viktoria Konya and Akos Heinemann from Institute of Experimental and Clinical Pharmacology, Medical University of Graz). Cells were incubated with mAbs against integrins αV (ITGAV), β3 (ITGB3) and β8 (ITGB8) and the endothelial marker VE-cadherin (VE-cad) as positive control. Open-colored graphs indicate the profile of cells positive for the different integrins after staining. The dotted vertical lines show the shift of integrin expression between VEC and AEC. The bar graphs represent the quantitative analysis of fluorescence values obtained in 3 experiments and normalized to AEC expression. Data are means ± SD of 3 different cell isolations each measured in duplicates. * $p<0.05$

6. Effect of ECM and oxygen on actin organization and cell shape of VEC and AEC

After ECM binding, integrins associate with the actin cytoskeleton and other structural proteins such as α-actinin, vinculin and talin, and signaling molecules such as paxillin, Src and Fak to form the focal adhesion complexes. One of the downstream effects of Fak phosphorylation after cell-ECM interaction is activation of the Rho subfamily of small GTPases, which will provide the signals for assembly and disassembly of the actin cytoskeleton. Actin assembly is a crucial step for lamellipodia and filopodia protusion (Cabodi et al., 2010, Ren et al., 2000, Aspenstrom, 1999). We aimed to determine possible effects on cell morphology and actin cytoskeleton organization as a consequence of VEC and AEC adhesion to the different ECM. Furthermore we wanted to investigate whether these possible changes could be oxygen dependent. To this end, VEC and AEC were grown in chamber slides coated with the different matrices at 12% and 21% oxygen for 48 hours and stained for actin filaments (F-actin) and vinculin (performed in collaboration with by Heidi Miedl, Department of Gynecology and Obstetrics, Medical University of Graz, Austria). Because effects of ECM and oxygen were similar between VEC and AEC, only pictures of AEC are shown. Both, AEC (Fig 23) and VEC had a flattened shape on fibronectin and laminin, with several actin bundles and high intensity F-actin staining (in red). Cells grown on collagen I showed an unorganized growing pattern, with more cells, smaller size and growing across each other, and also presented high intensity of F-actin staining when compared to gelatin and uncoated surfaces. On uncoated surfaces, some cells presented large membrane protusions (membrane ruffles), and the number of actin fibers appeared to be reduced. Membrane ruffles are formed when cells fail to establish a stable adhesion and start to detach from the matrix (Borm et al., 2005). Vinculin staining (green dots at the end of the actin fibers) revealed more focal adhesion complexes when both AEC (Fig 23) and VEC were grown on ECM-coated surfaces even though on laminin the focal adhesions were not as pronounced as on the other matrices. This observation parallels the weaker Fak phosphorylation when cells were seeded on laminin. These results confirmed the role of ECM in sustaining cell shape and activating focal adhesion complex formation. Oxygen was not a determinant of actin organization. However, there were always more cells and fewer visible membrane ruffles when both VEC and

AEC were grown on 21% oxygen. One interesting observation was that, again for both cell types, the stress fiber network was more disorganized when cells were grown on collagen I than on fibronectin or laminin; however, the actin bundles remained intact (Fig 23).

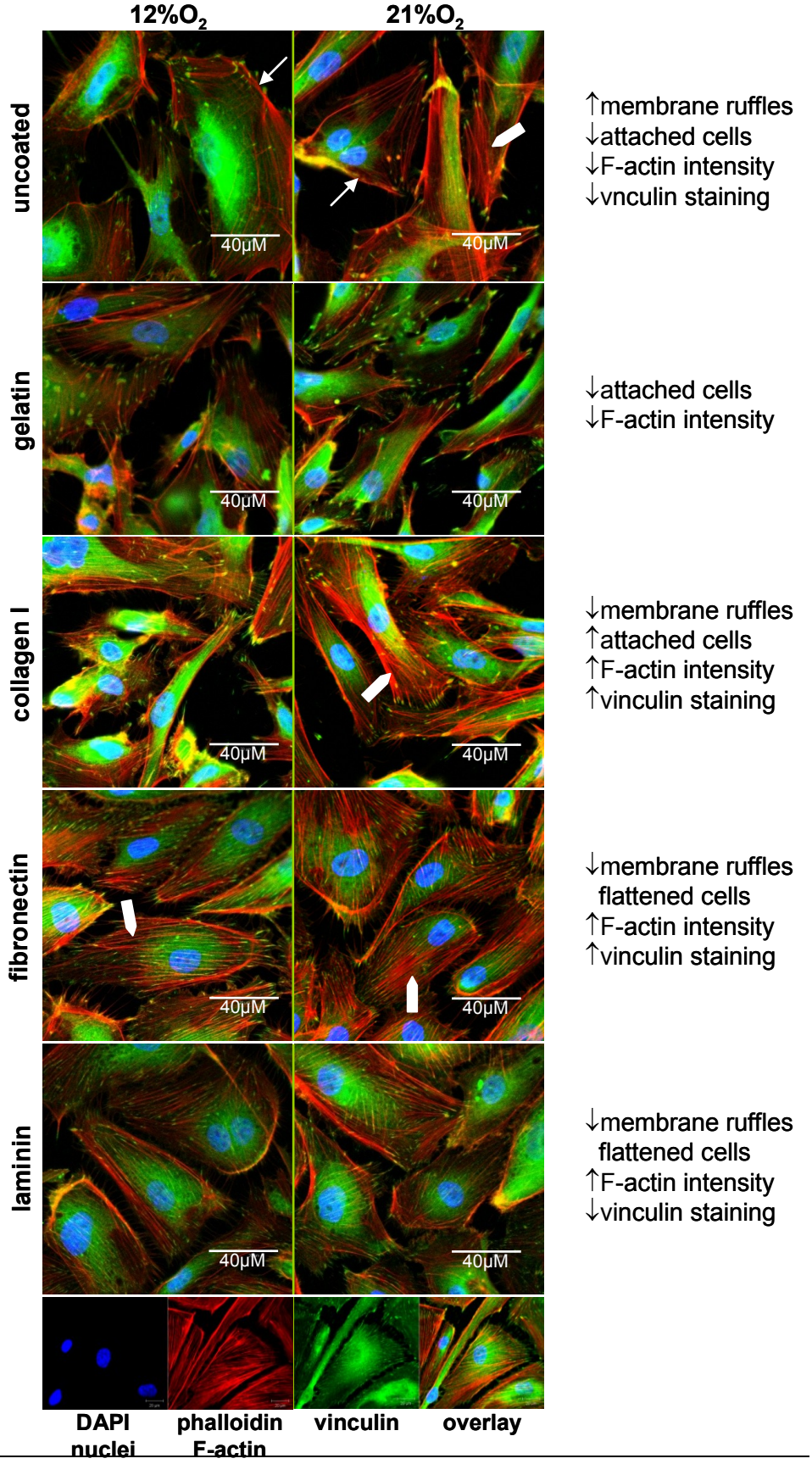


Figure 23: Assembly and organization of F-actin stress fibers and focal adhesions localization in primary placental AEC. Cells (50,000/well) were cultured on different ECM and on uncoated chamber slides at 12% and 21% oxygen for 48 hours. Thereafter they were washed, fixed, permeabilized, and stained with anti-vinculin antibody to detect focal adhesion complexes (green dots), and phalloidin Texas Red for F-actin staining (red fibers). Nuclei were stained with DAPI (blue). Note that cells grown on uncoated plates show fewer actin bundles (filled arrows) and more membrane ruffles (normal arrows) than cells grown on the other matrices. Cells grown on collagen I and gelatin are smaller than when they are grown in fibronectin and laminin. The actin fiber network shows a more organized and parallel organization on fibronectin and laminin than on collagen I, gelatin or on the uncoated slides. Cells are also arranged in a more organized monolayer on fibronectin and laminin whereas on collagen I cells grew to form multilayers. More focal adhesion complex staining can be oberserved with vinculin on gelatin, collagen I and fibronectin than on laminin and uncoated slides. Scale bar: 40 µm (performed in collaboration with by Heidi Miedl, Department of Gynecology and Obstetrics, Medical University of Graz, Austria).

Discussion

This study tested the hypothesis that anchorage-dependent processes differ between VEC and AEC from the human placenta and that these processes are modulated by oxygen. To the best of our knowledge this is the first study to investigate and compare the ECM effects on primary venous and arterial endothelial cells isolated from the same human organ and vascular loop.

Considerable progress has been made over the years in the understanding of anchorage-dependent cell proliferation and survival. Therefore, these processes have been chosen here as primary endpoints. A bulk of evidence has also demonstrated the strong influence of redox signaling and intracellular reactive oxygen species on the ECM-cell interaction (Chiarugi, 2008, Jean et al., 2011). However, this was beyond the scope of the present study. Rather we have concentrated on the influence of oxygen as a potential modulator of the ECM effect, because oxygen tension varies along the vascular tree (Nodwell et al., 2005). The ECM proteins were chosen on the basis of their presence in the basement membrane of the human placenta. Collagens, including collagen I and IV, laminins and fibronectin are present in the stroma of chorionic villi. These are the structural and functional units of the human placenta in which the feto-

placental vessels are embedded (Sati et al., 2008, Korhonen and Virtanen, 1997, Amenta et al., 1986, Yamada et al., 1987). In addition, gelatin was chosen as a widely used matrix for studying endothelial cell biology.

The data confirmed that there are not only phenotypic differences between VEC and AEC as previously described (Lang et al., 2003, Lang et al., 2008), but that the cells also respond differently to key components of the surrounding environment, i.e. ECM proteins and oxygen. The most important findings of this study are: 1) primary human placental VEC are more sensitive to changes in the matrix composition than AEC regarding proliferation, viability and survival; 2) these effects depend on Fak activation and are oxygen-modulated; 3) VEC are more susceptible to the oxygen-dependent ECM effects than AEC. Table 13 summarizes the findings of the effects of the different ECM in VEC and AEC on each process.

The extracellular fibrillar matrix of the placental villi manifests as a continuous network of collagen fibrils that have the role of sustaining the fetal arteries and veins (Vizza et al., 2001). Collagen IV is the major collagen present in the placental villi at term, but collagen I was also recently identified (Rukosuev et al., 1989, Sati et al., 2008). Both collagen I and IV increased similarly cell viability of primary term placental EC (Fig 18A). Therefore, subsequent experiments were performed with collagen I.

Fibronectin was the matrix with more consistent effects, affecting all of the processes studied in VEC in both oxygen conditions and promoting higher Fak phosphorylation in AEC at 21% oxygen. Collagen I, especially for VEC at 21% oxygen, also affected all of the processes analyzed under this condition. In fact, the effect size of collagen I was usually bigger than the one obtained for fibronectin, even though fibronectin showed more consistency. Intriguingly, AEC were less sensitive to changes in the ECM for the processes studied here. Laminin and gelatin were not as effective as fibronectin and collagen I.

Table 13 Summary of the processes affected by the different ECM in primary human placental VEC and AEC at distinct oxygen concentrations.

	VEC		AEC	
Process	**12% O2**	**21% O2**	**12% O2**	**21% O2**
Increases viable cells	fibronectin	collagen I fibronectin laminin gelatin	-	-
Increases % of cells in S phase	collagen I fibronectin laminin gelatin	collagen I fibronectin gelatin	collagen I fibronectin laminin gelatin	collagen I fibronectin laminin gelatin
Decreases apoptosis	collagen I gelatin	collagen I fibronectin gelatin	-	-
p-Fak	fibronectin ↑	collagen I ↑ fibronectin ↑ laminin ↓	-	laminin ↓
Fak inhibitor effects in viability	ND	collagen I ↓ fibronectin ↓	ND	collagen I ↓ gelatin/plastic ↓

As mentioned before, proliferation and survival of VEC were profoundly influenced by the ECM and oxygen conditions, whereas for AEC only proliferation (higher proportion of cells in S-phase) rather than survival was increased. All of these processes depend on the initial interaction of integrins with the ECM, which initiates a series of signals including Shc and Fak activation (Schwartz and Ginsberg, 2002, Chen et al., 1999). Fak activation by autophosphorylation at Tyr397 is one of the very early and crucial events that lead to exposure of the docking site for Src kinases, which phosphorylate additional sites ultimately resulting in full Fak activation. (Hanks et al., 1992, Schaller et al., 1994, Lipfert et

al., 1992, Guan et al., 1991). The strong Fak auto-phosphorylation in VEC induced by collagen I and fibronectin at 21% oxygen correlates with the increase in cell viability and proportion of cells in S-phase as well as with the reduction in apoptosis. Gelatin, which is not a component of the basement membrane itself, but a result of denaturation and fragmentation of collagen (Adachi et al., 2010), was also able to induce similar effects, although not as pronounced, on Fak phosphorylation and, consequently, proliferation, viability and survival of VEC.

