Dissertation

The role of the G protein-coupled receptor 55 (GPR55) in human placental endothelial cells

submitted by

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for the Academic Degree of

Doctor of Philosophy
(Ph.D.)

at the

Medical University of Graz

Institute of Cell Biology, Histology and Embryology

under Supervision of

Univ.-Prof. Dr. Berthold HUPPERTZ

submitted
2015

Statutory Declaration

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

Please note that parts of this thesis are already published:

KREMSHOFER, J., SIWETZ, M., BERGHOLD, V.M., LANG, I., HUPPERTZ, B. and GAUSTER, M., 2015. A role for GPR55 in human placental venous endothelial cells. *Histochem Cell Biol 44:49-58*

August 2015, Graz, Austria

Acknowledgements

First of all I want to thank Martin Gauster for being an encouraging supervisor. His door was always open for me and my questions, but he knew when to step back to make way for my own ideas and experiences. Thank you for not giving up on me, even if my “special nutrition” seems to be the reason for every problem arising during experiments.

My sincere thanks go to Berthold Huppertz for his astonishing knowledge and bottomless bucket full of ideas. I am proud to be your student.

Thanks to Rudi Schicho for the ideas and advice you gave me at the thesis committee meetings.

I also want to thank Gottfried Dohr for making it possible for me to carry out my work for this thesis at the Institute of Cell Biology, Histology and Embryology and for always encouraging his employees to never neglect being a good colleague.

Many thanks also go to

- Veronika, Stefan, Pablo, Shukun, Astrid and Amin for sharing both scientific knowledge and nice chats.
- Monika for always having the answer to every little lab problem and helping whenever she can.
- Beate Scheiber, Irmgard Ropitsch and Sandra Weber for their administrative support.
- Rudi and Daniel for their technical support.
- Ingrid for helping me out with endothelial cells and antibodies.
- Gernot, Heidi and Ate from the department of Gynecology and Obstetrics for both their theoretical and practical knowledge.
- Kerstin, Nina, Moni and Gerit for lunch and coffee breaks and many laughs to lighten up my days.
- Gregor for being there for me and helping with any lab and not-so-lab problems. I could write an entire thesis just about our inside jokes and things we shared.

- My family for giving me the opportunity to follow my path and whose belief in me gives me strength.
- The Krav girls for their patience and for reminding me of all the little beautiful things in life.

Contents

1 Abbreviations

2-AG	2-arachidonoyl glycerol
ABC	ATP-binding cassette transporter
ABCC	ATP-binding cassette, subfamily C
AD	aqua destillata
AEA	arachidonoylethanolamide
ATP	adenosine triphosphate
BMI	body mass index
C	celsius
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CD	cluster of differentiation
CT	cytotrophoblast
CV	cardiovascular
EBM	endothelial basal medium
ECS	endocannabinoid system
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGM-MV	microvascular endothelial growth medium
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
FBS	fetal bovine serum

FFPE	formalin-fixed paraffin-embedded
FT	first trimester
g	gravitational force
GDM	gestational diabetes mellitus
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GP(C)R	G protein-coupled receptor
GTP	guanosine triphosphate
HMVEC-L	human lung microvascular endothelial cells
ICC	immunocytochemistry
IFPA	International Federation of Placenta Associations
IHC	immunohistochemistry
IL	interleukin
IUFD	intrauterine fetal death
IUGR	intrauterine growth restriction
LDH	L-lactate hydrogenase
LBW	low birth weight
LPI	L-α-lysophosphatidylinositol
Lp-PLA2	Lipoprotein-associated phospholipase 2
MRP	multidrug resistance protein
MTS	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUG	Medical University of Graz
NaCl	sodium chloride

NAD^+	nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
OEA	*N*-oleoylethanolamine
OEGRM	Österreichische Gesellschaft für Reproduktionsmedizin und Endokrinologie
PAEC	placental arterial endothelial cells
PBS	phosphate buffer saline
PEA	*N*-palmitoylethanolamine
PECAM	platelet endothelial cell adhesion molecule
Pen-Strep	Penicillin-Streptomycin
PL	phospholipase
PVEC	placental venous endothelial cells
qPCR	quantitative polymerase chain reaction
RIN	RNA integrity number
RNA	ribonucleic acid
RPL30	ribosomal protein L30
Rpm	revolutions per minute
SD	standard deviation
SGA	small for gestational age
smA	smooth muscle actin
ST	syncytiotrophoblast

TNF	tumor necrosis factor
uNK	uterine natural killer cells
vWF	von Willebrand factor
µM	micromolar

2 Abstract

The endocannabinoid system (ECS) with its G protein-coupled receptors plays important roles throughout the human body. The system is also suggested to play a key role in human pregnancy, which includes processes like implantation, decidualization, placentation and labor. One particular receptor – the G protein-coupled receptor 55 (GPR55) – was previously postulated to be another cannabinoid receptor, because specific cannabinoids were found to act independently of the two classical cannabinoid receptors CB1 and CB2. Nonetheless, studies revealed great differences between the structure of these two receptors and GPR55. Even if GPR55 can be partly activated by certain endocannabinoids, its main agonist is the lysophospholipid L-α-lysophosphatidylinositol (LPI). Both GPR55 and LPI are known to be part of important pathways in the human body. Nonetheless, knowledge about the role of this receptor during pregnancy and placental development is limited, which motivated me to focus on this topic. In this study aims were the analysis of GPR55 expression in human placenta and the comparison to other human peripheral tissues. Furthermore, evaluation of spatiotemporal human placental GPR55 expression was one important aspect.

Analysis of the gene expression profile via qPCR showed that GPR55 levels in human placenta are relatively low compared to tissues with highest receptor expression, which are spleen and lung. When comparing first trimester and term placenta, the latter revealed a 5.8 fold higher expression. Localization of GPR55 via immunohistochemistry determined endothelial cells in first trimester and term placenta to be the only cells expressing the receptor. Strongest expression was found in endothelial cells of small vessels right underneath the trophoblast layer. Larger vessels revealed differential expression in arteries and veins, while umbilical cord vessels only showed very weak GPR55 expression. Receptor expression in endothelial cells was confirmed by immunocytochemistry of isolated primary placental arterial (PAEC) and venous endothelial cells (PVEC). These cells were also used for cell culture experiments analyzing the influence of LPI on endothelial cell functions. Incubation with LPI at a concentration of 1 µM significantly enhanced migration of venous, but not arterial endothelial cells. This LPI-induced migration was partly reduced by the GPR55 antagonist O-1918, leading to the suggestion that the GPR55/LPI axis might play a role in placental venous endothelial cell functions.

3 Zusammenfassung

Im menschlichen Körper spielt das sogenannte Endocannbinoidsystem (ECS) eine große Rolle in verschiedensten Geweben und bei den vielfältigsten Vorgängen. Grundsätzlich besteht das ECS aus den beiden klassischen Rezeptoren CB1 und CB2 und deren Liganden. Beide Rezeptoren sind – wie auch der GPR55 – G-Protein-gekoppelte Rezeptoren. Diese membranständigen Rezeptoren nehmen extrazelluläre Signale wahr und leiten diese ins Innere der Zelle weiter, damit diese entsprechend reagieren kann. Auch wenn GPR55 von Endocannabinoiden teilweise aktiviert werden kann, hat sich doch im Laufe der Zeit das Lysophospholipid L-α-Lysophosphatidylinositol (LPI) als sein wichtigster Agonist herauskristallisiert. Sowohl GPR55 als auch LPI nehmen einige wichtige Rollen im menschlichen Körper wahr, das Wissen um ihre konkreten Aufgaben rund um die Schwangerschaft und die Entwicklung der humanen Plazenta ist jedoch begrenzt. Aufgrund dessen have ich meinen Fokus auf diese Thematik gerichtet und mich mit dieser Arbeit der Aufgabe gewidmet, die Expression von GPR55 in der humanen Plazenta im Vergleich zu weiteren menschlichen Geweben zu analysieren und zusätzlich den Rezeptor zu lokalisieren und seine Expression im Plazentagewebe im Laufe der Schwangerschaft zu untersuchen.

Bei der Analyse der Genexpression via qPCR zeigte sich, dass GPR55 in Plazentagewebe verhältnismäßig gering exprimiert ist, wenn man mit Milz und Lunge vergleicht, die die beiden Organen mit der höchsten Expression darstellen. Die Expression steigt im Laufe der Schwangerschaft an, was sich mit einer 5.8-fach höheren Rezeptorexpression in reifem Plazentagewebe - verglichen mit Gewebe aus dem ersten Trimenon – niederschlägt. Bei der Lokalisierung des Rezeptors durch Immunhistochemie (IHC) zeigte sich deutlich, dass GPR55 einzig an plazentaren Endothelzellen exprimiert wird. Dieses Ergebnis konnte auch durch Expressionsanalysen an isolierten, primären plazentaren Endothelzellen (PAEC und PVEC) bestätigt werden. Interessanterweise war die Expression an kleinen Gefäßen unter der Trophoblastschicht am deutlichsten, während größere Arterien und Venen unterschiedliche Expressionsmuster zeigten. Die Blutgefäße der Nabelschnur zeigten nur eine sehr schwache IHC-Färbung mit dem GPR55-Antikörper. Da primäre plazentare Endothelzellen eine deutliche GPR55-Expression zeigten, wurden diese Zellen für Zellkulturexperimente verwendet, um damit den Einfluss des Liganden LPI

auf die Funktion von Endothelzellen zu erforschen. Es zeigte sich, dass LPI bei einer Konzentration von 1 µM einen deutlichen Einfluss auf die Migration von Endothelzellen venösen, nicht aber arteriellen Ursprungs aufweist. Dieser LPI-induzierte Effekt konnte durch den GPR55-Antagonisten O-1918 teilweise reduziert werden, was mich zu der Schlussfolgerung führte, dass das GPR55-LPI-System eine maßgebliche Rolle in der Funktion von plazentaren, venösen Endothelzellen spielen könnte.

4 Introduction

In this thesis, characterization of the G protein coupled-receptor 55 (GPR55) in human placenta and the role of its activating ligand L-α-lysophosphatidylinositol (LPI) in human placental endothelial cells should be disclosed.

4.1 The human placenta

The placenta is the supplying organ of the fetus of all life-bearing vertebrates during pregnancy. Its development starts after implantation of the blastocyst into the uterine wall (Hinrichsen 1990).

4.1.1 Structure and function of the human placenta

Towards the marternal uterus the placenta is confined by the basal plate which is embedded into the uterine wall. At the opposite side the chorionic plate with the umbilical cord is found, which is the connection to the fetus. The space in between is called the intervillous space. It contains maternal blood which is renewed three to four times per minute (Lüllmann-Rauch 2009, Ulfig 2005). The exchange of nutrients and gases in the human placenta takes place via diffusion between maternal and fetal blood while the two blood types are permanently separated and never come into direct contact (Hamilton, Boyd 1960). Maternal blood floods the intervillous space and surrounds villous trees where the previously mentioned diffusion takes place. Stem villi are anchored into the chorionic plate. Branches enlarge villous surface to provide more diffusion area. In a mature placenta the smallest branches at the end of villous trees are called terminal villi. Besides stroma and the trophoblast layer, fetal blood vessels constitute the major part of these villi (Moore, Persaud 1996). Fetal blood flows from capillaries in terminal villi to bigger vessels in stem villi and at the

chorionic plate to the umbilical cord to provide the fetus with nutrients and oxygen, then it flows back to return waste products to the maternal circulation (Fig. 1).

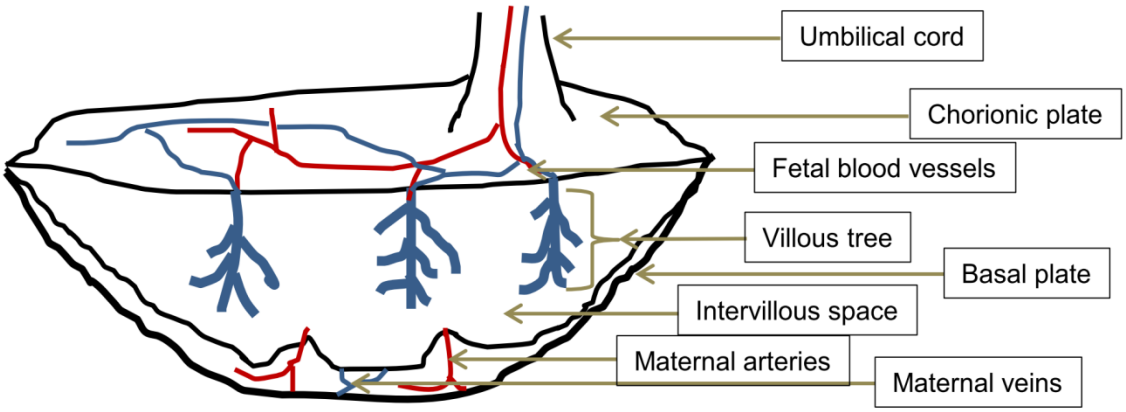

Figure 1 Scheme of the human placenta.
Maternal blood enters intervillous space through maternal arteries and leaves through maternal veins. In intervillous space it flows around villous trees and nutrients, oxygen and waste products are passed through diffusion between maternal and fetal blood with the latter flowing through fetal blood vessels within villous trees. Both blood types are never in direct contact.

4.1.2 Important steps in placental development

During first trimester, two outer trophoblast layers build the borders around villi (Fig.2). In the villous s trees, the multinucleated syncytiotrophoblast (ST) constitutes the only layer which comes into direct contact with maternal blood. Right underneath, mononucleated cells are found - the so called cytotrophoblast cells (CT). During placental development, CT cells fuse to become part of the ST. While during the first trimester of pregnancy villi have a continuous CT layer, this fusion leads to a disrupted layer in villi towards the end of gestation (Sadler, Langman 2003, Huppertz, Gauster 2011). Thereby layers between fetal and maternal blood are reduced to facilitate diffusion processes. The mesenchyme of placental villi conducts the fetal blood vessels (Fig. 2).

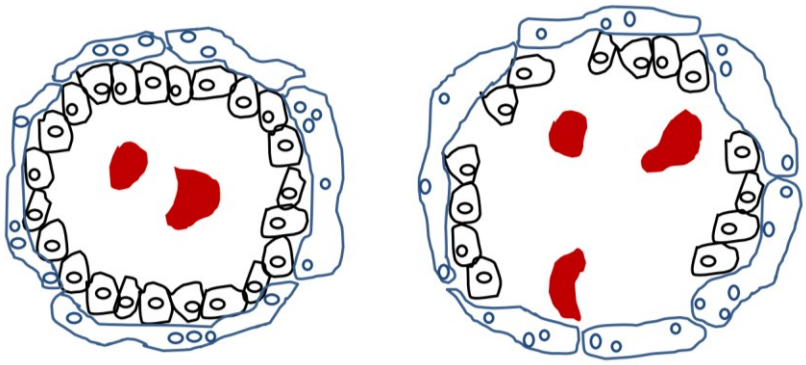

Figure 2 Cross section through a secondary (left) and a tertiary (right) villus. The multinucleated syncytiotrophoblast (blue) forms the outermost layer with mononucleated cytotrophoblasts cells (black) right underneath. Towards end of pregnancy this former continuous layer becomes disrupted to shorten the distance between maternal and fetal blood. Maternal blood flows around villi, while fetal blood is conducted in vessels inside villi (red).

Endothelial cells in these vessels are exposed to many different influencing substances from both the fetal and the maternal system (Shantsila, Watson et al. 2007). Factors released from placental macrophages, so-called Hofbauer cells, found in placental villi may further influence the endothelial cell cycle and development. During placental development two types of blood vessel formation can be distinguished. Angiogenesis on the one hand is the process of blood vessel formation from pre-existing ones. In this process endothelial cells from vessels start sprouting to form capillaries. The other process -vasculogenesis - takes place prior to angiogenesis. This term defines the process of *de novo* production of endothelial cells from cell precursors called angioblasts. Subsequently, final formation of blood vessels happens via angiogenesis (Demir, Kaufmann et al. 1989, Huppertz, Weiss et al. 2014, Lisman, van den Hoff et al. 2007, Benirschke, Kaufmann 2000, Boyd, Hamilton 1970). Vasculogenesis mainly occurs during embryonic development of the circulatory system. Furthermore there are some conditions when neovascularization is found in adult organisms too. Examples include tumor growth and revascularization following trauma (Shantsila, Watson et al. 2007, Gao, Nolan et al. 2008, Nolan, Ciarrocchi et al. 2007, Mellick, Plummer et al. 2010, Plummer, Freeman et al. 2013, Werner, Kosiol et al. 2005). Impairment in placental blood vessel development may

have adverse outcomes on the growing fetus and can even result in embryonic mortality (Moser, Li et al. 2004). When the hemal system of the placenta is underdeveloped, underdevelopment of the fetus can be a consequence due to malnourishment or oxygen deficiency. Intrauterine growth restriction (IUGR) is one well known fetal disorder caused by insufficient embryonic supply through reduced blow flow to the fetus (Arroyo, Winn 2008) and may even cause an increased risk for neonatal death (Lawn, Cousens et al. 2005).

