Dissertation

Effect of maternal platelets on trophoblasts

submitted by

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Statutory Declaration

I herby declare that this thesis 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 thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

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Désirée Forstner

Disclosures

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Foreword

The following pages of this thesis refers to the elucidation of the underlying mechanism of early placental development in presence of platelet-derived factors.

This project is based on the finding that alterations in coagulation, including platelet activation and platelet count, have been linked to the pathogenesis of preeclampsia. The application of low-dose aspirin as the state-of-the art treatment for the prevention of preeclampsia has raised the question of how disturbances in platelet activation and adhesion to villous trophoblast may contribute to the manifestation of this syndrome in very early stages of pregnancy.

Within this thesis we demonstrate the presence of maternal platelets at the early fetal-maternal interface. Our data contribute to a general better understanding of the interference between early placental development and maternal platelet activation as a potential source of a microinflammatory environment in the intervillous space.

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Abbreviations and Definitions

APC	Activated protein C
BMSCs	Bone marrow stromal cells
cAMP	Cyclic AMP
CBP	CREB-binding protein
circRNA	Circular RNA
CM-Ps	Common myeloid progenitors
CTGF	Connective tissue growth factor
EGF	Epidermal growth factor
EV	Extracellular vesicle
EVT	Extravillous trophoblast
eEVT	Endovascular trophoblast
EPO	Erythropoietin
ERM	Ezrin-Radixin-Moesin family proteins
ESC	Endometrial stromal cells
FBS	Fetal Bovine Serum
FFPE	Formol-fixed paraffin-embedded tissue
FSH	Follicle stimulating hormone
GP	Glycoprotein
GPIIb/IIIa	Glycoproteins IIb/IIIa
GPVI	Glycoprotein VI
hCG	Human chorionic gonadotropin
HLA-G	Human leucocyte antigen-G
hPGH	Human placental growth hormone
hPL	Human Placental Lactogen
HSCs	Hematopoietic stem cells
iEVT	Interstitial extravillous trophoblast
IVS	Intervillous space
LH	Luteinizing hormone
LH/CGR	Luteinizing hormone/choriogonadotropin hormone receptor
lncRNA	Non-coding RNA
ME-Ps	Megakaryocyte-erythrocyte bipotent progenitors
miRNA	MicroRNA

MK	Megakaryocytes
mRNA	Messenger RNA
OCS	Open canicular system
PAI-1	Plasminogen Activator Inhibitor
PAPP-A	Pregnancy associated plasma protein-A
PC	Protein C
P-Exos	Platelet-derived exosomes
pHPL	pooled Human Platelet Lysate
PIGF	Placental growth factor
PKA	Protein Kinase A
PMPs	Plasma membrane-derived microparticles
P-MVs	Platelet-derived microvesicles
PRP	Platelet rich plasma
SS	Shear stress
ST	Syncytiotrophoblast
TAFI	Activatable fibrinolytic inhibitors
TE	Trophectoderm
TF	Tissue factor
TGF-β	Transforming growth factor
TGFBR1	TGF-β type 1 receptor
TGFBR2	TGF-β type 2 receptor
TGFBR3	TGF-β type 3 receptor
TM	Thrombomodulin
tPA	Tissue-type plasminogen activator
TPO	Thrombopoietin
TSH	Thyroid-stimulating hormone
TXA_2	Thromboxane A_2
uPA	Urokinase-type plasminogen activator
vWF	Von Willebrand factor

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Kurzzusammenfassung

Bereits in der frühen Schwangerschaft wird die gesunde Entwicklung der Plazenta und des Fetus durch eine Reihe an komplexen und streng regulierten Prozessen gewährleistet. Trophoblastzellen besitzen die Fähigkeit sich entsprechend ihrer Funktion an der fetalen-maternalen Schnittstelle zu unterschiedlichen Subtypen zu differenzieren. Invasive Trophoblastzellen wandern als extravillöse Trophoblasten in die mütterlichen uterinen Arterien ein um diese zu verschließen und somit den mütterlichen Blutfluss in den intervillösen Raum in den ersten Wochen der Schwangerschaft zu verhindern. Ein Ultrafiltrat aus mütterlichem Blutplasma gewährleistet in dieser Zeit die Versorgung des embryonalen Gewebes.

Immunhistochemische Analysen von Plazentagewebe aus dem ersten Trimenon konnten bereits in sehr frühen Schwangerschaftswochen adhärente mütterliche Thrombozyten im intervillösen Raum nachweisen. Mütterliche Thrombozyten könnten in diesem frühen Stadium der Schwangerschaft durch winzige Kanäle in Trophoblast-Plugs, welche beginnen sich aufzulösen, in den intervillösen Raum gelangen. Durch Aktivierung der Thrombozyten kommt es zur Ausschüttung von verschiedenen inflammatorischen Mediatoren in den intervillösen Raum, was unter anderem die Entwicklung der Plazenta in der frühen Schwangerschaft beeinflussen kann. Darüber hinaus wird eine erhöhte Adhärenz und daher auch eine erhöhte Aktivierung der Thrombozyten vermehrt mit Schwangerschaftspathologien, wie der Präeklampsie, in Verbindung gebracht.

Um den Einfluss von Thrombozytenfaktoren auf die Differenzierung von villösen Trophoblastzellen zu untersuchen, wurde villöses Plazentagewebe aus dem ersten Trimenon und die Trophoblast-Zelllinie BeWo mit Thrombozytenfaktoren inkubiert und anschließend auf Genexpressions- als auch auf Proteinebene, mit immunohistochemischen Methoden und mit der Hilfe von Elektronenmikroskopie, analysiert. Die Expression und Synthese der beta-Untereinheit des Schwangerschaftshormons humanes Choriongonadotropin (hCG) wurde durch die Thrombozytenfaktoren signifikant verringert. Des Weiteren konnten wir zeigen, dass trotz der Verringerung der hCG Synthese, die biochemische und morphologische Differenzierung der Trophoblastzellen *per se* nicht beeinflusst wurde. Smad3 und sein Downstream-Target Plasminogen Aktivator Inhibitor (PAI)-1 wurden stark von den Thrombozytenfaktoren aktiviert. Durch die Verwendung von TGF-β/Smad Inhibitoren und eines Inhibitors für den Co-Aktivator Komplex CBP/p300, konnte die Interferenz des cAMP/CREB Signalwegs, welcher während der Differenzierung hochreguliert ist, mit dem aktivierten Smad-Signalweg nachgewiesen werden. Zusammenfassend konnte der Einfluss von Thrombozytenfaktoren auf die Entwicklung und den Metabolismus des villösen Trophoblasten in der frühen Schwangerschaft gezeigt und somit die Rolle aktivierter Plättchen in der frühen plazentaren Entwicklung näher erklärt werden.

Abstract

In early stage of pregnancy, a sequence of complex and tightly regulated processes guarantees the development of the placenta. Cytotrophoblast cells differentiate according to their functions at the fetal-maternal interface and extravillous trophoblasts invade into the maternal uterine arteries. The vessels are plugged to prevent maternal blood flow to reach the intervillous space in very early stages of pregnancy. Maternal blood plasma ultrafiltrate can pass the trophoblast plug to nourish the growing fetus.

Immunohistochemical analysis of first trimester placental tissue revealed adherent maternal platelets in the intervillous space from very early stages of gestation. Maternal platelets may be the first cells to enter the intervillous space through intertrophoblastic gaps, when trophoblast plugs become loosely cohesive. Platelet adherence and thus also activation in the intervillous space, as an event in health and disease, may contribute to early placental development, as they provide a source of inflammatory mediators. Increased activation of aggregated platelets at the fetal-maternal interface is implicated in serious pregnancy pathologies, such a preeclampsia.

In order to elucidate the influence of platelet-derived factors on trophoblast differentiation and endocrinology, first trimester placenta villi and the trophoblast cell line BeWo were incubated with platelet-derived factors, and subsequently analyzed by gene expression analysis, protein analysis, immunohistochemistry and scanning electron microscopy. The expression and synthesis of the beta-subunit of the human chorionic gonadotropin (hCG) in placental explants as well as in BeWo cells, were significantly downregulated due to stimulation with platelet-derived factors. Analysis of trophoblast fusion and differentiation markers showed that the downregulation of βhCG is not a consequence of hampered differentiation *per se*. Smad3 and its downstream-target plasminogen activator inhibitor (PAI)-1 was strongly activated after treatment with platelet-derived factors. Due to the usage of TGF-β/Smad inhibitors and an inhibitor for co-activator complex CBP/p300, our study suggests an interference of differentiation-induced cAMP/CREB signaling pathway and Smad3 signaling. Sequestration of transcription co-activators CBP/p300, known to bind both CREB and Smad3, may limit βhCG production.

In conclusion, this thesis will contribute to a deeper understanding of trophoblast differentiation under consideration of platelet activation in the intervillous space in early pregnancy.

1. Introduction

1.1. The human placenta

In early stage of human pregnancy, a sequence of complex and tightly regulated processes guarantee the development of the placenta and hence a healthy development of the embryo (2). Major defects in placental development may lead to placental dysfunctions, causing pregnancy complications such as fetal growth restriction, pre-eclampsia or even still birth. These pregnancy pathologies can further lead to a lifelong impact on the fetus (3).

The human placenta provides an immunologic barrier between mother and fetus and allows supply of maternal nutrients, as well as the exchange of respiratory gases to fulfil the needs of the developing fetus. As an endocrine organ, the placenta further synthesizes hormones, to sustain the pregnancy and to adapt the maternal physiology to the developing embryo (4).

The hemochorial placentation is reliant on the direct contact of fetal trophoblast cells with the maternal blood flow (5). Therefore trophoblast cells have been initially described by Ambrosius Arnold Willem Hubrecht in 1889 as a cell type, which is able to transport nutrients and form a protective barrier between mother and fetus (3).

The establishment of the uteroplacental circulation due to remodeling of spiral arteries is a key event in human pregnancy, but anyway the obstruction of maternal blood flow into the intervillous space in the first weeks of gestation is necessary to keep the intraplacental oxygen levels low, which is necessary for a healthy placental development (5,6).

1.1.1. Placental Development

1.1.1.1. Implantation and development of the placenta

The successful implantation of the embryo into the uterine endometrium, starts when the polar blastocyst, which at this time contains two lineages, the outlying trophectoderm (TE) and the inner cell mass, attaches to the endometrium with the inner cell mass facing towards the maternal side at day 6-7 post fertilization (5). It breaks through of the uterine luminal epithelium and invades into the endometrial stromal cells (ESC) (5). Previous to the successful implantation of the blastocyst, ESCs undergo decidualization, which is defined by the differentiation of elongated, fibroblast-like mesenchymal cells to rounded, epithelioid-like cells (4). This cyclic regulated process is mainly divided into a proliferative and a secretory phase, to finally supply the implanting blastocyst with nutrients, avoid immunological rejection and regulate the invasion of trophoblast cells. During invasion of trophoblast cells into the decidual

stroma, they are interacting with the extracellular matrix of the decidua, which mainly produces fibronectin, laminin and collagen type IV (7).

Due to fusion processes of the TE, the primary syncytium is formed, and it starts to invade into the maternal decidua, to be completely embedded at day 14 post fertilization. The primary syncytium starts to develop fluid-filled spaces, the so-called lacunae, which later become the intervillous space and organizes the primary syncytium into trabeculae. During the following villous stage of development, the cytotrophoblast cells rapidly proliferate and invade into the trabeculae of the primary syncytium to form primary villi, consistent of an inner cytotrophoblast core with an outlying syncytiotrophoblast. In the next stage of development, the secondary villi are formed by the intrusion of fetal mesenchymal cells into the primary villi. The development of fetal vessels within the villi core is defined as tertiary villi (3,5).

Soon afterwards, when cytotrophoblast cells penetrate through the primary syncytium (3) and come in contact with the maternal decidua, they develop multi-layered structures through reorganization and finally form trophoblastic cell columns (6). Those cells within the cell bond keep their proliferative capacity and still have stem cell character, whereas those cells which lose their contact with the basement membrane, invade towards the decidua as an invasive cell type, the extravillous trophoblasts (EVT) (3,6).

Different types of EVT have been described and discovered over the last couple of years. The endovascular EVT (eEVT) moves directly along the inside of the spiral artery, whereas the interstitial EVT (iEVT) reaches the spiral arteries due to the migration through the decidua (3). Beside the remodeling of the spiral artery, the iEVT also interacts with decidual stroma cells, which is important for the attachment of the placenta to the uterus (5). During that time of gestation and placentation, the developing embryo is histotrophical nourished by the glandular secretion products (8). The so-called endoglandular EVTs have the ability to invade uterine glands, replace the glandular epithelium and connect them to the intervillous space (8). Before the maternal blood flow is established, the ST is in direct contact with maternal blood plasma and glandular secretions and is nourished by those secretions (3).

1.1.1.2. Remodeling of uterine spiral arteries

Maternal spiral arteries are tightly coiled vessels, which arise from the uterine arteries and perfuse the intervillous space. Due to invasion of eEVT and iEVT into the maternal tissue, spiral arteries in the decidua are remodeled at an early stage of pregnancy. They finally dilate the vessels at the opening into the IVS into low resistance wide pore vessels (2).

The remodeling of the spiral arteries is mainly characterized by the 'fibrinoid' change, which is described with the replacement and loss of actin to an amorphous eosinophilic material in smooth muscle cells in the arterial media (3). Important in this regard is, that the remodeled arteries lose their vasoactivity, resulting in a constant blood flow into the intervillous space, even during times the maternal blood pressure increases (3).

Beside the remodeling process of the spiral arteries, invading EVTs also plug the lumen of the these arteries, resulting in an obstruction of maternal blood flow into the IVS (9). Maternal blood cells, such as erythrocytes are trapped within this plugs, allowing only maternal blood ultrafiltrate to pass through (6). A study from Roberts et al. in 2017 suggests maternal perfusion through the spiral arteries and hence microvascular filling of the IVS, from 6-7 weeks of gestation onwards. Microvascular flux rates are varying widely in the range of GA 6 and 7, but remain consistent with increasing GA up to 13th week (2). They also describe sharp-bordered channels within spiral artery trophoblastic plugs already by 7 weeks of gestation, which lead to the assumption, that blood flow to the IVS is not completely obstructed by EVT plugs. Those channels within trophoblast plugs have a diameter of 10-20 µM by 8 weeks of gestation and can reach a diameter of up to 100 µm by 10 weeks of gestation (2).

1.1.1.3. Trophoblast differentiation

Due to the hemochorial structure of the placenta, the development of a functional trophoblast layer is the key to a healthy and successful pregnancy.

The epithelial layer, which covers the placental villi over the whole gestation is the multinucleated syncytiotrophoblast (10). The syncytiotrophoblast is continuously supplied by the underlying proliferating cytotrophoblast through fusion and differentiation of the mononucleated cytotrophoblast cells into the terminally differentiated multinucleated syncytium (11). A key event in trophoblast differentiation is the ability of the CT to exit the cell cycle and the upregulation of fusion genes (3). Towards the end of pregnancy, the proliferative capacity of the CT is decreased, leading to a discontinuous layer of CT, which only covers 25% of the villous surface in the third trimester of pregnancy (3).

The highly polarized multinucleated ST has major endocrine functions and is an important immunological barrier. The ST is characterized to lack the expression of human leukocyte antigen (HLA) class I molecules, to be undetectable for the mother's immune system. To increase its tightly receptor packed surface area, it is densely covered with microvilli (3).

Trophoblast differentiation is accompanied with an increase of cyclic AMP due to the activation of adenylyl cyclase. This leads to the activation of cAMP-dependent protein kinase A (PKA), which resides in the basal state in the cytoplasm and upon activation, the catalytic subunit of

PKA diffuse into the nucleus. Subsequently, this leads to the phosphorylation of transcription factors such as cAMP-response element binding protein (CREB) (12,13). It also increases the transcription factor glial cell missing homolog 1 (GCM1), which in turn regulates the expression of its target genes, the fusion peptide Syncytin-1 (ERVWE1). This syncytialization results in an upregulation of ST specific proteins, such as the human chorionic gonadotropin (hCG) (14).

This differentiation process is influenced by a variety of signals, such as hormones, cytokines, oxygen levels as well as transcription factors (15,16). Beside those factors, mechanical stimuli, such as fluid shear stress (SS), has been described as a potential activator of signaling pathways, which are involved in trophoblast differentiation. Intracellular cAMP and CREB are shown to be increased under flow conditions in primary human trophoblast cells (17,18).

This syncytialization process is a continuous process, which is described as villous trophoblast turnover (19). It has been indicated, that a nucleus within the syncytial association shows signs of aging after 3 to 4 weeks and is finally packed into syncytial knots to be shed into the maternal circulation (19). An inappropriate and increased trophoblast deportation into the maternal circulation is indicated for example in preeclampsia (11).

1.1.1.3.1. Microvilli formation in the syncytiotrophoblast

Microvilli at the apical surface of cells are able to effectively sense and interact with the fluid environment. Due to their ability to enlarge the cell surface, one of their main functions are the secretion and absorption of material. Beside those functions, they also play an important role in mechanotransduction (20).

A range of different cell types have the ability to form microvilli, such as the nasal epithelia, intestinal epithelia, but also the placental syncytium (20). The densely organized microvilli on the surface of the syncytiotrophoblast (21) in the hemochorial placenta, are directly exposed to the maternal blood in order to fulfill the material transport between the mother and the fetus (20). It has been shown, that fluid shear stress triggers the microvilli formation on the surface of trophoblast cells (20).

1.1.1.4. Placental endocrinology

As an important endocrine organ during pregnancy, the placenta synthesizes hormones to adapt the maternal metabolism to the developing fetus and to maintain the pregnancy. Two main domains of hormones are synthesized by the placenta, the steroid hormones, which are comprised of the progesterone, estrogen and glucocorticoids and the peptide hormones, such as hCG, human placental lactogen (hPL) and human placental growth hormone (hPGH) (22).

HCG is the key player in the implantation of the blastocyst and the maintenance of the pregnancy (22), but is also contributes to processes such as trophoblast migration through the endometrium, the remodeling of the vascular bed (23) or the suppression of the maternal immunological system (24). HCG is mainly synthesized by the ST, but also EVTs have the ability to do so (22). HCG levels rise exponentially during the first 7 weeks, to peak at 10 weeks of gestation and decline slowly until term (22).

As a member of the glycoprotein family hormones, hCG shares its alpha subunit with the other members of this family, including the luteinizing hormone (LH), follicle stimulating hormone (FSH) and the thyroid-stimulating hormone (TSH). Their beta subunit are unique for each hormone (24). The subunits of the 237 amino acid heterodimer are non-covalently linked. The beta subunit of hCG and LH are 80-85% homologous, whereby they are able to bind to the same receptor, the G protein-coupled receptor luteinizing hormone/choriogonadotropin hormone receptor (LH/CGR) (23,24). The beta subunit of hCG is encoded by six non-allelic genes (*CGB1,2,3,5,7* and *8*), whereas the alpha subunit is encoded by just one single gene (*CGA*) (23).

The synthesis of hCG is triggered by the activation of the adenylyl cyclase, which is activated during trophoblast differentiation. Due to the binding to the LH/CG receptor, which is expressed at the CT, the ST and the EVT, the hCG synthesis is further triggered through a positive-feedback loop. The binding to LH/CG leads again to the increase of cAMP signaling and this in turn, leads to the subsequent production of hCG and syncytial genes (25).

1.2. Platelets in general

With a size of 1.5-3 µm in diameter (26) the anucleated freely circulating cell fragments are the smallest of all human blood cells (27). The most understood function of platelets, beside their indispensability in wound healing, angiogenesis and inflammation (28) is to arrest bleeding at site of vascular damage by activation, aggregation and secretion of their granule content (29). Platelets are key players in the innate as well as in the adaptive immune response, as they are able to attract for example macrophages and granulocytes at site of injury (30).

Due to their short life span of about 7-10 days (28,30), their constant blood count is ensured by constant platelet formation through megakaryopoiesis (30). Platelets are released from the cytoplasm of large progenitor cells, the megakaryocyte, which reside in the bone marrow (28,29). In contrast, macrophages in the spleen and liver are responsible for a constant clearance of platelets (31). A normal platelet count in the blood is defined by 1.5-4.0 x 10^5 platelets per µl (30) and the turnover rate per days amounts for about 10^{11} platelets per day (32).

Platelets are equipped with a variety cytokines, chemokine, adhesion molecules and coagulation factors, but also with mRNA and miRNA, which is transferred from the megakaryocyte to the mature platelets (30). Furthermore, several studies have demonstrated the ability of the anucleated platelets to synthesize proteins (33,34), by specific splicing of pre-mRNA to mature mRNA with their splicing machinery and subsequent translation into proteins (35).

1.2.1. Platelet Formation

The continuous platelet replenishment in the process of thrombopoiesis is based on the differentiation of hematopoietic stem cells (HSCs) to mature megakaryocytes (MK) (36).

The HSCs in the adult bone marrow differentiate into specialized blood cells due to their multipotency and self-renewal capability. The common myeloid progenitors (CM-Ps), which are initially separated from the common lymphoid progenitors, further divide into megakaryocyte-erythrocyte bipotent progenitors (ME-Ps) (37). During the maturation process, the developing MKs migrate from the osteoblastic niche to blood vessels at the vascular niche, where they release platelets into the blood stream (36).

Mature megakaryocytes are 50-100 µm in size and due to repeated cycles of endomitosis (replication of DNA without cell division) during their maturation they become polyploid (28,29). They also enlarge their cytoplasm in this phase of maturation (28). Their cytoplasm is packed

into multiple long reservoirs of membranes, which elongates into the sinusoidal blood vessels of the bone marrow to form the proplatelets. Once in vasculature, proplatelets undergo fission and release individual platelets from their tips (29). The whole process of MK maturation to the final release of platelets into the blood stream takes about 5 days in humans (28). Megakaryocyte granules and organelles are transported as streams of individual particles from the megakaryocyte cell body to the platelets (28). The platelet release can also occur via rupture of the mature MK membrane, but only under acute platelet demand or inflammatory environment (38).

The hematopoietic hierarchy is regulated by many different factors, but amongst these, the primary growth factor of MK proliferation and maturation is the thrombopoietin (TPO) (29,38). It is indispensable for the differentiation of megakaryocytes and a normal peripheral blood platelet level (36). TPO is a hematopoietic cytokine, which was discovered in 1958 by Kelemen and Tanos (39). TPO expression is mainly found in the liver, but also in the kidney and smooth muscles. It is a polypeptide of 353 amino acids, of which its amino terminal 154 residue domain is homologous to this of erythropoietin (EPO). The two functions of the carboxyl-terminal domain are the prolongation of its circulatory half-life and the function as a chaperone. The plasma concentration of TPO is inversely regulated with the platelet count (40). Once, TPO binds to the TPO receptors on the platelets, is gets internalized and degraded (41). The MK-specific surface receptor, c-mpl, interacts with TPO to finally initiate the megakaryopoiesis (28,42,43). TPO further triggers the expression of proteins such as CD42 (GPIb-V-IX complex) or CD41 (GPIIb) to commit HSCs to the platelet lineage (38).