Although viability, survival and Fak phosphorylation was not substantially increased by the different ECM in AEC, the proportion of cells in S-phase was higher than for VEC. A plausible scenario is the well established synergism between ECM-integrin interaction and growth-factor receptor activation by components present in the FCS (Alam et al., 2007, Bill et al., 2004, Ruoslahti, 2002, Kim et al., 2011), which may also activate the MAPK pathway independent of Fak (Juliano, 1996, Boudreau and Jones, 1999). However, this would need further investigation in our cell model. Another intriguing result was the decrease in Fak phosphorylation at 21% oxygen when cells were grown on laminin coated plates.

The results obtained in the presence of the Fak inhibitor confirmed the important role of Fak activation by cell-ECM interaction for both VEC and AEC viability and proliferation as well as in rescuing them from anoikis. Anoikis describes the process when cells undergo apoptosis by inappropriate cell-matrix interaction and, consequently, absent or incomplete Fak activation (Maubant et al., 2006, Frisch and Screaton, 2001). The Fak inhibitor mimicked this phenomenon leading to a decrease in the number of viable cells. The survival effect of Fak activation is a consequence of its binding to PI3K (Reiske et al., 1999) and subsequent activation of PkB/Akt kinase protecting cells from apoptosis (Frisch et al., 1996, Hennessy et al., 2005). For AEC, however, the effect of the Fak inhibitor was not significant when cells were grown on fibronectin, probably for the same reason as discussed before, a synergism of integrin-ECM interaction with growth factor receptor activation, rescuing them from apoptosis in a Fak-independent manner. Indeed, AEC had higher expression of many of the fibronectin binding integrins, such as subunits αv and β3. These participate in the integrin heterodimers αvβ3 and αvβ5, which can cooperate with different growth factor receptors, as FGFR and VEGFR (Hodivala-Dilke et al., 2003). The anti-apoptotic

function of integrin αvβ3 is known because its blocking interferes with cell anchorage and, thus, causes anoikis (Brooks et al., 1994). Despite the differential expression of some key integrins between VEC and AEC, their distinct response to the extracellular matrices cannot be explained by a straightforward mechanism. Table 12 shows the integrin subunits expression pattern in VEC and AEC when grown at 21% oxygen. As the microarray data is part of another study, the effect of different oxygen concentrations was not investigated. Therefore, the differences in global gene expression of VEC and AEC at 12 and 21% oxygen need further investigation.

It is well known that cell-interactions with ECM components can also cause alterations in cell shape and morphology (Jones et al., 1997, Wickstrom et al., 2001). These alterations result from signals downstream of the integrin-focal adhesion complexes and activate members of the Rho family of GTPases, such as Cdc42, Rac1 and RhoA, which control cytoskeleton organization (Clark et al., 1998, Vartanian et al., 2008). Different from the other processes analyzed in this study, the ECM effects on actin organization were similar in VEC and AEC. Both cell types changed their shape to a more rounded form when they were grown on uncoated plates. Under this condition they also displayed more membrane ruffling, which is a consequence of a non-stable adhesion to the surface leading to cell detachment (Borm et al., 2005). Collagen I stimulated formation of less organized actin bundles and also a less structured monolayer in both VEC and AEC. On fibronectin and laminin the actin bundles were more paralleled and cells grew as a homogeneous monolayer, whereas in collagen I cells proliferated more and, because of lack of space, grew in multiple layers. These collective data reinforce the role of ECM molecules in keeping cell shape and morphology also in primary placental EC. Oxygen did not cause changes in actin organization in both VEC and AEC, but increased the number of cells and decreased membrane ruffling.

One important study aim was to investigate the role of oxygen as modulator of anchorage-dependent processes. The data collectively demonstrate that oxygen can modulate ECM effects on proliferation, viability, survival and Fak activation, mainly in VEC. Exposure to higher oxygen concentrations improved VEC viability and survival as well as Fak activation especially when cells were grown on collagen I and fibronectin. So far, most of the studies on oxygen and ECM demonstrated higher proliferation levels, better adhesion and increased

angiogenesis under hypoxic conditions via HIF-1a activation as an adaptive response to oxygen deprivation (Jean et al., 2011, Chiarugi, 2008, Corley et al., 2005). Few studies demonstrated that higher oxygen levels increase ECM effects on cell proliferation and survival. For instance, hyperoxia increases VEGF protein expression in type II cell-like adenocarcinoma cell line A549 enhancing proliferation and survival rates (Shenberger et al., 2007) and stimulates proliferation as well as collagen I synthesis in rat lung fibroblasts (Chen et al., 2007, Lang et al., 2010, Adachi et al., 2010). In the present study, however, we could also demonstrate that hyperoxia increases the proliferative ECM effects in primary EC.

One limitation of this study was the occasional considerable variation of effect size of the ECM on the endpoints between different cell isolations, i.e. placentas (cf. collagen I effect in Fig 18). However, the response of each ECM relative to others was reproducible in all cell isolations tested. This is a limitation when working with primary cell cultures and reflects the biological variation of the tissue of origin. The disadvantage, however, is outweighed by the results closer reflecting the *in vivo* situation than using established cell lines, which after several passages can undergo a phenotypic drift (Hughes et al., 2007, Pastor et al., 2010, Adachi et al., 2010).

The present findings help understanding pathophysiological changes in the placenta associated with altered ECM composition. One of these is gestational diabetes mellitus, characterized by thickening of the placental basement membrane as a consequence of a higher production and accumulation of collagens, and by an increase in EC proliferation (Pietryga et al., 2004, Adachi et al., 2010). This parallels our *in vitro* results demonstrating higher proliferation of primary placental EC in the presence of collagen I and fibronectin, and highlights the biological relevance of our results.

In summary, our data provide the first evidence that primary human VEC and AEC from the same organ and vascular loop respond differently when exposed to different ECM components and oxygen conditions, and this may be modulated by differential Fak activation, integrin expression and, as a speculation, synergism with growth factor receptors.

Overall conclusions

The main objective of this study was to investigate the possible causes and consequences for the altered phenotype of placentas derived from gestational diabetes. Among them increased size/weight, thickening of the basement membrane and hypervascularization are best described (Winick and Noble, 1967, Naeye, 1987, Desoye and Shafrir, 1996, Molteni et al., 1978, Sherer and Divon, 1996, Jirkovska et al., 2002, Leach et al., 2004, Burstein et al., 1957, Pietryga et al., 2004, Giachini et al., 2008, Forsberg et al., 1998). In GDM the concentrations of insulin and IGF2, central growth factors for the placenta and the fetus, are elevated in the fetal circulation (Desoye and Shafrir, 1996, Desoye and Hauguel-de Mouzon, 2007). Their receptors are expressed on the placental endothelium, which is in contact with the fetal blood, but their cellular effects on the feto-placental vasculature have remained elusive. ECM composition is also altered in placentas from pregnancies complicated with GDM. Therefore the aims of the study were 1) to analyze whether insulin and IGF2 can modulate gene expression in AEC and VEC; 2) to identify placental processes regulated by fetal insulin and IGF2; 3) to delineate the major signalling pathways induced by insulin regulating these processes; 4) to investigate whether insulin has an effect on angiogenesis of placental EC and delineate the pathways that could be involved; 5) to study potential effects of ECM on EC proliferation, survival, and behaviour and 6) to determine whether oxygen plays a role on the insulin/IGF2 and ECM effects on EC.

Global expression profiling after insulin and IGF2 treatment revealed that insulin and IGF2 regulate different biological processes in AEC and VEC, and in AEC some of these processes are overlapping. Pathway analysis confirmed this differential effect of insulin and IGF2 in AEC and VEC. Insulin and IGF2 regulated more genes and affected more biological processes in AEC, which could be explained by the fact that AEC express more IR than VEC (Hiden, personal communication). In general, cell adhesion/junction, lipid metabolism, cell growth, angiogenesis and immuno-related pathways were the main targets of these growth factors on feto-placental EC. These processes are also often altered in diabetes, atherosclerosis and cardiovascular diseases.

As insulin regulated more biological processes in AEC than IGF2 and AEC express more IR than VEC, the further investigations were focused on insulin short-term effects on AEC. I found that insulin stimulates *in vitro* angiogenesis via the IRS1/PI3K/Akt signaling pathway in AEC and involves downstream eNOS and Rac1 activation. However, insulin did not induce proliferation even though G1/S-phase progression was observed. Insulin also induces actin rearrangements in AEC and this is mediated by the eNOS/Rac1 signaling pathway. These results show for the first time the pro-angiogenic effect of insulin on placental endothelial cells and delineate the signaling pathways that could result in increased placental vascularization in GDM.

As previously described, AEC and VEC present phenotypical differences and differential basal gene expression profiles (Lang et al., 2003, Lang et al., 2008), so they could also respond differently to the surrounding environment. As some basement membrane proteins, including collagens, fibronectin and laminin were found in higher amounts in placentas from diabetic pregnancies, I finally investigated the role of different ECM proteins on EC proliferation, survival and behavior. The most important findings were that VEC are more sensitive to changes in the matrix composition than AEC regarding proliferation, viability and survival. These effects depend on Fak activation and are oxygen-modulated. VEC are more susceptible to the oxygen-dependent ECM effects than AEC.

In summary, these data demonstrate that insulin and IGF2 have long term effects on placental AEC and VEC regulating gene expression. Insulin also has short term effects on AEC, acting as a pro-angiogenic factor increasing *in vitro* angiogenesis. Basement membrane components also influence placental EC behavior, but with differential effects depending on the vascular bed of origin and oxygen concentration. Taken together, these findings suggest that altered insulin/IGF2 levels and basement membrane constitution may contribute to the hypervascularization observed in placentas from diabetic pregnancies.

Acknowledgements

To Heidi Miedl, from the Department of Gynecology and Obstetrics, Medical University of Graz, for her support with the fluorescence microscopy and BrdU experiments.

To Josef Haas, from the Department of Gynecology and Obstetrics, Medical University of Graz, for his assistance with the statistics.

To Heimo Strohmaier and Heike Knausz, from the Flow Cytometry Core Facility, and to Eleonore Fröhlich and Markus Absenger from the Microscopy Core Facilitiy of the Center of Medical Research, Graz, Austria, for their expert technical assistance.

To Diego Guidolin from the Department of Human Anatomy and Physiology, Section of Anatomy, University of Padova, Italy, who developed and kindly provided us with the AngioJ-Matrigel assay plugin, for the ImageJ software (NIH).

References

ADACHI, T., WANG, X., MURATA, T., OBARA, M., AKUTSU, H., MACHIDA, M., UMEZAWA, A. & TOMITA, M. 2010. Production of a non-triple helical collagen alpha chain in transgenic silkworms and its evaluation as a gelatin substitute for cell culture. *Biotechnol Bioeng,* 106**,** 860-70.

AERTS, L., HOLEMANS, K. & VAN ASSCHE, F. A. 1990. Maternal diabetes during pregnancy: consequences for the offspring. *Diabetes Metab Rev,* 6**,** 147-67.

AHMAD, S., HEWETT, P. W., WANG, P., AL-ANI, B., CUDMORE, M., FUJISAWA, T., HAIGH, J. J., LE NOBLE, F., WANG, L., MUKHOPADHYAY, D. & AHMED, A. 2006. Direct evidence for endothelial vascular endothelial growth factor receptor-1 function in nitric oxide-mediated angiogenesis. *Circ Res,* 99**,** 715-22.

AL-OKAIL, M. S. & AL-ATTAS, O. S. 1994. Histological changes in placental syncytiotrophoblasts of poorly controlled gestational diabetic patients. *Endocr J,* 41**,** 355-60.

ALAM, N., GOEL, H. L., ZARIF, M. J., BUTTERFIELD, J. E., PERKINS, H. M., SANSOUCY, B. G., SAWYER, T. K. & LANGUINO, L. R. 2007. The integrin-growth factor receptor duet. *J Cell Physiol,* 213**,** 649-53.

AMENTA, P. S., GAY, S., VAHERI, A. & MARTINEZ-HERNANDEZ, A. 1986. The extracellular matrix is an integrated unit: ultrastructural localization of collagen types I, III, IV, V, VI, fibronectin, and laminin in human term placenta. *Coll Relat Res,* 6**,** 125-52.