4.2 G proteins and their receptors

G proteins together with their G protein-coupled receptors (GPCRs) play a key role in signal transduction systems by allowing or inhibiting mediation of molecular signals. Based on this functional principle the family of GPCRs together with their G proteins are often declared as molecular switches. Since the GPCRs are transmembrane receptors they fulfill their tasks by sensing extracellular molecules and activating intracellular signal transduction pathways to mediate cellular responses.

4.2.1 Structure and function

G nucleotide-binding proteins – the so called G proteins – are classified into two families: heterotrimeric G proteins composed of α-, β- and γ-subunit, and proteins belonging to the Ras superfamily of small GTPases. The latter are monomers homologous to the α-subunit of the heterotrimeric G proteins. Members of both G protein families enable signal transduction via binding of GTP guanosine triphosphate) and GDP (guanosine diphosphate), albeit their signaling pathways differ. Signal transduction takes place if a G protein binds GTP. By exchanging GTP to GDP, signal transduction is interrupted. Alteration between these two forms happens via hydrolysis of GTP through GTPase activity of the G proteins. In detail, the process starts with an inactive receptor and a complete G protein with bound GDP. The ligand attaches to the receptor and leads to a conformational change. The now activated GPCR is able to act as a guanine nucleotide exchange factor and

exchanges the bound GDP with GTP. As a consequence, the now activated G protein complex loses stability. Due to this instability the α-subunit with bound GTP dissociates and a signal transduction cascade is continued in this way by interacting with other intracellular proteins. Due to the intrinsic GTPase activity of the α-subunit, GTP is cleaved by hydrolysis into GDP and inorganic P_i. The free α-subunit is able to reassociate with the βγ-subunit and binds to the GPCR to inactivate the receptor. In this self-regulatory process, the cycle of activation and inactivation can now start all over again (Wettschureck, Offermanns 2005, Digby, Lober et al. 2006) (Fig. 3).

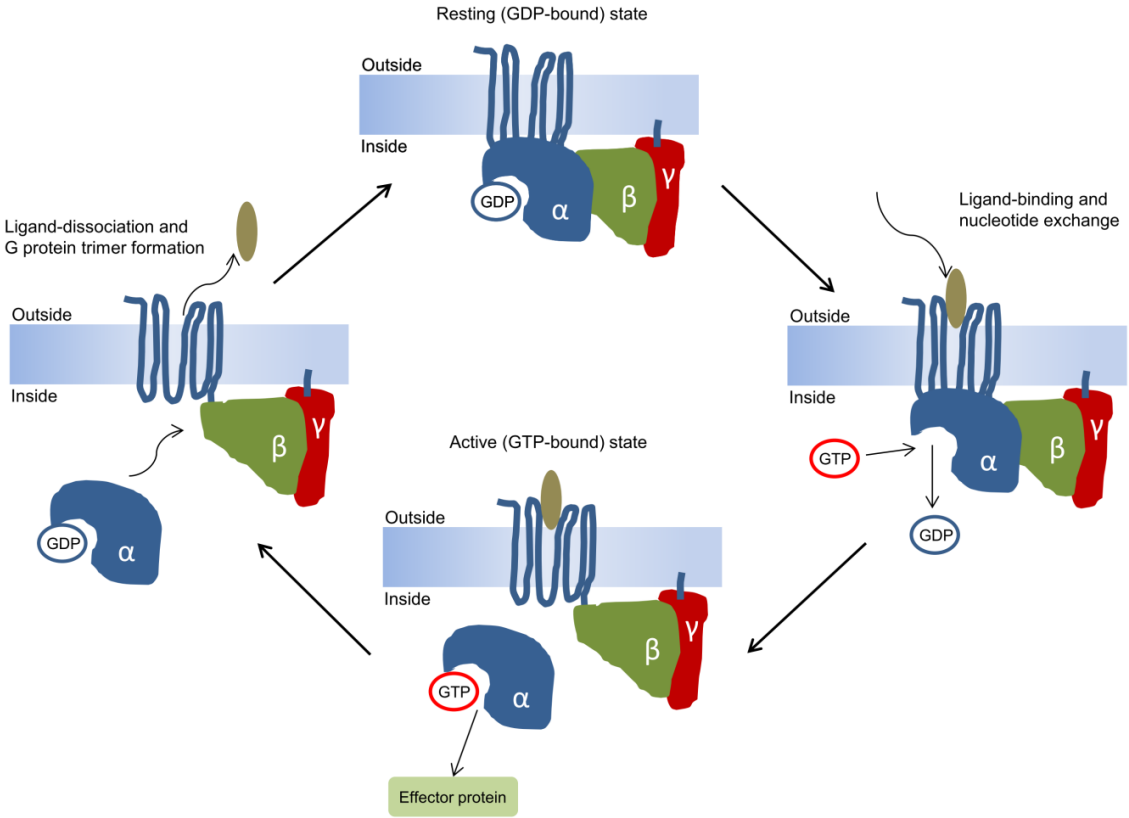

Figure 3 Cycle of GPCR activation and inactivation.
During resting state, GDP is bound to the α-subunit of the G protein which is attached to the GPCR. After ligand-binding and receptor activation, receptor conformation changes lead to the exchange of GDP to GTP. The α-subunit-GTP-complex dissociates off and interacts with different kinds of intercellular proteins to continue signal transduction. This subunit is also able to hydrolyze GTP into GDP and P_i via intrinsic GTPase activity and finally reassociates with the βγ-subunit of the G protein to restore inactive state of the GPCR.

Even though GPCRs are grouped into 6 different classes based on sequence homology and functional similarity, they all have a common structure and mechanism of signal transduction (Joost, Methner 2002, Attwood, Findlay 1994, Kolakowski 1994, Foord, Bonner et al. 2005).

4.2.2 The endocannabinoid system and GPR55 in the human body

The endocannabinoid system consists of the main two classical cannabinoid receptors CB1 and CB2 together with several natural ligands: arachidonoylethanolamine / anandamide (AEA), 2-arachidonoylgylcerol (2-AG), *N*-oleoylethanolamine (OEA), *N*-palmitoylethanolamine (PEA) and virodhamine (Taylor, Finney et al. 2011).

The G protein-coupled receptor 55 is a member of the rhodopsin-like 7TM/GPCR (seven-transmembrane/G protein-coupled receptor) family. The members of this family are characterized by α helices that are folded across the membrane seven times (Henstridge, Balenga et al. 2009, Moriconi, Cerbara et al. 2010, Pierce, Premont et al. 2002). Its gene is mapped to human chromosome 2q37 (Pertwee, Howlett et al. 2010). Although this receptor is lacking the typical cannabinoid binding pocket and shares only approximately 14% sequence homology with the classical cannabinoid receptors, GPR55 has been considered as endocannabinoid receptor (Baker, Pryce et al. 2006, Kotsikorou, Madrigal et al. 2011). The specific and functional endogenous ligand for GPR55 is found to be L-α-lysophosphatidylinositol (LPI) (Henstridge, Balenga et al. 2009, Kargl, Brown et al. 2013, Oka, Nakajima et al. 2007), albeit it can be activated partly by the endocannabinoids AEA (Habayeb, Taylor et al. 2008) and 2-AG. Although LPI-induced functions were first identified in the 1980's, it was not until 2007 that GPR55 was found to be activated by LPI (Henstridge, Balenga et al. 2009). Experiments with human platelets suggest that LPI derives from 1-stearoyl-2-arachidonoyl-phosphatidylinositol (PI) which is degraded by phospholipase PLA1 and cPLA2α activity (Ruban, Ferro et al. 2014). Synthesized LPI is released from the cell by AT-binding cassette transporter C1 (ABCC1) / multidrug resistance protein 1 (MRP1). cPLA2α activity leads to the production of 1-

stearoyl (18:0) LPI, which is the most abundant type in the human body. Intracellular phospholipase PLA1 is responsible for 2-arachidonoyl (20:4) LPI formation. This type of LPI is considered the most potent ligand for GPR55. In the healthy human body, LPI is found at levels between 0.3 and 1.51 µM. It takes on a key role in various physiological and pathophysiological processes, including cell proliferation, migration, apoptosis, reproduction, angiogenesis, inflammation and tumorigenesis (Ruban, Ferro et al. 2014, Choi, Lee et al. 2008). These functions are triggered on the one hand by activating GPR55 and on the other hand in both a paracrine and an autocrine manner (Ruban, Ferro et al. 2014, Pineiro, Maffucci et al. 2011). Both GPR55-dependent and –independent ion signaling pathways were shown in an endothelial cell line derived from human umbilical vein in response to LPI (Bondarenko, Waldeck-Weiermair et al. 2010). A role for GPR55/LPI system in platelet and endothelial cell function was suggested following wound healing experiments in primary human lung microvascular endothelial cells (HMVEC-L) (Kargl, Brown et al. 2013).

The current knowledge about the role of this receptor in placental tissue comprises gene expression studies of twenty human peripheral tissues and a functional study in rat (Fonseca, Correia-da-Silva et al. 2013, Henstridge, Balenga et al. 2009). In the human body, GPR55 is mainly found in various regions of the brain and nervous tissues. Furthermore, relatively high expression is found in tissues including adrenals, jejunum, ileum, spleen, and bone (Sanger 2007, Sawzdargo, Nguyen et al. 1999, Whyte, Ryberg et al. 2009). These findings could also be confirmed in my thesis study (Kremshofer, Siwetz et al. 2015). Expression analysis also detected GPR55 in metabolically important tissues such as liver, adipose tissue and pancreas. Functional studies in these tissues lead to the suggestion of a specific role of GPR55 in energy homeostasis (Liu, Song et al. 2015). In the nervous system, the receptor may play a role in incurrence of both inflammatory and neuropathic pain. In blood, it takes part in neutrophil migration regulation and prevents oxidative damage. Furthermore, GPR55 is known to play a role in vascular processes such as vasorelaxation and angiogenesis (Ho 2010). Additionally, the receptor was shown to influence cancer cell migration and proliferation (Henstridge, Balenga et al. 2009, Pineiro, Maffucci et al. 2011, Ford, Roelofs et al. 2010, Andradas, Caffarel et al. 2011).

4.2.3 The role of GPR55 in placental tissue

All three receptors – CB1, CB2 and GPR55 – are expressed in human placenta (Kremshofer, Siwetz et al. 2015, Chan, McKirdy et al. 2013, Fonseca, Correia-da-Silva et al. 2013). Various roles of the first two receptors have been described in human pregnancy – from processes like implantation, to decidualization, placentation and labour (Chan, McKirdy et al. 2013, Fonseca, Correia-da-Silva et al. 2013). The distinct role of GPR55 in placental development and physiology is not disclosed so far. In rat uterine tissues GPR55 is detected in decidual cells, uterine natural killer (uNK) cells and giant trophoblast cells. The receptor is assumed to trigger apoptosis of rat decidual cells and to play a role in decidual regression to accommodate the growing embryo (Henstridge, Balenga et al. 2009, Fonseca, Correia-da-Silva et al. 2013). Studies on the function of GPR55 and its agonist LPI in human placenta are lacking so far.

5 Aims

The aim of this thesis was to broaden basic knowledge about the G protein-coupled receptor 55 (GPR55) in the human placenta and to get a deeper insight into its functions during placental development.

The work was divided into two main parts. In the first part the focus was on exploration of the spatiotemporal expression pattern of GPR 55 in the human placenta. GPR55 is known to be expressed in lung endothelial cells (Kargl, Brown et al. 2013) and we were able to determine its expression in human placental endothelial cells (Kremshofer, Siwetz et al. 2015), hence the second part dealt with the influence of GPR55 and its agonist L-α-lysophosphatidylinositol on placental endothelial cell functions.

6 Methods

6.1 Immunohistochemistry

For immunohistochemistry, villous tissue of first trimester (6-12 weeks of gestation; n = 10) and term (>37 weeks of gestation; n = 10) placentas and umbilical cord tissue of six term placentas were fixed in formalin (4%) and embedded in paraffin. Serial tissue sections (5 µM) were mounted on Superfrost Plus slides (Menzel, Braunschweig, Germany). Sections were deparaffinized and rehydrated according to standard protocol (Tab. 1).

Solution	Time [min]
Xylene (1)	10
Xylene (2)	10
100% EtOH + Xylene (1:1)	3
100% EtOH	3
96% EtOH	3
80% EtOH	3
70% EtOH	3
50% EtOH	3
AD	3

Table 1 Deparaffinization steps as preparation for immunohistochemistry staining.

Antigen retrieval was performed by cooking slides under pressure at 120°C (pH 9, Eubio) for 7 min. After cooling down to RT immunohistochemistry was performed using the Ultravision LP detection system (Thermo Scientific, Fremont, USA) according to manufacturer's instructions. In this process hydrogen peroxidase block was used for 10 min to block endogenous peroxidase. The whole process was performed at RT. Next, slides were washed three times in AD prior to addition of Ultra Vision Protein Block for 5 min. Slides were incubated with a polyclonal anti-human GPR55 antibody (No. 10224, Cayman Chemical; 5 g/ml) for 45 min and with primary

antibody enhancer for 10 min afterwards. Slides were washed between those two steps three times with TBS including 0.05% Tween 20 (TBS/T; Merck). The same solution was taken for washing the slides thrice before the anti-mouse/rabbit Ultra Vision HRP-labeled polymer system and 3-amino-9-ethyl-carbacole (AEC, Thermo Scientific) was used for 10 min to enable detection. Sections were counterstained with hemalaun (12 min) and mounted with Kaiser's glycerol gelatin (Merck, Vienna, Austria). For negative controls, slides were incubated with rabbit Ig (Neo Markers) at the same concentration as mentioned above. To show specificity of the polyclonal anti-human GPR55 antibody slides were incubated with the antibody (5 µg/ml) pre-adsorbed with GPR55 blocking peptide (40 µg/ml, No. 10225, Cayman chemical).

6.2 Isolation of RNA, Reverse Transcription and RT-qPCR (Tissue and cells)

For RNA extraction small pieces of first trimester and term placental tissues were put into 1 ml Trizol and homogenized. After adding 100 µl (1/10 Trizol volume) of BCP (1-Bromo-3-Chloropane) the tube was inverted and incubated for 15 min at RT, followed by a 15 min centrifugation step at 12 000g. The clear upper phase containing total RNA was transferred into a new tube, 500 µl (1/2 Trizol volume) of 2-propanol was added, the tube inverted a few times and incubated for 10 min at RT. After a centrifugation step of 10 min at 12 000g the supernatant was discarded and the pellet washed in 1 ml cold ethanol (including another 10 min centrifugation at 12 000g). Removal of ethanol was followed by air drying of the pellet which was then dissolved by shaking for 15 min with 500 rpm in 30 µl RNase-free water at 55°C.

For RNA extraction out of primary cells, cells were cultivated in 6 well plates for 48 h (10^6 cells per well). 1 ml Trizol was used per well, isolation steps were the same as described above.

RNA quality was assessed on 1.5% denaturing agarose gels (Biozym, Vienna, Austria) and staining with GelRed (Biotium, USA). RNA quantity and purity were determined using a NanoDrop1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). After quality check, 500-2 000 ng of total RNA from each sample was

reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies).

Reverse Transcription was conducted with and without Reverse Transcriptase, respectively, to prove no genomic DNA was amplified during qPCR (Tab. 1).

Solution	**Amount [µl] (with RT)**	**Amount [µl] (without RT)**
Buffer	2	2
dNTPs	0.8	0.8
Primers	2	2
Reverse Transcriptase	1	0
H_2O	4.2	5.2

Table 2 Composition ratios for Reverse Transcription step.