1.2.2. Platelet activation

1.2.2.1. Changes in the cytoskeleton

Upon platelet activation by exposure to physical or biochemical stimuli (26), their highly organized cytoskeleton converts the disc shaped platelets through remodeling into hemisphere-shaped structures with extended filopodia (27). The shape change facilitates the propagation of platelet aggregate formation and further activation of the clotting cascade (26,27).

The main components of the platelet cytoskeleton are tubulin, actin, filamin and spectrin. In the first step of platelet activation, actin filaments get fragmented and spectrin networks, which are composed of filamin A, GP1b/IX and spectrin, are released. The cell membrane protrudes in the second step of activation as the actin filaments assembly and filopodia develops from the core (26).

1.2.2.2. Primary hemostasis

The balance between anti- and prothrombogenic components is a key player to sustain a proper platelet function. For example, the endothelial cells possess antithrombotic properties, including coagulation inhibitors and fibrinolysis activator, whereas in contrast the subendothelial layer contains highly thrombogenic factors such as vWF, collagen and thrombospondin, which are presented at site of injury (44). In this acute phase response, the first steps of primary hemostasis lead to the thrombin mediated cleavage of fibrinogen to fibrin, accompanied with an acute inflammatory response. The second phase is dominated by the dissolution of matrix proteins and fibrin and the employment of immune cells to promote tissue repair (45).

Once, subendothelial cells are exposed to the blood flow, primary hemostasis is initiated. Platelets interact with the platelet surface receptor complex GP1b/V/IX with von Willebrand factor (vWF) and furthermore the platelet receptors GPVI and integrin α2β1 bind to collagen in the initial step (44). This process results in a signal transduction within the platelets and enables them to incorporate new circulating platelets to the thrombus through platelet-platelet interaction via integrin receptor αIIbβ3 and the formation of a fibrin meshwork (44).

Subsequent release of TXA_2 and ADP from platelet granules, further promote platelet aggregation and stabilizes the platelet plug through the binding of fibrin, which finally results in the secondary hemostasis (46).

1.2.2.3. Coagulation cascade

The coagulation cascade is distinguished between two distinct mechanisms, the extrinsic pathway (or tissue factor pathway) and the intrinsic pathway, which converge both in the activation of factor X (**see Figure 1**) (46,47). Factors, involved in blood clotting cascade reside as inactive enzyme precursors, the zymogens, in the circulation and their activation through proteolysis leads to the activation of downstream enzymes (46,48). The activation of several enzymes of the coagulation cascade, results in the generation of the serine protease thrombin. It is the main effector in primary platelet activation, but also in secondary hemostasis, where it plays a central role in the conversion of fibrinogen to fibrin monomers (49,50). Factor X, as a prothrombinase interacts with membrane-bound factor V in presence of calcium and converts prothrombin to thrombin (46). Thrombin further activates the formation of factor V and boosts thereby the processing from fibrinogen into fibrin, which finally leads to the clot formation (48).

The extrinsic pathway is initiated by the formation of the extrinsic tenase complex TF/FVIIa, which triggers the activation of factor X (FXa) and finally results in the cleavage of prothrombin to thrombin. The membrane-bound tissue factor (TF) is expressed in keratinocytes in the skin, the adventitial cells of all blood vessels and in epithelial cells. Once exposed to the blood flow at site of injury, TF forms a complex with FVIIa, which circulates as an inert zymogen in the plasma and initiates the coagulation protease cascade (48,51). The TF/FVIIa complex, also activates factor IX, which serves therefore as a bridge between the extrinsic and intrinsic pathway (48).

The intrinsic pathway acts through the activation of factor XI by thrombin, which further activates factor IX. Together with its membrane-bound cofactor factor VIII, the tenase complex FVIIIa/FIXa, amplifies the clotting cascade by the activation of factor X (FXa) (52).

FXa and its activated cofactor FVa form the prothrombinase complex FVa/FXa to generate thrombin and finally amplify the coagulant response with a positive feedback-loop. Thrombin, as the central protease of the clotting cascade, cleaves fibrinogen into the soluble fibrin monomer, which finally results in the thrombus formation (51,52).

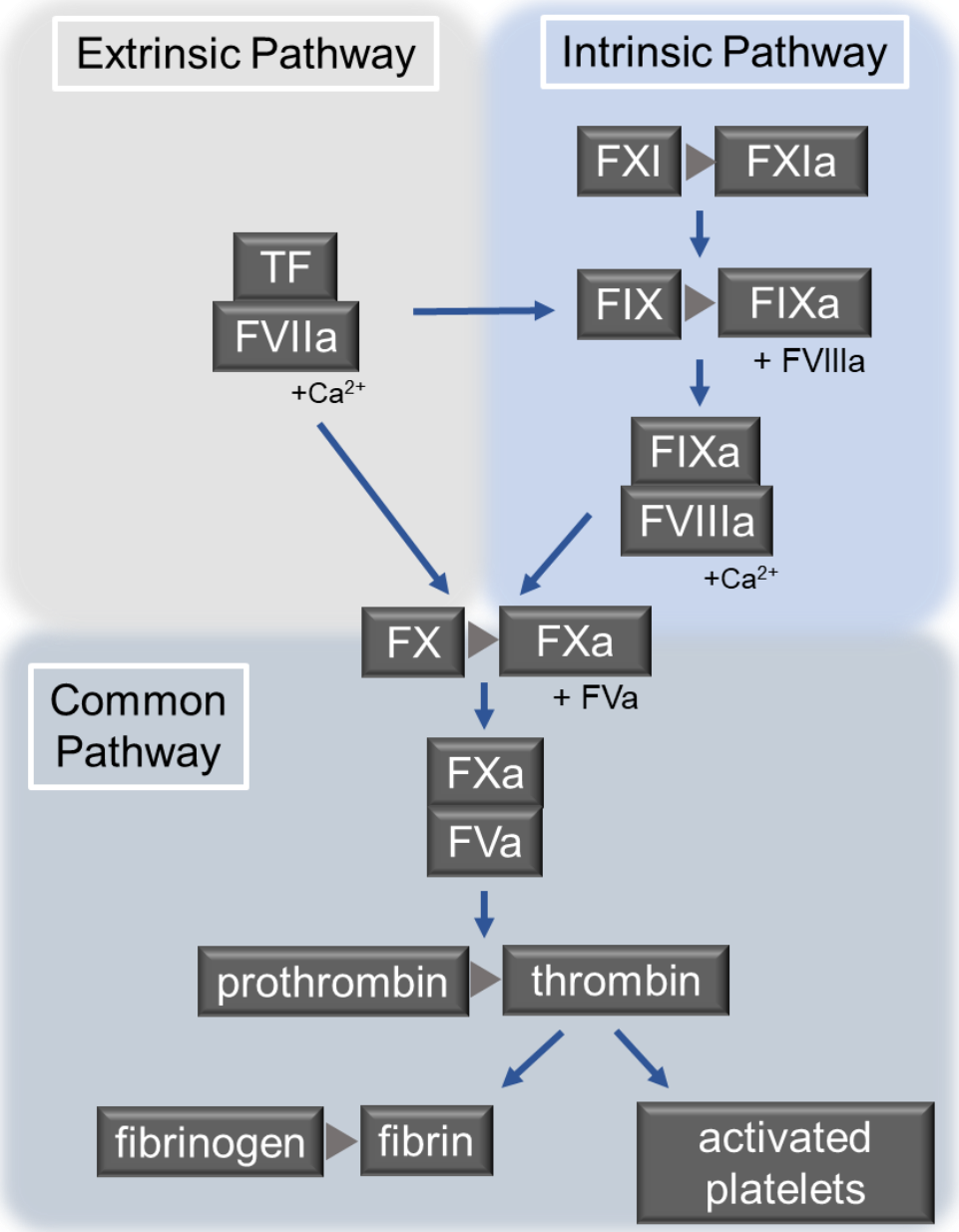

Figure 1: Simplified version of the clotting cascade. The extrinsic pathway comprises the complex formation of tissue factor (TF) and factor VIIa (FVIIa). Factor XI, IX and VIII are members of the intrinsic pathway. Both, extrinsic and intrinsic pathway, converge in the activation of factor X, which initiates the common pathway. The common pathway includes the complex formation of factor X (FXa) and factor VII (FVIIa), which indicates the conversion of prothrombin to thrombin. Thrombin subsequently cleaves fibrinogen to fibrin, which leads to fibrin clot formation and platelet activation. Factors involved in the blood clotting cascade reside as zymogens in the circulation and get activated due to proteolysis. Activated factors are indicated with a small **a**. Reproduced from Smith et al., 2015 with permission of publisher Taylor & Francis (53).

1.2.3. Platelet-derived factors

1.2.3.1. Platelet Granules

Platelets possess three types of intracellular secretory granules, the dense granules, the alpha-granules and the lysosomes, of which each has an unique molecular content (54). There morphological structure is determined by the their molecular content, which comprises growth factors, adhesion molecules, cytokines, integrins, proinflammatory molecules, cell-activating molecules, coagulation proteins and angiogenic factors (27). Lysosomes are ubiquitous, whereas alpha-granules and dense granules are only abundant in megakaryocytes and platelets (54) (**see Figure 2**).

The most abundant of the three secretory granules, are the alpha-granules, which play a key role in platelet activation (55). They are activated by strong agonists such as thrombin, but also by weak agonists such as adenosine diphosphate (ADP) (27). About 80 granules, with a size of 200-500 nm are present in one platelet and their protein content can be divided into “platelet-selective” molecules such as fibrinogen or vWF and “platelet-specific” factors, like platelet factor 4 (54). These molecules are either endogenous synthesized in the megakaryocyte or scavenged by endocytosis from the plasma (27). Alpha-granules release their protein content into the open canicular system (OCS) (27), which is an internal membrane structure and comprises a tunneling network of surface-connected channels (56). Alpha granules contain soluble factors, such as fibronectin, PF4, vWF and VEGF, as well as membrane-associated proteins such as or integrin αIIbβ3 and αVβ3 (30,57).

The slightly acid dense granules, which are less abundant than alpha-granules, mainly contain small molecules such as ADP, ATD, Ca2+ and serotonin (27,54). They directly fuse with the plasma membrane and release their content via exocytosis (27). Both, alpha and dense granules contain the type-1 cell glycoprotein P-selectin (54), which is rearranged to the cell surface membrane upon activation (27).

The membrane bound lysosomes are divided into primary and secondary lysosomes and they contain hydrolases, cathepsins and lysosomal membrane proteins (54). Hydrolyses are released upon thrombin activation of the platelets (27).

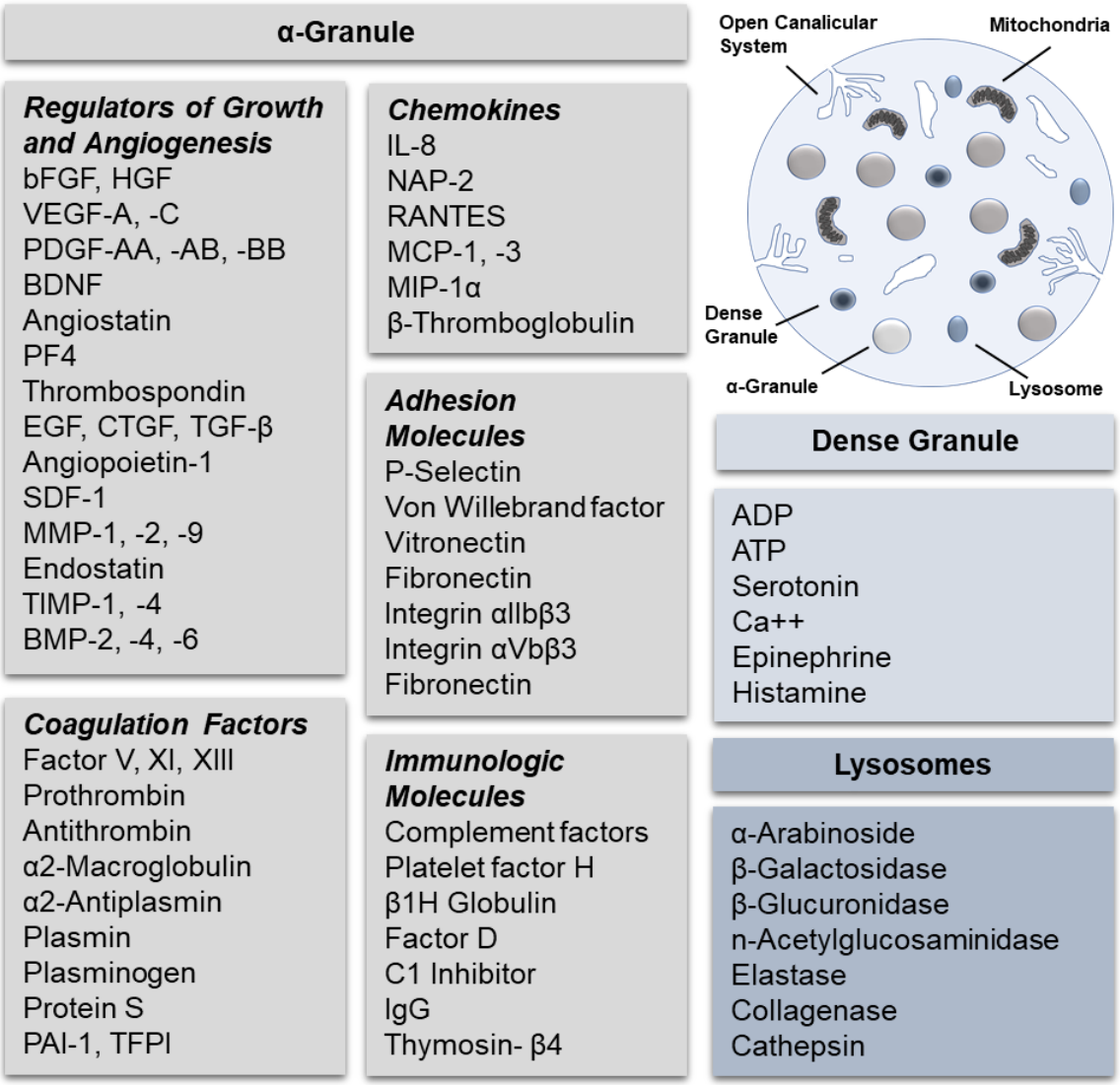

Figure 2: Schematic presentation of platelet granule content. Platelets contain three different types of granules to store their factors, which are released upon activation. Those granules are dense granules, lysosomes and α-granules. Factors, stored in α-granules are distinguished by their function into regulators of growth and angiogenesis, coagulation factors, chemokines, adhesion molecules and immunologic molecules. Platelets are organized in intracellular channel system, the so-called open canalicular system, which is connected with the platelet environment. Platelets also contain mitochondria. Figure according to Burnouf et al.,2016 (30) and reproduced from Fitch-Tewfik et al., 2013 with permission of publisher Frontiers (58).

1.2.3.2. Platelet Releasate

When it comes to platelet activation, a huge variety of adhesive and soluble agonists induce platelet activation through their respective receptors (59). Dependent on the mechanical or biochemical stimuli, a series of graded responses from shape change to aggregation is triggered and the amount of platelet secretion is dependent upon the strength of the agonist (27). The molecules released from the granules into the external milieu, act in an autocrine as

well as in a paracrine manner and comprise molecules such as growth factors, coagulation proteins, adhesion molecules, cytokines and proinflammatory molecules (27).

Over 350 proteins have been identified in thrombin activated platelet releasate of which most of them are attributed to platelets (27). Under pathological circumstance, the platelet secretion can be altered, like for example in cardiovascular diseases the platelet secretion is increased (54). It has also been reported, that the platelet releasate is altered in human pregnancy (60).

1.2.4. Platelet derived products

Since platelet derived factors contribute to a variety of biological processes *in vivo*, different methods have been developed to study their function in health and disease. So called platelet derived products are applied in clinical aspects like in advanced cell therapy and regenerative medicine as well as in basic research (61).

Platelet rich plasma (PRP) as one of the platelet products, is defined as platelet concentrate with a platelet count of 150.000-350.000/µl and is widely used as a treatment in regenerative medicine. Fields such as traumatology, orthopedics or sports medicine use the PRP as effector on cell proliferation, differentiation and migration in several cell types (61).

PRP is also the basic product for platelet products, such as the platelet lysate. After the spread in clinical applications, the human platelet lysate has been introduced to biomedical research as a human alternative to fetal bovine serum (30,61). It efficiently stimulates cell proliferation (30).

The platelet concentrate, which is the basis for the platelet lysate, can be produced of anticoagulated blood by two different procedures. The buffy coat method comprises the mixture of four buffy coat units plus one plasma unit and after centrifugation and leukocyte depletion, the platelet concentrate can be used. The platelet rich plasma (PRP) method is based on the separation of platelet poor plasma and platelet concentrates and subsequent leukocyte depletion (30).

In order to induce the release of platelet-derived factors in the platelet concentrates, four different methods can be performed. Platelets can either be lysed by repeated freeze/thaw cycles, by direct platelet activation via induction of endogenous thrombin, by sonication or by solvent/detergent treatment. For the freezing and thawing methods, the platelets within the platelet concentrate get activated with $CaCl_2$ to induce endogenous thrombin generation and are subsequent lysed by repeated freezing and thawing cycles (-80°C to 37°C). Fragments of platelets are depleted by final centrifugation (30,62).

The total protein concentration of the platelet lysate, which is a complex protein mixture, comprises about 50-55 mg protein per ml. Most abundant in the platelet lysate is albumin with 35-40 mg/mL, followed by immunoglobulin G and fibrinogen (30).

1.2.5. Platelet-derived extracellular vesicles

The consideration of extracellular vesicles (EVs) in a broad range of research fields as biologically active substances, has expanded over the last couple of years. Those double-layer phospholipid membrane vesicles, are released by different cell types in order to communicate with local or distant targets (63). More and more attractive features of EVs have been explored and it is widely accepted that EVs execute biological functions and contribute to intercellular communication (64,65). Extracellular vesicles are a heterogenous pool of vesicles, referred to as exosomes, microvesicles or extracellular vesicles (EVs) and they are further characterized by their size, cargo and origin (65–67).

Beside the platelet's ability to release their granule content upon activation, it has been reported, that they also release extracellular products into the external milieu (68). Cell activation via stimuli such as shear stress or cytokines leading to the reorganization of the cytoskeleton which in turn leads to the formation and shedding of microvesicles by membrane blebbing (65).

In order to isolate extracellular vesicles and further distinguish them, different centrifugation steps are necessary. According to a study by Raposo et al, intact cells, cell debris and dead cells can be removed by different centrifugation steps up to 10,000 g for 30 minutes by removing the cell pellet after each centrifugation step and further centrifuge the supernatant (65,69). To further distinguish between exosomes and microvesicles, it has been described, that microvesicles are isolated by centrifugation at 10,000-20,000 x g, whereas exosomes are usually isolated by centrifugation at 100,000-200,000 x g (65). Microvesicles have a size of 100-1000 nm, whereas exosomes are described as particles of under 100 nm in diameter (64). Platelet generated exosomes are about 50-100 nm in diameter and are endosomally derived multivesicular bodies (70).

Of all extracellular vesicles, present in the human blood, platelet derived EVs are the most abundant ones (64). EVs can contribute to signaling pathway via content delivery or the interaction of receptor-ligand binding (64). It is suggested, that the cargo of EVs consists of membrane and cytosolic proteins as well as of different RNAs such as messenger RNA (mRNA), microRNA (miRNA), circular RNA (circRNA) and non-coding RNA (lncRNA) (64).

Platelet-derived vesicles can generally be divided into plasma membrane-derived microparticles (PMPs) and platelet-derived exosomes (P-Exos) (65,66). Since the distinguish criteria for EVs in the blood are still unclear and under discussion, some potential EV markers for platelet-derived EVs, which are widely used, are CD31, CD41, CD42a, P-Selectin, GPIIb/IIIa (64). It has been revealed, that since annexin-V, factor X and prothrombin are restricted to platelet-derived microvesicles (P-MVs) rather than to platelet-derived exosomes (P-Exos), that P-MVs might play a more important procoagulatory role than P-Exos (64,71).

1.3. Coagulation system in the placenta

1.3.1. Pro- and anticoagulant changes in the placenta

Pregnancy has been described as a state of hypercoagulability and hypofibrinolysis (11), due to a decreased fibrinolytic activity and remarkable changes in hemostasis (72).

Hemostatic components such as tissue factor (TF) and thrombomodulin (TM) are expressed by the ST to maintain the local hemostatic balance in the placenta. TF, as one of the main key factors in coagulation, is a transmembrane protein, which is exposed on cell membrane at the site of injury (73). The TF-pathway is described as the extrinsic pathway of blood coagulation, since as soon as TF is exposed to the flowing blood, it binds the circulating factor VII (48). Subsequent activation of factor X by the TF/FVIIa complex leads to the generation of thrombin, which in turn processes fibrinogen to fibrin and finally triggers the blood clotting (**Figure 1**) (48).

TM in contrast, is important to prevent increased coagulation and plays therefore a major role in the coagulation system in the placenta. TM, which is expressed in various cell types, is a transmembrane glycoprotein and a ligand for thrombin. Once, the TM-thrombin complex is established, thrombin changes its substrate specificity from procoagulant to anticoagulant. The binding of thrombin further leads to the conversion of the zymogen protein C (PC) into the activated protein C (APC) (74). Activated protein C signaling in complex with protein S, results in a degradation of factor Va and factor VIIIa, to finally reduce the accumulation of active coagulation components (73,74). This process emphasizes the importance of a functional protein C or protein S, as patients with impaired functions suffer from increased risk of thrombosis (74). An increase in the expression of TF and fibrinolytic inhibitors in the placenta can therefore amongst others contribute to the initiation of coagulation (73).

1.3.2. Local hemostasis in the placenta

Beside the expression of anti- and proinflammatory mediators, the fibrinolytic system also plays a major role in the coagulation pathway and further contributes to the hypercoagulability during pregnancy. The fibrinolytic system is downregulated during pregnancy, which often presents a major risk factor for the development of intrauterine growth restriction (IUGR) and preeclampsia (PE) (72).

The fibrinolytic system is mainly regulated by the balance of the plasminogen activators urokinase- and tissue type (uPA/tPA) and its inhibitors, the plasminogen activator inhibitor type 1 (PAI-1) and type 2 (PAI-2). In order to guarantee the tissue homeostasis, PAI-1 is upregulated during wound healing and lack of PAI-1 protects different organs from fibrosis (72).

During pregnancy, the upregulation of PAI-1 (72), contributes to fibrin accumulation in the placenta towards term (73). The syncytiotrophoblast expresses PAI-1 in the cytoplasm as well as in the plasma membrane, whereas in cytotrophoblasts PAI-1 is only expressed in the cytoplasm (72). Overexpression of PAI-1 may also promote pathological fibrin deposition. Its plasma and placental levels are significantly increased in pregnant women with severe PE. These results suggest that localized elevated levels of PAI-1 may play a role in the thrombotic complications, but limited information is available on the factors that regulate the production of PAI-1 within healthy or pathological placentas (73).