ANASTASIOU, E., LEKAKIS, J. P., ALEVIZAKI, M., PAPAMICHAEL, C. M., MEGAS, J., SOUVATZOGLOU, A. & STAMATELOPOULOS, S. F. 1998. Impaired endothelium-dependent vasodilatation in women with previous gestational diabetes. *Diabetes Care,* 21**,** 2111-5.

ANNAS, A., GRANBERG, A. L. & BRITTEBO, E. B. 2000. Differential response of cultured human umbilical vein and artery endothelial cells to Ah receptor agonist treatment: CYP-dependent activation of food and environmental mutagens. *Toxicol Appl Pharmacol,* 169**,** 94-101.

ANTONETTI, D. A., BARBER, A. J., KHIN, S., LIETH, E., TARBELL, J. M. & GARDNER, T. W. 1998. Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. Penn State Retina Research Group. *Diabetes,* 47**,** 1953-9.

ARNAOUTOVA, I., GEORGE, J., KLEINMAN, H. K. & BENTON, G. 2009. The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis,* 12**,** 267-74.

ASPENSTROM, P. 1999. Effectors for the Rho GTPases. *Curr Opin Cell Biol,* 11**,** 95-102.

BABAWALE, M. O., LOVAT, S., MAYHEW, T. M., LAMMIMAN, M. J., JAMES, D. K. & LEACH, L. 2000. Effects of gestational diabetes on junctional adhesion molecules in human term placental vasculature. *Diabetologia,* 43**,** 1185-96.

BANERJEE, M. & CRUICKSHANK, J. K. 2006. Pregnancy as the prodrome to vascular dysfunction and cardiovascular risk. *Nat Clin Pract Cardiovasc Med,* 3**,** 596-603.

BARDEN, A., SINGH, R., WALTERS, B. N., RITCHIE, J., ROBERMAN, B. & BEILIN, L. J. 2004. Factors predisposing to pre-eclampsia in women with gestational diabetes. *J Hypertens,* 22**,** 2371-8.

BARTHA, J. L., MARTINEZ-DEL-FRESNO, P. & COMINO-DELGADO, R. 2000. Gestational diabetes mellitus diagnosed during early pregnancy. *Am J Obstet Gynecol,* 182**,** 346-50.

BASSAGANYA-RIERA, J., GURI, A. J. & HONTECILLAS, R. 2011. Treatment of Obesity-Related Complications with Novel Classes of Naturally Occurring PPAR Agonists. *J Obes,* 2011**,** 897894.

BAUER, J. W., BILGIC, H. & BAECHLER, E. C. 2009. Gene-expression profiling in rheumatic disease: tools and therapeutic potential. *Nat Rev Rheumatol,* 5**,** 257-65.

BAZZONI, G. & DEJANA, E. 2004. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev,* 84**,** 869-901.

BEGO, T., DUJIC, T., MLINAR, B., SEMIZ, S., MALENICA, M., PRNJAVORAC, B., OSTANEK, B., MARC, J. & CAUSEVIC, A. 2011. Association of PPARG and LPIN1 gene polymorphisms with metabolic syndrome and type 2 diabetes. *Med Glas Ljek komore Zenicko-doboj kantona,* 8**,** 76-83.

BEIERLE, E. A., MA, X., TRUJILLO, A., KURENOVA, E. V., CANCE, W. G. & GOLUBOVSKAYA, V. M. 2010. Inhibition of focal adhesion kinase and src increases detachment and apoptosis in human neuroblastoma cell lines. *Mol Carcinog,* 49**,** 224-34.

BELLAMY, L., CASAS, J. P., HINGORANI, A. D. & WILLIAMS, D. 2009. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. *Lancet,* 373**,** 1773-9.

BEN-HAROUSH, A., YOGEV, Y. & HOD, M. 2004. Epidemiology of gestational diabetes mellitus and its association with Type 2 diabetes. *Diabet Med,* 21**,** 103-13.

BILL, H. M., KNUDSEN, B., MOORES, S. L., MUTHUSWAMY, S. K., RAO, V. R., BRUGGE, J. S. & MIRANTI, C. K. 2004. Epidermal growth factor receptor-dependent regulation of integrin-mediated signaling and cell cycle entry in epithelial cells. *Mol Cell Biol,* 24**,** 8586-99.

BIRNBACHER, R., AMANN, G., BREITSCHOPF, H., LASSMANN, H., SUCHANEK, G. & HEINZ-ERIAN, P. 1998. Cellular localization of insulin-like growth factor II mRNA in the human fetus and the placenta: detection with a digoxigenin-labeled cRNA probe and immunocytochemistry. *Pediatr Res,* 43**,** 614-20.

BLASCHITZ, A., WEISS, U., DOHR, G. & DESOYE, G. 2000. Antibody reaction patterns in first trimester placenta: implications for trophoblast isolation and purity screening. *Placenta,* 21**,** 733-41.

BO, S., VALPREDA, S., MENATO, G., BARDELLI, C., BOTTO, C., GAMBINO, R., RABBIA, C., DURAZZO, M., CASSADER, M., MASSOBRIO, M. & PAGANO, G. 2007. Should we consider gestational diabetes a vascular risk factor? *Atherosclerosis,* 194**,** e72-9.

BORM, B., REQUARDT, R. P., HERZOG, V. & KIRFEL, G. 2005. Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization. *Exp Cell Res,* 302**,** 83-95.

BOSCO, E. E., MULLOY, J. C. & ZHENG, Y. 2009. Rac1 GTPase: a "Rac" of all trades. *Cell Mol Life Sci,* 66**,** 370-4.

BOUDREAU, N. J. & JONES, P. L. 1999. Extracellular matrix and integrin signalling: the shape of things to come. *Biochem J,* 339 (Pt 3)**,** 481-8.

BOYANOVSKY, B., ZACK, M., FORREST, K. & WEBB, N. R. 2009. The capacity of group V sPLA2 to increase atherogenicity of ApoE-/- and LDLR-/- mouse LDL in vitro predicts its atherogenic role in vivo. *Arterioscler Thromb Vasc Biol,* 29**,** 532-8.

BRENNAN, P., BABBAGE, J. W., BURGERING, B. M., GRONER, B., REIF, K. & CANTRELL, D. A. 1997. Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity,* 7**,** 679-89.

BROOKS, P. C., MONTGOMERY, A. M., ROSENFELD, M., REISFELD, R. A., HU, T., KLIER, G. & CHERESH, D. A. 1994. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell,* 79**,** 1157-64.

BURSTEIN, R., SOULE, S. D. & BLUMENTHAL, H. T. 1957. Histogenesis of pathological processes in placentas of metabolic disease in pregnancy. II. The diabetic state. *Am J Obstet Gynecol,* 74**,** 96-104.

CABODI, S., DI STEFANO, P., LEAL MDEL, P., TINNIRELLO, A., BISARO, B., MORELLO, V., DAMIANO, L., ARAMU, S., REPETTO, D., TORNILLO, G. & DEFILIPPI, P. 2010. Integrins and signal transduction. *Adv Exp Med Biol,* 674**,** 43-54.

CARPENTER, M. W. 2007. Gestational diabetes, pregnancy hypertension, and late vascular disease. *Diabetes Care,* 30 Suppl 2**,** S246-50.

CATALANO, P. M., TYZBIR, E. D., WOLFE, R. R., CALLES, J., ROMAN, N. M., AMINI, S. B. & SIMS, E. A. 1993. Carbohydrate metabolism during pregnancy in control subjects and women with gestational diabetes. *Am J Physiol,* 264**,** E60-7.

CERVAR, M., BLASCHITZ, A., DOHR, G. & DESOYE, G. 1999. Paracrine regulation of distinct trophoblast functions in vitro by placental macrophages. *Cell Tissue Res,* 295**,** 297-305.

CHEN, C. M., WANG, L. F., CHOU, H. C., LANG, Y. D. & LAI, Y. P. 2007. Up-regulation of connective tissue growth factor in hyperoxia-induced lung fibrosis. *Pediatr Res,* 62**,** 128-33.

CHEN, K. D., LI, Y. S., KIM, M., LI, S., YUAN, S., CHIEN, S. & SHYY, J. Y. 1999. Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. *J Biol Chem,* 274**,** 18393-400.

CHIARUGI, P. 2008. From anchorage dependent proliferation to survival: lessons from redox signalling. *IUBMB Life,* 60**,** 301-7.

CHRISTOPHE-HOBERTUS, C., SZPIRER, C., GUYON, R. & CHRISTOPHE, D. 2001. Identification of the gene encoding Brain Cell Membrane Protein 1 (BCMP1), a putative four-transmembrane protein distantly related to the Peripheral Myelin Protein 22 / Epithelial Membrane Proteins and the Claudins. *BMC Genomics,* 2**,** 3.

CLARK, E. A., KING, W. G., BRUGGE, J. S., SYMONS, M. & HYNES, R. O. 1998. Integrin-mediated signals regulated by members of the rho family of GTPases. *J Cell Biol,* 142**,** 573-86.

CONACCI-SORRELL, M., ZHURINSKY, J. & BEN-ZE'EV, A. 2002. The cadherin-catenin adhesion system in signaling and cancer. *J Clin Invest,* 109**,** 987-91.

CORLEY, K. M., TAYLOR, C. J. & LILLY, B. 2005. Hypoxia-inducible factor 1alpha modulates adhesion, migration, and FAK phosphorylation in vascular smooth muscle cells. *J Cell Biochem,* 96**,** 971-85.

COSO, O. A., CHIARIELLO, M., YU, J. C., TERAMOTO, H., CRESPO, P., XU, N., MIKI, T. & GUTKIND, J. S. 1995. The small GTP-binding proteins Rac1 and

Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell,* 81**,** 1137-46.

DALCIK, H., YARDIMOGLU, M., VURAL, B., DALCIK, C., FILIZ, S., GONCA, S., KOKTURK, S. & CEYLAN, S. 2001. Expression of insulin-like growth factor in the placenta of intrauterine growth-retarded human fetuses. *Acta Histochem,* 103**,** 195-207.

DAVIES, M. J., GORDON, J. L., GEARING, A. J., PIGOTT, R., WOOLF, N., KATZ, D. & KYRIAKOPOULOS, A. 1993. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *J Pathol,* 171**,** 223-9.

DAVIS, G. E. & SENGER, D. R. 2005. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circ Res,* 97**,** 1093-107.

DELA PAZ, N. G. & D'AMORE, P. A. 2009. Arterial versus venous endothelial cells. *Cell Tissue Res,* 335**,** 5-16.

DENLEY, A., WALLACE, J. C., COSGROVE, L. J. & FORBES, B. E. 2003. The insulin receptor isoform exon 11- (IR-A) in cancer and other diseases: a review. *Horm Metab Res,* 35**,** 778-85.

DESOYE, G., HARTMANN, M., BLASCHITZ, A., DOHR, G., HAHN, T., KOHNEN, G. & KAUFMANN, P. 1994. Insulin receptors in syncytiotrophoblast and fetal endothelium of human placenta. Immunohistochemical evidence for developmental changes in distribution pattern. *Histochemistry,* 101**,** 277-85.

DESOYE, G., HARTMANN, M., JONES, C. J., WOLF, H. J., KOHNEN, G., KOSANKE, G. & KAUFMANN, P. 1997. Location of insulin receptors in the placenta and its progenitor tissues. *Microsc Res Tech,* 38**,** 63-75.

DESOYE, G. & HAUGUEL-DE MOUZON, S. 2007. The human placenta in gestational diabetes mellitus. The insulin and cytokine network. *Diabetes Care,* 30 Suppl 2**,** S120-6.

DESOYE, G. & SHAFRIR, E. 1996. The human placenta in diabetic pregnancy. *Diabetes Rev,* 4**,** 70-89.

DIAMANT, Y. Z. 1991. The human placenta in diabetes mellitus. *Isr J Med Sci,* 27**,** 493-7.

DIEHL, J. A., CHENG, M., ROUSSEL, M. F. & SHERR, C. J. 1998. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev,* 12**,** 3499-511.

DUDEK, S. M., MUNOZ, N. M., DESAI, A., OSAN, C. M., MELITON, A. Y. & LEFF, A. R. 2011. Group V phospholipase A2 mediates barrier disruption of human pulmonary endothelial cells caused by LPS in vitro. *Am J Respir Cell Mol Biol,* 44**,** 361-8.