Thermocycler was programmed as follows:

10’ at 25°C – 120’ at 37°C – 5’’ at 85°C

Afterwards, cDNA was subjected to quantitative RT-PCR using TaqMan Universal PCR Mastermix (Applied Biosystems) with TaqMan Gene Expression Assays for human GPR55 and ribosomal protein L30 for normalization (Applied Biosystems). Kit components and cDNA were mixed in 20 µl total volume/well in a 96 well plate (Roche) according to manufacturer’s instructions and amplified using a Bio-Rad CFX96 Real-Time PCR System. Thermocycler was programmed as follows:

120’’ at 50°C

600’’ at 95°C

15’’ at 95°C

60’’ at 60°C

} Repeated for 40 cycles

6.3 Isolation and culture of primary endothelial cells from term placentas

Endothelial cells were isolated from term placental tissues by using an enzymatic solution consisting of Hank's balanced salt solution (HBSS; Gibco, Invitrogen) containing collagenase type II (0.1 U/ml; Roche), dispase (0.8 U/ml; Roche), penicillin (300 IU/ml) and streptomycin (300 µg/ml). First, arterial and venous chorionic plate blood vessels were dissected, washed with pure HBSS and perfused with the HBSS solution described above for 7 min at 37°C. Subsequently, cell suspension was centrifuged (200xg, 5 min) and resuspended with EBM medium (Endothelial Basal Medium; Lonza). Obtained cells were seeded on culture plates coated with gelatin. Therefore prewarmed gelatin (1%) was pipetted into the flasks, excess was aspirated and flasks were incubated for 1 h at 37°C. Cells were grown in EBM supplemented with the EGM-MV BulletKit (Endothelial Growth Medium; Lonza; supplements: 0.1% Human Epidermal Growth Factor, 0.1% Hydrocortisone, 0.1% Gentamicin Sulfate, 0.4% Bovine Brain Extract, 5% Fetal Bovine Serum).
To check identity and purity of primary endothelial cells they were stained immunohistochemically with the classical endothelial marker von Willebrand factor. For exclusion of contamination with other cell types, cells were stained with markers against fibroblasts (CD90) and smooth muscle cells (smooth muscle actin and desmin). Cells were only used at a purity of >99% between passages four and six to avoid phenotypic drift.

For treatments the same EGM-MV medium but without FBS was used.

For LDH and MTS assays cells were seeded 24 h prior to treatment (LDH: 6 000 cells/well; MTS: 10 000 cells/well). Cytotoxicity and mitochondrial activity were assessed after 8 h and 24 h of treatment. For the CytoSelect™ Cell Migration Assay cells were directly harvested in serum-free medium and applied to the inserts in the prescribed concentration. The same procedure was used for Wound Healing Assays.

For the Human Angiogenesis Antibody Array cells were treated the same way as for LDH and MTS assays. After 24 h incubation time supernatants were collected.

6.4 Cell pellets and immunocytochemistry

For immunocytochemistry cell pellets of PAEC and PVEC were prepared from three different isolations each. Therefore confluent cells of 75 cm² flasks were harvested, centrifuged, washed and fixed in formalin (4%) for 30 min at RT. After further washing steps warm gelatin (5%, 37°C) was added to cells and incubated for 30 min at 37°C. Subsequently cells were centrifuged again and the gelatin-cell-block was hardened for 20 min at 4°C. The block was overlaid with formalin (4%) for 1 h at 4°C. After formalin removal the resulting cell pellet was stored in formalin overnight. The last steps included paraffin embedding and immunohistochemical staining following the same protocol as described above (5.1).

6.5 Wound Healing Assay

Culture inserts (Culture-Insert 24, ibiTreat for 24 well plates; ibidi®) were used as another way to assess LPI's influence on primary endothelial cell migration. Therefore cells were harvested, seeded into both wells of the insert (3-5x10^6 cells/ml) and left overnight at 37°C. The next day inserts were carefully removed, cells were washed and pre-incubated for 30 min ± O-1918 [10 µM] in serum-free medium at 37°C. After pre-incubation time LPI was added in desired concentrations and cells were observed with a Cell-IQ life cell imaging system (Chip-Man Technologies; Tampere, Finland). Pictures were taken every 20 min for a total period of 24 h (Fig. 4).

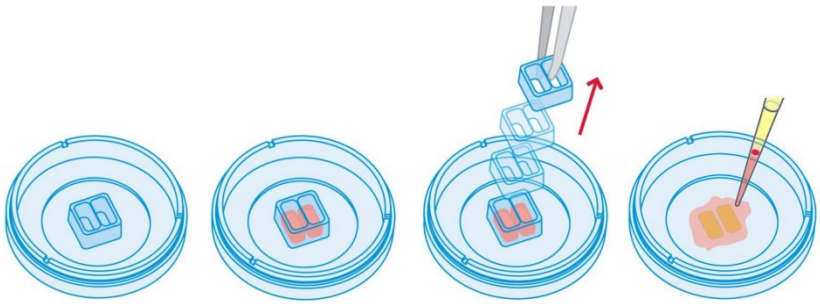

Figure 4 Functional principal of culture inserts for Wound Healing Assays. Endothelial cells are seeded in the required concentration into both chambers of the insert, which is removed after over-night incubation. Cells are treated with serum-free medium containing substances of interest and influence of these substances is observed by picturing cellular movement during a 24 h period. *With kind permission of ibidi®.*

6.6 Migration Assay

CytoSelect™ 24-Well Cell Migration Assay (8 µm, Colorimetric Format; Cell Biolabs, Inc.) was used according to the manufacturer's instructions to assess the influence of LPI on cell migration. Lower wells contained full medium (EGM-MV). Upper wells contained endothelial cells (0.5-1x10^6 cells/ml) in serum-free medium with 1 µM LPI. Cells were pre-incubated ± O-1918 [10 µM] in serum-free medium for 30 min at 37°C

following addition of LPI to upper and full medium to lower wells. After an incubation of 4 h non-migrating cells were removed from the upper side of the membrane which was then stained with provided Cell Stain Solution for 10 min at RT. Migrated cells were pictured with a light microscope and treated with Extraction Solution for 10 min while shaking. Absorption of extracted solution was measured at 560 nm (Fig. 5).

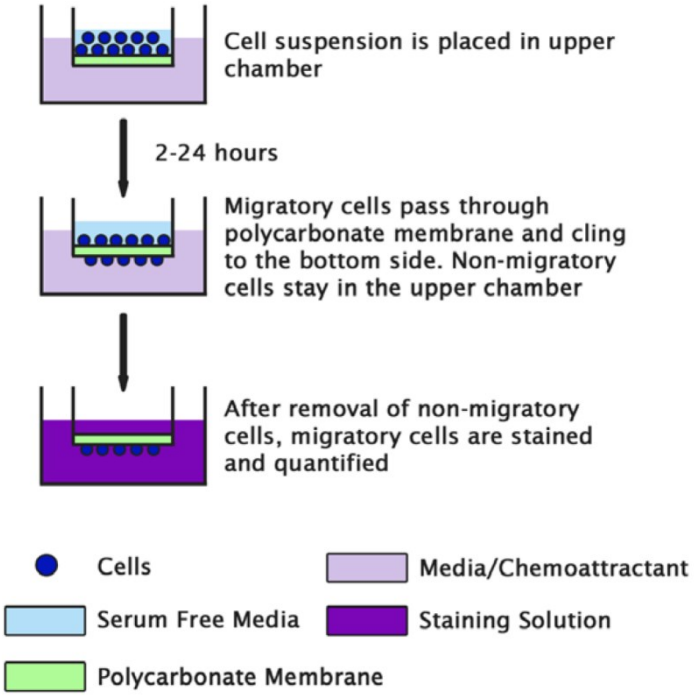

Figure 5 Image of the functional principle of Migration Assays.
Cell suspension is seeded into the upper chamber in serum-free medium containing substances of interest. Lower chambers contain complete medium. After a defined incubation time, non-migratory cells are removed and cells clinging to bottom side of the upper chamber are stained to enable evaluation and quantification.
With kind permission of Cell Biolabs.

6.7 Cell cytotoxicity assessment

Cell cytotoxicity was assessed by determination of LDH activity in supernatants of endothelial cells using an LDH Cytotoxicity Detection Kit (Takara Bio Inc., Eubio) according to the manufacturer's instructions. Cytotoxic compounds often comprise cell membrane integrity. In that way lactate dehydrogenase (LDH), which is normally in the inside of a cell, is released. LDH catalyzes the conversion of lactate to pyruvate while NAD^+ is reduced to $NADH/H^+$. A catalyst in the reaction solution (diaphorase) provided with the kit transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT, which is then reduced to a measurable Formazan dye (Fig. 6). If there is an increase in damaged cells, more LDH is released into the cell culture supernatant which directly correlates with the amount of Formazan product. For the evaluation of damage, a mixture of solution A and B (1:45) were mixed with supernatant (1:1) of cultured and treated cells (8 h or 24 h incubation) after centrifugation to remove cell debris (8 000 rpm, 10 min). After a 30 min incubation at RT in the dark, absorption was measured at 492 nm (reference wavelength 620 nm).

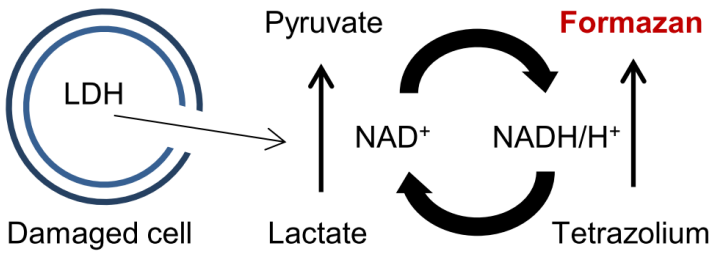

Figure 6 Scheme for cytotoxicity assessment assay.
Internal LDH is released through damaged cell membranes and catalyzes conversion of lactate and NAD^+ / $NADH/H^+$ to get a quantifiable dye product.

6.8 Assessment of mitochondrial activity

Mitochondrial activity was also assessed by using a colorimetric assay, the CellTiter 96® AQ_{ueous} One Solution Cells Proliferation Assay Kit (Promega). NADPH/NADH, which is produced in metabolically active cells, reduces the MTS tetrazolium compound of the assay into a colored formazan product. Therefore 20 µl of CellTiter 96® One Solution Reagent were pipetted into wells containing cultured cells in 100 µl of culture medium after 8 h or 24 h treatment and incubated for 2 h at 37°C. Afterwards absorption of supernatants was measured at 490 nm (reference wavelength 620 nm).

6.9 Human Angiogenesis Antibody Array

PAEC were cultivated and incubated with LPI (1 µM) in presence or absence of O-1918 (10 µM) and with antagonist alone for 24 h under standard cell culture conditions (37°C, 5% CO_2). Antibody Array membranes were treated with Blocking Buffer for 30 min at RT followed by incubation with the undiluted PAEC supernatant samples for 2 h at RT. Membranes were washed several times with both Wash Buffer I and Wash Buffer II at RT for 5 min each washing step. Subsequently, membranes were soaked with the Biotinylated Antibody Cocktail for 1.5 h at RT. After washing membranes the same way as described before, HRP-Streptavidin was added and incubated overnight at 4°C. Membranes were washed again and treated with a mixture of Detection Buffer C and Detection Buffer D (1:1) for 2 min at RT. Chemiluminescent imaging was performed using the FluorChem Q system (ProteinSimple™, USA) and signal densities were analysed with AlphaView software version 2.0.1.1 (AlphaInnotec, Cell Biosciences; Santa Clara, USA). Densities were normalized to the internal positive control (Fig. 7).

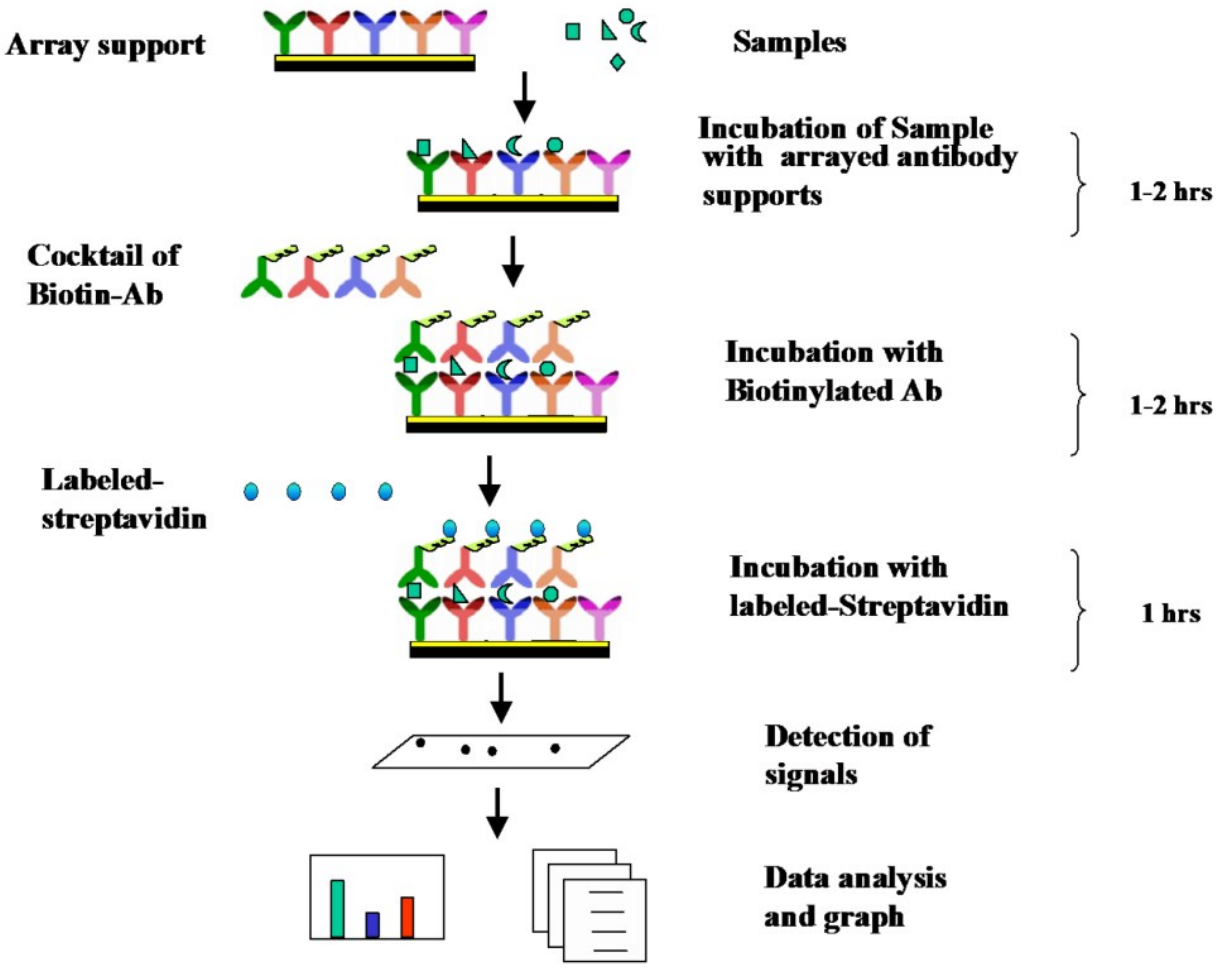

Figure 7 Principle of the Human Angiogenesis Antibody Array.

Cell culture supernatants are applied to membrane provided with defined antigens. After adding the second antibody, detection is enabled using labedled-Streptavidin.

With kind permission of RayBio®.

6.10 Isolation of primary trophoblasts

To extract the right fraction of cells, density gradients were used in this protocol. Therefore 36 ml of Percoll solution were mixed with 4 ml HBSS (10x) and gradients were prepared as indicated in table 3.

Gradient number	Percoll + 10x HBSS [ml]	1x HBSS [ml]
1	8.4	3.6
2	7.2	4.8
3	6.0	6.0
4	4.8	7.2
5	3.6	8.4
6	2.4	9.6
7	1.2	10.8

Table 3 Dilution information for the Percoll density gradients.

Furthermore, two washing solution have to be prepared: (A) consists of Keratinocyte-SFM (GIBCO®, Life technologies, Austria) with 1% Pen-Strep, while 1% Pen-Strep and 10% FBS are added for solution (B).
After preparing the Percoll density gradients, bead suspension has to be conjugated with the required antibodies. 200 µl of bead suspension were washed with the tenfold amount of 0.1% BSA solution (mixed with 4% of 0.5 M EDTA). 200 µl of BSA solution were put onto the washed beads and 52 µl of desired fibroblast antibody solution were added. After 1 h shaking at RT, fibroblast beads were washed again, suspended in 200 µL BSA solution, filled into a falcon and ready to use.