As a fibrinolytic inhibitor, PAI-1 is involved in the degradation of the non-cellular component, the extracellular matrix (ECM) (72), which mainly consists of proteoglycans and fibrous proteins. Regulation of the ECM in the wound healing process is very important, because deregulations can lead to fibrosis, which is defined as an abnormal fibroblast activation-related disease. Since inflammation is one of the key players in tissue repair, it contributes apparently to the synthesis of elevated ECM proteins. Accumulated collagen and other matrix proteins cause dysfunction and disruptions in tissue homeostasis (75).

Collagen, as the major component of the ECM (76), comprises a family of 28 proteins (77). The most abundant collagens types I, II and III form fibrils (78), whereas collagen type IV forms three-dimensional networks and collagen type VI forms beaded filaments (76,78). It has always been considered, that collagen type IV is mainly localized in the basement membranes, but Oefner et al. could show, that HLA-G positive EVT in the distal part of placental cell columns are also secreting collagen type IV (76).

In regard of platelet activation, collagen can induce platelet activation through the binding to the platelet membrane receptor glycoprotein VI (GPVI) and the integrin α2β1. The binding of vWF to the platelet surface glycoprotein Ib-V-IX complex, is important for the initial binding to

the exposed collagen. The activated platelets subsequently release further procoagulant factors, like ADP or thromboxane A_2 (TXA_2) to tighten the binding of integrin α2β1to collagen and increases the affinity of integrin αIIβ3 to fibrinogen (78). This process finally results in the thrombus formation and is therefore a procoagulant process.

1.3.3. Fibrinoids of the human placenta

Fibrin accumulation in placenta villi towards term is a normal finding and makes up about 7% of the villous surface at term (79). Increased fibrin accumulation at term can cause vessel occlusion and infarcts, resulting in placenta insufficiency and even late fetal loss (73).

As the balance between the conversion of fibrinogen to fibrin through the clotting cascade and the subsequent proteolytic degradation, the fibrinolysis, is disrupted, increased fibrin deposits are the result. In inflammation-driven acute phase response, the expression of fibrinogen genes is increased and is therefore considered to play a major role as a trigger for fibrin formation (80).

Tissue repair also plays a role in placenta development, as for example high-pressure blood flow into the intervillous space can lead to the disruption of syncytiotrophoblast, which is then replaced by the fibrin-type fibrinoid (79,81). The fibrin-type fibrinoid is a product of the coagulation cascade and is mainly composed of fibrin, whereas the matrix-type fibrinoid is secreted by EVT trophoblast and mainly composed of glycoproteins and collagen type IV (79). Fibrin-type fibrinoid in a perivillous position is referred to as perivillous fibrinoid (79) and fills gaps in the syncytiotrophoblast (82). As the trophoblastic basal lamina, exposed to the maternal blood, which contains collagen, fibronectin and laminin, it has procoagulant potential (81,83) and suggest that focal degeneration of syncytiotrophoblast results in local blood clotting (82).

1.3.4. Hemodynamic changes in pregnancy

During pregnancy, the female body undergoes a wide range of anatomical and physiological adaptions. Hemodynamic changes during pregnancy include an increase of the plasma volume of 30-50% and a total blood volume of 1-2 liters, resulting in a lower hematocrit. Pregnancy is described as a prothrombotic state, accompanied with an increase in protein-C resistance, decrease in fibrinolysis and an increase in procoagulant factors such as factor VII, VIII, IX, X, XII and vWF (84,85). Due to the hormonal changes during pregnancy, also the fibrinolytic pathway is downregulated by the increase of inhibitors such as PAI-1 and activatable fibrinolytic inhibitors (TAFI) (84).

Platelet count in healthy pregnancies is generally decreased towards the end of the first trimester (86) and is general lower by 10% at term (5). A lower platelet count at term is also often described as a gestational thrombocytopenia (86).

Studies reveal that a lower platelet count during pregnancy is linked to a higher sequestration of platelets in the intervillous space. This phenomenon is also observed in the spleen, but unlike in the spleen, the platelet in the placenta will not return to circulation, because they might get activated within the intervillous space and get adherent (87).

1.3.5. Placental Pathologies linked to platelet disorders

Changes in the coagulation system during pregnancy has been linked to pregnancy pathologies, such as preeclampsia. Important molecular mechanism and the major cause of the development of preeclampsia are still unknown. With a prevalence of about 2-8% of all pregnancies, preeclampsia is one of the most common pregnancy complication of which the placenta plays an essential role (88,89). Preeclampsia occurs during pregnancy and the postpartum period and the general knowledge about this syndrome is taking the placenta as major cause into account (16).

Preeclampsia is diagnosed in the mother due to hypertension (≥140/≥90 mmHg) and proteinuria (≥300 mg/24h). The only causal treatment so far is the removal of the placenta, but this is accompanied with increased rates of preterm birth and all associated risks for the mother and the new-born (88). Beside those long-established criteria, alterations in the serum creatinine levels, thrombocytopenia, increased levels of liver transaminases in blood or cerebral disturbances are considered in the specification of this syndrome (89).

Subtypes of preeclampsia are mainly categorized by the onset of the clinical symptoms, more specifically, the gestational age of delivery, which refers early-onset before the 34 weeks of gestation and late-onset preeclampsia after 34 weeks of gestation (89). With a prevalence of 5-20% out of all pregnancies, complicated with preeclampsia, the early-onset preeclampsia is further associated in most of the cases with fetal growth restriction, reduced remodeling of the spiral arteries and changes in fetal as well as placental perfusion (89).

The syncytiotrophoblast plays also an important role in the onset of the preeclampsia, as it has been described to trigger the maternal response (16). Especially the turnover of the syncytiotrophoblast has been implicated in preeclampsia (90).

In regard to the most widely prescribed treatment for this disease, the administration of low-dose aspirin, disturbances in the coagulation system have also been considered for the development of this disease.

Preeclampsia can be accompanied with alterations in platelet counts (91), but also with increased platelet aggregation (92). The molecular basis of the onset of preeclampsia is still not clear, but some studies have linked the ability of aspirin to inhibit cyclooxygenase and subsequently thromboxane A_2 (TXA_2) production, which has been reported to be increased in preeclampsia (93).

2. Aims and Objectives

The role of platelets during pregnancy has led to a considerable amount of research over the last decade and this has taken platelets into account for their contribution to pregnancy pathologies. Beside their well-known functions in coagulation during pregnancy, they are also involved in many inflammatory processes. Therefore, we hypothesized to consider them as a source of inflammatory mediators at the early fetal-maternal interface with the ability to affect early placental development. This hypothesis has led to following aims:

1.) To **identify adherent maternal platelets** at the early fetal-maternal interface in first trimester placental tissue

The time when platelets may enter the intervillous space is yet unclear and therefore this study was aiming to identify new possible routes, how platelets may enter the intervillous space at certain stages of development. For this purpose, first trimester placental tissue was subjected to immunohistochemistry to identify CD42b positive platelets on villous trophoblast cells, on cell columns of invading extravillous trophoblast cells as well as on trophoblast plugs of uterine vessels.

2.) To identify the **most abundant binding sites of maternal platelets** on trophoblasts

When platelets enter the intervillous space, they might get activated either from mechanical shear stress, by soluble platelet agonists, like ADP or thromboxane A_2, or by immobilized procoagulant factors within the ECM, exposed at site of injury. Therefore, we aimed to get more insights in platelet binding sites on villous trophoblast cells.

3.) To elucidate, whether platelet-derived factors influence the **endocrinology** of trophoblast cells

Trophoblast differentiation is tightly regulated by a series of downstream events, resulting in an endocrine active syncytiotrophoblast, which synthesizes, amongst other hormones, βhCG. In this study, we investigated the influence of platelet-derived factors on the expression and secretion of βhCG in first trimester placental tissue and a trophoblast cell line.

4.) To determine, the influence of platelet-derived factors on trophoblast **fusion and morphology**

Cytotrophoblast differentiation results in syncytial fusion and therefore fusogenic genes and proteins are used as trophoblast differentiation marker. Therefore, we have taken the influence of platelet-derived factors on trophoblast fusion markers on protein and gene expression level into account. Furthermore, trophoblast cells undergo morphological changes during differentiation. To elucidate the differentiation-dependent microvilli formation, trophoblast cells were subjected to scanning electron microscopy and protein as well as gene expression analysis.

5.) To investigate, whether platelet-derived factors impair **cAMP/CREB signaling**

HCG synthesis is induced with increased intracellular levels of cyclic AMP and the subsequent phosphorylation of CREB. In order to elucidate, whether impaired hCG signaling is a consequence of impaired cAMP/CREB signaling under influence of platelet-derived factors, we analyzed this signaling pathway in BeWo cells with enzyme-linked immunoassays.

6.) To **identify potential platelet-induced pathways**, which are interfering with hCG signaling pathway

Upon activation, platelet granules release a broad spectrum of chemokines and cytokines, such as EGF or TGF-β. These factors are known to regulate cell proliferation and differentiation and therefore we aimed to identify new potential platelet-induced pathways, which might interfere with βhCG signaling. For this purpose, we investigated the role of TGF-beta signaling in trophoblast cell line BeWo, by using different inhibitors for TGF-β induced signaling.

3. Methods

This chapter was taken and extended from Forstner, D., et al., “Platelet-derived factors impair placental chorionic gonadotropin beta-subunit synthesis.” J Mol Med 98, 193–207 (2020) (1).

3.1. Human Placenta Tissue

3.1.1. Human placenta tissue collection

Human first trimester placental tissue was collected between weeks 5 and 12 of gestation from women undergoing a legal elective pregnancy termination at a local gynaecologist’s practise (Dr.med.univ. Glasner; Femina Med) in Graz with written informed consent. Ethical approval was obtained from the Medical University of Graz Ethics Committee (31-019 ex 18/19; 26-132 ex 13/14).

3.1.2. First trimester placental explant culture

Placental villous tissue from human first trimester ($n = 14$, mean gestational week 8.26 ± 0.45) was processed at the Division of Cell Biology, Histology and Embryology within 1-4 hours after the medical intervention. Tissue samples were thoroughly rinsed in buffered saline and dissected under the stereoscopic microscope into small pieces of approximately 5 mg moist mass.

Placental explants were cultured in 6 well dishes (Nunc) and 4 ml/well DMEM (including low glucose, pyruvate, L-glutamine) supplemented with 10% FCS (HyCloneTM; Gibco), penicillin/streptomycin (Gibco), in a hypoxic workstation (BioSpherix LtD; Redfield, NY, USA) under 2.5% oxygen and 5% CO_2 for indicated time points at 37°C.

For treatments, culture medium was supplemented with 10% (v/v) pooled human platelet lysate (pHPL) and heparin (Merck, Darmstadt, Germany). Heparin served as an anticoagulant and was added to pHPL supplemented media as well as to the controls at a final concentration of 2 U/ml.

3.1.3. Placental tissue fixation and embedding

Human first trimester placenta tissue ($n = 31$, mean gestational week 8.01 ± 2.08, **Table 4**) was dissected into small pieces and fixed in 4% paraformaldehyde for 24h at RT. Afterwards fixed tissue was paraffin-embedded with Sakura Tissue-Tek VIP Series Tissue Processors (GMI, Ramsay, USA) and formalin-fixed paraffin-embedded (FFPE) first trimester placenta tissues were subjected to further immunohistological methods (chapter 3.8.2. Immunohistochemistry).

3.2. Cell Culture

For *in vitro* studies, the choriocarcinoma cell line BeWo, purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) was used. Cells were cultured in DMEM/F12 (1:1, Gibco, life technologies; Paisley, UK) supplemented with 10% (v/v) fetal calf serum (FCS, HyCloneTM; Gibco; heat inactivation for 1h at 56°C), 1% (v/v) L-Glutamine (Gibco; 20 mM 100X) and 0,1 U/ml Penicillin and 0,1 µg/ml Streptomycin (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C. Cells were detached with accutase (Biowest®) and cells between passage 10 and 30 were used for *in vitro* experiments. Cell count was determined with CASY® (SCHÄRFE SYSTEM) in 3 cycles to each 400 µl. The syncytialization of BeWo cells was induced with 20 µM forskolin (Tocris, Bio-techne, Abingdon, UK) (94).

3.2.1. Treatment of BeWo cells with pHPL

For experiments, cells were plated overnight in 12-well dishes (Nunc Lab-Tek; Thermo Fischer; NY, USA) with a density of 2 x 10^5 cells/well in the described media. After the overnight incubation, culture media was exchanged with fresh media, containing either 0,1% (v/v) dimethyl sulfoxide (DMSO) (Carl ROTH®) or 20 µM forskolin (Tocris, Bio-techne, Abingdon, UK) in presence or absence of 10% (v/v) human pooled platelet lysate (pHPL) for indicated time points under the above described culture conditions. DMSO served as the vehicle control for forskolin. Heparin (Merck, Darmstadt, Germany) was added to all conditions in a final concentration of 2U/ml as an anticoagulant.

3.2.2. Inhibitor treatments of BeWo cells

For inhibitor experiments, BeWo cells were pre-incubated for indicated period of time with following inhibitors and concentrations: 10 µM for selective Smad3 inhibitor SIS3 (Tocris Bio-techne, UK), 10 µM for TGF-β R1 inhibitor SB431542 (Sigma-Aldrich), 10 µM and 100 µM for TGF-β R3 inhibitor P144 and 20 µM for histone acetyltransferase inhibitor C646 (Sigma). Medium was removed after overnight incubation and exchanged with fresh medium supplemented with either 0,1% DMSO as solvent control or 20 µM forskolin in presence or absence of 10% pHPL and inhibitors, respectively, as indicated. Cells were incubated for indicated time points and experiments were repeated three or more times (as indicated in figure legends).

3.2.3. TGF-ß treatment of BeWo cells

BeWo cells were seeded overnight and afterwards pre-incubated with SIS3 (10 µM) for 1h. Subsequently cells were treated with recombinant human TGF-ß 1 (R&D Systems, Minneapolis) in final concentrations of 10 nM in presence or absence of SIS3 (10 µM) for another 1h.

3.3. Preparation of pooled human platelet lysate

Human pooled platelet lysate (pHPL) was produced at the Department of Transfusion Medicine, Paracelsus Medical University of Salzburg as previously described (62) and kindly provided for experiments.

In brief, the starting material for the preparation of pHPL is platelet rich plasma (PRP), derived from buffy coats of 40 blood donations. By performing one freeze/thaw cycle (-20°C / 37°C), the platelets are already lysed, but repeated freeze/thaw cycles are performed to increase the rate of fragmentation and the amount of platelet-derived factors. To deplete platelet fragments, the batch gets thawed, aliquoted and centrifuged at 4000g for 15 min. The supernatant is the platelet fragment-depleted pHPL, containing platelet-derived factors and can be used for further applications. Mean protein content of pHPL is shown in **Table 1**.

Table 1: *Mean protein content of human pooled platelet lysate in average*. Reproduced from Burnouf et al., 2016 (30).

Mean protein content of pHPL	
Total protein	55-65 mg/ml
Albumin	35-40 mg/ml
Immunoglobulin G	8-12 mg/ml
Fibrinogen	1,5-3 mg/ml
IGF-1	50-200 ng/ml
PDGF-AB	50-300 ng/ml
PDGF-BB	10-30 ng/ml
PDGF-AA	1-10 ng/ml
TGF- ß1	50-300 ng/ml
TGF- ß2	0,5 ng/ml
BDNF	10-50 ng/ml
VEGF	5-10 ng/ml
EGF	0,5-10 ng/ml
HGF	0,1-2 ng/ml
b-FGF	1-5 ng/ml

3.4. Isolation of human platelets

Citrated whole blood samples from healthy donors were collected at the Division of Pharmacology (Otto Loewi Research Center) at the Medical University of Graz with written informed consent. Blood samples were centrifuged at 300 x g and RT without breaks for 20min and supernatants were gently mixed with EDTA (2%) in a 1:20 ratio to the plasma volume. After centrifugation at 1000 x g and RT for 15min, the pellet was resuspended in 10 ml platelet wash buffer (**Table 6**) and centrifuged again at 1000 x g and RT for 15min. After another washing step, platelet number was determined using the Sysmex KX-21NTM. Platelets were resuspended in DMEM/F12 (1:1, Gibco, life technologies; Paisley, UK) supplemented with 10% FCS (Gibco), penicillin/streptomycin (Gibco) and L-glutamine (Gibco).

3.4.1. Co-incubation of platelets and BeWo cells

For co-incubation with platelets, 2 x 10^5 BeWo cells/well were seeded overnight in 12-well culture dishes. The next day, culture medium was exchanged with medium containing isolated platelets at a density of 1.5-3.5 x 10^8 platelets/ml in addition to 20 µM forskolin or 0.1 % DMSO as a vehicle control. Platelets were either pre-activated with the stable ADP analogue 2-Methylthioadenosine 5'-diphosphate (2-MeS-ADP) (Tocris, Bio-techne, Abingdon, UK) in a final concentration of 10 µM, or not. Cells were incubated for indicated time points in a humidified atmosphere of 5% CO_2 at 37°C.

3.5. Measurement of secreted βhCG

After indicated time points of treatment, conditioned culture media from cultured cells or *ex vivo* placental explants was collected and centrifuged at 1500 rpm at 4°C for 5 min, to remove any cellular debris. Samples were aliquoted and the supernatant was stored at -80°C until further use. βHCG concentrations in the cell culture supernatant were determined by routine immunoassay analysis (Dimension Xpand; Dade Behring Inc., Deerfield, Illinois) at the Department of Obstetrics and Gynecology at Medical University of Graz. Total hCG secretion was normalized to total cell or tissue protein, which was determined by Lowry protein assay.

3.6. Gene expression analysis

3.6.1. RNA Isolation and cDNA synthesis

Placental explant tissue was rinsed after incubation in buffered saline and afterwards homogenized by using the tissue homogenizer UltraTurrax (IKA) in peqGOLD TriFast (VWR, Radnor, Pennsylvania, USA or RNA-Lysis-Puffer T (peqGOLD Total RNA Kit; VWR, Radnor, Pennsylvania, USA) according to the manufacturer's instructions. For *in vitro* experiments, after the incubation time, cells were washed in PBS and lysed in peqGOLD TriFast or RNA-Lysis-Puffer T as indicated above.

After lysis, RNA from cells and tissue was isolated respectively with PeqGOLD Total RNA Kit (C-Line) (VWR, Radnor, Pennsylvania, USA) according to the manufacturer's manuals or with the phenol-chloroform method according to Chomczynski and Sacchi (95). Sample purification and concentrations were measured with NanoDrop® ND-1000 (peqlab Biotechnologie GmbH). Quality check was followed by reverse transcription of 1 µg total RNA per reaction using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's manual.

3.6.2. qPCR

qPCR was performed with Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) using a Bio-Rad CFX96 cycler as previously described with specific primers and the run protocol shown in **Table 2**. Cq values were automatically determined using single thresholds and normalized expression (ΔΔCq analysis) was automatically generated by the CFX Manager 2.0 Software (Bio-Rad). The expression of *GAPDH*, *CYC1*, and *YWHAZ* was used as reference, according to a previous evaluation of housekeeping genes in placental tissue (96).

Table 2: *Primer sequences for gene expression analysis*. Table reproduced from J Mol Med 98, 193–207 (2020) (1).

Primer Name	forward	reverse
ALPPL2	CCATACCTGGGATTTCCGCCT	CGGTTCCAGAAGTCCGGGTT
CDH1	CAGGATGGCTGAAGGTGACA	ACTGCATTCCCGTTGGATGA
CGB3_5_8	TGAGCCACTCCTGCGCCC	CAGCCCCTGGAACATCTCCA
CYC1	TAGAGTTTGACGATGGCACCC	CCCATGCGTTTTCGATGGTC
ERVFRD-1	ACCGCCATCCTGATTTCCC	GAGGCTGGATAAGCTGTCCC
ERVW-1	CCATGCCGCTGTATGACCAG	GGGTTCCCTTAGAAAGACTCCT
EZR	TTGGTTCCGCCCACTCATTC	CCACCTGCACATGGCATCTT
GAPDH	ACCCACTCCTCCACCTTTGA	CTGTTGCTGTAGCCAAATTCG
GCM1	TTCCCGGTCACCAACTTCTG	GTAAACTCCCCTGACTTTGTGTT
MSN	GTGCCTGACCTTGAGGAGTC	TTGCCCCACAATTCCAGGTT
NR4A2	CGATTTCAGAAGTGCCTGG	TAAACTGTCTGTGCGAACCAC
RDX	CGGAAAGTGATAACAGAATTCATTG	GGTTTCGGCATTTTCTTTCTCT
SERPINE1	GTTCTGCCCAAGTTCTCCCT	ACATGTCGGTCATTCCCAGG
TBP	TGACCCAGCATCACTGTTTC	CCAGCACACTCTTCTCAGCA
YWHAZ	GGTGGCCAATATGGGGATGT	TCCCTTTTATTCCCCGCCAG

All primers were run at initial 95 °C for 5 min, followed by 40 cycles of 95 °C for 5s and 60 °C for 30s. Melt Curve 60,0°C to 95,0°C: Increment 0,5°C 0:05 Plate Read

3.7. Protein Isolation

Placental explant tissue was rinsed after incubation in buffered saline and afterwards homogenized by using the tissue homogenizer UltraTurrax (IKA) in RIPA buffer, containing 1x protease inhibitor cocktail (PIC) (cOmplete Tablets EASYpack, Roche Diagnostics, Mannheim, Germany) and 1x phosSTOP (Roche Diagnostics, Mannheim, Germany). After the incubation time, cells were washed in PBS and lysed in RIPA Buffer containing PIC and phosSTOP as described above. After one freeze/thaw cycle, the homogenates were centrifuged at 8000 rpm for 10 minutes at 4°C and clear protein lysate was collected. Protein concentration was determined according to Lowry method (97). In short, the method is based on the complex formation of peptide bonds with divalent copper ions under alkaline conditions. Subsequent reduction of the Folin-Ciocalteu reagent is colorimetric detectable (98). Recipes for used solutions are shown in **Table 7**. OD-values were detected by using anthos 2010 Microplate Reader (Biochrom®)

3.7.1. Immunoblot

Protein concentrations of about 30 µg of total protein, together with 1x lithium dodecyl sulfate (LDS) (NuPAGE, Novex; Invitrogen) and 1x Reducing agent DTT-1M (NuPAGE, Novex; Invitrogen), were loaded on precast 10% Bis-Tris or 3-8% Tris-Acetate gels (NuPAGE, Novex; lifetechnologies). For subsequent SDS gel electrophoresis 1x MES SDS Running Buffer (NuPAGE, Novex; Invitrogen) or 1x MOPS SDS Running Buffer (NuPAGE, Novex; Invitrogen) was used. As standard protein ladder a mixture of MagicMark XP Western Protein Standard (Novex; Invitrogen) and PageRuler Prestained Protein Ladder (Thermo Scientific) was used.
Proteins were blotted on a 0.45-µm nitrocellulose membrane (Hybond, Amersham Biosciences, GE Healthcare Life Sciences, Little Chalfont, UK) by using PowerPacTM HC power supply (Bio-Rad) and blotting efficiency was determined with Ponceau staining (Ponceau S solution, Sigma Aldrich). Membranes were cut in horizontal strips at molecular weight ranges for target proteins and blocked for 1h with 5% milk (Carl Roth) in Tris-buffered saline with Tween20 (TBS-T) to avoid unspecific bindings of antibodies. Primary antibodies were diluted as described in **Table 3** and incubated on membranes overnight at 4 °C.