ENQUOBAHRIE, D. A., WILLIAMS, M. A., QIU, C., MELLER, M. & SORENSEN, T. K. 2009. Global placental gene expression in gestational diabetes mellitus. *Am J Obstet Gynecol,* 200**,** 206 e1-13.

ESPINOSA, E., GAMEZ-POZO, A., SANCHEZ-NAVARRO, I., PINTO, A., CASTANEDA, C. A., CIRUELOS, E., FELIU, J. & VARA, J. A. 2011. The present and future of gene profiling in breast cancer. *Cancer Metastasis Rev*.

FALKENHAIN A, L. T., BEHRENDT U & LEHMANN L 2002. *Dead cell estimation – a comparison of different methods, New Developments and New Applications in Animal Cell Technology*, Kluwer Academic Publishers.

FOLKMAN, J. 1985. Tumor angiogenesis. *Adv Cancer Res,* 43**,** 175-203.

FOLKMAN, J. 1987. What is the role of angiogenesis in metastasis from cutaneous melanoma? *Eur J Cancer Clin Oncol,* 23**,** 361-3.

FOLKMAN, J. & KLAGSBRUN, M. 1987. Angiogenic factors. *Science,* 235**,** 442-7.

FOLKMAN, J. & SHING, Y. 1992. Angiogenesis. *J Biol Chem,* 267**,** 10931-4.

FORBES, K. & WESTWOOD, M. 2010. Maternal growth factor regulation of human placental development and fetal growth. *J Endocrinol,* 207**,** 1-16.

FORSBERG, H., WENTZEL, P. & ERIKSSON, U. J. 1998. Maternal diabetes alters extracellular matrix protein levels in rat placentas. *Am J Obstet Gynecol,* 179**,** 772-8.

FORTI, F. L., SCHWINDT, T. T., MORAES, M. S., EICHLER, C. B. & ARMELIN, H. A. 2002. ACTH promotion of p27(Kip1) induction in mouse Y1 adrenocortical tumor cells is dependent on both PKA activation and Akt/PKB inactivation. *Biochemistry,* 41**,** 10133-40.

FRASCA, F., PANDINI, G., SCALIA, P., SCIACCA, L., MINEO, R., COSTANTINO, A., GOLDFINE, I. D., BELFIORE, A. & VIGNERI, R. 1999. Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol Cell Biol,* 19**,** 3278-88.

FRISCH, S. M. & SCREATON, R. A. 2001. Anoikis mechanisms. *Curr Opin Cell Biol,* 13**,** 555-62.

FRISCH, S. M., VUORI, K., RUOSLAHTI, E. & CHAN-HUI, P. Y. 1996. Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol,* 134**,** 793-9.

FUKUMOTO, S., HSIEH, C. M., MAEMURA, K., LAYNE, M. D., YET, S. F., LEE, K. H., MATSUI, T., ROSENZWEIG, A., TAYLOR, W. G., RUBIN, J. S., PERRELLA, M. A. & LEE, M. E. 2001. Akt participation in the Wnt signaling pathway through Dishevelled. *J Biol Chem,* 276**,** 17479-83.

FULTON, D., GRATTON, J. P., MCCABE, T. J., FONTANA, J., FUJIO, Y., WALSH, K., FRANKE, T. F., PAPAPETROPOULOS, A. & SESSA, W. C. 1999. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature,* 399**,** 597-601.

GAITHER, K., QURAISHI, A. N. & ILLSLEY, N. P. 1999. Diabetes alters the expression and activity of the human placental GLUT1 glucose transporter. *J Clin Endocrinol Metab,* 84**,** 695-701.

GAMBINERI, A., TOMASSONI, F., MUNARINI, A., STIMSON, R. H., MIONI, R., PAGOTTO, U., CHAPMAN, K. E., ANDREW, R., MANTOVANI, V., PASQUALI, R. & WALKER, B. R. 2011. A combination of polymorphisms in HSD11B1 associates with in vivo 11{beta}-HSD1 activity and metabolic syndrome in women with and without polycystic ovary syndrome. *Eur J Endocrinol,* 165**,** 283-92.

GAO, B., CURTIS, T. M., BLUMENSTOCK, F. A., MINNEAR, F. L. & SABA, T. M. 2000. Increased recycling of (alpha)5(beta)1 integrins by lung endothelial cells in response to tumor necrosis factor. *J Cell Sci,* 113 Pt 2**,** 247-57.

GAUSTER, M., DESOYE, G., TOTSCH, M. & HIDEN, U. 2011a. The Placenta and Gestational Diabetes Mellitus. *Curr Diab Rep*.

GAUSTER, M., HIDEN, U., VAN POPPEL, M., FRANK, S., WADSACK, C., HAUGUEL-DE MOUZON, S. & DESOYE, G. 2011b. Dysregulation of placental endothelial lipase in obese women with gestational diabetes mellitus. *Diabetes,* 60**,** 2457-64.

GEWOLB, I. H., MERDIAN, W., WARSHAW, J. B. & ENDERS, A. C. 1986. Fine structural abnormalities of the placenta in diabetic rats. *Diabetes,* 35**,** 1254-61.

GIACCO, F. & BROWNLEE, M. 2010. Oxidative stress and diabetic complications. *Circ Res,* 107**,** 1058-70.

GIACHINI, F. R., CARRIEL, V., CAPELO, L. P., TOSTES, R. C., CARVALHO, M. H., FORTES, Z. B., ZORN, T. M. & SAN MARTIN, S. 2008. Maternal diabetes affects specific extracellular matrix components during placentation. *J Anat,* 212**,** 31-41.

GOLDSTEIN, B. J., MAHADEV, K., WU, X., ZHU, L. & MOTOSHIMA, H. 2005. Role of insulin-induced reactive oxygen species in the insulin signaling pathway. *Antioxid Redox Signal,* 7**,** 1021-31.

GOLUBOVSKAYA, V. M., NYBERG, C., ZHENG, M., KWEH, F., MAGIS, A., OSTROV, D. & CANCE, W. G. 2008. A small molecule inhibitor, 1,2,4,5-benzenetetraamine tetrahydrochloride, targeting the y397 site of focal adhesion kinase decreases tumor growth. *J Med Chem,* 51**,** 7405-16.

GREGORY, W. B., JR. 1965. Effect of Insulin on the Healing of Bone Wounds in Albino Rats. *J Dent Res,* 44**,** 487-92.

GUAN, J. L., TREVITHICK, J. E. & HYNES, R. O. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Regul,* 2**,** 951-64.

GUNDUZ, D., THOM, J., HUSSAIN, I., LOPEZ, D., HARTEL, F. V., ERDOGAN, A., GREBE, M., SEDDING, D., PIPER, H. M., TILLMANNS, H., NOLL, T. & ASLAM, M. 2010. Insulin stabilizes microvascular endothelial barrier function via phosphatidylinositol 3-kinase/Akt-mediated Rac1 activation. *Arterioscler Thromb Vasc Biol,* 30**,** 1237-45.

GURAY, U., ERBAY, A. R., GURAY, Y., YILMAZ, M. B., BOYACI, A. A., SASMAZ, H., KORKMAZ, S. & KUTUK, E. 2004. Levels of soluble adhesion molecules in various clinical presentations of coronary atherosclerosis. *Int J Cardiol,* 96**,** 235-40.

HAN, R. N. & STEWART, D. J. 2006. Defective lung vascular development in endothelial nitric oxide synthase-deficient mice. *Trends Cardiovasc Med,* 16**,** 29-34.

HAN, V. K. & CARTER, A. M. 2000. Spatial and temporal patterns of expression of messenger RNA for insulin-like growth factors and their binding proteins in the placenta of man and laboratory animals. *Placenta,* 21**,** 289-305.

HANAM, S. R., SINGLETON, C. E. & RUDEK, W. 1983. The effect of topical insulin on infected cutaneous ulcerations in diabetic and nondiabetic mice. *J Foot Surg,* 22**,** 298-301.

HANKS, S. K., CALALB, M. B., HARPER, M. C. & PATEL, S. K. 1992. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc Natl Acad Sci U S A,* 89**,** 8487-91.

HANNEMANN, M. M., LIDDELL, W. G., SHORE, A. C., CLARK, P. M. & TOOKE, J. E. 2002. Vascular function in women with previous gestational diabetes mellitus. *J Vasc Res,* 39**,** 311-9.

HARRIS, L. K., CROCKER, I. P., BAKER, P. N., APLIN, J. D. & WESTWOOD, M. 2011. IGF2 actions on trophoblast in human placenta are regulated by the insulin-like growth factor 2 receptor, which can function as both a signaling and clearance receptor. *Biol Reprod,* 84**,** 440-6.

HAYDEN, M. R., SOWERS, J. R. & TYAGI, S. C. 2005. The central role of vascular extracellular matrix and basement membrane remodeling in metabolic syndrome and type 2 diabetes: the matrix preloaded. *Cardiovasc Diabetol,* 4**,** 9.

HE, C., CHOI, H. C. & XIE, Z. 2010. Enhanced tyrosine nitration of prostacyclin synthase is associated with increased inflammation in atherosclerotic carotid arteries from type 2 diabetic patients. *Am J Pathol,* 176**,** 2542-9.

HE, L., WU, Y., LIN, L., WANG, J., CHEN, Y., YI, Z., LIU, M. & PANG, X. 2011. Hispidulin, a small flavonoid molecule, suppresses the angiogenesis and growth of human pancreatic cancer by targeting vascular endothelial growth factor receptor 2-mediated PI3K/Akt/mTOR signaling pathway. *Cancer Sci,* 102**,** 219-25.

HEINONEN, S., TAIPALE, P. & SAARIKOSKI, S. 2001. Weights of placentae from small-for-gestational age infants revisited. *Placenta,* 22**,** 399-404.

HELSKE, S., VUORELA, P., CARPEN, O., HORNIG, C., WEICH, H. & HALMESMAKI, E. 2001. Expression of vascular endothelial growth factor receptors 1, 2 and 3 in placentas from normal and complicated pregnancies. *Mol Hum Reprod,* 7**,** 205-10.

HENNESSY, B. T., SMITH, D. L., RAM, P. T., LU, Y. & MILLS, G. B. 2005. Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov,* 4**,** 988-1004.

HERR, F., BAAL, N., WIDMER-TESKE, R., MCKINNON, T. & ZYGMUNT, M. 2010. How to study placental vascular development? *Theriogenology,* 73**,** 817-27.

HIDEN, U. & DESOYE, G. 2010. *The placenta in diabetes in pregnancy,* Oxford, Wiley-Blackwell.

HIDEN, U., GLITZNER, E., HARTMANN, M. & DESOYE, G. 2009a. Insulin and the IGF system in the human placenta of normal and diabetic pregnancies. *J Anat,* 215**,** 60-8.

HIDEN, U., GLITZNER, E., IVANISEVIC, M., DJELMIS, J., WADSACK, C., LANG, U. & DESOYE, G. 2008. MT1-MMP expression in first-trimester placental tissue is upregulated in type 1 diabetes as a result of elevated insulin and tumor necrosis factor-alpha levels. *Diabetes,* 57**,** 150-7.

HIDEN, U., LANG, U. & DESOYE, G. 2009b. [Fetoplacental disturbances in gestational diabetes mellitus]. *Gynakol Geburtshilfliche Rundsch,* 49**,** 224-9.

HIDEN, U., MAIER, A., BILBAN, M., GHAFFARI-TABRIZI, N., WADSACK, C., LANG, I., DOHR, G. & DESOYE, G. 2006. Insulin control of placental gene expression shifts from mother to foetus over the course of pregnancy. *Diabetologia,* 49**,** 123-31.

HILL, D. J., CLEMMONS, D. R., RILEY, S. C., BASSETT, N. & CHALLIS, J. R. 1993. Immunohistochemical localization of insulin-like growth factors (IGFs) and IGF binding proteins -1, -2 and -3 in human placenta and fetal membranes. *Placenta,* 14**,** 1-12.

HODIVALA-DILKE, K. M., REYNOLDS, A. R. & REYNOLDS, L. E. 2003. Integrins in angiogenesis: multitalented molecules in a balancing act. *Cell Tissue Res,* 314**,** 131-44.

HOEBEN, A., LANDUYT, B., HIGHLEY, M. S., WILDIERS, H., VAN OOSTEROM, A. T. & DE BRUIJN, E. A. 2004. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev,* 56**,** 549-80.