Placental tissue of three term placentas (>37 weeks of gestation) was washed with pre-warmed (37°C) solution (A) and patted to remove excess fluid. This step was important to determine actual tissue weight, but tissue should never be completely dry. Skin and vessels were removed from the tissue and 15 ml or 20 ml dispase DNase solution were added (depending on tissue weight). After stirring incubation for 15 min in water bath (37°C), tissues and cells are separated through a Cell Strainer

(Fisher Scientific, USA). 25 ml of solution (B) were added and cells were centrifuged for 6 min at 350g. Pelleted cells were suspended in 10 ml of solution (B), while residual tissue was incubated again to isolate more cells. These steps were repeated about three to four times, isolated cell fractions were pooled. Final cell suspension was centrifuged again and suspended in 20 ml solution (A). 5 ml were added to one prepared density gradient each. Separation of cell fractions took about 20 min at 2500 rpm (centrifugation is stopped without braking). Trophoblast fraction could be extracted from the gradient and washed with solution (B) (350g, 6 min). Cells were suspended in 12 ml BSA solution and added to prepared CD45 falcons (CD45 beads in 200 µl BSA solution). After 0.5 h incubation at 4°C, beads were separated by using a magnet. Steps were repeated with fibroblast beads. Supernatants without any beads were suspended in 45 ml of solution (B) and centrifuged for 6 min at 350g. Afterwards cells were suspended in 10 ml of solution (B) and were ready to be counted with the CASY® Technology Cell Counter and cultured under standard cell culture conditions (37°C, 5% CO_2). Two separated trophoblast isolations were conducted with each placenta.

6.11 Statistical analysis

Data were analyzed using SigmaPlot 12.5 and are presented as mean ± SD. Data were subjected to normality test (Shapiro-Wilk test) and equal variance test. With normally distributed data, differences between groups were tested using two-tailed *t* test. In other cases, Mann-Whitney rank-sum test was applied. For multiple comparison procedure, one-way repeated measures analysis of variance was followed by Holm-Sidak method to isolate groups that differ from the others. A *p* value of <0.05 was considered statistically significant.

7 Results

Parts of the results are already published by Kremshofer et al. (Kremshofer, Siwetz et al. 2015).

7.1 GPR55 mRNA is expressed in human placenta

Up to now, GPR55 expression is analyzed in tissues throughout the human body, but detailed expression studies on GPR55 in the placenta have only been conducted in rats (Fonseca, Correia-da-Silva et al. 2013). For this work, a human mRNA panel including mRNA extracted from human term placenta was used for quantitative gene expression analysis. Highest GPR55 gene expression could be found in spleen and lung, followed by salivary gland, trachea, small intestine and thymus (Tab. 4). Compared to these tissues, GPR55 expression in human term placenta showed relatively low levels with only 1.8% and 2.3% compared to spleen and lung, respectively. Other human tissues with GPR55 levels at the range of term placenta were prostate, thyroid gland, heart, uterus and adrenal gland. Lowest GPR55 gene expression was detected in skeletal muscle and cerebellum.

Human tissue	Fold change
Spleen (15)	54.74 ± 9.80
Lung (3)	44.06 ± 4.06
Salivary gland (24)	19.97 ± 1.85
Trachea (22)	18.95 ± 4.40
Small intestine (5)	17.64 ± 1.31
Thymus (2)	12.40 ± 0.75
Prostate (12)	1.68 ± 0.23
Thyroid gland (64)	1.65 ± 0.30
Heart (3)	1.64 ± 0.28
Uterus (8)	1.40 ± 0.29
Adrenal gland (64)	1.40 ± 0.10
Placenta, term (3)	*1.00 ± 0.12*
Skeletal muscle (7)	0.67 ± 0.22
Brain, cerebellum (10)	0.65 ± 0.04

Table 4 Quantitative gene expression analysis of GPR55 in human placenta and various other human tissues.
Expression of GPR55 was analyzed in pooled total RNA from indicated numbers of tissues given in parantheses. Data are presented as mean ± SD of fold change compared to placenta (cursively written).

To analyze placental expression patterns over the time course of gestation, GPR55 mRNA expression was investigated in first trimester placental tissues (n = 8) and compared with term placental tissues (n = 7). Quantitative gene expression analysis revealed a 5.8 fold ($p < 0.001$) increase in placental GPR55 expression at term, when compared to first trimester (Fig. 8).

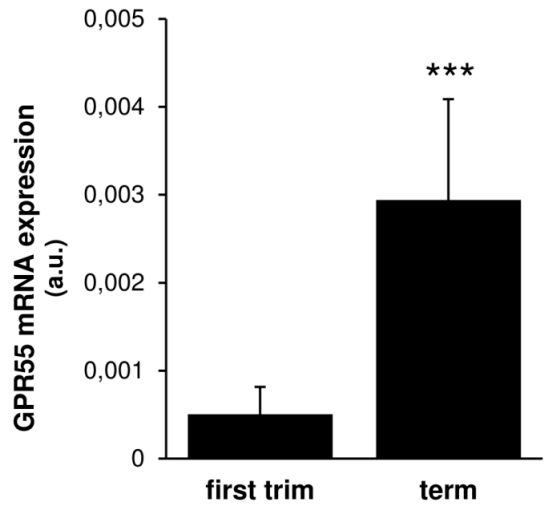

Figure 8 Analysis of GPR55 expression in human first trimester and term placental tissue.
Quantitative mRNA expression analysis revealed a 5.8 fold higher GPR55 gene expression in term (n = 7) compared to first trimester (n = 8) placental tissue. Values are given as mean ± SD. *** $p \leq 0.0001$

7.2 GPR55 protein is located at the fetal endothelium of human placenta

The next step after overall gene expression analysis was protein localization analysis in human placenta via immunohistochemistry (IHC). For this purpose we first checked different antibodies for their specificity by a pre-adsorption approach. Compared to the distinct staining with the anti-GPR55 antibody (Fig. 9a), pre-incubation of the primary antibody with a recombinant blocking peptide completely abolished staining which can be clearly shown by using adjacent serial sections (Fig. 9b). Together with examination of the right antibody we figured out a suitable antibody concentration.
After finding the appropriate antibody and its application concentration, we analyzed spatiotemporal expression of GPR55 in human placenta. 10 tissue sections of different samples from both first trimester and term placentas were stained

immunohistochemically to get an idea of GPR55 protein localization. In human first trimester placenta, GPR55 was detected in the fetal endothelium. In contrast, no staining was found in the villous stroma and the cell columns (Fig. 10a). Highest receptor expression was observed in newly emerging capillaries right underneath the villous trophoblast layer. Anti-CD34II (1:1000, 45 ng/ml), a marker for endothelial cells was used on adjacent serial sections to confirm identity of the fetal endothelium (10b, d and f).

In human term placenta, a similar staining was observed, showing most GPR55 expression in the fetal endothelium of smaller vessels in terminal villi. Compared to that, staining of larger vessels in the center of stem villi was less strong and we could see a differential GPR55 expression between arteries and veins. At protein level, arteries reveal a clear staining while veins showed a weak to absent staining (Fig. 10c). In contrast, qPCR results showed a higher GPR55 expression in veins (see 6.3 and Fig. 12). Like in first trimester, no GPR55 expression was found in the villous stroma, in villous cytotrophoblasts and syncytiotrophoblast. In umbilical cord, very weak staining was observed in the endothelium of both arteries and vein (Fig. 10e).

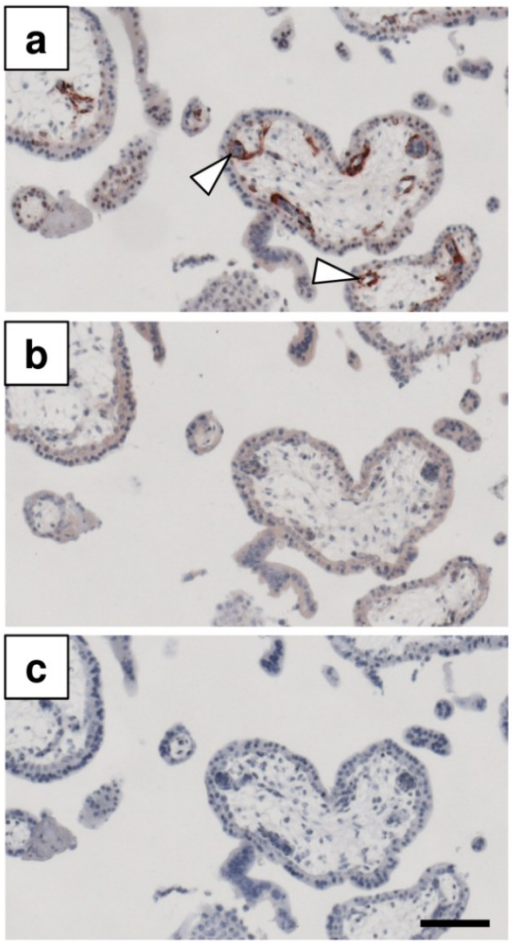

Figure 9 Evaluation of the used anti-GPR55 antibody.
Serial sections of first trimester placental tissue were stained with anti-GPR55 antibody. Specificity of the antibody was determined using an antibody pre-adsorption approach. **a** Incubation with the antibody alone showed clear staining of fetal vessels (*arrowheads*), whereas **b** pre-adsorption with recombinant GPR55 blocking peptide completely abolished staining. **c** Incubation with negative control rabbit IgG revealed no staining. *Scale bar* represents 100 µM.

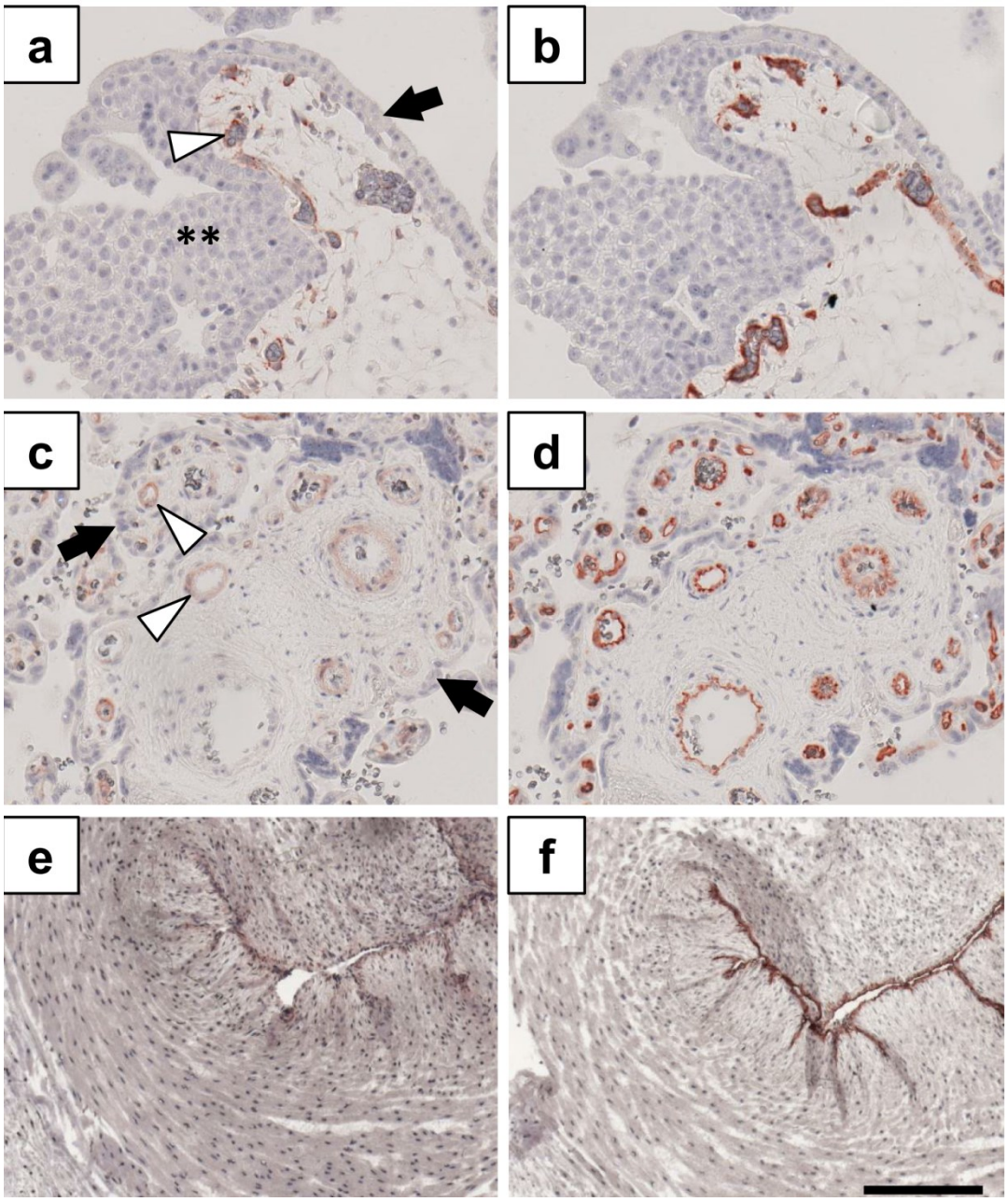

Figure 10 Immunohistochemical localization of GPR55 in human placenta.
a In human first trimester placenta, GPR55 protein expression was detected in the fetal endothelium (*arrowhead)*. The villous trophoblast compartment (*arrow*) and cell columns (*asterisks*) did not show any staining. **c** The same expression pattern was observed in human term placental villi. GPR55 staining show receptor expression in the fetal endothelium (*arrowheads*), whereas no staining was detected in the villous stroma, in villous cytotrophoblasts and syncytiotrophoblast (*arrows*). **e** In umbilical cord, very weak GPR55 staining was detected in the endothelium of both arteries and veins. **b, d** and **f** Adjacent serial sections were stained with anti-CD34II antibody to confirm identity of the fetal endothelium. *Scale bar* represents 100 µM.

7.3 GPR55 is expressed in primary placental endothelial cells

In order to confirm immunohistochemistry data and to determine an appropriate cell culture model for further experiments, we aimed for detection of GPR55 in primary placental arterial (PAEC) and venous endothelial cells (PVEC). Therefore, we isolated those cells from arteries and veins from the chorionic plate vessels of healthy human term placentas (n = 3). Purity of the endothelial cells was checked by immunohistochemical staining with the classical endothelial marker von Willebrand factor (vWF). For exclusion of contamination with other cell types, cells were stained with markers against fibroblasts (CD90) and smooth muscle cells (smooth muscle actin and desmin) (Fig. 11).

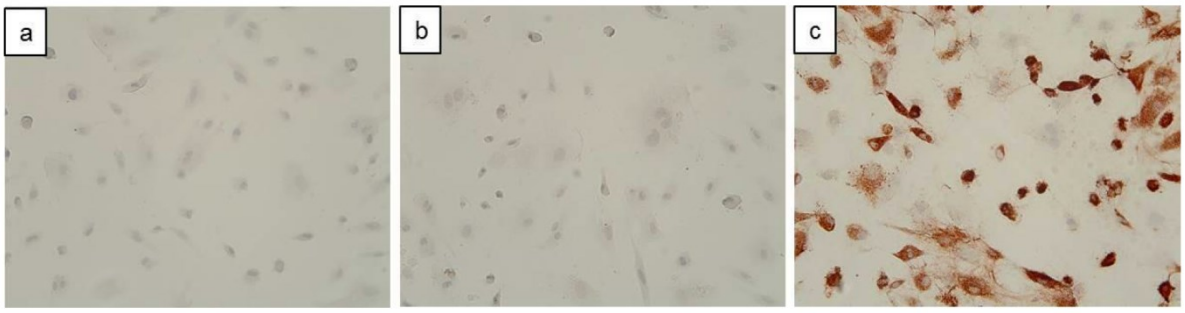

Figure 11 ICC stainings of isolated primary endothelial cells to prove their purity.
Use of **a** anti-desmin antibody and **b** anti-smooth muscle Actin (smA) antibody did not show any staining while **c** anti-von Willebrand factor (vWF) as a marker for endothelial cells revealed clear positive results (Kremshofer, Siwetz et al. 2015).

Beside placental endothelial cells we also checked human umbilical artery endothelial cells (HUAEC) and human umbilical vein endothelial cells (HUVEC) for GPR55 expression. Gene expression analysis via Real Time qPCR revealed GPR55 expression in all four cell types. We found a trend toward increased GPR55 expression in venous cells compared to arterial cells from the same origin (from placental vessels of the chorionic plate or umbilical vessels). Comparing all four cell types with each other, placental endothelial cells tend to have a higher GPR55 mRNA expression than endothelial cells from umbilical cord (Fig. 12a). In detail, GPR55 expression was 2.6 fold higher in PVEC, when compared to HUVEC, which, however, did not reach statistical significance ($p = 0.062$). A similar expression pattern was found when arterial endothelial cells were compared, with PAEC showing

a 1.6 fold higher GPR55 expression than HUAEC. Generally, GPR55 expression could be shown at mRNA level via qPCR in all evaluated cell types. Immunocytochemistry conducted with FFPE cell pellets revealed distinct staining at the plasma membrane of PVEC only (Fig 12b). All other cell types did not reveal any staining on protein level for GPR55.