HRP conjugated goat anti-rabbit (1:3000 Bio Rad) and goat anti-mouse IgG (1:3000, Bio Rad) were used as secondary antibodies and incubated on membranes for 2 h at RT. Before detecting the immunolabeled proteins with the FluorChem®Q System (Alpha Innotech, Cell Biosciences, Santa Clara, CA, USA) chemiluminescence was development with WesternBright Chemiluminescence Substrate Quantum Kit (Biozym Scientifc; Hessisch Oldendorf, Germany) according to the manufacturer's instructions. Images were acquired with FluorChem Q System (Alpha Innotech, Cell Bioscienes, Santa Clara, CA, USA) and iBright CL 1000 Imaging System (Thermo Fischer Scientific) and band densities were analyzed with Li-Cor Image Studio Lite 5.2. Results are presented as a ratio of band densities of target protein and reference proteins GAPDH and vinculin with control samples set to one.

3.8. Histological methods

3.8.1. Preparation of sections

Placental tissue samples were fixed in 4% paraformaldehyde (PFA) for 24h at RT and subsequently embedded in paraffin. Human formalin-fixed-paraffin-embedded (FFPE) placental tissue was sectioned (5 µm thickness) and tissue was mounted on Superfrost Plus slides (Thermo scientific). After deparaffinization (**Table 11**), antigen retrieval in a pressure-boiler for 7 minutes at 120°C either in citrate buffer at pH 6 (Thermo Scientific) or Tris EDTA Puffer at pH 9 (Novocostra, Leica) was subsequently performed.

3.8.2. Immunohistochemistry

Immunohistochemistry was performed using the UltraVision Large Volume Detection System HRP Polymer Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. In short, after blocking endogenous peroxidase for 10 min with hydrogen peroxidase block, slides were washed three times with tris-buffered saline including 0.05% Tween 20 (TBS-T). Afterwards, sections were incubated for 5 min with Ultra Vision Protein Block to perform background blocking followed by a 45min incubation step at RT with the primary antibody (see **Table 3**), diluted in Antibody Diluent (Dako). Subsequently, slides were washed three times and incubated with anti-rabbit Large Volume HRP Polymer for 15 minutes. Primary anti-mouse antibodies required an additional incubation step with Enhancer for 15 min. To achieve detection, slides were incubated for 10 minutes with the substrate 3-amino-9-ethylcarbacole (AEC, Chromogen Single Solution; Thermo Scientific). After three washing steps in aqua dest., nuclei were stained with Mayer's Hemalaun (Thermo scientific). Slides were mounted in Kaiser's Glycerin Gelatine (Merck).

For the detection of adherent platelets on villous trophoblast in the FFPE first trimester placenta cohort ($n = 31$, mean gestational week 8.01 ± 2.08, see Table 4), sections were stained using a staining robot (Autostainer 360; Thermo Fisher Scientific) with primary antibodies as indicated in Table 3, using the UltraVision Large Volume Detection System HRP Polymer Kit (Thermo Fisher Scientific) as previously described.

3.8.2.1. Immunohistochemical double staining

Immunohistochemical double staining was performed with the Multivision Polymer Detection system (Thermo Scientific, Fremont, USA) using mouse monoclonal anti-HLA-G and rabbit polyclonal anti-vWF antibodies using dilutions as indicated in **Table 3** according to the manufacturer´s instructions (99).

Table 3: *Antibodies and their working concentrations used for immunostaining and immunoblots* Table reproduced from Forstner et al., J Mol Med 98, 193–207 (2020) (1).

			IHC	Immunoblot
ALPPL2	polyclonal rabbit	PA5-22336, ThermoFisher Scientific		1:500
CBP	monoclonal rabbit	Clone D6C5, #7389, Cell signaling		1:1000
CD42b	polyclonal rabbit	12860-1-AP, proteintech	1:1000	
Cytokeratin 7	Rabbit	Acris		1:500
Ezrin	Rabbit	Cell Signaling		1:1000
GAPDH	monoclonal rabbit	Clone 14C10, #2118, Cell Signaling		1:5000
GCM1	polyclonal rabbit	P100836_P050, Aviva Systems Biology		1:250
HLA-G	monoclonal mouse	clone 4H84; BD-Pharmingen	1:2000	
P300	monoclonal rabbit	Clone D8Z4E, #86377, Cell Signaling	1:500	1:1000
pCREB(Ser133)	monoclonal rabbit	Clone 87G3, #9198, Cell Signaling		1:1000
pSmad2(S465/467) / pSmad3(S423/425)	monoclonal rabbit	Clone D27F4, #8828, Cell Signaling		1:500
pSmad3(S423/425)	monoclonal rabbit	Clone EP823Y, ab52903, abcam		1:2000
vWF	polyclonal rabbit	F3520, Sigma-Aldrich	1:1000	
Vinculin	polyclonal rabbit	#PA5-29638 Invitrogen		1:1000

3.9. Scanning electron microscopy

BeWo cells were seeded in 12-well culture dishes containing round cover slips (15mm, Thermo Fischer Scientific) at a density of 2 x 10^5 cells/well. Next day, cells were stimulated with either 20 µM forskolin or 0.1% DMSO as a vehicle control in presence or absence of 10% pHPL. After 48h treatment, cells were washed with PBS and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde solved in 0.1 M sodium-phosphate buffer (pH7.4). After rinsing in sodium-phosphate buffer, specimens were post-fixed in 2% osmium tetroxide (Electron Microscopy Sciences) solved in 0.1 M sodium-phosphate buffer (pH7.4) and rinsed again. Each step was performed for 30min at RT. After dehydration in a graded series of ethanol and critical point drying (CPD 030; Bal-Tec, Balzers, Liechtenstein), samples were sputter coated with gold palladium (SCD 500; Bal-Tec, Balzers, Liechtenstein). Images were acquired using a Zeiss Sigma 500 field emission scanning electron microscope (Zeiss, Oberkochen, Germany), operated at an acceleration of 3 kV with an Everhart-Thornley-secondary electron detector.

3.10. Analysis of intracellular cAMP and pCREB levels

Intracellular cAMP levels were measured in BeWo cell lysates, which were obtained by lysis with 0,1M HCl solution after indicated time points. Lysates were measured in duplicates without acetylation step using the Direct cAMP ELISA kit (Enzo Life Sciences, Switzerland), according to the manufacturer's manual.

For analysis of pCREB levels, BeWo cells were lysed with Lysis Buffer after indicated time points and lysates were subjected to Human Phospho-CREB (S133) DuoSet IC ELISA (R&D Systems, Bio-techne, Abingdon, UK) according to the manufacturer's protocol.

3.11. Human TGF-β pathway phosphorylation array

By using a membrane based Human TGF-β Pathway Phosphorylation Array (C1 Series, RayBiotech; Norcross, GA, USA) relative levels of phosphorylation of eight candidate proteins involved in TGF-β signaling were determined.
BeWe cells were lysed with ready-to-use lysis buffer (RayBiotech) after 1h incubation with either 0.1% DMSO or 20 µM forskolin in presence or absence of 10% pHPL. BeWo cell lysates were subjected to membrane arrays according to the manufacturer's instructions. Chemiluminescent imaging was performed using the FluorChemQ System (Alpha Innotech, Cell Bioscienes; Santa Clara, CA, USA) and signal densities were analyzed with AlphaView software version 3.4.0.

3.12. Statistical analysis

Data were analyzed using GraphPad Prism Version 8.1.0 and are presented as means ± SEM. Data were subjected to Normality Test (D'Agostino & Pearson omnibus normality test) and Equal Variance Test. In case of normally distributed data differences between groups were tested using two-tailed t-test. Otherwise Wilcoxon signed rank test was applied. For multiple comparison procedure One-way analysis of variance was followed by Tukey's multiple comparisons test to isolate groups that differ from the others. One sample t test was used when controls were set as 1. A p-value of less than 0.05 was considered statistically significant.

4. Results

4.1. Adherent maternal platelets at the early fetal-maternal interface

First trimester placental tissue was subjected to immunohistochemistry in order to detect maternal platelets upon their transition from maternal blood flow towards the intervillous space. For this purpose, placental tissue was stained with the platelet marker CD42b (**Figure 3b-d**), a surface glycoprotein, which functions as a receptor for von Willebrand factor (vWF), while adjacent sections were double-stained for the extravillous trophoblast marker HLA-G and vWF (**Figure 3a**), as a marker for endothelial cells. Cross-sections of maternal vessels revealed adherent platelets, trapped within loosely cohesive HLA-G positive trophoblast plugs (**Figure 3b**). Further investigations on an archival first trimester placenta *in utero* from hysterectomy showed CD42b positive platelets in cell columns of extravillous trophoblasts as well as at the adjacent lining of the intervillous space (**Figure 3c+d**), suggesting the presence of platelets at the early fetal-maternal interface from very early weeks of gestation.

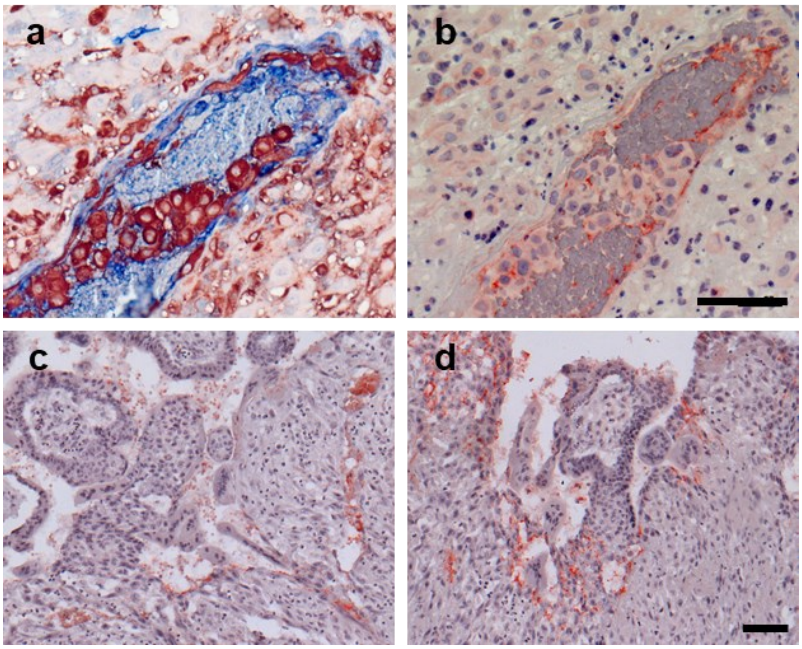

Figure 3: Detection of adherent maternal platelets at the early fetal-maternal interface. Human first trimester decidua sections were double stained for extravillous trophoblast marker HLA-G (brownish) and von Willebrand factor (vWF, blue) (**a**), suggesting remnants of trophoblast plugs in uteroplacental vessels. Adjacent sections stained for the platelet marker CD42b showed staining on the surface of dissolving trophoblast plugs (**b**). Immunohistochemistry of an archival placenta in utero from hysterectomy showed CD42b positive platelets in cell columns as well as at the adjacent lining of the intervillous space (**c+d**). Images represent different areas of the same section (**c+d**). Nuclei were stained with Hemalaun in (**b-d**), no nuclear counterstain in (**a**). Tissue samples are from 7 weeks of gestation. Scale bars represent 100µm. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.2. Localization of adherent platelets on trophoblast

Since we identified a possible new route of how platelets may enter the intervillous space through narrow channels within trophoblast plugs, we further analyzed 31 first trimester placenta samples from week 5 to 12 of gestation to clarify the adherence of platelets on placental villi over the first trimester (summarized in **Table 4**).

In 93,6% of all cases, CD42b positive platelets were detected on the surface of placental villi, suggesting the presence of platelets in the intervillous space from very early stages of gestation onwards is a common phenomenon. Noteworthy, platelets were either detected on the apical surface of villous trophoblasts (**Figure 4a+b**) or on initial perivillous fibrinoid deposits (**Figure 4c**). Furthermore, we also detected adherent platelets between the layer of cytotrophoblast cells and the syncytiotrophoblast (**Figure 4d**), with a prevalence of 48,4% of all cases. We next investigated the presence of platelets in proximity to HLA-G positive EVTs (**Figure 4f+h**) and CD42b positive platelets (**Figure 4e+g**) were detected within the anchoring parts of trophoblast cell columns in 82,8%. This finding was consistent over the whole time span of the first trimester starting from week 5 of gestation onwards.

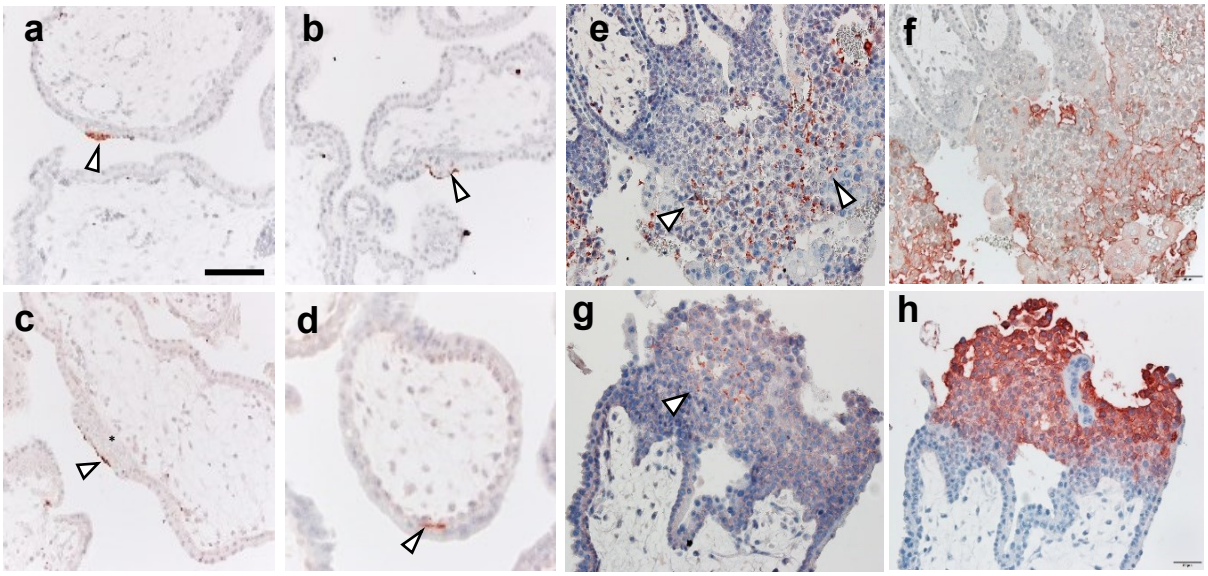

Figure 4: Adherent maternal platelets on trophoblasts. First trimester placenta tissue (n=31) was subjected to immunohistochemistry and staining for platelet marker CD42b revealed adherent maternal platelets on the surface of placental villi (**a-c**). Platelets attached either directly to the apical surface of syncytiotrophoblast (**a + b**, arrowheads) or on initial perivillous fibrinoid deposits (**c**, asterisk). Platelets were also detected between the cytotrophoblast and the syncytiotrophoblast layer (**d**, arrowhead). Staining for CD42b (**e + g**) and the extravillous trophoblast marker HLA-G (**f + h**) in adjacent sections showed platelets (**e + g**, arrowhead) within intercellular clefts of extravillous trophoblasts. Nuclei were stained with Hemalaun in (a-h). Images are representative for gestational

week 7 in **a-c** and **e + f** and for gestational week 6 in d and **g + h**. Scale bars represent 100µm. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

Table 4: Immunohistochemistry of platelet marker CD42b in human first trimester placenta *Table used from Forstner et al., J Mol Med 98, 193–207 (2020) (1).*

gestational week (mean ± SD)	number of cases showing platelets on the surface of villi	number of cases showing platelets between ST and VCT layer	number of cases showing platelets in intercellular gaps of EVT cell column
5.31 ± 0.43 (n=5)	4/5	2/5	3/5
6.54 ± 0.38 (n=4)	3/4	2/4	4/4
7.17 ± 0.26 (n=5)	5/5	3/5	5/5
8.29 ± 0.29 (n=5)	5/5	2/5	3/5*
9.34 ± 0.28 (n=5)	5/5	4/5	5/5
10.39 ± 0.46 (n=4)	4/4	2/4	3/4
11.38 ± 0.54 (n=3)	3/3	0/3	1/3*

Extravillous trophoblast (EVT), syncytiotrophoblast (ST), villous cytotrophoblast (VCT); *one sample did not contain EVT cell column

4.3. pHPL reduces forskolin-induced βhCG expression and secretion in human first trimester placental explants and in BeWo cells

Upon activation, platelets release their granule content into their environment and factors such as chemokines, cytokine and inflammatory mediators, allowing them to interact with vascular or circulating cells (100). Platelet degranulation at the early fetal-maternal interface may thus effect trophoblast function and endocrinology. Therefore, βhCG levels of placental villi explant cultures and the trophoblast cell line BeWo were analyzed after incubation with pooled human platelet lysate (pHPL). Gene expression levels of the β-subunit of hCG, encoded by *CGB*, in placental explant cultures after an 48h incubation with pHPL were significantly downregulated by 35.4% in comparison to the controls (**Figure 5a**). In line with this finding, βhCG secretion of *ex-vivo* cultures, measured in culture supernatant, was also reduced by 25.9% after pHPL treatment (**Figure 5b**). In order to verify these results with a trophoblast cell line, BeWo cells were stimulated with forskolin, which is known to stimulate intracellular cAMP and thus leading to the expression of hCG (101), in presence or absence of pHPL. As expected, forskolin significantly induced βhCG mRNA expression (**Figure 5c**) as well as βhCG secretion (**Figure 5d**) after 48h. Incubation with pHPL led to significant reduction of βhCG on gene expression level as well as on secretion level.

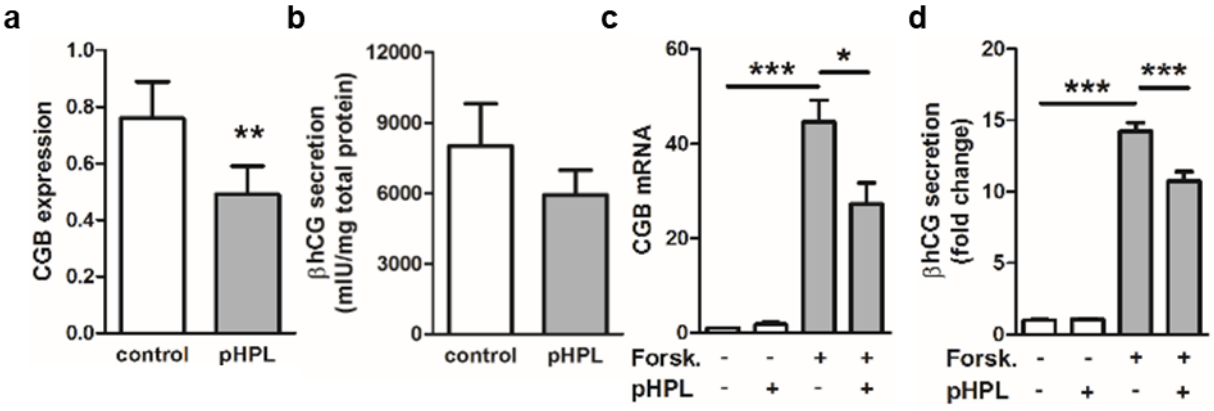

Figure 5: pHPL impairs βhCG synthesis. Expression of hCG beta-subunit *CGB* (**a**) and secretion of βhCG (**b**) was analyzed in first trimester placental villi (n = 14, mean gestational week 8.26 ± 0.45) after 48h of incubation under 2.5% oxygen at 37 °C in presence or absence of 10% pHPL. Heparin in a final concentration of 2 U/ml was administered in control samples as well as in presence of pHPL. Secretion levels of βhCG were normalized to total protein concentration of tissue lysates. BeWo cells were stimulated with 20 µM forskolin in presence or absence of 10% pHPL for 48h under 5% CO_2 at 37 °C. DMSO, as vehicle control, was applied in a concentration of 0.1% (v/v). Gene expression levels of hCG beta-subunit *CGB* (**c**) and secretion of βhCG (**d**) of BeWo cells are presented as means ± SEM from three independent experiments using different cell passages and differences between groups were identified using One-way analysis of variance followed by Tukey's multiple comparisons test. Data in (**a**) and (**b**) are presented as means ± SEM from 14 different cases and were tested for differences using two-tailed Wilcoxon signed rank test. *p≤0.05, **p≤0.01, ***p<0.001. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.4. ADP-activated platelets impair hCG synthesis

Since the platelet lysate is a platelet product, which was obtained by repeatedly freeze/thaw cycles in order to release a sufficient amount of platelet-derived factors, we wanted to verify our initial results (described in chapter 4.3.) with ADP-activated platelets. Previous, to the analysis of hCG expression in presence of activated platelets, we subjected isolated platelets to scanning electron microscopy as a proof of concept for platelet isolation method as well as for co-incubation with trophoblast cells. Microscopy revealed ADP-induced activation of isolated platelets (**Figure 6b**) in comparison to controls (**Figure 6a**). Next, BeWo cells were co-incubated with pre-activated platelets for 1h and activated platelets, adherent to the surface of BeWo cells, have been detected (**Figure 6c+d,** arrowhead).

In order to test, whether ADP-activated platelets may also impair hCG synthesis, BeWo cells were incubated with isolated platelets, which have been either pre-stimulated with 2-MeS-ADP or not. In line with our observation on the pHPL-induced impaired hCG synthesis, ADP-

activated platelets significantly reduced *CGB* expression in forskolin stimulated cells (**Figure 6e**). In undifferentiated cells, neither ADP alone, nor activated or non-activated platelets have influenced the hCG expression.