HOEGH, A. M., BORUP, R., NIELSEN, F. C., SORENSEN, S. & HVIID, T. V. 2010. Gene expression profiling of placentas affected by pre-eclampsia. *J Biomed Biotechnol,* 2010**,** 787545.

HONORE, S., PICHARD, V., PENEL, C., RIGOT, V., PREV T, C., MARVALDI, J., BRIAND, C. & ROGNONI, J. B. 2000. Outside-in regulation of integrin clustering processes by ECM components per se and their involvement in

actin cytoskeleton organization in a colon adenocarcinoma cell line. *Histochem Cell Biol,* 114**,** 323-35.

HOOD, J. D., FRAUSTO, R., KIOSSES, W. B., SCHWARTZ, M. A. & CHERESH, D. A. 2003. Differential alphav integrin-mediated Ras-ERK signaling during two pathways of angiogenesis. *J Cell Biol,* 162**,** 933-43.

HOU, Y., YE, R. D. & BROWNING, D. D. 2004. Activation of the small GTPase Rac1 by cGMP-dependent protein kinase. *Cell Signal,* 16**,** 1061-9.

HU, J., NORMAN, M., WALLENSTEEN, M. & GENNSER, G. 1998. Increased large arterial stiffness and impaired acetylcholine induced skin vasodilatation in women with previous gestational diabetes mellitus. *Br J Obstet Gynaecol,* 105**,** 1279-87.

HUANG DA, W., SHERMAN, B. T. & LEMPICKI, R. A. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc,* 4**,** 44-57.

HUGHES, P., MARSHALL, D., REID, Y., PARKES, H. & GELBER, C. 2007. The costs of using unauthenticated, over-passaged cell lines: how much more data do we need? *Biotechniques,* 43**,** 575, 577-8, 581-2 passim.

HUPPERTZ, B., KERTSCHANSKA, S., FRANK, H. G., GAUS, G., FUNAYAMA, H. & KAUFMANN, P. 1996. Extracellular matrix components of the placental extravillous trophoblast: immunocytochemistry and ultrastructural distribution. *Histochem Cell Biol,* 106**,** 291-301.

HYNES, R. O. 2009. The extracellular matrix: not just pretty fibrils. *Science,* 326**,** 1216-9.

ISENOVIC, E. R., FRETAUD, M., KORICANAC, G., SUDAR, E., VELEBIT, J., DOBUTOVIC, B. & MARCHE, P. 2009. Insulin regulation of proliferation involves activation of AKT and ERK 1/2 signaling pathways in vascular smooth muscle cells. *Exp Clin Endocrinol Diabetes,* 117**,** 214-9.

JANG, C., OBEYESEKERE, V. R., DILLEY, R. J., KROZOWSKI, Z., INDER, W. J. & ALFORD, F. P. 2007. Altered activity of 11beta-hydroxysteroid dehydrogenase types 1 and 2 in skeletal muscle confers metabolic protection in subjects with type 2 diabetes. *J Clin Endocrinol Metab,* 92**,** 3314-20.

JANSSON, T., WENNERGREN, M. & POWELL, T. L. 1999. Placental glucose transport and GLUT 1 expression in insulin-dependent diabetes. *Am J Obstet Gynecol,* 180**,** 163-8.

JEAN, C., GRAVELLE, P., FOURNIE, J. J. & LAURENT, G. 2011. Influence of stress on extracellular matrix and integrin biology. *Oncogene*.

JIRKOVSKA, M., KUBINOVA, L., JANACEK, J., MORAVCOVA, M., KREJCI, V. & KAREN, P. 2002. Topological properties and spatial organization of villous capillaries in normal and diabetic placentas. *J Vasc Res,* 39**,** 268-78.

JONES, P. L., CRACK, J. & RABINOVITCH, M. 1997. Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the alpha v beta 3 integrin to promote epidermal growth factor receptor phosphorylation and growth. *J Cell Biol,* 139**,** 279-93.

JULIANO, R. 1996. Cooperation between soluble factors and integrin-mediated cell anchorage in the control of cell growth and differentiation. *Bioessays,* 18**,** 911-7.

JUNG, K. H., CHOI, M. J., HONG, S., LEE, H., HONG, S. W., ZHENG, H. M., LEE, H. S. & HONG, S. S. 2011. HS-116, a novel phosphatidylinositol 3-kinase inhibitor induces apoptosis and suppresses angiogenesis of

hepatocellular carcinoma through inhibition of the PI3K/AKT/mTOR pathway. *Cancer Lett*.

KALTER, H. 1998. The non-teratogenicity of gestational diabetes. *Paediatr Perinat Epidemiol,* 12**,** 456-8.

KAMATH, K., OROUDJEV, E. & JORDAN, M. A. 2010. Determination of microtubule dynamic instability in living cells. *Methods Cell Biol,* 97**,** 1-14.

KARAR, J. & MAITY, A. 2011. PI3K/AKT/mTOR Pathway in Angiogenesis. *Front Mol Neurosci,* 4**,** 51.

KELLERER, M., LAMMERS, R., ERMEL, B., TIPPMER, S., VOGT, B., OBERMAIER-KUSSER, B., ULLRICH, A. & HARING, H. U. 1992. Distinct alpha-subunit structures of human insulin receptor A and B variants determine differences in tyrosine kinase activities. *Biochemistry,* 31**,** 4588-96.

KIM, C. 2010. Gestational diabetes: risks, management, and treatment options. *Int J Womens Health,* 2**,** 339-51.

KIM, F., GALLIS, B. & CORSON, M. A. 2001. TNF-alpha inhibits flow and insulin signaling leading to NO production in aortic endothelial cells. *Am J Physiol Cell Physiol,* 280**,** C1057-65.

KIM, S. H., TURNBULL, J. & GUIMOND, S. 2011. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. *J Endocrinol,* 209**,** 139-51.

KNOCK, G. A., MCCARTHY, A. L., LOWY, C. & POSTON, L. 1997. Association of gestational diabetes with abnormal maternal vascular endothelial function. *Br J Obstet Gynaecol,* 104**,** 229-34.

KOHLHAAS, C. F., MORROW, V. A., JHAKRA, N., PATIL, V., CONNELL, J. M., PETRIE, J. R. & SALT, I. P. 2011. Insulin rapidly stimulates L-arginine transport in human aortic endothelial cells via Akt. *Biochem Biophys Res Commun,* 412**,** 747-51.

KOJIMA, N., SATO, M., IMAI, K., MIURA, M., MATANO, Y. & SENOO, H. 1998. Hepatic stellate cells (vitamin A-storing cells) change their cytoskeleton structure by extracellular matrix components through a signal transduction system. *Histochem Cell Biol,* 110**,** 121-8.

KONDO, T., VICENT, D., SUZUMA, K., YANAGISAWA, M., KING, G. L., HOLZENBERGER, M. & KAHN, C. R. 2003. Knockout of insulin and IGF-1 receptors on vascular endothelial cells protects against retinal neovascularization. *J Clin Invest,* 111**,** 1835-42.

KORHONEN, M. & VIRTANEN, I. 1997. The distribution of laminins and fibronectins is modulated during extravillous trophoblastic cell differentiation and decidual cell response to invasion in the human placenta. *J Histochem Cytochem,* 45**,** 569-81.

KOSAKI, A., NELSON, J. & WEBSTER, N. J. 1998. Identification of intron and exon sequences involved in alternative splicing of insulin receptor pre-mRNA. *J Biol Chem,* 273**,** 10331-7.

KOSAKI, A., PILLAY, T. S., XU, L. & WEBSTER, N. J. 1995. The B isoform of the insulin receptor signals more efficiently than the A isoform in HepG2 cells. *J Biol Chem,* 270**,** 20816-23.

KOSAKI, A. & WEBSTER, N. J. 1993. Effect of dexamethasone on the alternative splicing of the insulin receptor mRNA and insulin action in HepG2 hepatoma cells. *J Biol Chem,* 268**,** 21990-6.

KRYWICKI, R. F. & YEE, D. 1992. The insulin-like growth factor family of ligands, receptors, and binding proteins. *Breast Cancer Res Treat,* 22**,** 7-19.

KUBOKI, K., JIANG, Z. Y., TAKAHARA, N., HA, S. W., IGARASHI, M., YAMAUCHI, T., FEENER, E. P., HERBERT, T. P., RHODES, C. J. & KING, G. L. 2000. Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and in vivo : a specific vascular action of insulin. *Circulation,* 101**,** 676-81.

KUEHN K, S. H.-H., TIMPL R 1982. *Eds: New trends in Basement Membrane Research,* New York Raven Press.

KUHL, C. 1991. Insulin secretion and insulin resistance in pregnancy and GDM. Implications for diagnosis and management. *Diabetes,* 40 Suppl 2**,** 18-24.

KUMAR, P., AMIN, M. A., HARLOW, L. A., POLVERINI, P. J. & KOCH, A. E. 2003. Src and phosphatidylinositol 3-kinase mediate soluble E-selectin-induced angiogenesis. *Blood,* 101**,** 3960-8.

KUROKAWA, K., ITOH, R. E., YOSHIZAKI, H., NAKAMURA, Y. O. & MATSUDA, M. 2004. Coactivation of Rac1 and Cdc42 at lamellipodia and membrane ruffles induced by epidermal growth factor. *Mol Biol Cell,* 15**,** 1003-10.

KUUSISTO, J., ANDRULIONYTE, L. & LAAKSO, M. 2007. Atherosclerosis and cardiovascular risk reduction with PPAR agonists. *Curr Atheroscler Rep,* 9**,** 274-80.

KVETNY, J. & POULSEN, H. F. 2003. Incidence of gestational hypertension in gestational diabetes mellitus. *Arch Gynecol Obstet,* 267**,** 153-7.

LANG, I., PABST, M. A., HIDEN, U., BLASCHITZ, A., DOHR, G., HAHN, T. & DESOYE, G. 2003. Heterogeneity of microvascular endothelial cells isolated from human term placenta and macrovascular umbilical vein endothelial cells. *Eur J Cell Biol,* 82**,** 163-73.

LANG, I., SCHWEIZER, A., HIDEN, U., GHAFFARI-TABRIZI, N., HAGENDORFER, G., BILBAN, M., PABST, M. A., KORGUN, E. T., DOHR, G. & DESOYE, G. 2008. Human fetal placental endothelial cells have a mature arterial and a juvenile venous phenotype with adipogenic and osteogenic differentiation potential. *Differentiation,* 76**,** 1031-43.

LANG, Y. D., HUNG, C. L., WU, T. Y., WANG, L. F. & CHEN, C. M. 2010. The renin-angiotensin system mediates hyperoxia-induced collagen production in human lung fibroblasts. *Free Radic Biol Med,* 49**,** 88-95.

LAO, T. T., LEE, C. P. & WONG, W. M. 1997. Placental weight to birthweight ratio is increased in mild gestational glucose intolerance. *Placenta,* 18**,** 227-30.

LAPPAS, M., MITTON, A. & PERMEZEL, M. 2010. In response to oxidative stress, the expression of inflammatory cytokines and antioxidant enzymes are impaired in placenta, but not adipose tissue, of women with gestational diabetes. *J Endocrinol,* 204**,** 75-84.

LARSEN, M., ARTYM, V. V., GREEN, J. A. & YAMADA, K. M. 2006. The matrix reorganized: extracellular matrix remodeling and integrin signaling. *Curr Opin Cell Biol,* 18**,** 463-71.

LEACH, L. 2011. Placental vascular dysfunction in diabetic pregnancies: intimations of fetal cardiovascular disease? *Microcirculation,* 18**,** 263-9.

LEACH, L., GRAY, C., STATON, S., BABAWALE, M. O., GRUCHY, A., FOSTER, C., MAYHEW, T. M. & JAMES, D. K. 2004. Vascular endothelial cadherin and beta-catenin in human fetoplacental vessels of pregnancies complicated by Type 1 diabetes: associations with angiogenesis and perturbed barrier function. *Diabetologia,* 47**,** 695-709.

LEACH, L., TAYLOR, A. & SCIOTA, F. 2009. Vascular dysfunction in the diabetic placenta: causes and consequences. *J Anat,* 215**,** 69-76.

LEE, O. H., BAE, S. K., BAE, M. H., LEE, Y. M., MOON, E. J., CHA, H. J., KWON, Y. G. & KIM, K. W. 2000. Identification of angiogenic properties of insulin-like growth factor II in in vitro angiogenesis models. *Br J Cancer,* 82**,** 385-91.