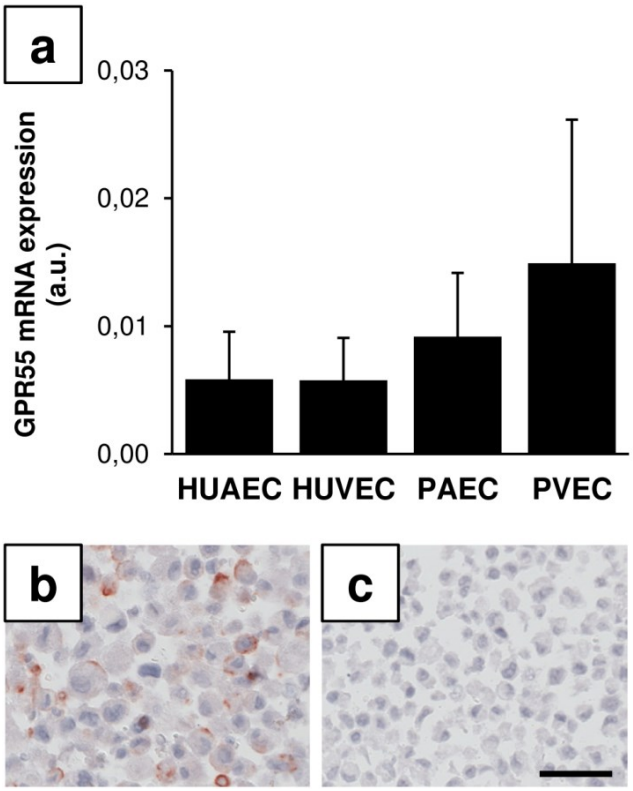

Figure 12 GPR55 expression in primary placental endothelial cells.
a Primary arterial (PAEC, n = 8) and venous endothelial cells (PVEC, n = 8) were isolated from healthy human term placentas and expression of GPR55 mRNA levels were compared with human umbilical artery (HUAEC, n = 3) and human umbilical vein endothelial cells (HUVEC, n = 3). **b** Immunocytochemistry of FFPE PVEC pellets revealed GPR55 expression at the plasma membrane. **c** Incubation with control rabbit IgG showed no staining. Values are given as mean ± SD. *Scale bar* represents 50 µM.

7.4 GPR55 is not expressed in isolated trophoblasts

IHC with tissue from both first trimester and term placenta did not show GPR55 expression neither at villous cytotrophoblasts nor syncytiotrophoblast (Fig. 10a and c). ICC with isolated primary trophoblast cells confirmed these results (data not shown).

7.5 Wound Healing Assays

Fig. 13 shows the first picture of a Wound Healing experiment with PAEC after removal of the culture insert and adding the serum-free medium containing LPI (1 µM). FBS contains a relatively high amount of different LPI types. Since these amounts are higher than our designated LPI concentration of 1 µM, it covers any influences the added LPI might have. Therefore we decided to perform functional experiments under serum-free conditions. Unfortunately cells did not move at all without serum hence we tried Cell Migration Assays as another approach.

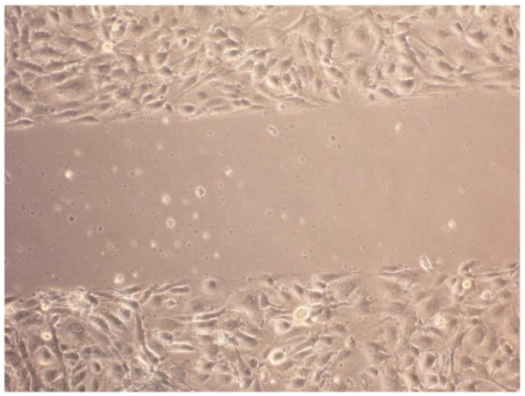

Figure 13 Primary placental arterial endothelial cells seeded into culture inserts after removal of the insert.

7.6 Effect of LPI on endothelial cell migration

Ford et al. could previously show that LPI enhanced a migratory response in human breast cancer cells through GPR55 (Ford, Roelofs et al. 2010). Based on this observation we wanted to analyze the effect of LPI on migration of primary placental arterial and venous endothelial cells. Incubation of these cells with LPI (1 µM) enhanced cell migration towards complete medium. Figure 14 shows that LPI revealed the same influential tendency for both arterial (left graph) and venous (right graph) endothelial cells, albeit only the latter reached statistical significance. PAEC showed an increased migration of 26.2% ($p = 0.177$) while LPI enhanced PVEC migration by 42.5% (Fig. 14).

To demonstrate that these effects are mediated through GPR55, we co-incubated PAEC and PVEC with LPI (1 µM) and the GPR55 antagonist O-1918 (10 µM). This approach partly, but not significantly reduced the effects of LPI in both arterial and

venous endothelial cells, while PVEC again showed a greater response. More precisely, co-incubation with LPI (1 µM) and O-1918 (10 µM) reduced LPI-enhanced migration in PVEC by 37.5% (Fig. 14, right).

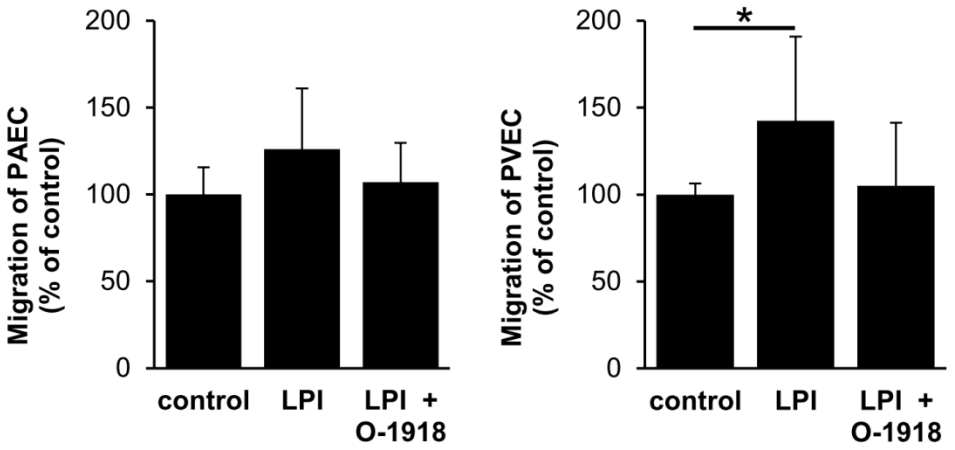

Figure 14 Effect of LPI on primary placental endothelial cell migration.
PAEC (n = 3) and PVEC (n = 3) were incubated with LPI (1 µM) in the presence or absence of GPR55 antagonist O-1918 (10 µM). Quantification of PAEC (left) and PVEC (right) migration followed after 4 h incubation. LPI revealed a tendency to increase endothelial cell migration towards compelete medium which only reached significance for PVEC. Values are given as mean ± SD. *p ≤ 0.05.

Qualitative evaluation of the cell migration assay is shown in Fig. 15.

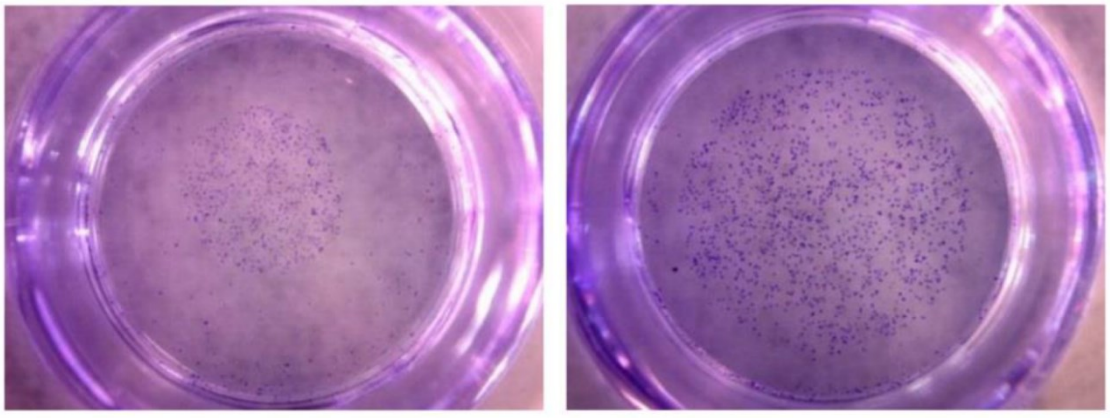

Figure 15 Effect of LPI on primary endothelial cell migration.
Comparison of untreated PAEC (left side) and PAEC incubated with LPI (1 µM) for 4 h.
The pictures show cells which are migrated through the membrane, non-migratory cells at the upper side of the membrane were removed. Cells were stained with provided staining solution and photographed with a reflected light microscope.

7.7 Effect of LPI on endothelial cell viability

Elevated cell migration triggered by LPI could also be based on higher cell proliferation or a decrease of cell death through LPI. To exclude these side effects and confirm the effect of LPI on actual migration we investigated the impact of LPI treatment (1 µM) in the presence or absence of GPR55 antagonist O-1918 (10 µM) on cell death and cell viability. Cell death was analyzed through LDH assays. A rise of LDH activity in cell culture supernatants indicates an increase in cell membrane damages which leads to cell death. Neither treatment with LPI alone nor co-incubation of LPI and O-1918 revealed any changes in released LDH activity (Fig. 16).

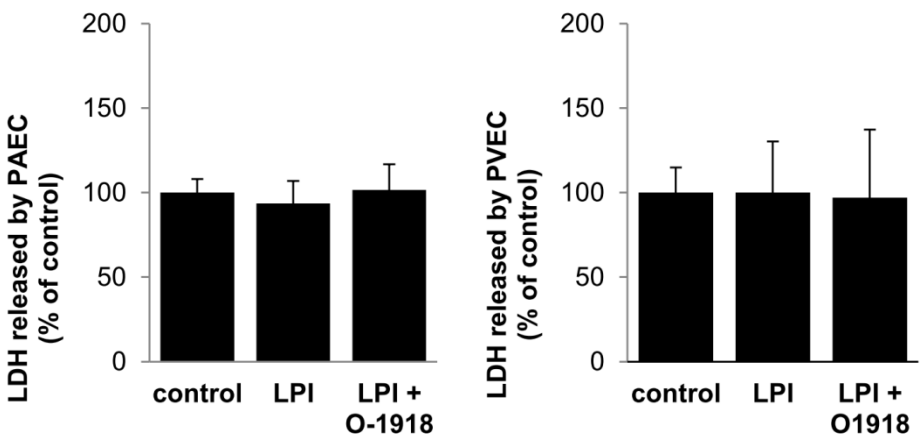

Figure 16 Effect of LPI on primary placental endothelial cell death.
PAEC (n = 3) and PVEC (n = 3) were incubated with LPI (1µM) in the presence or absence of GPR55 antagonist O-1918 (10 µM). LDH activity in supernatants was measured after 24 h incubation to determine endothelial cell damage and death. LPI did not show cytotoxic effects for neither cell type. Values are given as mean ± SD. *p ≤ 0.05.

Similar results were seen with endothelial cell viability analysis through measurement of mitochondrial activity. Therefore MTS assays were used on PAEC and PVEC after 24 h of treatment with LPI (1 µM) again in presence of absence of O-1918 (19 µM). None of these conditions showed an influence on endothelial cell viability (Fig. 17).

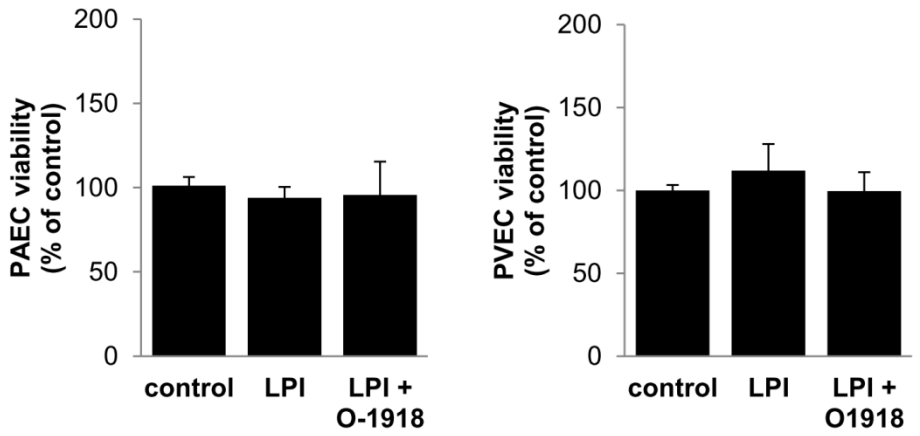

Figure 17 Effect of LPI on primary placental endothelial cell viability.
PAEC (n = 3) and PVEC (n =3) were incubated with LPI (1 µM) in the presence or absence of GPR55 antagonist O-1918 (10 µM). Mitochondrial activity was measured by MTS assay after 24 h incubation time. Neither PAEC nor PVEC revealed a difference in cell viability in response to treatments.
Values are given as mean ± SD. *p ≤ 0.05.

7.8 LPI increases secretion of PECAM-1 and TNF-α from PAEC

To get an insight into secretion of various angiogenic factors we performed Human Angiogenesis Antibody Array with supernatants of PAEC treated for 24 h with LPI (1 µM) ± O-1918 (10 µM) compared to untreated controls (pooled supernatants of 3 different cell isolations). Fig. 18 shows one of the membranes after performing the process. Evaluation of the membranes revealed the greatest change between different conditions for platelet endothelial cell adhesion molecule (PECAM-1; blue ovals) and tumor necrosis factor (TNF-α; red ovals), even if these changes were not significant.

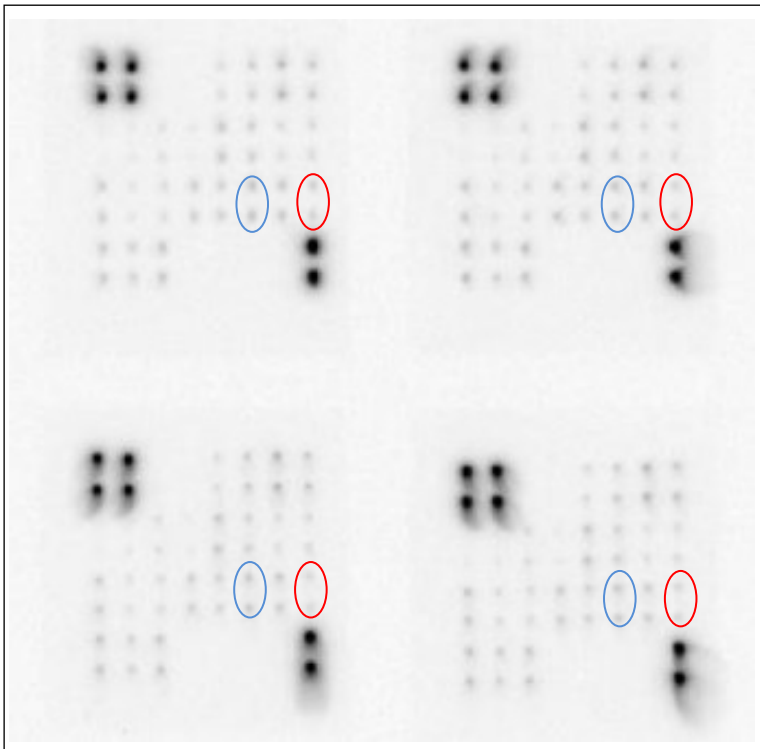

Figure 18 Membrane from Human Angiogenesis Antibody Array with spots for the different angiogenic factors.
Ovals mark the spots for PECAM-1 (blue) and TNF-α (red).

Quantification is accomplished by measurement of spot densities and normalization to positive controls. Results showed an increase of PECAM-1 and TNFα levels in PAEC supernatants after treatment with LPI (1 µM).
Co-incubation with O-1918 (10 µM) partly reduced those levels as well as incubation with the antagonist alone (Fig. 19).