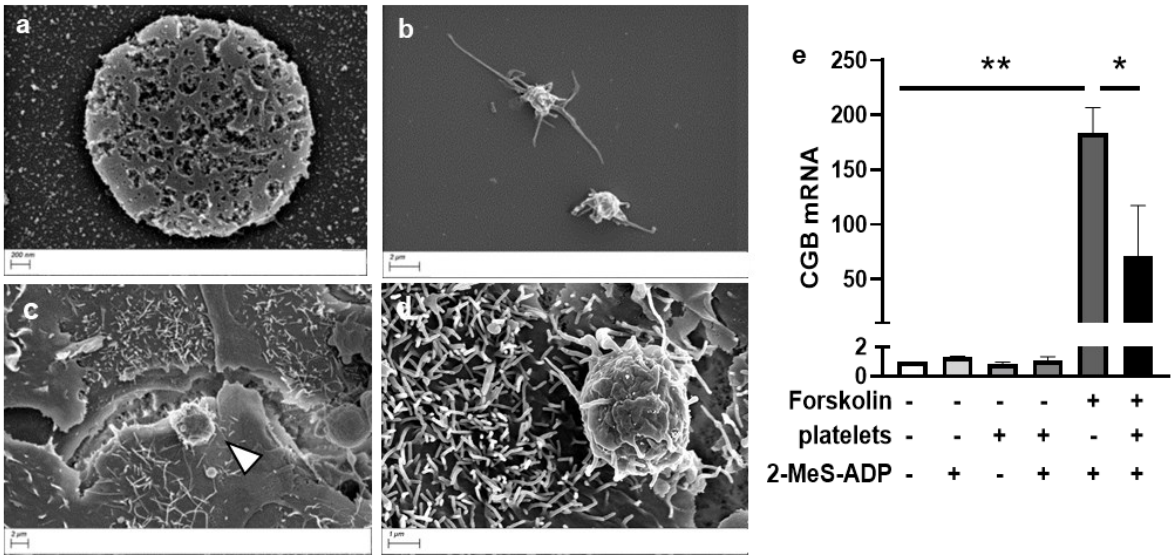

Figure 6: ADP-activated platelets impair hCG expression. Human platelets from healthy donors were isolated and subsequently either activated with the stable ADP analogue 2-Methylthioadenosine 5'-diphosphate (2-MeS-ADP) in a final concentration of 10 µM (a) or not (b) and subjected to standard scanning electron microscopy (SEM) procedure. Pictures revealed efficient activation of ADP (**b**) in comparison to control samples (**a**). BeWo cells were co-cultured with isolated platelets, which were either pre-activated with ADP (10 µM) or not and SEM pictures showed adherent platelets on trophoblast cells (**c + d**, arrowhead). BeWo cells were stimulated with forskolin (20 µM) or vehicle control DMSO (0.1%) and were cultured for 48h in presence or absence of isolated platelets in a concentration of 1,5-3.0*10^5 platelets/µl, which were either pre-activated with 2-MeS-ADP or not. Cell lysates were subsequently subjected to gene expression analysis of hCG beta-subunits *CGB-3*, *-5* and *-8* (**e**). Data are presented as means ± SEM from three independent experiments using different cell passages and blood donors, differences between groups were identified using One-way analysis of variance followed by Tukey's multiple comparisons test. *p≤0.05, **p≤0.01. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.5. Platelet-derived factors do not substantially affect villous trophoblast differentiation

HCG is mainly synthesized in the highly differentiated syncytiotrophoblast and therefore often used as a marker for trophoblast differentiation (102). In order to elucidate, whether the pHPL-induced downregulation of hCG in differentiated cells is a consequence of impaired syncytialisation, we analyzed the effect of pHPL on biochemical trophoblast differentiation, trophoblast fusion and morphological changes during trophoblast differentiation.

4.5.1. Biochemical trophoblast differentiation markers are slightly influenced by pHPL

The transcription factor GCM1 is a major regulator of differentiation and important in the regulation of hCG (14,103). As expected, gene expression analysis of forskolin-induced trophoblast cells showed a 3.1-fold higher GCM1 expression after 3h stimulation than in control cells (**Figure 7a**). The presence of pHPL did not significantly alter GCM1 expression neither in undifferentiated cells nor in forskolin-stimulated cells. Immunoblot analysis of GCM1 verified observation and showed no significant influence of pHPL on GCM1 protein levels (**Figure 7b+c**).

Another marker of biochemical villous trophoblast differentiation, the alkaline phosphatase, placental-like 2 (*ALPPL2*) (104), was as expected significantly upregulated upon forskolin treatment on gene expression level as well as on protein level. Treatment with pHPL has shown a significant reduction of mRNA levels of *ALPPL2* in differentiated cells (**Figure 7d**). Protein levels of *ALPPL2* were reduced by 33.5% upon pHPL treatment in forskolin-stimulated BeWo cells (**Figure 7e+f**).

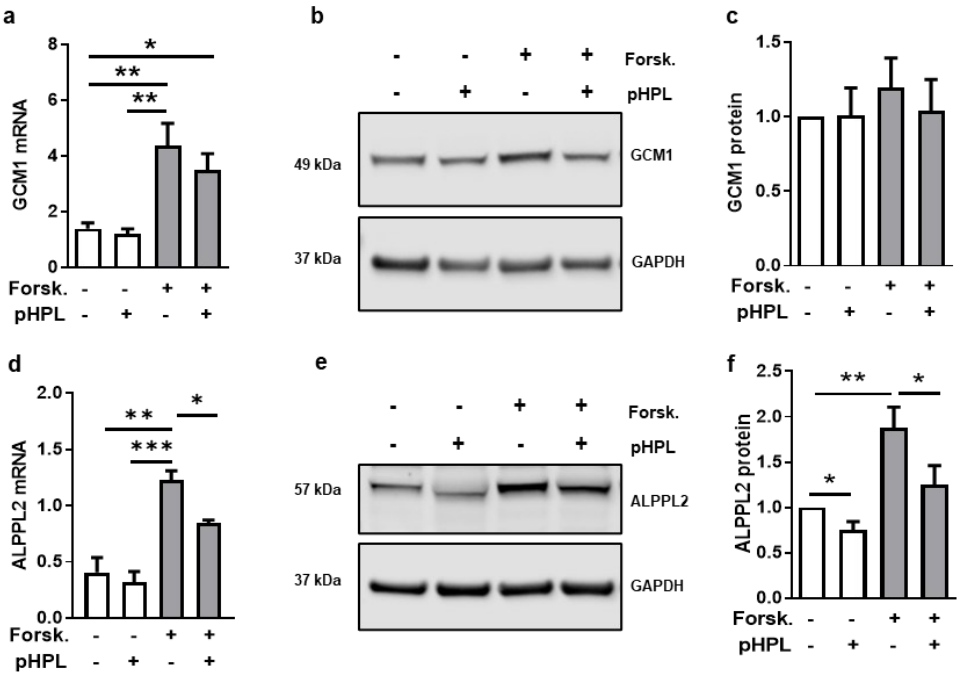

Figure 7: Biochemical trophoblast differentiation markers are slightly influenced by pHPL. BeWo cells were treated either with solvent control DMSO (0,1%) or forskolin (20µM) in presence or absence of 10% (v/v) pHPL for 3h (**a-c**) and 48h (**d-e**) and cell lysates were subjected to gene expression analysis of GCM1 (**a**) and *ALPPL2* (**d**) and immunoblot analysis for GCM1 (**b**) and *ALPPL2* (**e**). Data in bar graphs are presented as means ± SEM from six (**a**), four (**c**) or three (all others) independent experiments using different cell passages. Differences between groups were identified using One-way analysis of variance and Tukey's multiple comparisons test (**a + d**). Western blots are representative for four (**b**) and three (**e**) different experiments. For band densitometry (**c** and **f**), controls

were set to one and data were tested using one sample t-test. *p≤0.05, **p≤0.01, ***p<0.001. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.5.2. Trophoblast fusion is not affected by pHPL

The fusogenic retroviral envelope proteins, syncytin-1 (*ERVW-1*) and syncytin-2 (*ERVFRD*), are downstream targets of GCM1 and are known to induce trophoblast fusion (105). As expected, Syncytin-1 (**Figure 8a**) and Syncytin-2 (**Figure 8b**) are both upregulated on gene expression level upon forskolin treatment, of which Syncytin-2 was significantly increased. After an incubation of 48h in the presence of pHPL, there was no significant effect of pHPL on both markers detectable.

The further elucidation of the cell junction protein E-cadherin (CDH1), which is known to be downregulated upon cell fusion, showed a 4.5-fold decrease in gene expression after forskolin treatment (**Figure 8c**). In line with our previous results, pHPL did not affect the expression of E-cadherin after a 48h treatment.

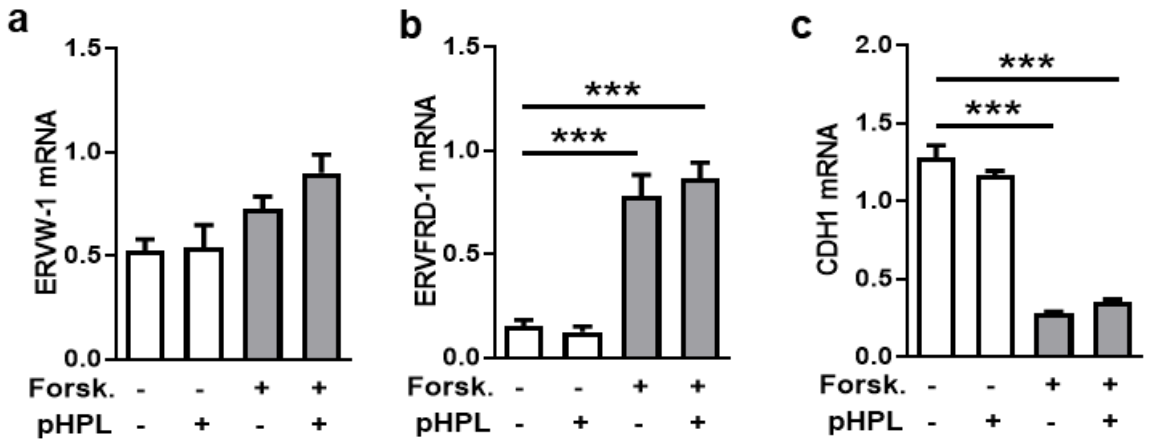

Figure 8: Trophoblast fusion is not affected by pHPL. Gene expression of trophoblast fusion markers syncytin-1 (**a**, ERVW-1), syncytin-2 (**b**, ERVFRD-1), and E-cadherin (**c**, CDH1) were analyzed in BeWo cells after stimulation with 20 µM forskolin in presence or absence of pHPL after 48h. DMSO (0.1%) served as vehicle control. Fusion marker were strongly upregulated (**b**) or downregulated (**c**) by forskolin. Data in bar graphs are presented as means ± SEM from three independent experiments using different cell passages. Differences between groups were identified using One-way analysis of variance and Tukey's multiple comparisons test. ***p<0.001. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.5.3. Differentiation-dependent microvilli formation in trophoblast cells is not impaired by pHPL

Trophoblast differentiation is accompanied with morphological changes and therefore we determined the effect of pHPL on microvilli formation by using scanning electron microscopy. After a forskolin stimulation of 48h, BeWo cells developed notable closely spaced microvilli on their surface (**Figure 9c+d**) in comparison to the vehicle control, where mainly reef-like membrane ruffles have been observed (**Figure 9a+b**). The presence or absence of pHPL seems to neither impair the membrane ruffles of undifferentiated cells (**Figure 9b**), nor the differentiation dependent microvilli formation (**Figure 9d**).

Beside this imaging procedure, in order to substantiate this observation, we analyzed the influence of pHPL on the ezrin-radixin-moesin (ERM) proteins, which are known to play a central role in the organization of the cortical actin-based cytoskeletons and thus also in the formation of microvilli (106). Gene expression levels of ERM proteins in BeWo cells revealed a 5.5-fold higher expression of ezrin (EZR), compared to radixin (RDX) ((**Figure 9e**). Moesin (MSN) was only marginally expressed in BeWo cells, leading to the exclusion of moesin for further analysis. Analysis of ezrin expression in forskolin-stimulated cells, revealed a 1.9-fold upregulation compared to the control (**Figure 9f**), which is in line with our observations under the scanning electron microscope. In forskolin-stimulated cells in presence of pHPL, only a slightly downregulation of 14.2% was detectable, whereas in undifferentiated cells ezrin expression in presence of pHPL was reduced by 28%, however, not significantly. Results of immunoblot analysis verified the significant forskolin-induced upregulation of ezrin (**Figure 9g+h**) and suggests only a slight influence of pHPL on this microvilli formation involved protein.

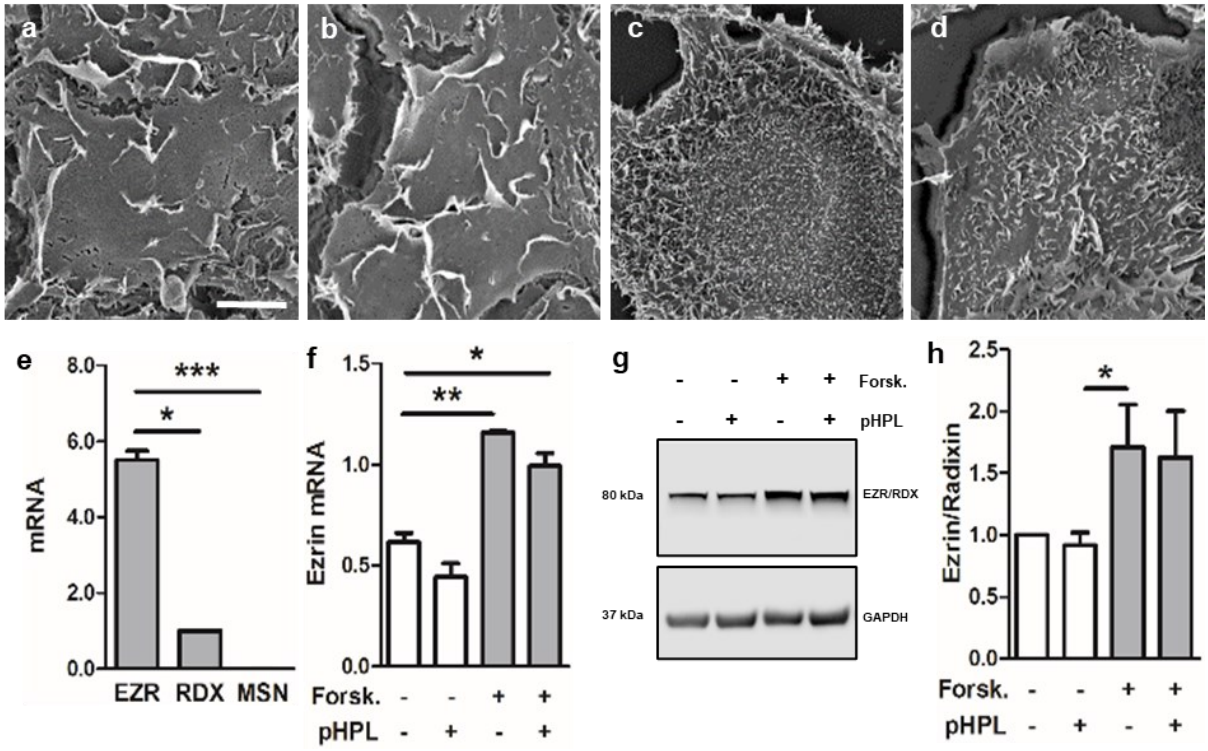

Figure 9: Effect of pHPL on differentiation-dependent microvilli formation. BeWo cells were incubated for 48h in presence or absence of 10% pHPL with either 20 µM forskolin or 0.1% DMSO as vehicle control and subsequently subjected to standard procedure for scanning electron microscopy (SEM). Forskolin stimulated samples revealed an extensive formation of microvilli (**c + d**) compared to controls, where mainly membrane-ruffles (**a + b**) were detected. Presence of pHPL did not influence the formation of those membrane structures (**b + d**). Gene expression level of the the ezrin (EZR), radixin (RDX) and moesin (MSN) family was analyzed in unstimulated BeWo cells after 48h (e) and revealed high expression of ezrin. Expression of ezrin mRNA (**f**) and protein levels (**g** + **h**) were analyzed in BeWo cells after forskolin induction in presence and absence of 10% pHPL after 48h. Data in bar graphs are represented as means ± SEM from three independent experiments using different cell passages. Scale bar in (**a**) represents 10µm. *p≤0.05, **p≤0.01, ***p<0.001. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.5.4. pHPL impairs hCG synthesis and alkaline phosphatase, placental-like 2, in pre-differentiated trophoblasts

In order to verify our assumptions, that pHPL impairs hCG synthesis independently of syncytialization, we analyzed the effect of pHPL on pre-differentiated BeWo cells. Cells were stimulated with forskolin and pHPL was administered either simultaneously with forskolin at experimental start or after a preceding 48h treatment with forskolin for another 24h. The forskolin-induced upregulation of hCG expression was downregulated in both conditions (**Figure 10a**), suggesting different pHPL-induced regulatory mechanisms for hCG synthesis and syncytialization. Interestingly, the differentiation marker *ALPPL2* was also downregulated by pHPL in forskolin-stimulated cells in both conditions (**Figure 10b**), however not significantly.

Considering the results on the effect of pHPL on trophoblast differentiation, our data suggests, that pHPL does not have a substantial impact on syncytialization and pHPL-induced downregulation of hCG in differentiated cells is not a consequence of impaired trophoblast differentiation *per se*.

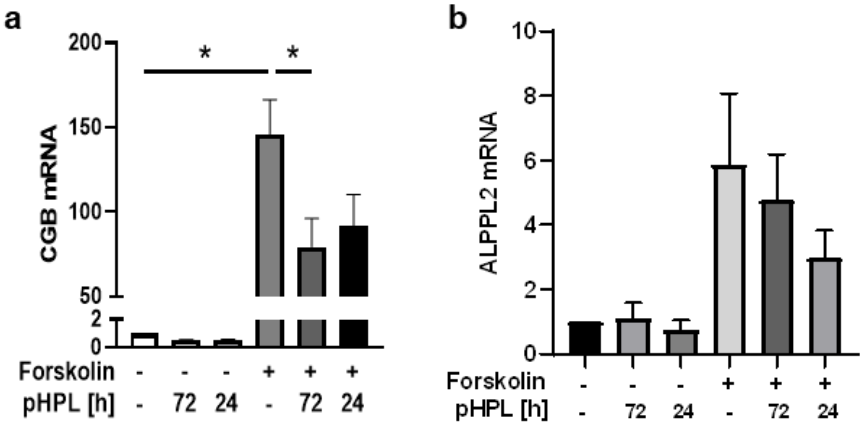

Figure 10: Effect of pHPL on pre-differentiated trophoblast cells. Gene expression of hCG beta-subunits *CGB-3*, *-5* and *-8* (**a**) and alkaline phosphatase, placental-like 2 (*ALPPL2*) (**b**) were analyzed in forskolin-stimulated (20 µM) BeWo cells, when pHPL was either administered at experimental start (72h total incubation) or after a 48h preceding stimulation with forskolin (24h incubation with pHPL). DMSO (0.1%) served as vehicle control. Forskolin-induced upregulation of *CGB* and *ALLPL2* was also impaired in pre-differentiated cells. Data are presented as means ± SEM from three independent experiments using different cell passages and were tested for differences using One-way analysis of variance followed by Tukey's multiple comparisons test. *p≤0.05. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.6. Effect of pHPL on forskolin induced cAMP/CREB signaling in BeWo cells

As previously described, trophoblast differentiation is accompanied with an increase of intracellular cyclic AMP (cAMP) (12) and the subsequent phosphorylation of CREB at serine 133 (14,107). Since the previous results on trophoblast differentiation did not suggest a substantial effect of pHPL on syncytialization, we next focused on cAMP/CREB signaling.

Enzyme-linked immunoassay revealed a steep forskolin-induced increase of intracellular cAMP after 30 minutes with a peak after 1h treatment and a decrease to nearly control levels within 24h (**Figure 11a**). At none of those time points, a significant impact of pHPL on cAMP levels in forskolin-stimulated cells was detectable. Next, the influence of pHPL on the phosphorylation of CREB, as a downstream event of increased cAMP levels, was determined. Upon forskolin treatment, levels of phosphorylated CREB (pCREB) were, as expected, 2.4-fold increased after 30 minutes and 2.5-fold after 1h treatment (**Figure 11b**). Interestingly,

treatment of pHPL without forskolin, induced pCREB levels 2.4-fold and 2.1-fold after 30 minutes and 1h. Treatment of pHPL in combination with forskolin has also led to a slide increase compared to forskolin-treatment alone.

In order to confirm the effect of pHPL on the phosphorylation of CREB, BeWo cells were subjected to immunoblot analysis after 1h treatment with forskolin in presence or absence of pHPL (**Figure 11c+d**). In line with our observation, treatment of pHPL alone has significantly increased pCREB levels, whereas the combinational treatment with forskolin and pHPL only slightly increased pCREB levels compared to forskolin treatment alone.

Since the nuclear receptor subfamily 4 group A member 2 (NR4A2) has been previously described as a downstream target of cAMP/CREB signaling (108,109), the effect of pHPL on its gene expression level was elucidated. Forskolin treatment significantly induced NR4A2 expression in BeWo cells after 1h treatment (**Figure 11e**). The administration of forskolin and pHPL has slightly increased the expression levels of NR4A2 in comparison to forskolin alone, which is in line with the pHPL induced phosphorylation of CREB. However, these results show, that cAMP/CREB signaling is not significantly impaired by pHPL in BeWo cells.