LEGATE, K. R., WICKSTROM, S. A. & FASSLER, R. 2009. Genetic and cell biological analysis of integrin outside-in signaling. *Genes Dev,* 23**,** 397-418.

LESZCZYNSKA, K., KAUR, S., WILSON, E., BICKNELL, R. & HEATH, V. L. 2011. The role of RhoJ in endothelial cell biology and angiogenesis. *Biochem Soc Trans,* 39**,** 1606-11.

LIANG, J. & SLINGERLAND, J. M. 2003. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle,* 2**,** 339-45.

LIANG, J., ZUBOVITZ, J., PETROCELLI, T., KOTCHETKOV, R., CONNOR, M. K., HAN, K., LEE, J. H., CIARALLO, S., CATZAVELOS, C., BENISTON, R., FRANSSEN, E. & SLINGERLAND, J. M. 2002. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med,* 8**,** 1153-60.

LIPFERT, L., HAIMOVICH, B., SCHALLER, M. D., COBB, B. S., PARSONS, J. T. & BRUGGE, J. S. 1992. Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. *J Cell Biol,* 119**,** 905-12.

LIU, Y., PETREACA, M. & MARTINS-GREEN, M. 2009. Cell and molecular mechanisms of insulin-induced angiogenesis. *J Cell Mol Med,* 13**,** 4492-504.

LIU, Y., ZHANG, X., ZHANG, Z., FANG, P. Y. & XU, W. S. 2004. [Effects of topical application of insulin on the wound healing in scalded rats]. *Zhonghua Shao Shang Za Zhi,* 20**,** 98-101.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

LOEFFLER, I., HOPFER, U., KOCZAN, D. & WOLF, G. 2011a. Type VIII Collagen Modulates TGF-{beta}1-induced Proliferation of Mesangial Cells. *J Am Soc Nephrol,* 22**,** 649-63.

LOEFFLER, I., HOPFER, U., KOCZAN, D. & WOLF, G. 2011b. Type VIII collagen modulates TGF-beta1-induced proliferation of mesangial cells. *J Am Soc Nephrol,* 22**,** 649-63.

LOPPNOW, H., BUERKE, M., WERDAN, K. & ROSE-JOHN, S. 2011. Contribution of vascular cell-derived cytokines to innate and inflammatory pathways in atherogenesis. *J Cell Mol Med,* 15**,** 484-500.

MACCIONI, R. B. & CAMBIAZO, V. 1995. Role of microtubule-associated proteins in the control of microtubule assembly. *Physiol Rev,* 75**,** 835-64.

MADRI, J. A. & PRATT, B. M. 1986. Endothelial cell-matrix interactions: in vitro models of angiogenesis. *J Histochem Cytochem,* 34**,** 85-91.

MAENO, Y., LI, Q., PARK, K., RASK-MADSEN, C., GAO, B., MATSUMOTO, M., LIU, Y., WU, I. H., WHITE, M. F., FEENER, E. P. & KING, G. L. 2011. Inhibition of insulin signaling in endothelial cells by protein kinase C induced phosphorylation of P85 subunit of PI3-kinase. *J Biol Chem*.

MAGAZINE, H. I., CHANG, J., GOUMON, Y. & STEFANO, G. B. 2000. Rebound from nitric oxide inhibition triggers enhanced monocyte activation and chemotaxis. *J Immunol,* 165**,** 102-7.

MARTINEZ-FRIAS, M. L., BERMEJO, E., RODRIGUEZ-PINILLA, E., PRIETO, L. & FRIAS, J. L. 1998. Epidemiological analysis of outcomes of pregnancy in gestational diabetic mothers. *Am J Med Genet,* 78**,** 140-5.

MAUBANT, S., SAINT-DIZIER, D., BOUTILLON, M., PERRON-SIERRA, F., CASARA, P. J., HICKMAN, J. A., TUCKER, G. C. & VAN OBBERGHEN-SCHILLING, E. 2006. Blockade of alpha v beta3 and alpha v beta5 integrins by RGD mimetics induces anoikis and not integrin-mediated death in human endothelial cells. *Blood,* 108**,** 3035-44.

MEHTA, D., MALIK, A. B. 2006. Signaling Mechanisms Regulating Endothelial Permeability. *Physiol Rev,* 86**,** 279-367.

MENG, D., LV, D. D. & FANG, J. 2008. Insulin-like growth factor-I induces reactive oxygen species production and cell migration through Nox4 and Rac1 in vascular smooth muscle cells. *Cardiovasc Res,* 80**,** 299-308.

MEREDITH, J. E., JR., FAZELI, B. & SCHWARTZ, M. A. 1993. The extracellular matrix as a cell survival factor. *Mol Biol Cell,* 4**,** 953-61.

MIEHLE, K., STEPAN, H. & FASSHAUER, M. 2012. Leptin, adiponectin and other adipokines in gestational diabetes mellitus and pre-eclampsia. *Clin Endocrinol (Oxf),* 76**,** 2-11.

MILLAN, J., CAIN, R. J., REGLERO-REAL, N., BIGARELLA, C., MARCOS-RAMIRO, B., FERNANDEZ-MARTIN, L., CORREAS, I. & RIDLEY, A. J. 2010. Adherens junctions connect stress fibres between adjacent endothelial cells. *BMC Biol,* 8**,** 11.

MLINAR, B., MARC, J., JENSTERLE, M., BOKAL, E. V., JERIN, A. & PFEIFER, M. 2011. Expression of 11beta-hydroxysteroid dehydrogenase type 1 in visceral and subcutaneous adipose tissues of patients with polycystic ovary syndrome is associated with adiposity. *J Steroid Biochem Mol Biol,* 123**,** 127-32.

MOLTENI, R. A., STYS, S. J. & BATTAGLIA, F. C. 1978. Relationship of fetal and placental weight in human beings: fetal/placental weight ratios at various gestational ages and birth weight distributions. *J Reprod Med,* 21**,** 327-34.

MONCADA, S. & HIGGS, A. 1993. The L-arginine-nitric oxide pathway. *N Engl J Med,* 329**,** 2002-12.

MONTAGNANI, M., CHEN, H., BARR, V. A. & QUON, M. J. 2001. Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser(1179). *J Biol Chem,* 276**,** 30392-8.

MONTAGNANI, M., GOLOVCHENKO, I., KIM, I., KOH, G. Y., GOALSTONE, M. L., MUNDHEKAR, A. N., JOHANSEN, M., KUCIK, D. F., QUON, M. J. & DRAZNIN, B. 2002. Inhibition of phosphatidylinositol 3-kinase enhances mitogenic actions of insulin in endothelial cells. *J Biol Chem,* 277**,** 1794-9.

MOSTHAF, L., GRAKO, K., DULL, T. J., COUSSENS, L., ULLRICH, A. & MCCLAIN, D. A. 1990. Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *EMBO J,* 9**,** 2409-13.

MUFSON, E. J., COUNTS, S. E., CHE, S. & GINSBERG, S. D. 2006. Neuronal gene expression profiling: uncovering the molecular biology of neurodegenerative disease. *Prog Brain Res,* 158**,** 197-222.

MUROHARA, T., HOROWITZ, J. R., SILVER, M., TSURUMI, Y., CHEN, D., SULLIVAN, A. & ISNER, J. M. 1998. Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. *Circulation,* 97**,** 99-107.

NAEYE, R. L. 1987. Do placental weights have clinical significance? *Hum Pathol,* 18**,** 387-91.

NAKAE, J., KIDO, Y. & ACCILI, D. 2001. Distinct and overlapping functions of insulin and IGF-I receptors. *Endocr Rev,* 22**,** 818-35.

NAKAYAMA, T. 2010. Genetic polymorphisms of prostacyclin synthase gene and cardiovascular disease. *Int Angiol,* 29**,** 33-42.

NARASIMHAN, P., LIU, J., SONG, Y. S., MASSENGALE, J. L. & CHAN, P. H. 2009. VEGF Stimulates the ERK 1/2 signaling pathway and apoptosis in cerebral endothelial cells after ischemic conditions. *Stroke,* 40**,** 1467-73.

NARITA, Y., NAGANE, M., MISHIMA, K., HUANG, H. J., FURNARI, F. B. & CAVENEE, W. K. 2002. Mutant epidermal growth factor receptor signaling down-regulates p27 through activation of the phosphatidylinositol 3-kinase/Akt pathway in glioblastomas. *Cancer Res,* 62**,** 6764-9.

NISATO, R. E., TILLE, J. C., JONCZYK, A., GOODMAN, S. L. & PEPPER, M. S. 2003. alphav beta 3 and alphav beta 5 integrin antagonists inhibit angiogenesis in vitro. *Angiogenesis,* 6**,** 105-19.

NISHIZAWA, H., PRYOR-KOISHI, K., KATO, T., KOWA, H., KURAHASHI, H. & UDAGAWA, Y. 2007. Microarray analysis of differentially expressed fetal genes in placental tissue derived from early and late onset severe pre-eclampsia. *Placenta,* 28**,** 487-97.

NOBES, C. D. & HALL, A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell,* 81**,** 53-62.

NOBES, C. D., LAURITZEN, I., MATTEI, M. G., PARIS, S., HALL, A. & CHARDIN, P. 1998. A new member of the Rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion. *J Cell Biol,* 141**,** 187-97.

NODWELL, A., CARMICHAEL, L., ROSS, M. & RICHARDSON, B. 2005. Placental compared with umbilical cord blood to assess fetal blood gas and acid-base status. *Obstet Gynecol,* 105**,** 129-38.

NOLD, J. L. & GEORGIEFF, M. K. 2004. Infants of diabetic mothers. *Pediatr Clin North Am,* 51**,** 619-37, viii.

NOMURA, D. K., MORRISON, B. E., BLANKMAN, J. L., LONG, J. Z., KINSEY, S. G., MARCONDES, M. C., WARD, A. M., HAHN, Y. K., LICHTMAN, A. H., CONTI, B. & CRAVATT, B. F. 2011. Endocannabinoid hydrolysis generates brain prostaglandins that promote neuroinflammation. *Science,* 334**,** 809-13.

OH, I. Y., YOON, C. H., HUR, J., KIM, J. H., KIM, T. Y., LEE, C. S., PARK, K. W., CHAE, I. H., OH, B. H., PARK, Y. B. & KIM, H. S. 2007. Involvement of E-selectin in recruitment of endothelial progenitor cells and angiogenesis in ischemic muscle. *Blood,* 110**,** 3891-9.

OISHI, Y., WAKATSUKI, T., NISHIKADO, A., OKI, T. & ITO, S. 2000. Circulating adhesion molecules and severity of coronary atherosclerosis. *Coron Artery Dis,* 11**,** 77-81.

OLSON, M. F., ASHWORTH, A. & HALL, A. 1995. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science,* 269**,** 1270-2.

OTROCK, Z. K., MAHFOUZ, R. A., MAKAREM, J. A. & SHAMSEDDINE, A. I. 2007. Understanding the biology of angiogenesis: review of the most important molecular mechanisms. *Blood Cells Mol Dis,* 39**,** 212-20.

PADMANABHAN, R. & AL-ZUHAIR, A. G. 1990. Ultrastructural studies on the placentae of streptozotocin induced maternal diabetes in the rat. *Z Mikrosk Anat Forsch,* 104**,** 212-30.

PARK, J. J., BERGGREN, J. R., HULVER, M. W., HOUMARD, J. A. & HOFFMAN, E. P. 2006. GRB14, GPD1, and GDF8 as potential network collaborators in weight loss-induced improvements in insulin action in human skeletal muscle. *Physiol Genomics,* 27**,** 114-21.

PARSONS, J. T., SCHALLER, M. D., HILDEBRAND, J., LEU, T. H., RICHARDSON, A. & OTEY, C. 1994. Focal adhesion kinase: structure and signalling. *J Cell Sci Suppl,* 18**,** 109-13.

PARVEZ, B. & DARBAR, D. 2011. The "missing" link in atrial fibrillation heritability. *J Electrocardiol,* 44**,** 641-4.

PASTOR, D. M., PORITZ, L. S., OLSON, T. L., KLINE, C. L., HARRIS, L. R., KOLTUN, W. A., CHINCHILLI, V. M. & IRBY, R. B. 2010. Primary cell lines: false representation or model system? a comparison of four human colorectal tumors and their coordinately established cell lines. *Int J Clin Exp Med,* 3**,** 69-83.

PEDERSEN, J. 1952. Diabetes and pregnancy; blood sugar of newborn infants during fasting and glucose administration. *Nord Med,* 47**,** 1049.