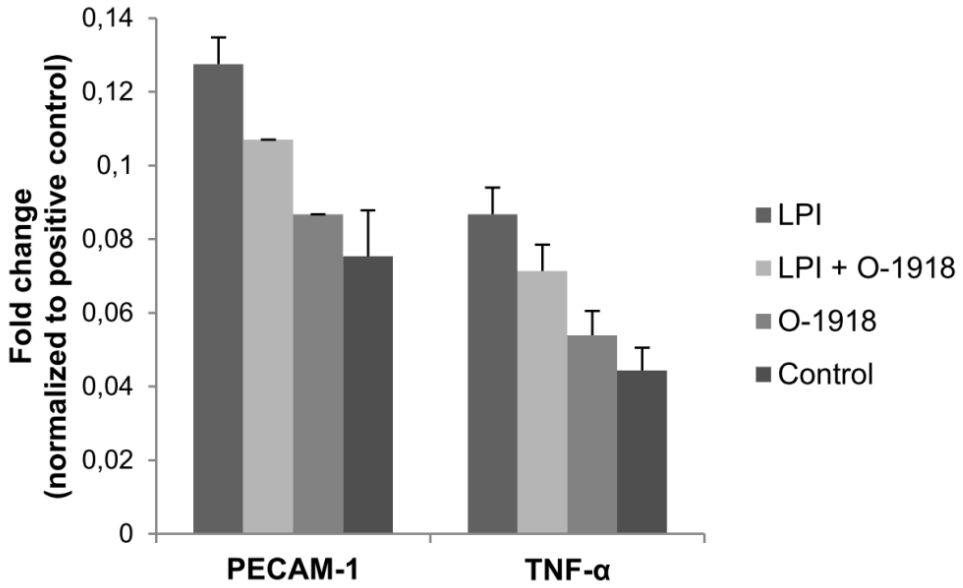

Figure 19 Spot analysis of PECAM-1 and TNF-α (normalized to positive control). Treatment with LPI (1 µM) lead to elevated levels of both angiogenic factors compared to untreated controls. This LPI-induced effect was partly reduced by co-incubation with the GPR55 antagonist O-1918 (19 µM).

8 Discussion

In this study GPR55 expression in human placenta was shown, although gene expression levels were relatively low in comparison to human tissues with highest receptor expression, namely spleen and lung (Kremshofer, Siwetz et al. 2015). This result is in line with a previous gene expression analysis of primary peripheral tissues (Henstridge, Balenga et al. 2009). An explanation for the high GPR55 expression in spleen may be the white pulp consisting of lymphatic tissue, since lymphocytes are well known for a very high receptor expression. Also, GPR55 shows a distinct gene expression in microvascular lung endothelial cells, hence GPR55 expression in pulmonary capillaries might be the reason for the high expression levels found in lung tissue (Kargl, Brown et al. 2013).

To confirm gene expression results and to localize GPR55 protein in human placental tissues, immunohistochemistry was used with both first trimester and term placental tissues collected from healthy women. GPR55 was shown to be expressed solely in endothelial cells. These results were confirmed by immunocytochemistry of isolated primary placental endothelial cells showing membrane-associated staining for GPR55 (Kremshofer, Siwetz et al. 2015). The antibody we used for both IHC and ICC has been successfully used in several other studies which lends credence to our immunostaining results (Bouskila, Javadi et al. 2013, Li, Feng et al. 2013, Lin, Yuece et al. 2011). Furthermore, we conducted experiments to confirm the specificity of the used antibody. We stained adjacent serial sections with either antibody alone or antibody pre-adsorbed with a blocking peptide. Staining was completely abolished when using the blocking peptide, thus the specificity of the antibody was proven.

Comparison of GPR55 mRNA expression between first trimester and term placental tissues showed a significant increase of a 5.8 fold higher expression in the latter. According to our immunostaining results only endothelial cells contribute to GPR55 expression, thus two suppositions can be made to explain this difference. On the one hand, increased villous vascularization over gestation (Kaufmann, Mayhew et al. 2004) can lead to an increasing GPR55 expression towards end of gestation. On the other hand, individual endothelial cells might show a higher receptor expression, which results in rising GPR55 levels in a consistent quantity of vessels. In addition, a

combination of these two hypotheses would a possible explanation for higher GPR55 expression levels at the end of gestation compared to the first trimester.
The increase in GPR55 expression towards end of gestation was found in villous tissue whereas receptor levels in decidual tissue were not investigated. There is a possibility that these levels might peak in mid trimester, since this is the case in uterine rat tissue. In rodents, GPR55 protein levels in decidual cells peak on day 14 of a 20-22 days lasting gestation period. Experiments have led to the suggestion that GPR55 might be associated with important uterine remodeling processes via apoptosis of decidual cells. The idea behind is, that the decline of decidual cells in rats causes tissue regression to accommodate the growing embryo (Fonseca, Correia-da-Silva et al. 2013). Thus, human GPR55 expression peaks may occur at different time points during gestation, not necessarily in term placenta. Unfortunately the possibility of investigating receptor expression during the progress of gestation is limited, since we only have access to placental tissue either from first trimester placentas (6-12 weeks of gestation) or after delivery (>37 weeks of gestation) resulting in an uncovered period of about 26-28 weeks.

Results of our investigations concerning GPR55 localization are in line with studies that show receptor expression in primary endothelial cells from multiple vascular beds, including endothelial cells from lung, dermis, brain, liver and coronary arteries (Wilhelmsen, Khakpour et al. 2014, Zhang, Maor et al. 2010). For endothelial cells of the umbilical cord IHC revealed only weak staining. These results could be confirmed through qPCR where HUVECs and HUAECs showed lower GPR55 expression compared to placental endothelial cells. Two further studies have shown similar results for HUVECs and their derived cell lines (Waldeck-Weiermair, Zoratti et al. 2008, Wilhelmsen, Khakpour et al. 2014). This leads to the conclusion that primary placental endothelial cells are an adequate model for cell culture experiments and pharmacological studies. In addition to stable GPR55 expression in placental endothelial cells, these cells are relatively easy to isolate from chorionic plate vessels. Since neither IHC nor ICC could show any GPR55 expression in trophoblasts cells, these cells could be excluded from cell culture work.

Evidence was found that expression and activation of GPR55 in endothelial cells is associated with angiogenesis and endothelial wound-healing capacity (Kargl, Brown et al. 2013, Zhang, Maor et al. 2010). No correlation is known between GPR55

activation and endothelial cell function in human placenta so far. Experiments we conducted on this topic were performed to observe the effect of LPI treatments at physiological level on endothelial cell migration. In our first attempt we tried so called Wound Healing Assays. The first part of these experiments was conducted with an LPI concentration of 1 µM ± O-1918 (10 µM) within complete culture medium. These trials did not reveal any differences between any of the chosen conditions. The underlying problem may be the fact that FBS contains various types of phospholipids including different kinds of LPI. The LPI concentration in FBS is relatively high which leads to the problem that an addition of 1 µM may not evoke any considerable change. Therefore another trial was performed with serum-free EGM-MV. Unfortunately, without FBS cells did not move at all. We considered to try growth factors to stimulate cellular movement without overlaying LPI influences, but decided to try another migration assay first. Using the CytoSelect™ 24-Well Cell Migration Assay revealed differences in endothelial cell migration under different conditions; hence we continued experiments with this assay.

Experiments were conducted with LPI (1µM) ± O-1918 (10 µM) and an untreated medium-only control. This approach revealed an LPI-enhanced endothelial cell migration towards complete medium, which was partly reduced through co-incubation with the GPR55 antagonist. In both arterial and venous cells, LPI tended to show the same influence, albeit the latter revealed more pronounced results. Calling immunocytochemistry results to mind, the explanation for this difference may be found in the higher GPR55 expression in venous endothelial cells compared to those obtained from arteries. If we look for underlying reasons we suggest that there are differences between these two cell types referring both their physiology and the degree of maturity. Placental arterial endothelial cells display a fully differentiated arterial phenotype, whereas placental venous endothelial cells have a juvenile, less differentiated phenotype with a high degree of plasticity (Lang, Schweizer et al. 2008). Not alone, further differences are found at DNA level. If one compares global DNA methylation levels one will find that endothelial cells obtained from veins are hypermethylated compared to arterial endothelial cells (Joo, Hiden et al. 2013). It is interesting that DNA methylation is inversely correlated with CB1 expression when looking at human colon cancer cells (Di Francesco, Falconi et al. 2015). However, the correlation between different DNA methylation levels and differential GPR55 expression in arteries and veins is still not clear.

In vitro, different migratory behaviors of endothelial cells can be caused by several reasons. First, LPI could have an influence on cell viability. If there are actually more or less cells over time in the upper chamber, more or less cells could migrate through the membrane. Second, if cells are migrating much faster under a certain condition compared to others, these cells might stick no longer to the lower side of the membrane, but drop off towards the bottom of the cell culture well. Third, LPI could have an influence on actual migration processes.

Pursuing possibility one, we conducted LDH and MTS assays to exclude cell number as an underlying reason for altered migration levels. Both assays clearly showed that LPI does not have an influence on cell viability at a concentration of 1 µM. Only LPI concentrations above 10 µM lead to a higher number of damaged endothelial cells. Other studies showed similar results, with LPI levels of 10 µM leading to decreased proliferation of human dermal microvascular endothelial cells. The same study indicated LPI concentrations of 0.01 and 0.1 µM to significantly increase proliferation of cells mentioned above. However, LPI at a concentration of 1 µM had no effect on human dermal microvascular endothelial cell proliferation (Zhang, Maor et al. 2010). The same concentration was also used for our experiments, since circulating LPI levels in normal-weight humans range between 0.3 and 1.51 µM. Obese subjects showed higher levels and especially female patients revealed a positive correlation between LPI levels, and fat percentage and body mass index, respectively (Moreno-Navarrete, Catalan et al. 2012, Sutphen, Xu et al. 2004).

To exclude possibility two, we analyzed the bottoms of the wells after 4 h treatments and before evaluation of the assay. In none of our experiments endothelial cells could be found below the membrane (pictures not shown). This strategy leads to the assumption that LPI is able to increase actual migration movements of placental endothelial cells over a time course of 4 h.

Initial experiments were performed to detect LPI influences on different angiogenic factors released from endothelial cells. Differences in GPR55 expression between PAEC and PVEC were not fully discovered at this point. Since PAEC show better viability and proliferation we started using these cells. Two factors were identified to be released from PAEC in a slightly higher amount when treated with LPI compared to untreated controls. Furthermore, co-incubation with the GPR55 antagonist O-1918 partly reduced the LPI-induced effect. Those two factors are PECAM-1 and TNF-α. PECAM-1 is the platelet endothelial cell adhesion molecule also known as cluster of

differentiation (CD) 31 (Jackson 2003, Wong, Jackson 2004, Ilan, Madri 2003). It is a member of the immunoglobulin superfamily. The protein is mainly expressed on the surface of endothelial cells, platelets, monocytes, macrophages, neutrophils and some types of T-cells. On endothelial cells it is found at connection points between adjacent cells. PECAM-1 is involved in angiogenic processes, thrombocyte functions, leukocyte transmigration and integrin activation (Jackson 2003).

Tumor necrosis factor (TNF)-α is a cytokine which is mainly involved in inflammation processes. It is primarily released by activated macrophages (Hehlgans, Pfeffer 2005). TNF-α is chiefly regulating immune cells and it is able to induce fever, apoptosis, and inflammation and is also able to inhibit tumorigenesis. Dysregulation of TNF-α expression is involved in several human diseases including cancer (Locksley, Killeen et al. 2001). Furthermore, TNF-α is found in elevated levels in blood plasma of obese patients compared to lean controls. Studies revealed elevated GPR55 levels in visceral adipose tissue of obese patients compared to lean controls. These levels were even higher if obese subjects were diabetic. In addition to this rise in GPR55, a positive correlation between LPI levels and both fat percentage and body mass index was found, with more pronounced results in female subjects compared to age-matched males (Moreno-Navarrete, Catalan et al. 2012, Sutphen, Xu et al. 2004). Due to these data we consider that there might be a correlation between obesity and GPR55 functions.

Based on this assumption, GPR55 effects on pregnancy of obese women and women exhibiting gestational diabetes mellitus (GDM) would be of special interest. In these days obesity is one of the most dangerous health problems amongst the western civilization and numbers of deaths caused by secondary diseases are constantly increasing. Evidence is rising that obesity not only impacts health of the adult population but adverse influences occur as early as during pregnancy. GDM, preeclampsia and intrauterine growth restriction (IUGR) are three of the main health issues with an established correlation to maternal obesity (Radulescu, Munteanu et al. 2013, Chu, Callaghan et al. 2007, Roberts, Bodnar et al. 2011, Jeyabalan 2013, Itoh, Kanayama 2014).

GDM is characterized by glucose intolerance with onset or first recognition during pregnancy (Dhulkotia, Ola et al. 2010). The world-wide incidence has constantly increased over time (Ben-Haroush, Yogev et al. 2004, Ferrara, Kahn et al. 2004).

Growth disturbances, exaggerated weight gain, high blood pressure and an increased risk of intrauterine fetal death (IUFD) are some issues the fetus will be faced with (Gunter, Tzialidou et al. 2006). Obese women who exhibit GDM are at high risk to develop type 2 diabetes post-pregnancy (Ben-Haroush, Yogev et al. 2004, Catalano, Huston et al. 1999). Adding another interesting fact, levels and composition of plasma lysophospholipids are known to correlate with glycemic state of pregnant women. Studies revealed decreasing levels of various LPIs in women with GDM (Dudzik, Zorawski et al. 2014). These data lead to the speculation that fetal LPI levels are influenced too when mothers exhibit GDM, with changes in GPR55-mediated placental endothelial functions as a further consequence.

Preeclampsia is a gestational disorder which is characterized by high blood pressure and a large amount of protein in the urine, known as proteinuria (Eiland, Nzerue et al. 2012). Usually it occurs during the third trimester with severe disease progression. Women with a preeclamptic pregnancy are at an increased risk for later-life cardiovascular (CV) diseases. The idea behind is, that these disorders share common risk factors, rather than preeclampsia being a cause for CV diseases (Al-Jameil, Aziz Khan et al. 2014).

In IUGR, remodeling of spiral arteries through extravillous trophoblast cells is impaired. This leads to an altered blood flow to the placenta and subsequently the fetus has to face a diminished supply with oxygen and nutrients (Arroyo, Winn 2008, Huppertz 2014). Consequences are - like the term IUGR reveals - disorders in fetal development and growth. Over 60% of neonatal deaths worldwide are associated with low birth weight, with IUGR being one of the three major causes (Lawn, Cousens et al. 2005).

Not only LPI and GPR55 are directly influenced by body fat and body weight, but also members upstream the LPI pathway show changes in correlation to obesity. As mentioned before, LPI is formed by degradation of 1-stearoyl-2-arachidonoyl-phosphatidylinositol by either PLA1 or Lp-PLA2. Studies conducted with obese children revealed higher plasma Lp-PLA2 levels in these subjects compared to lean controls (Sakka, Siahanidou et al. 2015). Furthermore obese women also showed higher Lp-PLA2 levels compared to age-matched normal-weight controls (Paik, Kim et al. 2015).

This work provides basic knowledge in the wide field of human placental physiology and the GPR55/LPI axis, but still there is a lot of ground to cover.

9 Outlook

This work covered GPR55 expression at both mRNA and protein levels in healthy human placental tissues from first trimester and term placentas. Additionally, functional studies suggest a role of GPR55/LPI axis in endothelial cell migration. Expansion of knowledge about the role of placental GPR55 in complicated pregnancies would clarify its consequences on pregnancy and outcome.

Future studies will investigate the influences of obesity and GDM on the GPR55/LPI axis at three stages. Measurement of LPI levels in cord blood of pregnancies complicated by obesity and GDM will be one part. Additionally PLA1 and PLA2 activities will be analyzed in human cord blood. In this work we covered analysis of the GPR55 expression pattern in healthy human first trimester and term placental tissues at both mRNA und protein level. Prospective studies will change focus on receptor expression analysis in placentas from pathological pregnancies. Furthermore, functional assays could be repeated and extended performing cell culture experiments with previously used primary endothelial cells under altered cell culture conditions. GDM environment could be simulated by adding glucose (25 mmol/l) and insulin (0.1 and 1 nmol/l) to culture media prior to LPI treatment to observe potential influences of diabetes on the role of GPR55 on endothelial functions. Obese conditions are more difficult to simulate since a rise in body weight and composition will have an influence at many different levels throughout the body. One possibility is to view on obesity as the low-grade systemic inflammation it is. During inflammation processes several pro-inflammatory cytokines reveal elevated levels in human blood due to increased adipose tissue, since adipose tissue functions as a highly active endocrine organ releasing such cytokines. Some of these cytokines could be used at different concentrations to mimic those inflammatory processes, including TNF-α (10 and 25 ng/ml) and IL-6 (10 ng/ml). These concentrations are well established by other groups working with human placenta (Hiden, Glitzner et al. 2008). Calling to mind, that TNF-α is increasingly released from PAEC treated with LPI, this cytokine is of special interest. Preliminary experiments done by our work group lead to the suggestion, that these two cytokines may elevate GPR55 gene expression (Fig. 20).