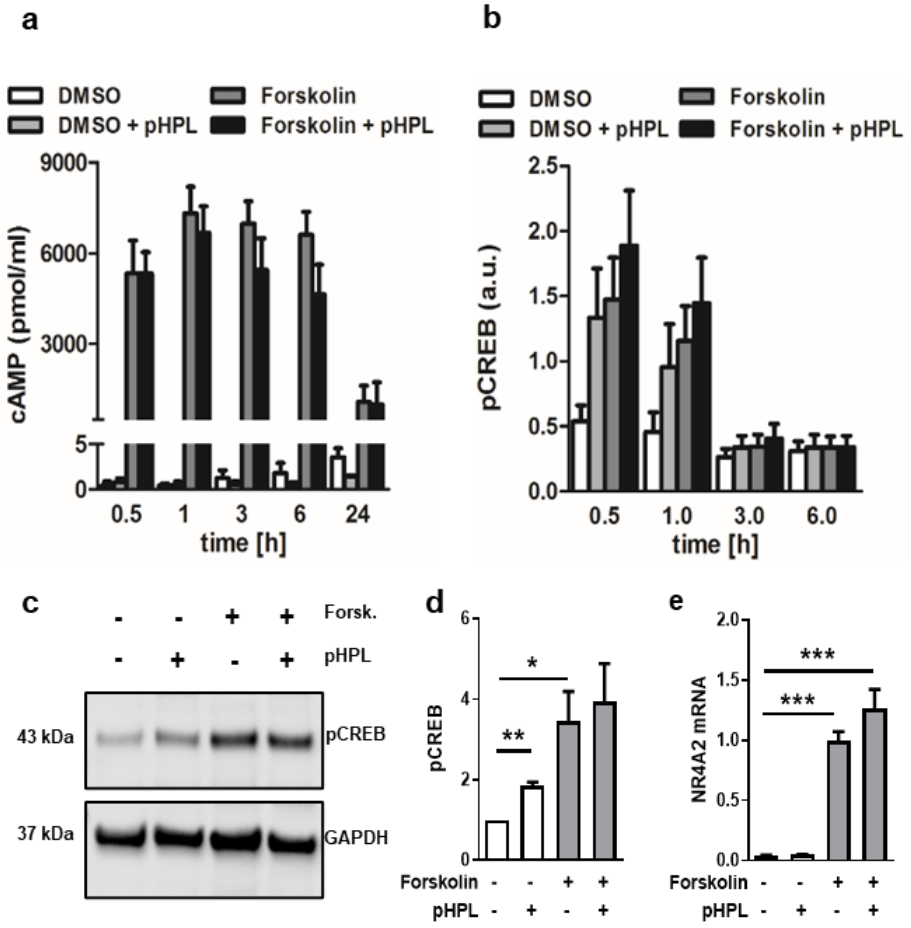

Figure 11: Effect of pHPL on forskolin induced cAMP/CREB signaling in BeWo cells. Levels of intracellular cAMP (**a**) and phosphorylated CREB (**b**) were analyzed by enzyme-linked immunosorbent assay in forskolin-stimulated (20µM) and vehicle (DMSO, 0.1% v/v) treated BeWo cells in presence or absence of 10% pHPL at indicated time points. Furthermore, BeWo cell lysates were subjected to immunoblotting for analysis of phosphorylation of CREB (pCREB) after 1h treatment (**c**). Control in band densitometry for pCREB (**d**) was set to one. Gene expression of NR4A2 (**e**) was analyzed in BeWo cells after 1h incubation with forskolin (20 µM) in presence or absence of 10% pHPL. Data in bar graphs (**a**) and (**b**) are presented as means ± SEM from five independent experiments using different cell passages and were tested for differences using Two-way analysis of variance followed by Tukey's multiple comparisons test. Western blot is representative for three (**c**) different experiments. Data for band densitometry (**d**), were tested using one sample t-test. Data in (**e**) were analyzed using One-way analysis of variance and Tukey's multiple comparisons test. *p≤0.05, **p≤0.01, ***p<0.001. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.7. Effect of pHPL on phosphorylation of TGF-β pathway proteins

The protein content of platelet granules comprises factors, such as transforming growth factor (TGF)-β superfamily members (110) and epidermal growth factor (EGF). These are known to be involved in cell proliferation and differentiation (111). Protein analysis of platelet lysate according to Burnouf et al. (30), which is shown in **Table 1**, revealed an abundant amount of TGF-β 1. In order to elucidate the effect of pHPL on TGF-β signaling in BeWo cells, cell lysates were subjected to a membrane-based immunoblot array after stimulation with forskolin in presence or absence of pHPL (**Figure 12a-d**). Analysis of this array revealed, that neither forskolin nor pHPL have a significant impact on the phosphorylation of Smad1(**Figure 12e**), Smad2 (**Figure 12f**), Smad4 (**Figure 12g**) and Smad5 (**Figure 12h**). Also the transcription factor ATF2 (**Figure 12j**) or TGF-β-activated kinase (TAK)1(**Figure 12i**) were not influenced by forskolin or pHPL. Forskolin stimulation did not show effects on the transcription factors c-Jun (**Figure 12k**) and c-Fos (**Figure 12l**), which dimerize and form the AP-1 transcription factor complex (112,113). Interestingly, pHPL did not induce the phosphorylation of c-Jun, whereas activation of c-Fos was 1.5-fold increased upon pHPL treatment in controls as well as in forskolin-stimulated cells.

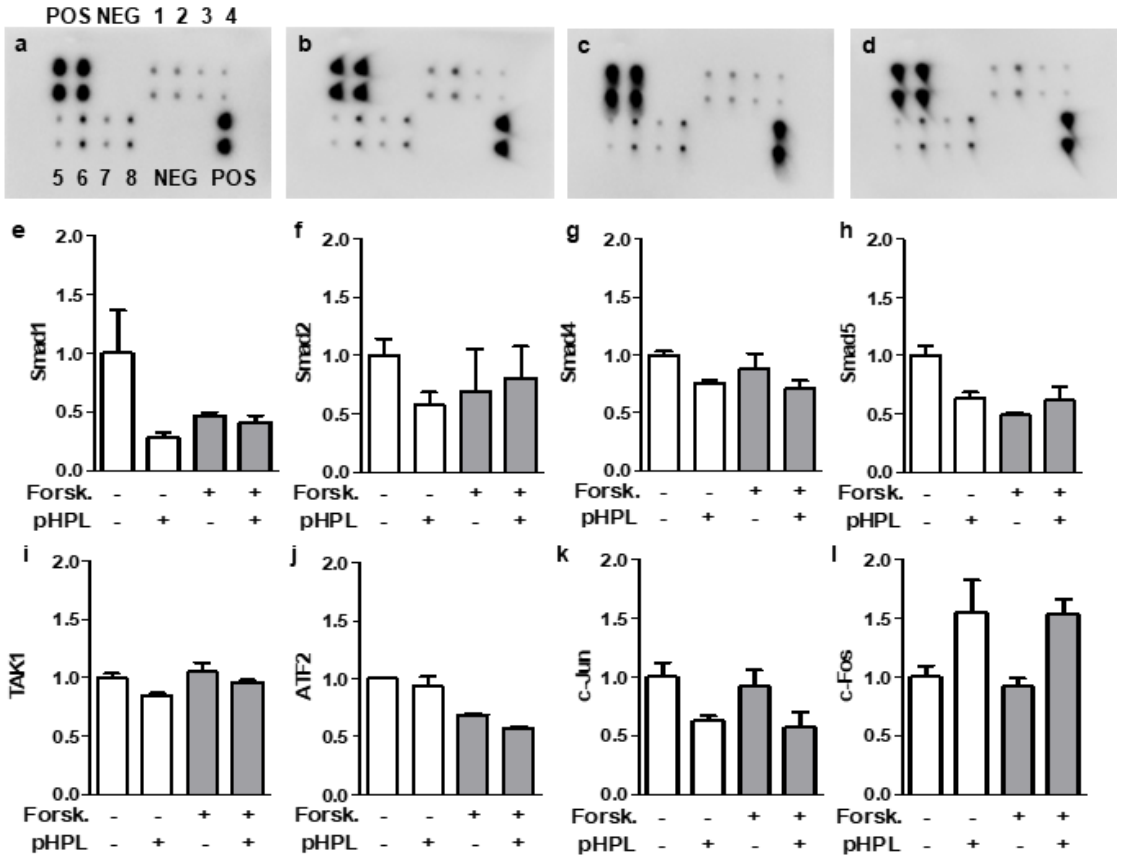

Figure 12: Effect of pHPL on phosphorylation of TGF-β pathway proteins. BeWo cells were treated either with solvent control DMSO (0.1%) (**a**), DMSO and 10% pHPL (**b**), with 20 µM forskolin alone (**c**) and with forskolin and 10% pHPL (**d**) for 1h. Afterwards, cell lysates were subjected to a human TGF-β Pathway Phosphorylation Array, which is a membrane-based immunoblot array for the analysis of phosphorylation status of eight TGF-β pathway proteins. Each membrane includes positive control (**POS**), negative control (**NEG**), ATF2 (**1**), C-Fos (**2**), c-Jun (**3**), Smad1 (**4**), Smad2 (**5**), Smad4 (**6**), Smad5 (**7**) and TAK1 (**8**). Data for band densitometry (**i-l**) were presented as means ± SEM and controls were set to one. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.8. Platelet-derived factors activate Smad3 in BeWo cells

Since, pHPL did not induce the activation of Smad1, Smad2, Smad4 and Smad5, we further elucidated the influence of pHPL on the phosphorylation of Smad3 in BeWo cells. Cell lysates were subjected to immunoblot analysis and interestingly, phosphorylation of Smad3 was 2.5-fold higher upon pHPL treatment compared to controls (**Figure 13a+b**), independently of forskolin-stimulation. The pHPL-induced activation of Smad3 was also confirmed by a second antibody clone, which is shown in **Figure 20**.

In order to confirm this findings on gene expression levels, the effect of pHPL on TGF-β signaling was analyzed with the TGF-β downstream target plasminogen activator inhibitor (PAI)-1, which is encoded by the gene *SERPINE1* (114). In undifferentiated cells, *SERPINE1* expression was 3.3-fold upregulated upon pHPL-treatment as compared to the solvent control (**Figure 12c**), whereas in forskolin-stimulated cells, a significant activation of Smad3 due to pHPL could not be detected. In summary, the effect of pHPL on phosphorylation of TGF-β signaling pathway proteins revealed, that platelet-derived factors may induce TGF-β signaling in BeWo cells through activation of Smad3.Undifferentiated as well as differentiated BeWo cells were also incubated with isolated platelets, which were either pre-activated with ADP or not. Gene expression analysis of *SERPINE1* revealed a strong platelet-induced upregulation in undifferentiated cells, whereby treatment with ADP-stimulated platelets increased this upregulation even more. ADP-activated platelets also induced *SERPINE1* expression in differentiated cells, but however, its expression was lower in comparison to undifferentiated cells.

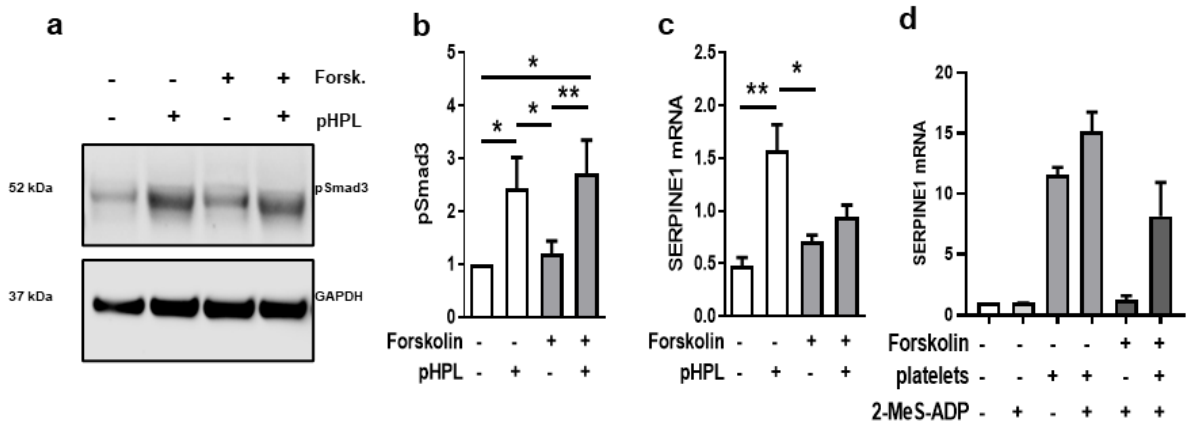

Figure 13: Platelet-derived factors activate Smad3. BeWo cells were stimulated with forskolin (20 µM) in presence or absence of 10% pHPL for 1h. DMSO (0.1%) served as vehicle control. Cell lysates were subsequently subjected to immunoblotting for analysis of the phosphorylation of Smad3 (pSmad3) (**a**). Following band densitometry (**b**) showed strong pSmad3 activation upon pHPL treatment. Smad3 downstream target *SERPINE1* was analyzed after 48h treatment (**c**). Furthermore, BeWo cells were stimulated with forskolin (20 µM) or vehicle control DMSO (0.1%) in presence or absence of isolated platelets with a final concentration of 1,5-3.0*10^5 PLT/µl, which were either pre-activated with 2-MeS-ADP or not for 48h. Subsequent gene expression analysis for *SERPINE1* showed a strong upregulation upon treatment with platelets. Data are presented as means ± SEM from eight (**a + b**) and three (**c + d**) independent experiments using different cell passages and blood donors. Differences between groups were identified using One-way analysis of variance followed by Tukey's multiple comparisons test. *p≤0.05, **p≤0.01. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.9. TGF-β type 1 receptor inhibitor SB431542 does not restore impaired βhCG synthesis

Since the previous results suggested a strong pHPL-induced activation of Smad3 in BeWo cells, we used the well-known TGF-β type 1 receptor inhibitor SB431542 to analyze, whether the pHPL-induced downregulation of hCG in forskolin-stimulated BeWo cells is a consequence of activated TGF-β signaling.

First of all, we tested the efficiency of the inhibitor by incubating BeWo cells with forskolin in the presence or absence of pHPL and either with or without SB431542. Gene expression levels of *SERPINE1* were significantly reduced nearly to control levels by the TGF-β type 1 receptor inhibitor (**Figure 14a**), suggesting an efficient sufficiency of the inhibitor. Interestingly, immunoblot analysis of Smad3 (**Figure 14b**) revealed only a slight decrease in pHPL-induced Smad3 levels after incubation with SB431542.

The expected pHPL-induced downregulation of forskolin-induced *CGB* expression in BeWo cells (**Figure 14c**), was interestingly not abrogated by the treatment with SB431542. Increased expression levels of *ALPPL2* (**Figure 14d**) due to forskolin treatment, were downregulated with pHPL by 40% and in that case, we could also not detect any effects of SB431542. In summary, the absence of any significant SB431542-induced effects on expression levels of *CGB* and *ALPPL2* as well as on pHPL-induced Smad3 activation in forskolin-stimulated cells, does not suggest an involvement of TGF-β type 1 receptor in the pHPL-mediated impaired hCG synthesis.

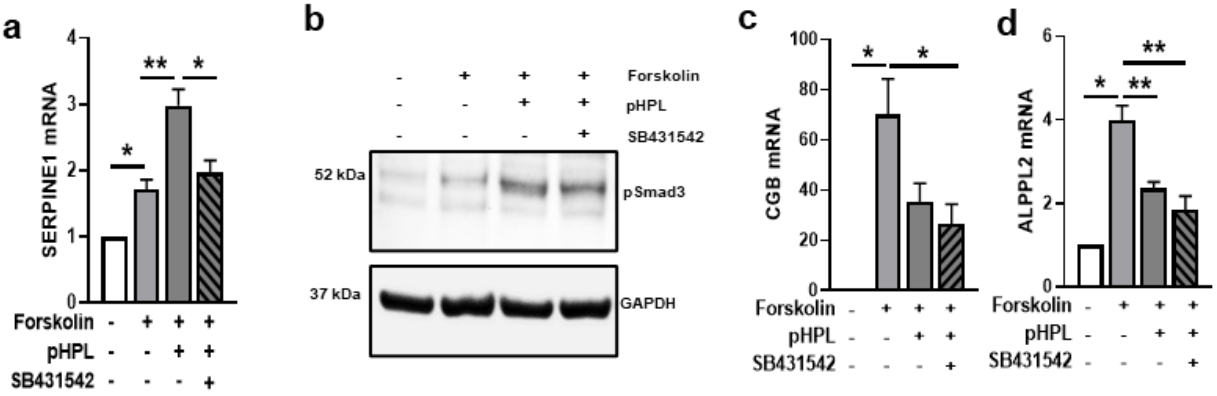

Figure 14: TGF-β type 1 receptor inhibitor do not restore impaired βhCG synthesis. BeWo cells were pre-incubated with SB431542 at final concentrations of 10µM for 2h. Subsequently, cells were treated either with 0,1% DMSO solvent control or 20 µM forskolin in presence or absence of 10% pHPL and in addition with or without TGF-β type 1 receptor inhibitor SB431542 in a final concentration of 10 µM for 1h (**b**) or 48h (**a**, **c** and **d**). Inhibitor efficiency was tested by gene expression analysis of *SERPINE1* (**a**) and immunoblot analysis for pSmad3 (**b**).

Thereafter, cell lysates were subjected to gene expression analysis for *CGB* (**c**) and *ALPPL2* (**d**). Data in bar graphs are presented as means ± SEM from three (**a-d**) independent experiments using different cell passages. Data in **a, c and d** and were tested for differences using one-way analysis of variance followed by Tukey's multiple comparisons test. Western blot (b) is representative for three different experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p < 0.001$. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.10. Platelet-derived factors induce placental plasminogen activator inhibitor-1 expression via TGFBR3

Since analysis for TGF-β type 1 receptor did not suggest the involvement of TGFBR1 in the regulation of hCG, we further analyzed the abundance of the different types of TGF-β receptors in BeWo cells.

Gene expression analysis of TGF-β receptor type 1, 2 and 3 revealed a predominant expression of TGFBR3, also described as betaglycan, in untreated BeWo cells (**Figure 15a**). Therefore, we next used a specific TGF- β receptor type 3 inhibitor, the peptide P144, which encompasses amino acids 730–743 from the membrane-proximal ligand-binding domain of betaglycan (115,116). BeWo cells were stimulated with forskolin in addition to pHPL and two different concentrations of the inhibitor P144. Gene expression levels of Smad3 downstream target *SERPINE1* (**Figure 15b**) were as expected substantially upregulated in response to pHPL, whereas peptide P144 blocked this effect to control levels in a concentration dependent manner in both undifferentiated and differentiated BeWo cells. The expression of the TGFBR3 *per se* was not affected by pHPL (**Figure 15c**). The pHPL-induced downregulation of *CGB* in forskolin-stimulated BeWo cells, was not restored after administration of different concentrations of P144, arguing rather against an involvement of TGFBR3 in the regulation of hCG. However, these results suggest that platelet-derived factors induce trophoblastic PAI-1 expression via TGFBR3.

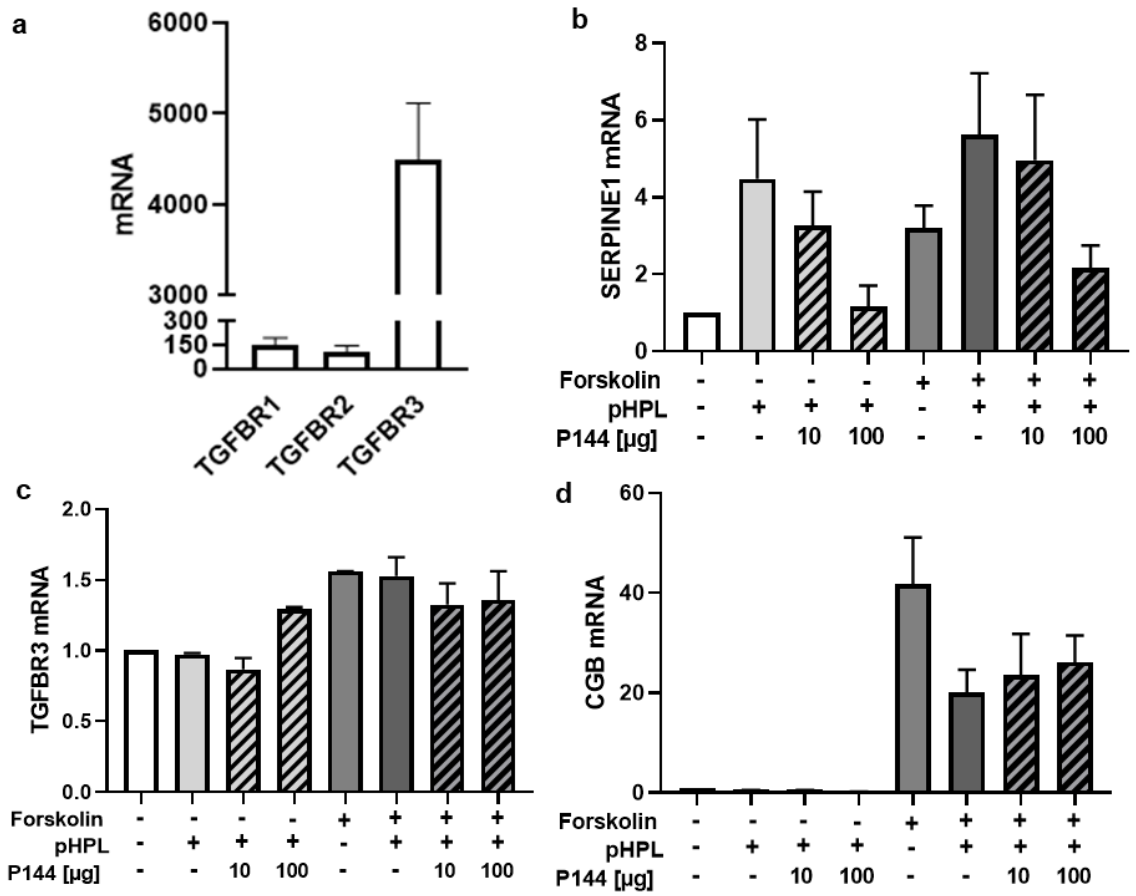

Figure 15: pHPL induces PAI-1 via TGFBR3. Expression of TGF-β receptor type 1, 2 and 3 (a) was analyzed in untreated BeWo cells after 48h incubation. BeWo cells were stimulated with either forskolin (20 µM) or vehicle control DMSO (0.1%) in presence or absence of 10% pHPL in addition to TGF-β receptor type 3 (TGFBR3) inhibitor peptide 144 in a final concentration of 10 µg/ml and 100 µg/ml for 24h and cell lysates were subjected to gene expression analysis for *SERPINE1* (**b**), TGFBR3 (**c**), and hCG beta-subunits *CGB-3*, *-5* and *-8* (**d**). Data are presented as means ± SEM from three independent experiments (**a-d**) using different cell passages.

4.11. Smad3 inhibitor SIS3 do not restore impaired βhCG synthesis, but interferes with cAMP/CREB signaling

Since we detected a strong Smad3 activation upon pHPL treatment and inhibition of TGFBR1 and TGFBR3 did not restore the pHPL-induced downregulation of hCG, we elucidated the involvement of Smad3 in the regulation of hCG synthesis by using the specific TGF-β/Smad3 signaling inhibitor SIS3 (117).

In order to test the efficiency of the SIS3 inhibitor, BeWo cells were incubated with recombinant TGF-β 1 protein and co-incubation with SIS3 considerably reduced the phosphorylation of Smad3 (**Figure 16a**). Gene expression analysis of *CGB* revealed, that SIS3 did not restore the pHPL-induced downregulation of hCG in forskolin-stimulated cells, whereas interestingly SIS3 *per se*, induced *CGB* expression 11.0-fold in undifferentiated cells (**Figure 16b**). Additionally, hCG secretion was as expected significantly reduced upon pHPL treatment in differentiated cells, but SIS3 did not inhibit this effect (**Figure 16c**).

In the next step, we analyzed the effect of SIS3 on Smad3 activation (**Figure 16d+e**) in response to pHPL in forskolin-stimulated cells and their controls. The pHPL-induced phosphorylation of Smad3 was detectable in undifferentiated as well as in differentiated cells. Interestingly SIS3 inhibited the phosphorylation of Smad3 in undifferentiated cell, whereas in differentiation cells SIS3 did not influence the phosphorylation of Smad3.

Since forskolin-induced cAMP/CREB signaling is involved in the regulation of βhCG synthesis, the effect of SIS3 on the phosphorylation of CREB was elucidated. As expected, levels of pCREB (**Figure 16f+g**) were strongly increased upon forskolin treatment, but interestingly, SIS3 did significantly inhibit CREB phosphorylation in presence as well as in absence of pHPL in differentiated cells. In undifferentiated cells, SIS3 alone induce phosphorylation of CREB, which leads to the assumption of an interference between Smad3 signaling and the phosphorylation of CREB.