PELENGARIS, S., KHAN, M. & EVAN, G. 2002. c-MYC: more than just a matter of life and death. *Nat Rev Cancer,* 2**,** 764-76.

PERSSON, J., EKELUND, U. & GRANDE, P. O. 2003. Endogenous nitric oxide reduces microvascular permeability and tissue oedema during exercise in cat skeletal muscle. *J Vasc Res,* 40**,** 538-46.

PICHARD, V., HONORE, S., KOVACIC, H., LI, C., PREVOT, C., BRIAND, C. & ROGNONI, J. B. 2001. Adhesion, actin cytoskeleton organisation and the spreading of colon adenocarcinoma cells induced by EGF are mediated by alpha2beta1 integrin low clustering through focal adhesion kinase. *Histochem Cell Biol,* 116**,** 337-48.

PIETRYGA, M., BICZYSKO, W., WENDER-OZEGOWSKA, E., BRAZERT, J., BIEGANSKA, E. & BICZYSKO, R. 2004. [Ultrastructural examination of the placenta in pregnancy complicated by diabetes mellitus]. *Ginekol Pol,* 75**,** 111-8.

POPOFF, M. R. & GENY, B. 2009. Multifaceted role of Rho, Rac, Cdc42 and Ras in intercellular junctions, lessons from toxins. *Biochim Biophys Acta,* 1788**,** 797-812.

POULAKI, V., QIN, W., JOUSSEN, A. M., HURLBUT, P., WIEGAND, S. J., RUDGE, J., YANCOPOULOS, G. D. & ADAMIS, A. P. 2002. Acute intensive insulin therapy exacerbates diabetic blood-retinal barrier breakdown via hypoxia-inducible factor-1alpha and VEGF. *J Clin Invest,* 109**,** 805-15.

PRIYA, S. & SUDHAKARAN, P. R. 2008. Cell survival, activation and apoptosis of hepatic stellate cells: modulation by extracellular matrix proteins. *Hepatol Res,* 38**,** 1221-32.

QI, Y., LIU, J., WU, X., BRAKEBUSCH, C., LEITGES, M., HAN, Y., CORBETT, S. A., LOWRY, S. F., GRAHAM, A. M. & LI, S. 2011. Cdc42 controls vascular network assembly through protein kinase Ciota during embryonic vasculogenesis. *Arterioscler Thromb Vasc Biol,* 31**,** 1861-70.

QIAO, J. G., WU, L., LEI, D. X. & WANG, L. 2005. Insulin promotes sinusoidal endothelial cell proliferation mediated by upregulation of vascular endothelial growth factor in regenerating rat liver after partial hepatectomy. *World J Gastroenterol,* 11**,** 5978-83.

RADAELLI, T., LEPERCQ, J., VARASTEHPOUR, A., BASU, S., CATALANO, P. M. & HAUGUEL-DE MOUZON, S. 2009. Differential regulation of genes for

fetoplacental lipid pathways in pregnancy with gestational and type 1 diabetes mellitus. *Am J Obstet Gynecol,* 201**,** 209 e1-209 e10.

RADAELLI, T., VARASTEHPOUR, A., CATALANO, P. & HAUGUEL-DE MOUZON, S. 2003. Gestational diabetes induces placental genes for chronic stress and inflammatory pathways. *Diabetes,* 52**,** 2951-8.

RATH, S., KALOGERIS, T., MAI, N., ZIBARI, G., ALEXANDER, J. S., LEFER, D. & TURNAGE, R. H. 2006. Insulin prevents oxidant-induced endothelial cell barrier dysfunction via nitric oxide-dependent pathway. *Surgery,* 139**,** 82-91.

READ, M. A., BOURA, A. L. & WALTERS, W. A. 1995. Effects of variation in oxygen tension on responses of the human fetoplacental vasculature to vasoactive agents in vitro. *Placenta,* 16**,** 667-78.

REIMER, T., KOCZAN, D., GERBER, B., RICHTER, D., THIESEN, H. J. & FRIESE, K. 2002. Microarray analysis of differentially expressed genes in placental tissue of pre-eclampsia: up-regulation of obesity-related genes. *Mol Hum Reprod,* 8**,** 674-80.

REISKE, H. R., KAO, S. C., CARY, L. A., GUAN, J. L., LAI, J. F. & CHEN, H. C. 1999. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. *J Biol Chem,* 274**,** 12361-6.

REN, X. D., KIOSSES, W. B., SIEG, D. J., OTEY, C. A., SCHLAEPFER, D. D. & SCHWARTZ, M. A. 2000. Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J Cell Sci,* 113 (Pt 20)**,** 3673-8.

RISAU, W. 1997. Mechanisms of angiogenesis. *Nature,* 386**,** 671-4.

RITCHIE, S. A., KOHLHAAS, C. F., BOYD, A. R., YALLA, K. C., WALSH, K., CONNELL, J. M. & SALT, I. P. 2010. Insulin-stimulated phosphorylation of endothelial nitric oxide synthase at serine-615 contributes to nitric oxide synthesis. *Biochem J,* 426**,** 85-90.

ROBINSON, J., CANAVAN, J. P., EL HAJ, A. J. & GOLDSPINK, D. F. 1988. Maternal diabetes in rats. I. Effects on placental growth and protein turnover. *Diabetes,* 37**,** 1665-70.

ROLLI-DERKINDEREN, M., TOUMANIANTZ, G., PACAUD, P. & LOIRAND, G. 2010. RhoA phosphorylation induces Rac1 release from guanine dissociation inhibitor alpha and stimulation of vascular smooth muscle cell migration. *Mol Cell Biol,* 30**,** 4786-96.

ROSENTHAL, S. P. 1968. Acceleration of primary wound healing by insulin. *Arch Surg,* 96**,** 53-5.

ROSSIG, L., JADIDI, A. S., URBICH, C., BADORFF, C., ZEIHER, A. M. & DIMMELER, S. 2001. Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells. *Mol Cell Biol,* 21**,** 5644-57.

RUKOSUEV, V. S., FOKIN, E. I. & MILOVANOV, A. P. 1989. [Immunofluorescence study of the extracellular matrix of the human placenta]. *Ontogenez,* 20**,** 171-8.

RUOSLAHTI, E. 2002. Specialization of tumour vasculature. *Nat Rev Cancer,* 2**,** 83-90.

SACCO, A., MORCAVALLO, A., PANDINI, G., VIGNERI, R. & BELFIORE, A. 2009. Differential signaling activation by insulin and insulin-like growth factors I and II upon binding to insulin receptor isoform A. *Endocrinology,* 150**,** 3594-602.

SACHARIDOU, A., STRATMAN, A. N. & DAVIS, G. E. 2011. Molecular Mechanisms Controlling Vascular Lumen Formation in Three-Dimensional Extracellular Matrices. *Cells Tissues Organs*.

SALT, I. P., MORROW, V. A., BRANDIE, F. M., CONNELL, J. M. & PETRIE, J. R. 2003. High glucose inhibits insulin-stimulated nitric oxide production without reducing endothelial nitric-oxide synthase Ser1177 phosphorylation in human aortic endothelial cells. *J Biol Chem,* 278**,** 18791-7.

SANZ, L. & ALVAREZ-VALLINA, L. 2003. The extracellular matrix: a new turn-of-the-screw for anti-angiogenic strategies. *Trends Mol Med,* 9**,** 256-62.

SASAOKA, T., ISHIKI, M., WADA, T., HORI, H., HIRAI, H., HARUTA, T., ISHIHARA, H. & KOBAYASHI, M. 2001. Tyrosine phosphorylation-dependent and -independent role of Shc in the regulation of IGF-1-induced mitogenesis and glycogen synthesis. *Endocrinology,* 142**,** 5226-35.

SATI, L., DEMIR, A. Y., SARIKCIOGLU, L. & DEMIR, R. 2008. Arrangement of collagen fibers in human placental stem villi. *Acta Histochem,* 110**,** 371-9.

SAWADA, N., SALOMONE, S., KIM, H. H., KWIATKOWSKI, D. J. & LIAO, J. K. 2008. Regulation of endothelial nitric oxide synthase and postnatal angiogenesis by Rac1. *Circ Res,* 103**,** 360-8.

SCHALLER, M. D., HILDEBRAND, J. D., SHANNON, J. D., FOX, J. W., VINES, R. R. & PARSONS, J. T. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol,* 14**,** 1680-8.

SCHIFFRIN, E. L. 2001. Small artery remodeling in hypertension: can it be corrected? *Am J Med Sci,* 322**,** 7-11.

SCHMIDT, A. & HALL, A. 2002. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev,* 16**,** 1587-609.

SCHNEEBERGER, E. E. & LYNCH, R. D. 2004. The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol,* 286**,** C1213-28.

SCHWARTZ, M. A. & GINSBERG, M. H. 2002. Networks and crosstalk: integrin signalling spreads. *Nat Cell Biol,* 4**,** E65-8.

SCIACCA, L., MINEO, R., PANDINI, G., MURABITO, A., VIGNERI, R. & BELFIORE, A. 2002. In IGF-I receptor-deficient leiomyosarcoma cells autocrine IGF-II induces cell invasion and protection from apoptosis via the insulin receptor isoform A. *Oncogene,* 21**,** 8240-50.

SEINO, S. & BELL, G. I. 1989. Alternative splicing of human insulin receptor messenger RNA. *Biochem Biophys Res Commun,* 159**,** 312-6.

SELL, S. M., REESE, D. & OSSOWSKI, V. M. 1994. Insulin-inducible changes in insulin receptor mRNA splice variants. *J Biol Chem,* 269**,** 30769-72.

SENGER, D. R. & DAVIS, G. E. 2011. Angiogenesis. *Cold Spring Harb Perspect Biol,* 3**,** a005090.

SESSA, W. C. 2009. Molecular control of blood flow and angiogenesis: role of nitric oxide. *J Thromb Haemost,* 7 Suppl 1**,** 35-7.

SFERRUZZI-PERRI, A. N., OWENS, J. A., STANDEN, P. & ROBERTS, C. T. 2008. Maternal insulin-like growth factor-II promotes placental functional development via the type 2 IGF receptor in the guinea pig. *Placenta,* 29**,** 347-55.

SHAI, R. M. 2006. Microarray tools for deciphering complex diseases. *Front Biosci,* 11**,** 1414-24.

SHARMA, J., TURK, J., MANCUSO, D. J., SIMS, H. F., GROSS, R. W. & MCHOWAT, J. 2011. Activation of group VI phospholipase A2 isoforms in cardiac endothelial cells. *Am J Physiol Cell Physiol,* 300**,** C872-9.

SHENBERGER, J. S., ZHANG, L., POWELL, R. J. & BARCHOWSKY, A. 2007. Hyperoxia enhances VEGF release from A549 cells via post-transcriptional processes. *Free Radic Biol Med,* 43**,** 844-52.

SHERER, D. M. & DIVON, M. Y. 1996. Prenatal ultrasonographic assessment of the ductus arteriosus: a review. *Obstet Gynecol,* 87**,** 630-7.

SHIGEMATSU, S., YAMAUCHI, K., NAKAJIMA, K., IIJIMA, S., AIZAWA, T. & HASHIZUME, K. 1999. IGF-1 regulates migration and angiogenesis of human endothelial cells. *Endocr J,* 46 Suppl**,** S59-62.

SHORT, K., WIRADJAJA, F. & SMYTH, I. 2007. Let's stick together: the role of the Fras1 and Frem proteins in epidermal adhesion. *IUBMB Life,* 59**,** 427-35.

SHRADER, C. D., BAILEY, K. M., KONAT, G. W., CILENTO, E. V. & REILLY, F. D. 2009. Insulin enhances proliferation and viability of human umbilical vein endothelial cells. *Arch Dermatol Res,* 301**,** 159-66.

SIDDLE, K. 2011. Signalling by insulin and IGF receptors: supporting acts and new players. *J Mol Endocrinol,* 47**,** R1-10.

SILVA, I. T., MELLO, A. P. & DAMASCENO, N. R. 2011. Antioxidant and inflammatory aspects of lipoprotein-associated phospholipase A2 (Lp-PLA2): a review. *Lipids Health Dis,* 10**,** 170.

SIMIONESCU, M. 2000. Morphogenesis of Endothelium. *In:* RISAU, W., RUBANYI G. M. (ed.) *Structural, Biochemical and Functional Differentiation of the Vascular Endothelium.* Harwood Academic Publishers.