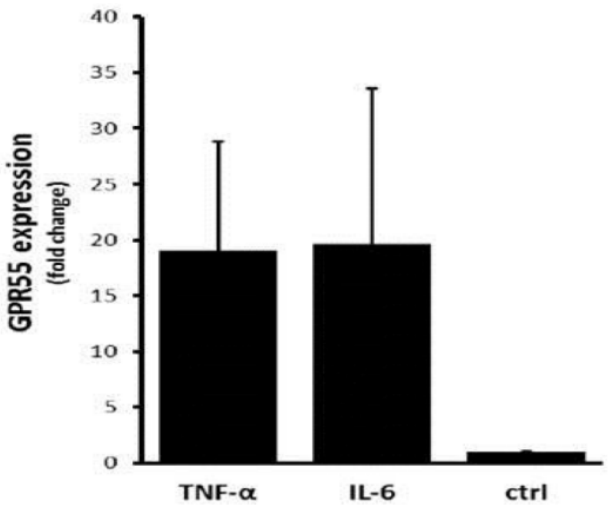

Figure 20 GPR55 mRNA expression in PAEC (n = 3) after 24 h treatment with 10 ng/ml of either TNF-α or IL-6 compared to untreated controls.

Moreover, it would be interesting to work with primary endothelial cells isolated directly from placentas of complicated pregnancies. However, if these cells are cultured under normal cell culture conditions their altered characteristics might be lost after a few passages. In addition to that it is easier to determine specific influencing factors on endothelial functions if those are added one by one to cell culture media.

10 References

AL-JAMEIL, N., AZIZ KHAN, F., FAREED KHAN, M. and TABASSUM, H., 2014. A brief overview of preeclampsia. *Journal of clinical medicine research,* **6**(1), pp. 1-7.

ANDRADAS, C., CAFFAREL, M.M., PEREZ-GOMEZ, E., SALAZAR, M., LORENTE, M., VELASCO, G., GUZMAN, M. and SANCHEZ, C., 2011. The orphan G protein-coupled receptor GPR55 promotes cancer cell proliferation via ERK. *Oncogene,* **30**(2), pp. 245-252.

ARROYO, J.A. and WINN, V.D., 2008. Vasculogenesis and angiogenesis in the IUGR placenta. *Seminars in perinatology,* **32**(3), pp. 172-177.

ATTWOOD, T.K. and FINDLAY, J.B., 1994. Fingerprinting G-protein-coupled receptors. *Protein engineering,* **7**(2), pp. 195-203.

BAKER, D., PRYCE, G., DAVIES, W.L. and HILEY, C.R., 2006. In silico patent searching reveals a new cannabinoid receptor. *Trends in pharmacological sciences,* **27**(1), pp. 1-4.

BEN-HAROUSH, A., YOGEV, Y. and HOD, M., 2004. Epidemiology of gestational diabetes mellitus and its association with Type 2 diabetes. *Diabetic medicine : a journal of the British Diabetic Association,* **21**(2), pp. 103-113.

BENIRSCHKE, K. and KAUFMANN, P., 2000. Architecture of normal villous trees. *Pathology of the Human Placenta.* USA: Springer, pp. 116-154.

BONDARENKO, A., WALDECK-WEIERMAIR, M., NAGHDI, S., POTESER, M., MALLI, R. and GRAIER, W.F., 2010. GPR55-dependent and -independent ion signalling in response to lysophosphatidylinositol in endothelial cells. *British journal of pharmacology,* **161**(2), pp. 308-320.

BOUSKILA, J., JAVADI, P., CASANOVA, C., PTITO, M. and BOUCHARD, J.F., 2013. Rod photoreceptors express GPR55 in the adult vervet monkey retina. *PloS one,* **8**(11), pp. e81080.

BOYD, J.D. and HAMILTON, W.J., 1970. *The Human Placenta.* London: Heffer.

CATALANO, P.M., HUSTON, L., AMINI, S.B. and KALHAN, S.C., 1999. Longitudinal changes in glucose metabolism during pregnancy in obese women with normal glucose tolerance and gestational diabetes mellitus. *American Journal of Obstetrics and Gynecology,* **180**(4), pp. 903-916.

CHAN, H.W., MCKIRDY, N.C., PEIRIS, H.N., RICE, G.E. and MITCHELL, M.D., 2013. The role of endocannabinoids in pregnancy. *Reproduction (Cambridge, England),* **146**(3), pp. R101-9.

CHOI, J.W., LEE, C.W. and CHUN, J., 2008. Biological roles of lysophospholipid receptors revealed by genetic null mice: an update. *Biochimica et biophysica acta,* **1781**(9), pp. 531-539.

CHU, S.Y., CALLAGHAN, W.M., KIM, S.Y., SCHMID, C.H., LAU, J., ENGLAND, L.J. and DIETZ, P.M., 2007. Maternal obesity and risk of gestational diabetes mellitus. *Diabetes care,* **30**(8), pp. 2070-2076.

DEMIR, R., KAUFMANN, P., CASTELLUCCI, M., ERBENGI, T. and KOTOWSKI, A., 1989. Fetal vasculogenesis and angiogenesis in human placental villi. *Acta Anatomica,* **136**(3), pp. 190-203.

DHULKOTIA, J.S., OLA, B., FRASER, R. and FARRELL, T., 2010. Oral hypoglycemic agents vs insulin in management of gestational diabetes: a systematic review and metaanalysis. *American Journal of Obstetrics and Gynecology,* **203**(5), pp. 457.e1-457.e9.

DI FRANCESCO, A., FALCONI, A., DI GERMANIO, C., MICIONI DI BONAVENTURA, M.V., COSTA, A., CARAMUTA, S., DEL CARLO, M., COMPAGNONE, D., DAINESE, E., CIFANI, C., MACCARRONE, M. and D'ADDARIO, C., 2015. Extravirgin olive oil up-regulates CB1 tumor suppressor gene in human colon cancer cells and in rat colon via epigenetic mechanisms. *The Journal of nutritional biochemistry,* **26**(3), pp. 250-258.

DIGBY, G.J., LOBER, R.M., SETHI, P.R. and LAMBERT, N.A., 2006. Some G protein heterotrimers physically dissociate in living cells. *Proceedings of the National Academy of Sciences of the United States of America,* **103**(47), pp. 17789-17794.

DUDZIK, D., ZORAWSKI, M., SKOTNICKI, M., ZARZYCKI, W., KOZLOWSKA, G., BIBIK-MALINOWSKA, K., VALLEJO, M., GARCIA, A., BARBAS, C. and RAMOS, M.P., 2014. Metabolic fingerprint of Gestational Diabetes Mellitus. *Journal of proteomics,* **103**, pp. 57-71.

EILAND, E., NZERUE, C. and FAULKNER, M., 2012. Preeclampsia 2012. *Journal of pregnancy,* **2012**, pp. 586578.

FERRARA, A., KAHN, H.S., QUESENBERRY, C.P., RILEY, C. and HEDDERSON, M.M., 2004. An increase in the incidence of gestational diabetes mellitus: Northern California, 1991-2000. *Obstetrics and gynecology,* **103**(3), pp. 526-533.

FONSECA, B.M., CORREIA-DA-SILVA, G., ALMADA, M., COSTA, M.A. and TEIXEIRA, N.A., 2013. The Endocannabinoid System in the Postimplantation Period: A Role during Decidualization and Placentation. *International journal of endocrinology,* **2013**, pp. 510540.

FOORD, S.M., BONNER, T.I., NEUBIG, R.R., ROSSER, E.M., PIN, J.P., DAVENPORT, A.P., SPEDDING, M. and HARMAR, A.J., 2005. International Union of Pharmacology. XLVI. G protein-coupled receptor list. *Pharmacological reviews,* **57**(2), pp. 279-288.

FORD, L.A., ROELOFS, A.J., ANAVI-GOFFER, S., MOWAT, L., SIMPSON, D.G., IRVING, A.J., ROGERS, M.J., RAJNICEK, A.M. and ROSS, R.A., 2010. A role for L-alpha-lysophosphatidylinositol and GPR55 in the modulation of migration, orientation and polarization of human breast cancer cells. *British journal of pharmacology,* **160**(3), pp. 762-771.

GAO, D., NOLAN, D.J., MELLICK, A.S., BAMBINO, K., MCDONNELL, K. and MITTAL, V., 2008. Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. *Science (New York, N.Y.),* **319**(5860), pp. 195-198.

GUNTER, H.H., TZIALIDOU, I., SCHARF, A., WENZLAFF, P., MAUL, H. and HILLEMANNS, P., 2006. Intrauterine fetal death in pregnancies of women with preconceptional and gestational diabetes mellitus and of women without glucose

tolerance disorders. Results of the perinatal registry of Lower Saxony, Germany. *Zeitschrift fur Geburtshilfe und Neonatologie,* **210**(6), pp. 193-199.

HABAYEB, O.M., TAYLOR, A.H., BELL, S.C., TAYLOR, D.J. and KONJE, J.C., 2008. Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. *Endocrinology,* **149**(10), pp. 5052-5060.

HAMILTON, W.J. and BOYD, J.D., 1960. Development of the human placenta in the first three months of gestation. *Journal of anatomy,* **94**, pp. 297-328.

HEHLGANS, T. and PFEFFER, K., 2005. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. *Immunology,* **115**(1), pp. 1-20.

HENSTRIDGE, C.M., BALENGA, N.A., FORD, L.A., ROSS, R.A., WALDHOER, M. and IRVING, A.J., 2009. The GPR55 ligand L-alpha-lysophosphatidylinositol promotes RhoA-dependent Ca2+ signaling and NFAT activation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* **23**(1), pp. 183-193.

HIDEN, U., GLITZNER, E., IVANISEVIC, M., DJELMIS, J., WADSACK, C., LANG, U. and 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**(1), pp. 150-157.

HINRICHSEN, K., 1990. *Humanembryologie.* Springer Verlag Heidelberg.

HO, W.S., 2010. Angiogenesis: a new physiological role for N-arachidonoyl serine and GPR55? *British journal of pharmacology,* **160**(7), pp. 1580-1582.

HUPPERTZ, B., 2014. Oxygenation of the placenta and its role in pre-eclampsia. *Pregnancy hypertension,* **4**(3), pp. 244-245.

HUPPERTZ, B. and GAUSTER, M., 2011. Trophoblast fusion. *Advances in Experimental Medicine and Biology,* **713**, pp. 81-95.

HUPPERTZ, B., WEISS, G. and MOSER, G., 2014. Trophoblast invasion and oxygenation of the placenta: measurements versus presumptions. *Journal of reproductive immunology,* **101-102**, pp. 74-79.

ILAN, N. and MADRI, J.A., 2003. PECAM-1: old friend, new partners. *Current opinion in cell biology,* **15**(5), pp. 515-524.

ITOH, H. and KANAYAMA, N., 2014. Obesity and Risk of Preeclampsia. *Medical Journal of Obstetrics and Gynecology,* .

JACKSON, D.E., 2003. The unfolding tale of PECAM-1. *FEBS letters,* **540**(1-3), pp. 7-14.

JEYABALAN, A., 2013. Epidemiology of preeclampsia: impact of obesity. *Nutrition reviews,* **71 Suppl 1**, pp. S18-25.

JOO, J.E., HIDEN, U., LASSANCE, L., GORDON, L., MARTINO, D.J., DESOYE, G. and SAFFERY, R., 2013. Variable promoter methylation contributes to differential expression of key genes in human placenta-derived venous and arterial endothelial cells. *BMC genomics,* **14**, pp. 475-2164-14-475.

JOOST, P. and METHNER, A., 2002. Phylogenetic analysis of 277 human G-protein-coupled receptors as a tool for the prediction of orphan receptor ligands. *Genome biology,* **3**(11), pp. RESEARCH0063.

KARGL, J., BROWN, A.J., ANDERSEN, L., DORN, G., SCHICHO, R., WALDHOER, M. and HEINEMANN, A., 2013. A selective antagonist reveals a potential role of G protein-coupled receptor 55 in platelet and endothelial cell function. *The Journal of pharmacology and experimental therapeutics,* **346**(1), pp. 54-66.

KAUFMANN, P., MAYHEW, T.M. and CHARNOCK-JONES, D.S., 2004. Aspects of human fetoplacental vasculogenesis and angiogenesis. II. Changes during normal pregnancy. *Placenta,* **25**(2-3), pp. 114-126.

KOLAKOWSKI, L.F.,Jr, 1994. GCRDb: a G-protein-coupled receptor database. *Receptors & channels,* **2**(1), pp. 1-7.

KOTSIKOROU, E., MADRIGAL, K.E., HURST, D.P., SHARIR, H., LYNCH, D.L., HEYNEN-GENEL, S., MILAN, L.B., CHUNG, T.D., SELTZMAN, H.H., BAI, Y., CARON, M.G., BARAK, L., ABOOD, M.E. and REGGIO, P.H., 2011. Identification of the GPR55 agonist binding site using a novel set of high-potency GPR55 selective ligands. *Biochemistry,* **50**(25), pp. 5633-5647.

KREMSHOFER, J., SIWETZ, M., BERGHOLD, V.M., LANG, I., HUPPERTZ, B. and GAUSTER, M., 2015. A role for GPR55 in human placental venous endothelial cells. *Histochemistry and cell biology,* .

LANG, I., SCHWEIZER, A., HIDEN, U., GHAFFARI-TABRIZI, N., HAGENDORFER, G., BILBAN, M., PABST, M.A., KORGUN, E.T., DOHR, G. and DESOYE, G., 2008. Human fetal placental endothelial cells have a mature arterial and a juvenile venous phenotype with adipogenic and osteogenic differentiation potential. *Differentiation; research in biological diversity,* **76**(10), pp. 1031-1043.

LAWN, J.E., COUSENS, S., ZUPAN, J. and LANCET NEONATAL SURVIVAL STEERING TEAM, 2005. 4 million neonatal deaths: when? Where? Why? *Lancet (London, England),* **365**(9462), pp. 891-900.

LI, K., FENG, J.Y., LI, Y.Y., YUECE, B., LIN, X.H., YU, L.Y., LI, Y.N., FENG, Y.J. and STORR, M., 2013. Anti-inflammatory role of cannabidiol and O-1602 in cerulein-induced acute pancreatitis in mice. *Pancreas,* **42**(1), pp. 123-129.

LIN, X.H., YUECE, B., LI, Y.Y., FENG, Y.J., FENG, J.Y., YU, L.Y., LI, K., LI, Y.N. and STORR, M., 2011. A novel CB receptor GPR55 and its ligands are involved in regulation of gut movement in rodents. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society,* **23**(9), pp. 862-e342.

LISMAN, B.A., VAN DEN HOFF, M.J., BOER, K., BLEKER, O.P., VAN GRONINGEN, K. and EXALTO, N., 2007. The architecture of first trimester chorionic villous vascularization: a confocal laser scanning microscopical study. *Human reproduction (Oxford, England),* **22**(8), pp. 2254-2260.

LIU, B., SONG, S., JONES, P.M. and PERSAUD, S.J., 2015. GPR55: from orphan to metabolic regulator? *Pharmacology & therapeutics,* **145**, pp. 35-42.

LOCKSLEY, R.M., KILLEEN, N. and LENARDO, M.J., 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell,* **104**(4), pp. 487-501.

LÜLLMANN-RAUCH, R., 2009. Weibliche Geschlechtsorgane. *Histologie.* 3 edn. Georg Thieme Verlag KG, .

MELLICK, A.S., PLUMMER, P.N., NOLAN, D.J., GAO, D., BAMBINO, K., HAHN, M., CATENA, R., TURNER, V., MCDONNELL, K., BENEZRA, R., BRINK, R., SWARBRICK, A. and MITTAL, V., 2010. Using the transcription factor inhibitor of DNA binding 1 to selectively target endothelial progenitor cells offers novel strategies to inhibit tumor angiogenesis and growth. *Cancer research,* **70**(18), pp. 7273-7282.

MOORE, K. and PERSAUD, T., 1996. *Embryologie.* F.K. Schattauer Verlagsgesellschaft Stuttgart.

MORENO-NAVARRETE, J.M., CATALAN, V., WHYTE, L., DIAZ-ARTEAGA, A., VAZQUEZ-MARTINEZ, R., ROTELLAR, F., GUZMAN, R., GOMEZ-AMBROSI, J., PULIDO, M.R., RUSSELL, W.R., IMBERNON, M., ROSS, R.A., MALAGON, M.M., DIEGUEZ, C., FERNANDEZ-REAL, J.M., FRUHBECK, G. and NOGUEIRAS, R., 2012. The L-alpha-lysophosphatidylinositol/GPR55 system and its potential role in human obesity. *Diabetes,* **61**(2), pp. 281-291.

MORICONI, A., CERBARA, I., MACCARRONE, M. and TOPAI, A., 2010. GPR55: Current knowledge and future perspectives of a purported "Type-3" cannabinoid receptor. *Current medicinal chemistry,* **17**(14), pp. 1411-1429.

MOSER, M., LI, Y., VAUPEL, K., KRETZSCHMAR, D., KLUGE, R., GLYNN, P. and BUETTNER, R., 2004. Placental failure and impaired vasculogenesis result in embryonic lethality for neuropathy target esterase-deficient mice. *Molecular and cellular biology,* **24**(4), pp. 1667-1679.