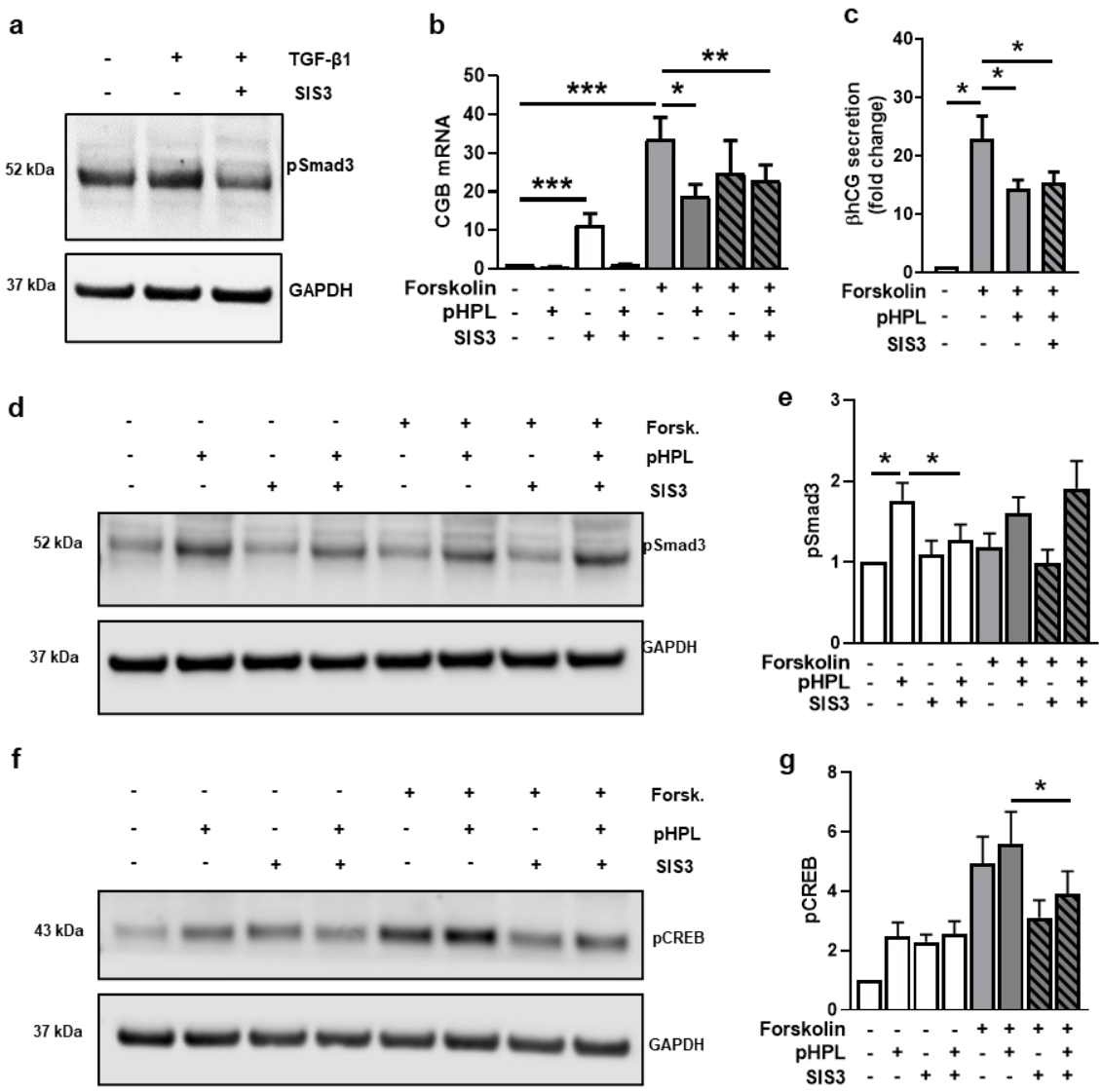

Figure 16: Smad3 signaling interferes with cAMP/CREB signaling. Phosphorylation of Smad3 (pSmad3) was analyzed by immunoblotting in BeWo cells after a preceding pre-incubation with SIS3 (10 µM) for 1h and subsequent treatment with recombinant TGF-β1 in presence or absence of SIS3 for 1h (**a**). Furthermore gene expression analysis of hCG beta-subunits *CGB-3*, *-5* and *-8* (**b**) and β-hCG secretion levels (**c**) were analyzed in BeWo cells after forskolin induction (20µM) in presence and absence of pHPL and with or without SIS3 (10µM) after 48h. Immunoblots and band densitometry for pSmad3 (**d + e**) and pCREB (**f + g**) were obtained from BeWo cells after pre-incubation with SIS3 (10 µM) for 1h and subsequent forskolin-stimulation in presence or absence of 10% pHPL and with or without SIS3 for another 1h. Data in bar graphs are presented as means ± SEM from five (**b**) and three (all others) independent experiments using different cell passages. Data in **b** were tested for differences using One-way analysis of variance followed by Tukey's multiple comparisons test. Western blots are representative for five (**d + f**) different experiments. For data analysis of secreted hCG (**c**) and band densitometry (**e + g**), controls were set one and data were tested using one sample t-test. *p≤0.05, **p≤0.01, ***p<0.001. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

4.12. Inhibition of CBP/p300 impairs *CGB* expression in BeWo cells

Due to our findings on the interference between the phosphorylation of CREB and the activation of Smad3, we investigated the role of the transcription co-activators CREB binding protein (CBP) and p300, which interact with a variety of transcription factors (118). Amongst those many different interaction partners, CBP/p300 has been recently referred to interact with both, Smad3 and CREB signaling (119).

Immunohistochemistry of villous placental explant cultures after 1h incubation with (**Figure 17b**) or without pHPL (**Figure 17a**) demonstrated the predominantly location of p300 in nuclei of cytotrophoblast cells. Only a few p300 positive nuclei were detected within the syncytiotrophoblast. Treatment of pHPL did neither change localization nor the amount of p300 positive nuclei.

Immunoblot analysis of CBP (**Figure17c+e**) and p300 (**Figure 17d+f**) protein levels after treatment with forskolin in presence and absence of pHPL revealed no significant changes in protein levels after treatment with pHPL. Only a slight decrease of p300 by 30% in forskolin-treated cells was observed.

To further elucidate the role of CBP/p300 in βhCG synthesis, we used the selective histone acetyltransferase (HAT) inhibitor for CBP/p300, the C646 inhibitor (120). BeWo cells were stimulated with forskolin and either treated with pHPL, C646 or co-treated with both. Expression levels of *CGB* were as expected, significantly upregulated by forskolin, whereas this induction was significantly downregulated by pHPL (**Figure 17g**). Interestingly, inhibition of CBP/p300 with C646 has led to a reduction of forskolin-induced *CGB* expression by 57.1%, suggesting the involvement of CBP/p300 in hCG synthesis. Co-administration of C646 and pHPL did show similar effects on hCG expression as C646 or pHPL did alone. These data suggest an interference of activated Smad3 signaling and cAMP/CREB signaling, by sequestration of limited amounts of the co-activator complex CBP/p300 (**Figure 18**).

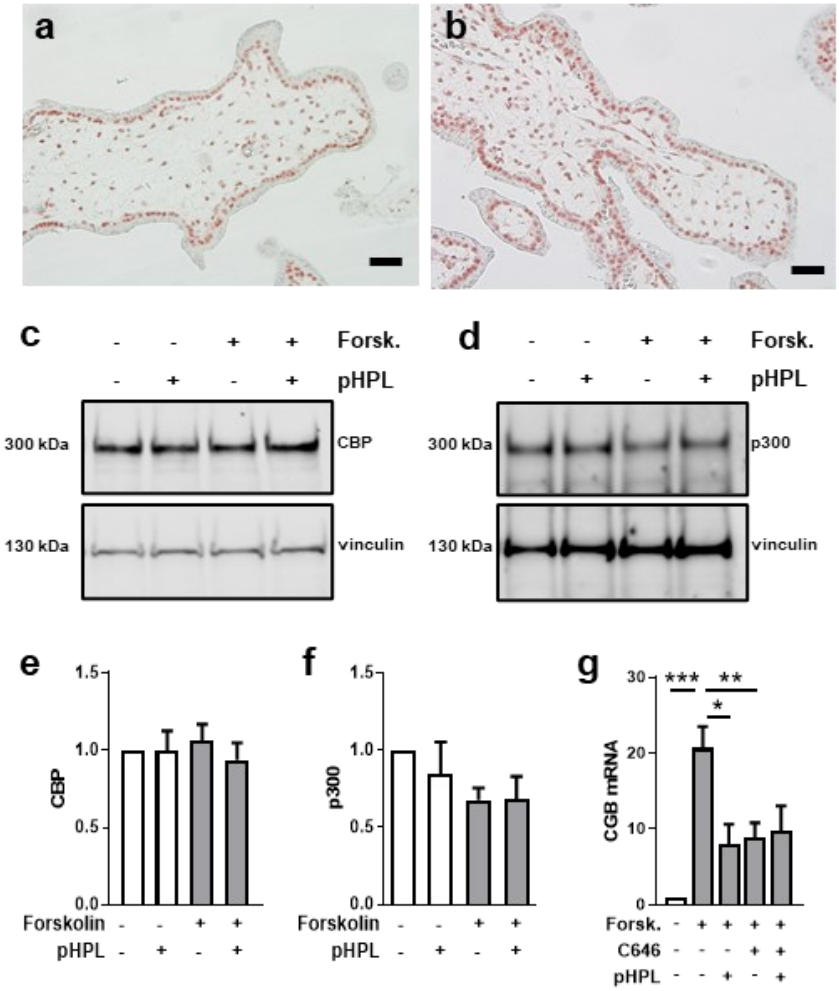

Figure 17: Inhibition of CBP/p300 impairs *CGB* expression in BeWo cells. First trimester placental villi were incubated in absence (**a**) or presence of 10% pHPL (**b**) and heparin in a final concentration of 2U/ml for 1h at 2.5% oxygen at 37 °C. FFPE tissue was subjected to standard immunohistochemistry for staining of p300. Positive nuclei were predominantly detected in cytotrophoblast cells. Scale bar represents 50 µM. Pictures are representative for 3 independent experiments. Forskolin-stimulated (20 µM) BeWo cells were either incubated in presence or absence of 10% pHPL for 1h. DMSO (0.1%) served as vehicle control. Samples were subjected to immunoblotting for CREB-binding protein (CBP) (**c**) and p300 (**d**) and quantification of protein levels were obtained by band densitometry (**e + f**) by setting control samples to one. Gene expression of hCG beta-subunits *CGB-3*, *-5* and *-8* (**g**) were analyzed in forskolin-stimulated (20 µM) BeWo cells after incubation with the CBP/p300 inhibitor C646 in a final concentration of 20 µM in presence or absence of pHPL for 24h. Data in (**e**) and (**f**) are presented as means ± SEM from four, and those in (**g**) from three independent experiments using different cell passages. Western blots are representative for four different experiments. Data of band densitometry (**e + f)** were tested using one sample t-test. Data in **g** were tested for differences using One-way analysis of variance followed by Tukey's multiple comparisons test. *p≤0.05, **p≤0.01, ***p<0.001. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

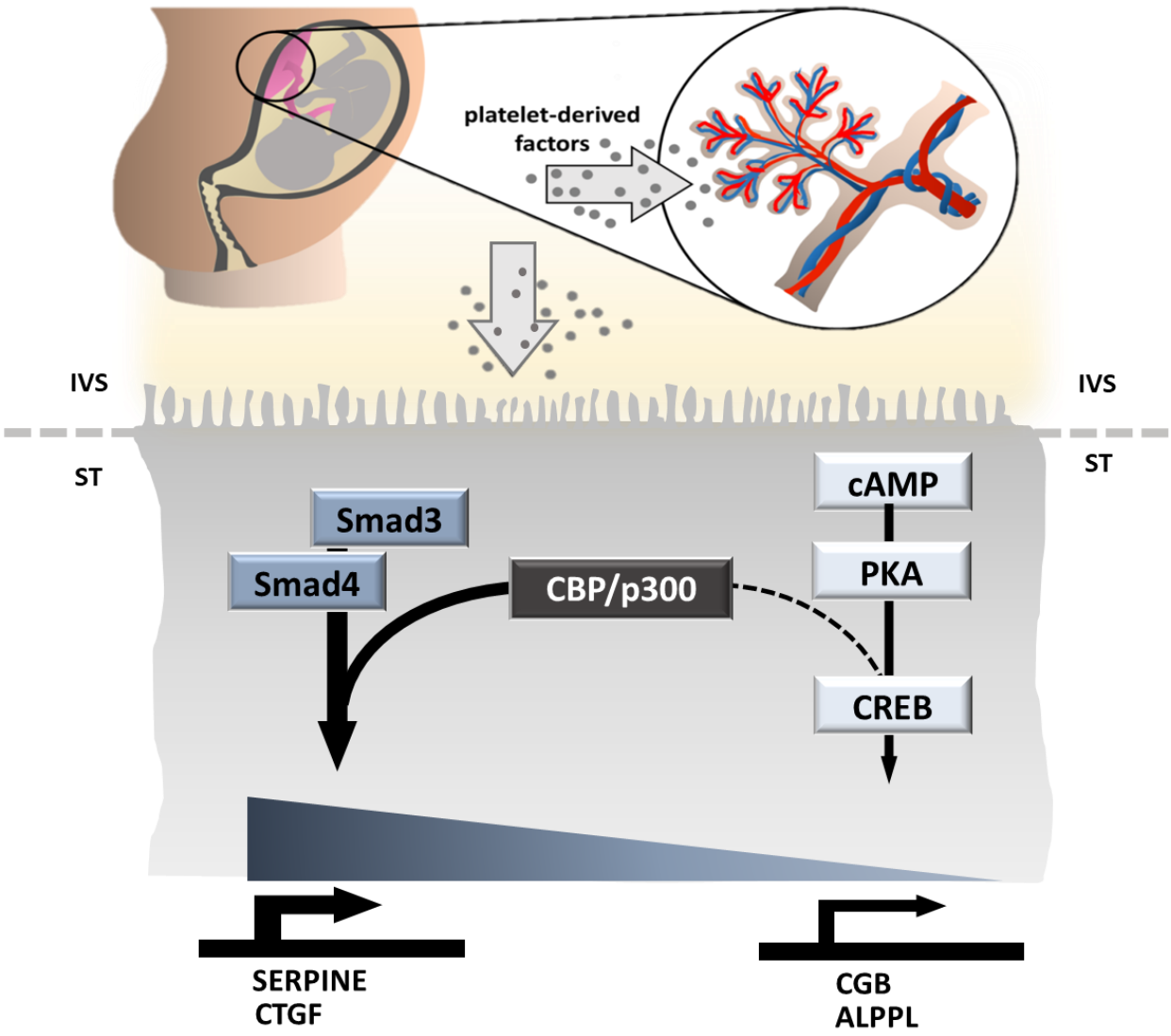

Figure 18: Proposed concept how platelet-derived factors impair placental βhCG synthesis. Activation of maternal platelets at the maternal-fetal interface is followed by degranulation of granule-stored factors, which then could easily be transported into the intervillous space (IVS) where they can act on the syncytiotrophoblast (ST). Activation of Smad-signaling in response to platelet-derived factors induces expression of Smad3 targets, such as plasminogen activator inhibitor 1 (PAI-1, encoded by *SERPINE1*) and connective tissue growth factor (*CTGF*). At the same time, activation of Smad-signaling abrogates CREB-dependent expression of βhCG (*CGB*) and alkaline phosphatase, placental-like 2 (*ALPPL2*) by sequestrating the transcriptional co-activators CBP/p300. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

5. Discussion

As the early placental development has been put in perspective as the critical step in the development of a healthy pregnancy, in this thesis we addressed the role of platelets in this context. We provide evidence, that maternal platelets may be the first maternal blood cells to enter the intervillous space and upon activation, platelet-derived factors can impair trophoblast function.

Presence of platelets at the early fetal-maternal interface

Although the human placenta is structured as hemochorial organ and is reliant on direct contact with maternal blood, in the first weeks of gestation, uterine arteries are plugged to avoid maternal blood flow into the intervillous space and to keep oxygen levels low. The time, when trophoblast plugs become loose and the first maternal perfusion into the intervillous space is established, is suggested to take place by six weeks of gestation as Roberts *et al.* provided evidence for (2).

Moreover, we detected in fragmentary trophoblast plugs of uterine vessels, maternal platelets, which were trapped in the EVT plugs (1). This finding is in line with Roberts et al., who described, that EVT trophoblast plugs in spiral arteries are intermixed with maternal blood cells, like red blood cells (2). Platelets, attaching to the vessel walls of uterine spiral arteries and attached to endovascular trophoblasts have also been described by Sato *et al.* (121). Platelets can potentially be activated by fibronectin or collagen type IV, which are expressed on the surface of endovascular trophoblasts (121). This process is followed by platelet granule release and represents a potential source of cytokines and inflammatory proteins at this interface between mother and fetus.

Vascular channels within the EVT plugs in spiral arteries from seven weeks of gestation onwards have been described by Roberts *et al.* (2). Those microchannels within loosely cohesive trophoblasts can reach a diameter of 10-20 µm by 8 weeks of gestation (2), which leads us to speculate, that platelets are able to pass through the narrow channels into the intervillous space and may be the first cells to reach the intervillous space (**Figure 19**),

In this work, we further detected platelets in extracellular gaps within HLA-G positive EVT in cell columns towards the maternal tissue (1). In regard of this finding, a study, published by Sato *et al.* in 2005, described platelet-derived soluble factors as a potential driver for EVT migration and vice versa the capability of EVTs to potentially activate platelets (121). This finding gives hints to speculate, whether platelet derived soluble factors, released from activated platelets are involved in the migration of EVTs into the maternal tissue. This will

probably provide a new route, how platelets may enter the intervillous space and a possible mechanism to interact with HLA-G positive EVTs, migrating towards the maternal tissue.

Immunohistochemical analyzed placenta tissue from week 5 to 12 of gestation, provided evidence, that platelets may enter the intervillous space from very early stages of pregnancy, as we found adherent platelets on placental villi from 5 weeks of gestation onwards (1).

Our findings of adherent platelets on regions of damaged syncytiotrophoblast is in line with the procoagulant capacity of the subendothelial/subepithelial tissue. Exposure of extracellular matrix to the fluid environment potentially activates platelets in the intervillous space. Furthermore, we are the first to describe platelets between the cytotrophoblast layer and the syncytiotrophoblast, which leads us to speculate, that platelets are involved in the re-epithelialization process of damaged trophoblast cells (1)

The detection of adherent platelets on perivillous fibrinoid on discontinuous syncytiotrophoblast in our study (1), is supported by transmission electron microscopy pictures, where Benirschke and Kaufmann (1995) show several platelets attached to the fibrinoids within damaged villi, which is summarized by Kaufmann *et al.* in 1996 (79). The origin of fibrinoids was described by several groups in contrasting views. The degeneration of the syncytiotrophoblast and the development of fibrinoids is described by Fox *et al.* (1967) as a consequence of aggregated platelets on the intact surface of the syncytiotrophoblast. In contrast, fibrin-type fibrinoids are free of cells and are mainly located in areas facing the maternal blood flow, which supports the assumption that the damaged syncytiotrophoblast induces blood clotting, when exposed to maternal platelets (122).

A potential cause of lesions in the surface of the syncytiotrophoblast, is increased shear stress within the intervillous space and subsequent wall shear stress on the ST (18,123). These findings are for example linked with pregnancy complications such as IUGR, where increased placental villous damage was found (123).

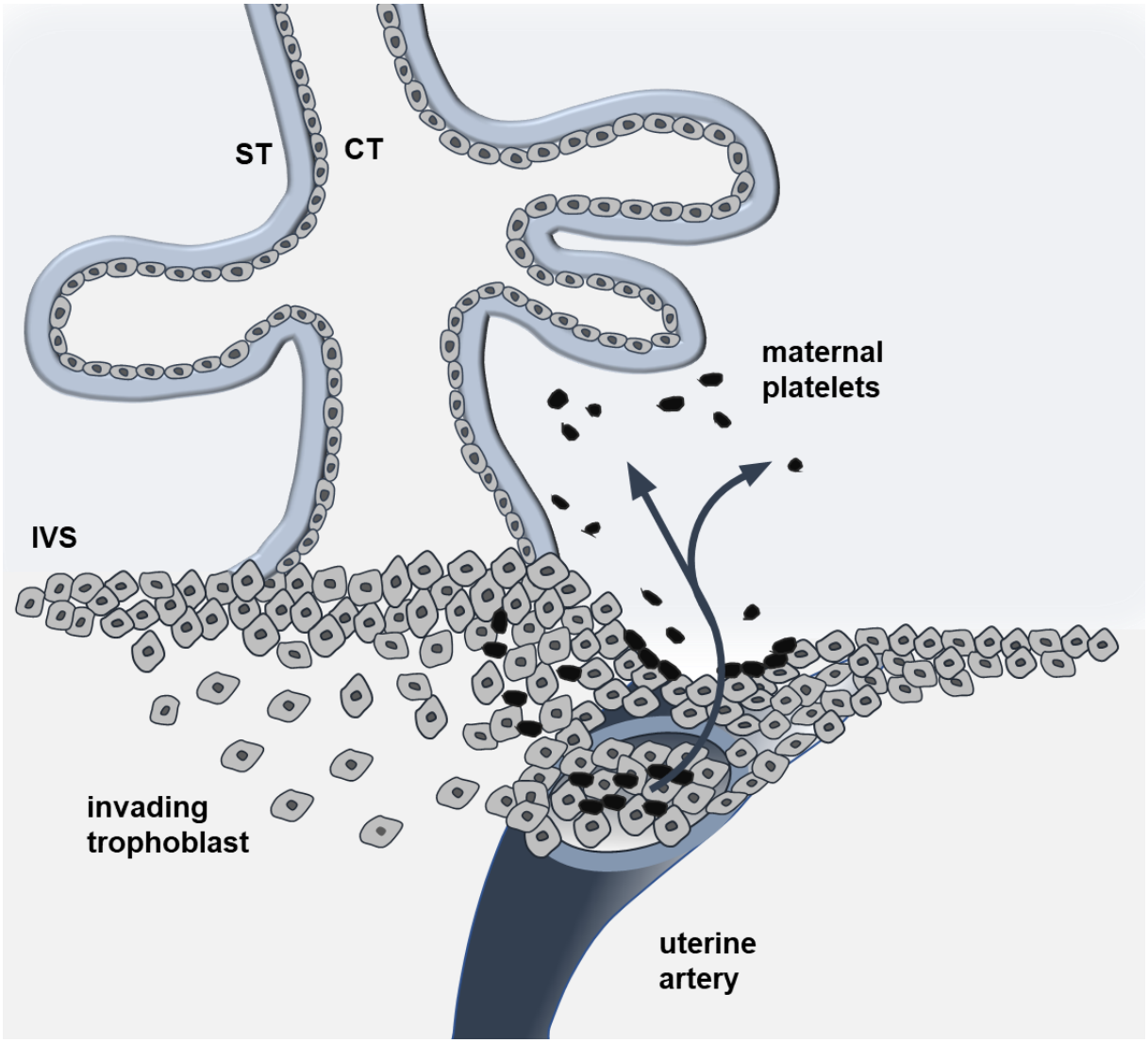

Figure 19: Schematic presentation of how platelets may enter the intervillous space. During the early placental development, extravillous trophoblasts (EVTs) invade into maternal vessels and form a trophoblast plug within the spiral arteries. This trophoblast plug obstructs maternal blood to flow into the intervillous space (IVS). At around 7 weeks of gestation, trophoblast plugs become loosely cohesive and intercellular channels within the trophoblast plugs may allow maternal platelets to enter the intervillous space as one of the first maternal blood cells. ST = Syncytiotrophoblast, CT = Cytotrophoblast, IVS = Intervillous space; Reproduced from Moser et al., 2019 with permission of publisher MDPI (5).