SIMON, M. C. & KEITH, B. 2008. The role of oxygen availability in embryonic development and stem cell function. *Nat Rev Mol Cell Biol,* 9**,** 285-96.

SIMPSON, M., SNELL-BERGEON, J. K., KINNEY, G. L., LACHE, O., MILLER-LOTAN, R., ANBINDER, Y., REWERS, M. J. & LEVY, A. P. 2011. Haptoglobin genotype predicts development of coronary artery calcification in a prospective cohort of patients with type 1 diabetes. *Cardiovasc Diabetol,* 10**,** 99.

SINHA, S. & YANG, W. 2008. Cellular signaling for activation of Rho GTPase Cdc42. *Cell Signal,* 20**,** 1927-34.

SITRAS, V., PAULSSEN, R. H., GRONAAS, H., LEIRVIK, J., HANSSEN, T. A., VARTUN, A. & ACHARYA, G. 2009. Differential placental gene expression in severe preeclampsia. *Placenta,* 30**,** 424-33.

SKJOT-ARKIL, H., BARASCUK, N., REGISTER, T. & KARSDAL, M. A. 2010. Macrophage-mediated proteolytic remodeling of the extracellular matrix in atherosclerosis results in neoepitopes: a potential new class of biochemical markers. *Assay Drug Dev Technol,* 8**,** 542-52.

SKURK, C., MAATZ, H., ROCNIK, E., BIALIK, A., FORCE, T. & WALSH, K. 2005. Glycogen-Synthase Kinase3beta/beta-catenin axis promotes angiogenesis through activation of vascular endothelial growth factor signaling in endothelial cells. *Circ Res,* 96**,** 308-18.

SMYTH, G. K. 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol,* 3**,** Article3.

SPENCER, T. E. & BAZER, F. W. 2002. Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. *Front Biosci,* 7**,** d1879-98.

STENGEL, K. & ZHENG, Y. 2011. Cdc42 in oncogenic transformation, invasion, and tumorigenesis. *Cell Signal,* 23**,** 1415-23.

STROMBLAD, S. & CHERESH, D. A. 1996. Cell adhesion and angiogenesis. *Trends Cell Biol,* 6**,** 462-8.

STURN, A., QUACKENBUSH, J. & TRAJANOSKI, Z. 2002. Genesis: cluster analysis of microarray data. *Bioinformatics,* 18**,** 207-8.

TAKAHASHI, Y., TOBE, K., KADOWAKI, H., KATSUMATA, D., FUKUSHIMA, Y., YAZAKI, Y., AKANUMA, Y. & KADOWAKI, T. 1997. Roles of insulin receptor substrate-1 and Shc on insulin-like growth factor I receptor signaling in early passages of cultured human fibroblasts. *Endocrinology,* 138**,** 741-50.

TAN, W., PALMBY, T. R., GAVARD, J., AMORNPHIMOLTHAM, P., ZHENG, Y. & GUTKIND, J. S. 2008. An essential role for Rac1 in endothelial cell function and vascular development. *FASEB J,* 22**,** 1829-38.

TARICCO, E., RADAELLI, T., ROSSI, G., NOBILE DE SANTIS, M. S., BULFAMANTE, G. P., AVAGLIANO, L. & CETIN, I. 2009. Effects of gestational diabetes on fetal oxygen and glucose levels in vivo. *BJOG,* 116**,** 1729-35.

TERAMOTO, H., COSO, O. A., MIYATA, H., IGISHI, T., MIKI, T. & GUTKIND, J. S. 1996. Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. *J Biol Chem,* 271**,** 27225-8.

THOMSEN, B. M., CLAUSEN, H. V., LARSEN, L. G., NURNBERG, L., OTTESEN, B. & THOMSEN, H. K. 1997. Patterns in expression of insulin-like growth factor-II and of proliferative activity in the normal human first and third trimester placenta demonstrated by non-isotopic in situ hybridization and immunohistochemical staining for MIB-1. *Placenta,* 18**,** 145-54.

TOFT, J. H., LIAN, I. A., TARCA, A. L., EREZ, O., ESPINOZA, J., EIDE, I. P., BJORGE, L., DRAGHICI, S., ROMERO, R. & AUSTGULEN, R. 2008. Whole-genome microarray and targeted analysis of angiogenesis-regulating gene expression (ENG, FLT1, VEGF, PlGF) in placentas from pre-eclamptic and small-for-gestational-age pregnancies. *J Matern Fetal Neonatal Med,* 21**,** 267-73.

TREINIES, I., PATERSON, H. F., HOOPER, S., WILSON, R. & MARSHALL, C. J. 1999. Activated MEK stimulates expression of AP-1 components independently of phosphatidylinositol 3-kinase (PI3-kinase) but requires a PI3-kinase signal To stimulate DNA synthesis. *Mol Cell Biol,* 19**,** 321-9.

TZIMA, E. 2006. Role of small GTPases in endothelial cytoskeletal dynamics and the shear stress response. *Circ Res,* 98**,** 176-85.

VAMBERGUE, A. & FAJARDY, I. 2011. Consequences of gestational and pregestational diabetes on placental function and birth weight. *World J Diabetes,* 2**,** 196-203.

VAN AELST, L. & D'SOUZA-SCHOREY, C. 1997. Rho GTPases and signaling networks. *Genes Dev,* 11**,** 2295-322.

VAN RIJEN, H., VAN KEMPEN, M. J., ANALBERS, L. J., ROOK, M. B., VAN GINNEKEN, A. C., GROS, D. & JONGSMA, H. J. 1997. Gap junctions in human umbilical cord endothelial cells contain multiple connexins. *Am J Physiol,* 272**,** C117-30.

VARTANIAN, K. B., KIRKPATRICK, S. J., HANSON, S. R. & HINDS, M. T. 2008. Endothelial cell cytoskeletal alignment independent of fluid shear stress on micropatterned surfaces. *Biochem Biophys Res Commun,* 371**,** 787-92.

VIZZA, E., GORANOVA, V., HEYN, R., CORRER, S. & MOTTA, P. M. 2001. Extracellular fibrillar matrix architecture of human placental villi at term. *Ital J Anat Embryol,* 106**,** 317-23.

WANG, G., LIEM, D. A., VONDRISKA, T. M., HONDA, H. M., KORGE, P., PANTALEON, D. M., QIAO, X., WANG, Y., WEISS, J. N. & PING, P. 2005.

Nitric oxide donors protect murine myocardium against infarction via modulation of mitochondrial permeability transition. *Am J Physiol Heart Circ Physiol,* 288**,** H1290-5.

WASSERMAN, L., SHLESINGER, H., ABRAMOVICI, A., GOLDMAN, J. A. & ALLALOUF, D. 1980. Glycosaminoglycan patterns in diabetic and toxemic term placentas. *Am J Obstet Gynecol,* 138**,** 769-73.

WEIDEMANN, A. & JOHNSON, R. S. 2008. Biology of HIF-1alpha. *Cell Death Differ,* 15**,** 621-7.

WEISS, U., CERVAR, M., PUERSTNER, P., SCHMUT, O., HAAS, J., MAUSCHITZ, R., ARIKAN, G. & DESOYE, G. 2001. Hyperglycaemia in vitro alters the proliferation and mitochondrial activity of the choriocarcinoma cell lines BeWo, JAR and JEG-3 as models for human first-trimester trophoblast. *Diabetologia,* 44**,** 209-19.

WELCH, C., SANTRA, M. K., EL-ASSAAD, W., ZHU, X., HUBER, W. E., KEYS, R. A., TEODORO, J. G. & GREEN, M. R. 2009. Identification of a protein, G0S2, that lacks Bcl-2 homology domains and interacts with and antagonizes Bcl-2. *Cancer Res,* 69**,** 6782-9.

WENDUM, D., COMPERAT, E., BOELLE, P. Y., PARC, R., MASLIAH, J., TRUGNAN, G. & FLEJOU, J. F. 2005. Cytoplasmic phospholipase A2 alpha overexpression in stromal cells is correlated with angiogenesis in human colorectal cancer. *Mod Pathol,* 18**,** 212-20.

WICKSTROM, S. A., VEIKKOLA, T., REHN, M., PIHLAJANIEMI, T., ALITALO, K. & KESKI-OJA, J. 2001. Endostatin-induced modulation of plasminogen activation with concomitant loss of focal adhesions and actin stress fibers in cultured human endothelial cells. *Cancer Res,* 61**,** 6511-6.

WILLIAMSON, J. R., TILTON, R. G., CHANG, K. & KILO, C. 1988. Basement membrane abnormalities in diabetes mellitus: relationship to clinical microangiopathy. *Diabetes Metab Rev,* 4**,** 339-70.

WINICK, M. & NOBLE, A. 1967. Cellular growth in human placenta. II. Diabetes mellitus. *J Pediatr,* 71**,** 216-9.

WOJCIAK-STOTHARD, B. & RIDLEY, A. J. 2003. Shear stress-induced endothelial cell polarization is mediated by Rho and Rac but not Cdc42 or PI 3-kinases. *J Cell Biol,* 161**,** 429-39.

WOJCIAK-STOTHARD, B., TORONDEL, B., ZHAO, L., RENNE, T. & LEIPER, J. M. 2009. Modulation of Rac1 activity by ADMA/DDAH regulates pulmonary endothelial barrier function. *Mol Biol Cell,* 20**,** 33-42.

WRIGHT, T. J., LEACH, L., SHAW, P. E. & JONES, P. 2002. Dynamics of vascular endothelial-cadherin and beta-catenin localization by vascular endothelial growth factor-induced angiogenesis in human umbilical vein cells. *Exp Cell Res,* 280**,** 159-68.

XIE, X., MA, Y. T., FU, Z. Y., YANG, Y. N., MA, X., CHEN, B. D., WANG, Y. H. & LIU, F. 2009. Association of polymorphisms of PTGS2 and CYP8A1 with myocardial infarction. *Clin Chem Lab Med,* 47**,** 347-52.

YAMADA, T., ISEMURA, M., YAMAGUCHI, Y., MUNAKATA, H., HAYASHI, N. & KYOGOKU, M. 1987. Immunohistochemical localization of fibronectin in the human placentas at their different stages of maturation. *Histochemistry,* 86**,** 579-84.

YAMAGUCHI, Y., FLIER, J. S., YOKOTA, A., BENECKE, H., BACKER, J. M. & MOLLER, D. E. 1991. Functional properties of two naturally occurring isoforms of the human insulin receptor in Chinese hamster ovary cells. *Endocrinology,* 129**,** 2058-66.

ZALEWSKI, A. & MACPHEE, C. 2005. Role of lipoprotein-associated phospholipase A2 in atherosclerosis: biology, epidemiology, and possible therapeutic target. *Arterioscler Thromb Vasc Biol,* 25**,** 923-31.

ZENG, G., NYSTROM, F. H., RAVICHANDRAN, L. V., CONG, L. N., KIRBY, M., MOSTOWSKI, H. & QUON, M. J. 2000. Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation,* 101**,** 1539-45.

ZHANG, W., WANG, X., JIN, H., QIAN, R., ZHANG, G., CHEN, S. & HU, R. 2008. Effects of high glucose plus high insulin on proliferation and apoptosis of mouse endothelial progenitor cells. *Inflamm Res,* 57**,** 571-6.

ZHOU, B. P., LIAO, Y., XIA, W., SPOHN, B., LEE, M. H. & HUNG, M. C. 2001. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol,* 3**,** 245-52.

ZHOU, J., DEHNE, N. & BRUNE, B. 2009. Nitric oxide causes macrophage migration via the HIF-1-stimulated small GTPases Cdc42 and Rac1. *Free Radic Biol Med,* 47**,** 741-9.

ZHU, J., MOTEJLEK, K., WANG, D., ZANG, K., SCHMIDT, A. & REICHARDT, L. F. 2002. beta8 integrins are required for vascular morphogenesis in mouse embryos. *Development,* 129**,** 2891-903.

ZICHE, M., MORBIDELLI, L., CHOUDHURI, R., ZHANG, H. T., DONNINI, S., GRANGER, H. J. & BICKNELL, R. 1997. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest,* 99**,** 2625-34.

ZORN, T. M., ZUNIGA, M., MADRID, E., TOSTES, R., FORTES, Z., GIACHINI, F. & SAN MARTIN, S. 2011. Maternal diabetes affects cell proliferation in developing rat placenta. *Histol Histopathol,* 26**,** 1049-56.