NOLAN, D.J., CIARROCCHI, A., MELLICK, A.S., JAGGI, J.S., BAMBINO, K., GUPTA, S., HEIKAMP, E., MCDEVITT, M.R., SCHEINBERG, D.A., BENEZRA, R. and MITTAL, V., 2007. Bone marrow-derived endothelial progenitor cells are a major determinant of nascent tumor neovascularization. *Genes & development,* **21**(12), pp. 1546-1558.

OKA, S., NAKAJIMA, K., YAMASHITA, A., KISHIMOTO, S. and SUGIURA, T., 2007. Identification of GPR55 as a lysophosphatidylinositol receptor. *Biochemical and biophysical research communications,* **362**(4), pp. 928-934.

PAIK, J.K., KIM, M., KIM, M., YEN, Y., AHN, H.Y., LEE, S.H. and LEE, J.H., 2015. Circulating Lp-PLA(2) activity correlates with oxidative stress and cytokines in overweight/obese postmenopausal women not using hormone replacement therapy. *Age (Dordrecht, Netherlands),* **37**(2), pp. 32.

PERTWEE, R.G., HOWLETT, A.C., ABOOD, M.E., ALEXANDER, S.P., DI MARZO, V., ELPHICK, M.R., GREASLEY, P.J., HANSEN, H.S., KUNOS, G., MACKIE, K., MECHOULAM, R. and ROSS, R.A., 2010. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2). *Pharmacological reviews,* **62**(4), pp. 588-631.

PIERCE, K.L., PREMONT, R.T. and LEFKOWITZ, R.J., 2002. Seven-transmembrane receptors. *Nature reviews.Molecular cell biology,* **3**(9), pp. 639-650.

PINEIRO, R., MAFFUCCI, T. and FALASCA, M., 2011. The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation. *Oncogene,* **30**(2), pp. 142-152.

PLUMMER, P.N., FREEMAN, R., TAFT, R.J., VIDER, J., SAX, M., UMER, B.A., GAO, D., JOHNS, C., MATTICK, J.S., WILTON, S.D., FERRO, V., MCMILLAN, N.A., SWARBRICK, A., MITTAL, V. and MELLICK, A.S., 2013. MicroRNAs regulate tumor angiogenesis modulated by endothelial progenitor cells. *Cancer research,* **73**(1), pp. 341-352.

RADULESCU, L., MUNTEANU, O., POPA, F. and CIRSTOIU, M., 2013. The implications and consequences of maternal obesity on fetal intrauterine growth restriction. *Journal of medicine and life,* **6**(3), pp. 292-298.

ROBERTS, J.M., BODNAR, L.M., PATRICK, T.E. and POWERS, R.W., 2011. The Role of Obesity in Preeclampsia. *Pregnancy hypertension,* **1**(1), pp. 6-16.

RUBAN, E.L., FERRO, R., ARIFIN, S.A. and FALASCA, M., 2014. Lysophosphatidylinositol: a novel link between ABC transporters and G-protein-coupled receptors. *Biochemical Society transactions,* **42**(5), pp. 1372-1377.

SADLER, T. and LANGMAN, J., 2003. *Medizinische Embryologie.* 10 edn. Georg Thieme Verlag.

SAKKA, S., SIAHANIDOU, T., VOYATZIS, C., PERVANIDOU, P., KAMINIOTI, C., LAZOPOULOU, N., KANAKA-GANTENBEIN, C., CHROUSOS, G.P. and PAPASSOTIRIOU, I., 2015. Elevated circulating levels of lipoprotein-associated phospholipase A2 in obese children. *Clinical chemistry and laboratory medicine : CCLM / FESCC,* .

SANGER, G.J., 2007. Endocannabinoids and the gastrointestinal tract: what are the key questions? *British journal of pharmacology,* **152**(5), pp. 663-670.

SAWZDARGO, M., NGUYEN, T., LEE, D.K., LYNCH, K.R., CHENG, R., HENG, H.H., GEORGE, S.R. and O'DOWD, B.F., 1999. Identification and cloning of three novel human G protein-coupled receptor genes GPR52, PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain. *Brain research.Molecular brain research,* **64**(2), pp. 193-198.

SHANTSILA, E., WATSON, T. and LIP, G.Y., 2007. Endothelial progenitor cells in cardiovascular disorders. *Journal of the American College of Cardiology,* **49**(7), pp. 741-752.

SUTPHEN, R., XU, Y., WILBANKS, G.D., FIORICA, J., GRENDYS, E.C.,Jr, LAPOLLA, J.P., ARANGO, H., HOFFMAN, M.S., MARTINO, M., WAKELEY, K., GRIFFIN, D., BLANCO, R.W., CANTOR, A.B., XIAO, Y.J. and KRISCHER, J.P., 2004. Lysophospholipids are potential biomarkers of ovarian cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* **13**(7), pp. 1185-1191.

TAYLOR, A.H., FINNEY, M., LAM, P.M. and KONJE, J.C., 2011. Modulation of the endocannabinoid system in viable and non-viable first trimester pregnancies by

pregnancy-related hormones. *Reproductive biology and endocrinology : RB&E,* **9**, pp. 152-7827-9-152.

ULFIG, N., 2005. *Kurzlehrbuch Embryologie.* Georg Thieme Verlag.

WALDECK-WEIERMAIR, M., ZORATTI, C., OSIBOW, K., BALENGA, N., GOESSNITZER, E., WALDHOER, M., MALLI, R. and GRAIER, W.F., 2008. Integrin clustering enables anandamide-induced Ca2+ signaling in endothelial cells via GPR55 by protection against CB1-receptor-triggered repression. *Journal of cell science,* **121**(Pt 10), pp. 1704-1717.

WERNER, N., KOSIOL, S., SCHIEGL, T., AHLERS, P., WALENTA, K., LINK, A., BOHM, M. and NICKENIG, G., 2005. Circulating endothelial progenitor cells and cardiovascular outcomes. *The New England journal of medicine,* **353**(10), pp. 999-1007.

WETTSCHURECK, N. and OFFERMANNS, S., 2005. Mammalian G proteins and their cell type specific functions. *Physiological Reviews,* **85**(4), pp. 1159-1204.

WHYTE, L.S., RYBERG, E., SIMS, N.A., RIDGE, S.A., MACKIE, K., GREASLEY, P.J., ROSS, R.A. and ROGERS, M.J., 2009. The putative cannabinoid receptor GPR55 affects osteoclast function in vitro and bone mass in vivo. *Proceedings of the National Academy of Sciences of the United States of America,* **106**(38), pp. 16511-16516.

WILHELMSEN, K., KHAKPOUR, S., TRAN, A., SHEEHAN, K., SCHUMACHER, M., XU, F. and HELLMAN, J., 2014. The endocannabinoid/endovanilloid N-arachidonoyl dopamine (NADA) and synthetic cannabinoid WIN55,212-2 abate the inflammatory activation of human endothelial cells. *The Journal of biological chemistry,* **289**(19), pp. 13079-13100.

WONG, M.X. and JACKSON, D.E., 2004. Regulation of B cell activation by PECAM-1: implications for the development of autoimmune disorders. *Current pharmaceutical design,* **10**(2), pp. 155-161.

ZHANG, X., MAOR, Y., WANG, J.F., KUNOS, G. and GROOPMAN, J.E., 2010. Endocannabinoid-like N-arachidonoyl serine is a novel pro-angiogenic mediator. *British journal of pharmacology,* **160**(7), pp. 1583-1594.

11 Publications

1: Kremshofer J, Siwetz M, Berghold VM, Lang I, Huppertz B, Gauster M
A role for GPR55 in human placental venous endothelial cells. Histochem Cell Biol. 2015 Apr 14. [Epub ahead of print]

2: Siwetz M, Dieber-Rotheneder M, Cervar-Zivkovic M, Kummer D, Kremshofer J, Weiss G, Herse F, Huppertz B, Gauster M
Placental Fractalkine Is Up-Regulated in Severe Early-Onset Preeclampsia. Am J Pathol. 2015 Mar 11. doi: 10.1016/j.ajpath.2015.01.019. [Epub ahead of print]

3: Berghold VM, Gauster M, Hemmings DG, Moser G, Kremshofer J, Siwetz M, Sundl M, Huppertz B
Phospholipid scramblase 1 (PLSCR1) in villous trophoblast of the human placenta. Histochem Cell Biol. 2015 Apr;143(4):381-96. doi: 10.1007/s00418-014-1294-y. Epub 2014 Nov 2.

4: Siwetz M, Blaschitz A, Kremshofer J, Bilic J, Desoye G, Huppertz B, Gauster M
Metalloprotease Dependent Release of Placenta Derived Fractalkine. Mediators of Inflammation. Volume 2014 (2014), Article ID 839290; http://dx.doi.org/10.1155/2014/839290

12 Materials

12.1 Ethics statement

This study was approved by the ethical committees of the Medical University of Graz, Austria. Written informed consent was obtained from each participant. Placental tissues were obtained from legal pregnancy terminations for psychosocial reasons (6-12 weeks of gestation) and from pregnancies after normal term delivery or caesarean section (>37 weeks of gestation). Only tissues from healthy pregnancies were included in this study.

12.2 General materials

Falcons 15 and 50 ml	VWR® International, Germany
Freezer	Liebherr Comfort, Austria
Fridge	Liebherr Premium, Austria
Nitril gloves	Kimtech Science Brand, USA
Pipettes	PeQLab Biotechnologie GmbH, Austria
Pipettes 5, 10, 25 ml	Sterilin® Limited, UK
Pipetus®	Hirschmann®, Germany
RNase-free water	QIAGEN GmbH, Germany GmbH Hilden, Germany
Tips for pipettes	Eppendorf, Austria
Tubes 1.5 and 2 ml	Eppendorf, Austria

12.3 Cell culture

Medium:

Cells were grown in EBM (Endothelial Growth Medium) supplemented with the EGM-MV BulletKit (Lonza):

0.1% Human Epidermal Growth Factor

0.1% Hydrocortisone

0.1% Gentamicin Sulfate

0.4% Bovine Brain Extract

5% Fetal Bovine Serum

Solutions and reagents

Accutase	PAA Laboratories, Austria
Bovine Pituitary Extract	GIBCO®, Life technologies, Austria
Collagenase Type II	Roche, Germany
Dispase	Roche, Germany
DNase I from bovine pancreas	Sigma Aldrich, Austria
EBM	Lonza, Austria
EGF Human Recombinant	GIBCO®, Life technologies, Austria
EGM-MV BulletKit	Lonza, Austria
Formol 4%	Merck KGaA, Germany
Hank's buffered salt solution (HBSS)	GIBCO®, Invitrogen™, Austria
Keratinocyte-SFM (serum free medium)	GIBCO®, Life technologies, Austria
Lysophosphatidylinositol (LPI)	Sigma Aldrich, Austria
O-1918	Tocris bioscience, UK
Pasteur pipettes	ROTH GmbH, Germany
Penicillin-Streptomycin (10 000 U/ml)	Life technologies, Austria
Percoll	Sigma Aldrich, Austria

Materials, equipment and kits

6-,12-, 24- and 96-well plates	NUNC™, Denmark
CASY® Casyton solution	Roche, Germany
CASY® Cups	Roche, Germany
CASY® Technology Cell Counter	Roche, Germany
CellTiter 96® AQ_{ueous} One Solution Cells Proliferation Assay	Promega, USA
Centrifuge	Thermo Scientific Germany
Chamber Slides	Bartolt/ Nunc
Culture-Insert 24, ibiTreat for 24 well plates	ibidi®, Germany
CytoSelect™ 24-Well Cell Migration Assay	Cell Biolabs, Inc., USA
Dynabeads Goat Anti-mouse	Invitrogen™, Austria
Falcon™ Cell Strainers	Fisher Scientific, USA
Flasks 75 cm^2	NUNC™, Denmark
FluorChem Q System	ProteinSimple™, USA
Human Angiogenesis Antibody Array	RayBio®, Austria
Incubator Herracell 150	Thermo Scientific, Germany
LDH Cytotoxicity Detection Kit	TaKaRa, Germany
Working bench, LaminAir	Thermo Scientific, Germany

Cell lines and tissues

First trimester placenta	Dr. Glasner, Graz, Austria
HUAEC (human umbilical artery endothelial cells)	Primary cells kindly provided by Gynaecological clinic, Graz, Austria
HUVEC (human umbilical vein endothelial cells)	Primary cells kindly provided by Gynaecological clinic, Graz, Austria
PAEC (placental arterial endothelial cells)	Isolated at the Gynaecological clinic, Graz, Austria
PVEC (placental venous endothelial cells)	Isolated at the Gynaecological clinic, Graz, Austria
Term placenta	Gynaecological clinic, Graz, Austria

Human placental tissue samples:

The study was approved by the ethics committee of the Medical University of Graz. Patients were informed and gave their written consent. First trimester placental tissues originated from terminated pregnancy between 7 and 10 weeks of gestation.

12.4 Reagents and equipment for RNA isolation and purification

Solutions and equipment

1-Bromo-3-Chloropropane (BCP)	MRC, Inc.; USA
2-Propanol	Merck KGaA, Germany
Agarose	Bioenzym® Scientific GmbH, Austria
GelRed Nucleic Acid Gel Stain	Biotium, USA
Homogenizer	IKA®-Werke, Germany
Loading Dye Solution (6 x)	Fermentas, Thermo Scientific Germany
NanoDrop-1000 V3.5.2	peQLab Biotechnologie GmbH, Austria
RNeasy Micro Kit	QIAGEN GmbH, Germany
TRIzol® Reagent	Molecular Research Center Inc.; USA
β-mercaptoethanol	ROTH GmbH, Germany

12.5 Real Time qPCR

Kits and primers

GPR55 Hs00271662_s1	Applied biosystems™, CA
High Capacity cDNA Reverse Transcription Kit	Applied biosystems™, CA
Human total RNA Master Panel II	TaKaRa, Clontech
RPL30 Hs00265497_m1	Applied biosystems™, CA
TaqMan Gene Expression Assays:	Applied biosystems™, CA
TaqMan Universal PCR Mastermix	Applied biosystems™, CA

Materials, equipment and software

1.5% denaturing agarose gels	Biozym, Vienna, Austria
96 well plates	Roche, Mannheim, Germany
Bio-Rad CFX96 Real-Time PCR System	Bio-Rad, Austria
CFX Manager 2.0 Software	Bio-Rad, Austria
E-centrifuge	Wealtec, USA
GelRed™ Nucleic Acid Gel Stain	Biotium, USA
Labofuge 400R	Heraeus, Germany
NanoDrop1000 Spectrophotometer	Thermo Scientific, Waltham, MA, USA
PCR Tubes	Biozym® Scientific GmbH, Austria

12.6 Immunohistochemistry

Solutions and reagents

Ammonium solution	Merck KGaA, Germany
Antibody Diluent	Dako, Austria
Antigen retrieval buffer pH9	Eubio, Vienna, Austria
Citrate buffer	Sigma Aldrich, Austria
Ethanol	Merck KGaA, Germany
GPR55 blocking peptide No. 10225	Cayman Chemicals, USA
Hematoxylin/ Eosin	Merck KGaA, Germany
TBS	Pro Taqstura, Germany
Tween 20	Sigma-Aldrich, Austria
UltraVision HRP-labeled polymer system	Thermo Scientific, USA
Ultravision LP detection system	Thermo Scientific, Fremont, USA
Xylene	Merck KGaA, Germany

Antibodies

CD34II clone QBEnd-10	Dako, Austria
CD90	Dianova, Hamburg, Germany
Desmin	Neomarkers, USA
GPR55 polyclonal antibody No.10224	Cayman Chemicals, USA
Negative control mouse IgG1	Dako, Austria
Smooth muscle actin	Dako, Austria
Von Willebrand factor	Dako, Austria

Materials and equipment

Chambers for staining	Bertoni, Austria
Embedding Cassettes Turboflow II	Histocom AG, Austria
Embedding station Microm AP280-1	Histocom AG, Austria
Kaiser's glycerol gelatine	Merck KGaA, Vienna, Austria
Lab Vision Autostainer 360	Thermo Scientific, Germany
Metal bowls for paraffin outpouring	Sanova®, Austria
Microscope	Leica DM 6000 B, Germany
Microscope cover glass 24 x 32 mm	Menzel, France
Microtom Microm HM 440E	Histocom AG, Austria
Microwave	Severin, Austria
Moistened dark chamber	LABOnord
Object Slides Superfrost Plus	Menzel, Braunschweig, Germany
Pap Pen	Dako, Austria
Paraffin automatic embedding Tissue-Tek® VIP™	Sakura®, Germany
Paraffin Histo-Comp 56°C	Sanova®, Austria