Impaired βhCG synthesis

The differentiation of cytotrophoblast cells to the syncytiotrophoblast, as the cell type, which is in direct contact with the maternal blood, underlies a lot of different regulatory mechanisms. Impaired trophoblast differentiation has been repeatedly linked to pregnancy pathologies such as preeclampsia or IUGR (124), implicating the relevance of a functional syncytiotrophoblast from very early stages of gestation.

Since preeclampsia has also been described with increased platelet activation and procoagulant changes in the last couple of years (125), we elucidated a possible crosstalk between platelet derived factors present in the early intervillous space and the development and differentiation of the trophoblast.

Trophoblast differentiation is a tightly regulated process, to which factors such as vascular endothelial factor (VEGF) (126), epidermal growth factor (EGF) (127), tumor necrosis factor (TNF)-α (128), TGF-β (16) or hCG in an autocrine positive feedback loop (23), are contributing (16). Some of these factors, like TGF-β or EGF, are also abundant in platelet granules and are released upon platelet activation (27,129) and may constitute therefore as potential regulators of trophoblast differentiation (1).

The fusion of cytotrophoblast cells to the syncytiotrophoblast is a key point in the development of a functional syncytiotrophoblast, which is reliant on the permanent supply of fresh cellular components (124), since studies indicate that nuclei of syncytiotrophoblast are barely able to replicate (130). However, the syncytiotrophoblast is highly metabolic active, synthesizes and secretes large amounts of protein and steroid hormones and is mainly responsible for the transport in the placenta (83,131).

In this study, we could show, that platelet-derived factors decrease βhCG synthesis in *ex vivo* cultures of first trimester placental villi as well as in the trophoblast cell line BeWo. The βhCG synthesis was significantly affected on mRNA as well as on protein level (1). The differentiation dependent upregulation of the well-known biochemical differentiation marker such as GCM-1 and its downstream target Syncytin-1 (132) as well as *ALLPL2*, was not significantly impaired by the platelet lysate (1). Interestingly, also the differentiation induced cAMP/CREB signaling was only slightly influenced by the platelet lysate (1).

Regarding the experimental approach to investigate the influence of pHPL on the trophoblast differentiation signaling pathway, we used the BeWo cell as a model organism for trophoblast differentiation upon forskolin stimulation. In order to verify the obtained results in first trimester placental tissue, it has to be taken into account, that villous explants contain numerous cell types, like mesenchymal stroma cells, endothelial cells, placental immune cells and blood cells. Therefore explant cultures are mainly used to investigate trophoblast function in a cellular

context rather than investigating different signaling pathways in a heterogeneous cell population without the ability to identify the activity of individual cell types (133).

Investigating the influence of platelet derived factors on morphological changes during trophoblast differentiation, we found, that the platelet lysate did not significantly impair the differentiation dependent reduction of the fusion marker E-cadherin (1). The gene expression of Syncytin-1 and -2 was as upregulated upon forskolin treatment as expected but not significantly impaired by pHPL. Additionally, beside the analysis of cell fusion on gene expression levels of well-known fusion markers, we also tried to investigate the cell fusion with histological techniques. BeWo cells were subjected to immunofluorescence for E-cadherin and desmosomal protein to identify the percentage of fused cells in presence of pHPL. Since the cell membranes could not be identified as sharp borders to clearly demarcate individual cells, a quantitative analysis of cell fusion was inconclusive.

Further elucidations with scanning electron microscopy on morphological changes during trophoblast differentiation such as the differentiation dependent microvilli formation and the gene expression analysis of the ERM family proteins (ezrin, radixin and moesin) (134), which are involved in the microvilli formation, did not show any responses to the platelet lysate (1).

However, in line with our findings, syncytialization and the synthesis of hCG has been described to be differently regulated, as for example EGF treatment triggers syncytialization, with simultaneously inhibiting hCG secretion (127). It has also been shown, that EGF stimulates the metabolic rate in CT cells and maintains those metabolic rates even in the differentiation from CT to ST (131).

Trophoblast differentiation is accompanied with increased intracellular cAMP levels due to the activation of adenylyl cyclase, which can also be induced by forskolin *in vitro* (14,132). However, it has been postulated by Ordeni et al. (14) that the CGB protein expression is not necessarily linked to syncytial fusion. They could show, that the protein kinase A inhibitor H-89, significantly reduced the fusion marker LGALS13, while levels of hCG synthesis seemed to be unaffected in forskolin induced BeWo cells (14). Whether this *in vitro* situation on differentially regulated syncytialisation and hCG synthesis also reflects the *in vivo* situation, remains speculative. Furthermore, it also has to be taken into account, that forskolin, as a strong inducer of intracellular cyclic AMP, might also have an impact on other cAMP regulated signalling pathways in trophoblast cells.

These findings suggest, different regulatory mechanisms for hCG synthesis and morphological differentiation, implicating that these pathways might interfere but its downstream pathways may not necessarily be activated together (1).

Furthermore in agreement with this theory, we could show that platelet-derived factors downregulate the forskolin-induced hCG synthesis independently, whether platelet-derived factors were administered simultaneously with forskolin or after a 48h pre-differentiation period (1). This leads to the suggestion, that platelet derived factors may directly act on the syncytiotrophoblast independently of trophoblast differentiation (1).

The significant downregulation of βhCG synthesis upon treatment with platelet lysate was verified by the co-incubation of 2-MeS-ADP-activated isolated platelets with forskolin-stimulated BeWo cells. The *CGB* expression of undifferentiated BeWo cells were neither influenced by ADP alone nor by co-treatment with platelets, which is also in line with our previous findings. Whether the release of the granule content upon ADP-stimulation of isolated platelets is indispensable for the interference with βhCG synthesis or only the presence of isolated platelets can induce this effect, remains speculative.

In a non-ADP-stimulated platelet control, it has to be considered, that during the isolation process, a certain amount of platelets might get activated due to mechanical stimulation. Furthermore, we have to take into account that non-activated platelets might get activated as soon as they come in contact with trophoblast cells or during the period of co-culture due to trophoblast-platelet interactions. Therefore, we mainly wanted to show the effect of activated platelets on *CGB* expression in differentiated cells to verify our observation with the platelet lysate. However, in further approaches non-activated platelet controls could possibly obtained by using appropriate platelet activation and aggregation inhibitors.

The role of TGF-β in hCG synthesis

TGF-β, as one of the most abundant factors in platelet granules (55), is involved in a variety of functions, such as cell proliferation, apoptosis and differentiation (90).

TGF-β is well known for its function as a cell proliferation initiator, but also for its functions in wound healing, the immune system and cancer (135). Nowadays, TGF-β's role as a proliferation inhibitor is also well described, which suggests a very context dependent nature of TGF-β activity (135). Elevated TGF-β 1 and TGF-β 2 serum concentrations (136), have been linked to a higher risk to develop preeclampsia (90). It has been reported, that preeclamptic placentas show higher expression levels of TGF-β 3 (90).

The involvement of TGF-β in trophoblast differentiation was described by Morrish et al., who showed the ability of TGF-β 1 to reduce hCG and human placental lactogen (hPL) while inhibiting trophoblast differentiation (137).

In this thesis, we showed, that platelet lysate triggers the activation of pSmad3 and the upregulation of its downstream target *SERPINE1*, independently of forskolin-induction in BeWo cells. In line with this, it has been reported that TGF-β 1 and TGF-β 3 treatment increases phosphorylation of Smad2 in explant cultures of first trimester placenta, whereas GCM1 and Syncytin-1 levels were decreased. This was verified in BeWo cells as well, suggesting a negative regulation of TGF-β on the expression of differentiation marker GCM1 and Syncytin-1 (90).

During pregnancy, phosphorylation of Smad2 levels decrease towards term with peaking at gestational week 6 to 7. In early gestation, Smad2 is located in both, the cytoplasm as well as in the nuclei of CT and ST, but predominantly in the nuclei of ST (90). Regarding the phosphorylation of Smad2 in preeclampsia, it has been shown, that pSmad2 is increased in early-onset preeclampsia in comparison to age-matched preterm controls (90).

So far, seven TGF-β type 1 and five type 2 receptors on the cell membrane are known. Those serine and threonine kinase receptors form a heterodimeric receptor complex subsequent to TGF-β binding (138). The threonine and serine residues of the TGF-β type 1 receptor (TGFBR1) are phosphorylated by the TGFBR2 (138). Subsequent to the receptor binding and the activation, the TGF-β signal is transduced via phosphorylation of the serine residues on the carboxy-terminal of the R-Smad proteins (138).

In order to elucidate the involvement of TGF-β 1 receptor in trophoblast differentiation, we used the well-known TGF-β receptor 1 inhibitor SB431542. Since the pHPL-induced downregulation of hCG was not restored by using the inhibitor, we assume that TGF-β 1 receptor does not play a major role in this process (1).

The TGF-β receptor complex contains, beside type 1 and 2, which constitute the core signaling complex, the type 3 receptors, which include endoglin and betaglycan. Betaglycan can bind to the ligand and to the TGF-β type 1 and 2 receptors to modulate the signaling (139). Investigations of the involvement of TGFBR3 in the expression of PAI-1 in trophoblast cells, showed a strong inhibitory response to the TGFBR3 inhibitor P144. Platelet lysate induced upregulation of PAI-1 was blocked to control levels upon treatment with inhibitor, suggesting the induction of PAI-1 via TGFBR3 in trophoblast cells. However, pHPL-induced downregulation of hCG was not influenced by the inhibition of TGFBR3.

Next, by inhibiting specifically the phosphorylation of Smad3 with SIS3 inhibitor (117), we interestingly identified an interference with CREB signaling, as SIS3 *per se* activated CREB. Furthermore, Smad3 phosphorylation was only inhibited in undifferentiated cells, whereas in forskolin-stimulated cells the effect was abrogated (1). So far, it remains unclear whether the activating effect of SIS3 on CREB was a side-effect of the treatment or if there is an upstream interference between those pathways.

Regarding a possible interaction between the phosphorylation of CREB, which is a downstream event of increased intracellular cAMP levels, and TGF-β pathway, it has been reported, that TGF-β treatment leads to the phosphorylation of CREB ser-133 (140). These assumptions, that cAMP-regulated pathways may be involved in mediating TGF-β induced signals (140), were further described by Schiller *et al.* with an antagonistic effect of cAMP on TGF-β signaling (119).

The responses to TGF-β factors are regulated via Smad-dependent and Smad-independent pathways (141). The Smad protein family consists of 8 Smad proteins, of which Smad 1/2/3/5/8 are receptor-activated Smads (R-Smads) and Smad 6/7 are inhibitory Smads. R-Smads form a complex with the common Smad 4 and translocate into the nucleus, where they regulate transcription of target genes (141). The C-terminal domain of R-Smads can recruit and interact with the acetyltransferases CREB-binding protein (CBP) or p300 (141). Other coactivators, beside CBP and p300 can also interact with Smad and thus regulate the level of transcription activation (141). It has been reported, that CBP is strongly expressed in the nuclei of the syncytiotrophoblast, whereas p300 is predominantly located in cytotrophoblast cells, but is also found in the syncytium in low numbers (142). In detail, in this study from Schiller et al., it has been described, that the interaction of Smad3 with the transcriptional co-activator CBP/p300 was abrogated with increasing intracellular cAMP levels in human dermal fibroblasts (119).

Beside the activity of fibroblasts to sustain the balance of ECM degradation and synthesis, TGF-β is also a key regulator of the ECM. It regulates the expression of protease inhibitor, such as PAI-1, and the expression of ECM components, such as fibronectin or fibrillar

collagens (143,144). They could show that activation of cAMP reduces TGF-β induced collagen type 1 expression as well as the expression of Smad-dependent regulated connective tissue growth factor (CTGF) (119).

Furthermore, the TGF-β induced upregulation of PAI-1 was strongly repressed by forskolin or dibutyryl-cAMP (119), which is in line with our observation, that platelet lysate induced upregulation of PAI-1 was higher in undifferentiated cells, than in forskolin-stimulated BeWo cells (1). Since cAMP neither effected the phosphorylation of Smad, nor the translocation to the nucleus, Schiller et al. further investigated if cAMP possibly interferes with Smad3-CBP interaction (119). Their study finally suggests, that the association of Smad3 with the co-activator CBP/p300 is impaired by the activation of cAMP/CREB signaling (119).

This linkage between activated cAMP/CREB signaling and TGF-β induced Smad signaling, might also be an explanation for our observations, when trophoblast cells were co-treated with forskolin and platelet lysate. The availability of limited amounts of nuclear CBP/p300 (145) possibly lead to disturbances in the interaction of both, Smad3-CBP/p300 and CREB-CBP/p300 and might be a key explanations for the downregulation of βhCG under co-treatment with pHPL for the inference of those two signaling pathways (1,119).

An appropriate approach for analyzing such interactions, is the well-known co-immunoprecipitation. In our initial experimental design, we were unfortunately not able to get results, maybe the levels of endogenous protein were too low.

Therefore we used the well-known P300 inhibitor C646 (146) and could verify these assumptions by showing a significant reduction of forskolin-induced βhCG expression in presence or absence of platelet lysate (1).

Regarding the platelet response upon activation, platelets are able to release their granule content, but they also shed extracellular vesicles from their membrane into the environment. What was initially described as “platelet dust” (147) is nowadays presumed to contribute to cellular signaling, hemostasis and coagulation (148). These assumptions indicate the importance, to consider the action of platelet-derived EVs in platelet-derived products such as platelet rich plasma (PRP), platelet-lysate or platelet releasate.

Studies from Torreggiani et al. has demonstrated, that platelet-derived EVs might be one of the effectors of the platelet lysate and PRP (149). In this study incubation of bone marrow stromal cells (BMSCs) with platelet-derived EVs showed significant increases in cell proliferation and migration. Regarding the effect of platelet-derived TGF-β, this study demonstrated higher amounts of TGF-β 1 in platelet-derived EVs as compared to platelet lysate (149).

These findings raise the question, whether the effects of platelet lysate on hCG synthesis in our study can be ascribed to platelet-derived factors or partially also to the action of platelet-derived EVs. Treatment with platelet-derived EVs and EV-depleted platelet releasate will give more insights and are a potential outlook for follow up studies.

Platelet activation in disease

Pregnancies, complicated with preeclampsia, have been linked to altered hCG serum levels during first trimester as well as towards term. HCG, as the central hormone in pregnancy and synthesized from the very beginning, is used in different pregnancy pathologies, such as gestational diabetes or down syndrome as predicting marker (23). Also in preeclampsia, it has been observed, that hCG serum levels differ from normal pregnancies and therefore the application of hCG as a predicting marker in combination with other measurements (23), such as pregnancy associated plasma protein-A (PAPP-A) and placental growth factor (PIGF) (150), has been implicated (23).

Studies have shown, that low hCG levels in maternal serum during the first trimester is significantly associated with the subsequent risk to develop preeclampsia, especially early-onset preeclampsia (151,152). This points out the importance of hCG regulation from the very beginning of pregnancy and the possible consequences of impaired hCG levels.

Whether the downregulation of hCG in pregnancies with risk of preeclampsia is a consequence of impaired syncytiotrophoblast differentiation, and if so, whether this a result of increased platelet activation at the fetal-maternal interface, remains speculative.

However, anti-platelet therapy has been found to prevent the risk to develop serve preeclampsia (153,154), leading to the assumption that this has a protective effect on pathological blood coagulation in the placenta (155). A metaanalysis reported, that the administration of low dose aspirin (50-150 mg/day) before the 16th week of gestation leads to a significant reduction and a dose-response effect for the prevention of preeclampsia. Also the risk to develop severe preeclampsia and fetal growth restriction was reduced (156). Another study showed an association with aspirin and the onset of preterm preeclampsia, but no significant reduction in development of term preeclampsia (157).

However, the role of anti-platelet therapy in the development of this syndrome remains still unclear and raises the question whether lower levels of platelet activation at the fetal-maternal interface can prevent the platelet factor-mediated impairment of hCG synthesis in early pregnancy.

Conclusion

In conclusion, our study suggests new insights of the role of platelets in the early placental development. We detected adherent platelets from the 5th week of gestation onwards in the intervillous space. Platelets may enter the intervillous space as the first maternal blood cells through narrow channels in trophoblast plugs of uterine vessels, but also the presence of platelets in intercellular clefts of the extravillous trophoblast plugs and cell columns gives hints on a new possible route to enter the intervillous space.

Furthermore, our study shows, that platelet-derived factors significantly impair the βhCG synthesis in human first trimester placental explants as well as in trophoblast cells, without substantially influencing trophoblast fusion or differentiation-dependent morphological changes. Elucidations of the involvement of TGF-β 1, as a factor, which is abundant in platelet releasate, suggests an interference between activated Smad3 signaling and cAMP/CREB pathway by sequestration of limited amounts of nuclear co-activator complex CBP/p300.

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7. Appendix

Table 5: *Human TGF-β Pathway Phosphorylation Array* Data are represented as means ± SD from two independent experiments, values from the DMSO alone experiments were set to 1. Table used from Forstner et al., 2019 (1).

Target & Phosphorylation Site		DMSO	DMSO + pHPL	Forskolin	Forskolin + pHPL
C-Fos	T232	1 ± 0.13	1.55 ± 0.40	0.92 ± 0.10	1.54 ± 0.17
TAK1	S412	1 ± 0.06	0.85 ± 0.03	1.05 ± 0.11	0.96 ± 0.03
SMAD2	S245/ S250/ S255	1 ± 0.20	0.57 ± 0.16	0.69 ± 0.51	0.81 ± 0.38
SMAD4	T277	1 ± 0.04	0.76 ± 0.04	0.88 ± 0.18	0.71 ± 0.10
SMAD5	S463/ S465	1 ± 0.12	0.63 ± 0.07	0.49 ± 0.03	0.61 ± 0.17
ATF2	T69/ T71	1 ± 0.01	0.94 ± 0.11	0.69 ± 0.02	0.58 ± 0.02
c-Jun	S73	1 ± 0.17	0.63 ± 0.06	0.92 ± 0.20	0.57 ± 0.18
SMAD1	S463/ S465	1 ± 0.52	0.28 ± 0.06	0.47 ± 0.03	0.41 ± 0.08

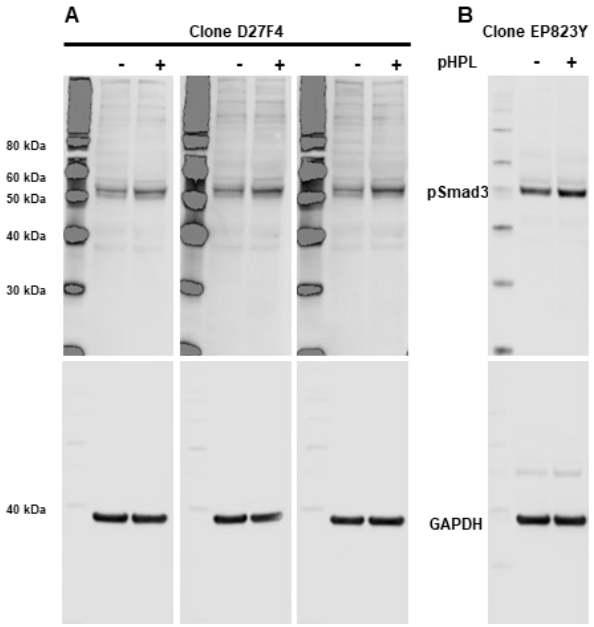

Figure 20: Detection of pHPL-induced Smad3 activation by two different antibody clones. BeWo cells were treated with pHPL for 1h and subsequently subjected to immunoblot analysis. Phosphorylated Smad3 (pSmad3) was detected with either monoclonal rabbit anti-pSmad2(S465/467)/pSmad3(S423/425) antibody (Clone D27F4) (A), or with monoclonal rabbit anti-pSmad3(S423/425) antibody (Clone EP823Y) (B). Three upper panels in A present Western Blots from three different purchase dates, performed with anti-pSmad2(S465/467)/pSmad3(S423/425) antibodies (Clone D27F4). A single band of pSmad3 increases was detected with both antibody and it increases upon pHPL treatment. Reproduced from Forstner et al., 2020 with permission of publisher Springer Nature (1).

Buffers and Solutions

Table 6: *Recipe of platelet wash buffer*

140 mM	NaCl
10 mM	$NaHCO_3$
2.5 mM	KCl
0.9 mM	Na_2HPO_4 * $2H_20$
2.1 mM	$MgCl_2$
22 mM	$C_6H_5Na_3O_7$
0.055 mM	D(+)-Glucose monohydrate
0.35%	Bovine Serum Albumin

Table 7: *Solutions for Lowry Protein Determination*

Solution A	2 % Na2CO3 in 0,1M NaOH
Solution B	1 % CuSO4 x 5H2O in Aqua dest.
Solution C	2 % KNOC4H4O8 x 4H2O in Aqua dest.

Table 8: *Recipe for Ammonium water*

2.5 ml	Ammoniac (25%)
ad 1l	Aqua bidest.

Table 9*: Recipe for 10x PBS*

81mM	Na2HPO4; H20x12
19mM	KH2PO4
1.54M	NaCl
ad 1l	Aqua bidest.
adjust pH to 7.2	

Table 10*: Recipe for 10x TBS*

1.5 mM	NaCl
0.5 M	Tris pH 8.0
ad 1l	Aqua bidest.
adjust pH to 7.4	

Protocols

Table 11: *Protocol for Deparaffinization of paraffine sections*

after sectioning of FFPE tissue samples, slides are deparaffinized by a series of graded alcohols	
Tissue Clear (Tissue-Tek; Sakura) 1	10 min
Tissue Clear (Tissue-Tek; Sakura) 2	10 min
Tissue Clear / 100% EtOH	1 min
100% EtOH	1 min
96% EtOH	1 min
70% EtOH	1 min
50% EtOH	1 min
Aqua dest.	3x

Table 12*: Protocol for Immunohistochemistry for paraffine sections*

after deparaffinization, slides are subjected to heat-induced antigen retrieval in a pressure cooker for 7 min at 120°C in citrate buffer at pH6 or Tris EDTA Puffer at pH 9	
Wash cooled down slides with TBS-T	3x
Incubate with Hydrogen Peroxidase Block	12 min
Wash with TBS-T	3x
Incubate with Ultravision Protein Block	5 min
Tab off UV-Block	
Incubate with diluted primary antibody at RT	45 min
Wash with TBS-T	3x
For mouse antibody: incubate with enhancer	15 min
Wash with TBS-T	3x
Incubate with HRP-Polymer	15 min
Wash with TBS-T	3x
Incubate with AEC substrate	10 min
Wash with Aqua dest.	3x
Counterstaining in Hemalaun	10 min
Rinse with Aqua dest.	3x
Blueing of nuclei in NH3 water	30 sec
Wash with Aqua dest.	3x
Remove excessive water	
Mount with Kaiser’s glycerol gelatine	