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MASTERARBEIT

Titel der Masterarbeit

„Localization of the human neonatal Fc receptor (hFcRn) in the syncytiotrophoblast and fetal endothelial cells of human term placental chorionic villi *in situ* and in isolated human placental endothelial cells *in vitro* “

verfasst von

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I dedicate this thesis to my beloved family: my husband, Sergiy;

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I love you all dearly.

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1. ABSTRACT

The immune system of the human fetus and newborn is immature and during the first month of life the baby is unable to produce the amount of antibodies needed for protection from infections and toxins. Therefore, already during pregnancy a passive immune-protection is provided by active transport of maternal IgG across the placental barrier.

In the full term placenta, where the major transfer of IgG occurs, the placental barrier consists of two cell layers, the multinucleated epithelial syncytiotrophoblast (STB) and fetal endothelial cells (FEC) of the fetal capillaries. Transplacental IgG transfer clearly involves the MHC class I-like human neonatal Fc-receptor, hFcRn, composed of a transmembrane α-chain and soluble ß2-microglobulin. It is well established that transcytosis across the STB is mediated by hFcRn, but the identity of the receptor involved in IgG-transport across the FEC remains unclear due to contradictory data on hFcRn expression in the FEC *in situ* and isolated and *in vitro* cultured FECs. While in isolated FECs, expression and involvement of hFcRn in IgG-transcytosis were demonstrated, hFcRn expression in FECs in terminal villi *in situ* was not described. In contrast, FECs *in situ* were shown to express another Fc-receptor, FcγRIIb2, which was co-localized with IgG in intracellular vesicles of FECs. Thus, while *in vitro* data support the involvement of the hFcRn in IgG transcytosis across the entire placental barrier, *in situ* expression of hFcRn in FECs remains to be unequivocally demonstrated.

In this work, expression of hFcRn α-chain was re-investigated both in term placental chorionic tissue samples as well as in isolated and cultured human placental FECs using Immunofluorescence Microscopy, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and Western Blotting. Protein expression was analyzed using a specific affinity-purified rabbit antiserum previously prepared against a peptide-sequence of hFcRn α-chain.

First, expression of hFcRn α-chain in total placental tissue lysates was confirmed by Western Blotting. Localization of hFcRn α-chain in placental tissue *in situ* was then re-investigated by Immunofluorescence Microscopy. In contrast to the published data both the STB (Cytokeratin 7 positive) and FECs (CD31 positive), were found to express hFcRn α-chain, and surprisingly, higher expression of the protein in the FEC as compared to the STB was found. This pattern of hFcRn α-chain expression was observed in all placentas investigated. In collaboration with the Medical University of Graz, arterial and venous FECs were then isolated from human term placentas, and hFcRn α-chain mRNA and protein expression in both cell types was confirmed by RT-PCR and Western Blotting, respectively. As analyzed by Immunofluorescence Microscopy, cultured FECs (CD31 positive) were able to internalize IgG and exhibited a predominant intracellular localization of hFcRn α-chain. A predominant endosome localization of hFcRn, which is also observed in the trophoblast-like BeWo cell line, is in line with hFcRn function, which binds IgG predominantly in these acidic organelles.

In conclusion, this study demonstrated for the first time expression of hFcRn α-chain in both cell layers (STB, FEC) of the term human placental barrier *in situ* and confirmed FEC-expression in isolated and *in vitro* cultured FECs. Presence of the receptor in STB and FECs suggests that hFcRn mediates IgG-transcytosis across the entire placental barrier. As expression of hFcγRIIb2 was also observed in FECs *in situ* and *in vitro*, future studies need to clarify the individual contributions of hFcRn and hFcγRIIb2 in IgG transport by FECs.

2. ZUSAMMENFASSUNG

Das Immunsystem menschlicher Föten und Neugeborener ist unreif und während der ersten Lebensmonate sind Babies nicht in der Lage genügend Antikörper zum Schutz vor Infektionen zu produzieren. Vorsorglich erhalten Föten daher bereits während der Schwangerschaft eine natürliche passive Immunisierung mit mütterlichen Immunglobulinen der Klasse G (IgGs). Maternales IgG wird dabei in einem Rezeptor-mediierten Prozess über die Plazenta zum Föten transportiert.

Der IgG-Transfer erfolgt hauptsächlich in der reifen menschlichen Plazenta, in der die Plazentaschranke aus zwei Zellschichten besteht. Einerseits dem multinukleären epithelialen Synzytiotrophoblast (STB) und andererseits den fötalen Endothelzellen (FEC) der fötalen Kapillaren. Funktionelle Studien zeigten, dass der MHC Klasse I-homologe humane neonatale Fc-Rezeptor (hFcRn) in den transplazentaren IgG Transport involviert ist. hFcRn besteht aus zwei Untereinheiten, einer Membran-verankerten α-Kette und einem löslichen Protein, ß2-Mikroglobulin. Während viele Studien bestätigen, dass hFcRn in die IgG Transzytose durch den STB involviert ist, ist die Identität des Rezeptors, welcher IgG über die FECs transportiert nicht geklärt. Dies liegt an der widersprüchlichen Datenlage hinsichtlich hFcRn Expression in FEC *in situ* einerseits und in isolierten und *in vitro* kultivierten FECs andererseits. hFcRn Expression und die Involvierung von hFcRn in die IgG Transzytose in isolierten plazentaren FECs wurde gezeigt, dagegen konnte die hFcRn Expression in FECs in der terminalen Plazentaschranke *in situ* bisher nicht beschrieben werden. Stattdessen wurde ein anderer Fc-Rezeptor, FcγRIIb2, in FECs *in situ* nachgewiesen und auch mit IgG in FECs ko-lokalisiert. Während also Befunde aus Studien mit isolierten plazentaren FECs die Beteiligung von hFcRn an der IgG Transzytose über die gesamte plazentare Barriere unterstützen, fehlt eine Bestätigung der hFcRn-Expression in FECs im Gewebsverband (*in situ*).

In dieser Arbeit wurde die Expression und Lokalisation der hFcRn α-Kette sowohl im reifen plazentaren Gewebe wie auch in daraus isolierten und kultivierten humanen plazentaren FECs unter Verwendung von Immunfluoreszenz Mikroskopie, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), und Western Blotting untersucht. Die Proteinexpression wurde unter Verwendung eines spezifischen Affinitäts-gereinigten Kaninchen Antiserums, welches gegen eine Peptid-Sequenz der hFcRn α-Kette hergestellt worden war, untersucht.

Zuerst wurde die Expression von hFcRn α-Kette in totalen plazentaren Gewebslysaten mittels Western Blotting bestätigt. Die Lokalisation der hFcRn α-Kette im plazentaren Gewebe *in situ* wurde danach mit Immunfluoreszenz Mikroskopie untersucht. Im Gegensatz zu publizierten Daten wurde die hFcRn α-Kette sowohl in STB (Zytokeratin 7 positiv) wie auch in FECs (CD31 positiv) nachgewiesen, wobei überraschenderweise FEC eine höhere Proteinexpression aufwiesen als STB. Dieses Expressionsmuster der hFcRn α-Kette wurde in allen untersuchten Plazentaproben beobachtet. In einer Zusammenarbeit mit der Medizinischen Universität Graz, wurden danach sowohl arterielle wie auch venöse FECs aus humanen reifen Plazenten isoliert. hFcRn α-Kette mRNA und Proteinexpression wurde per RT-PCR und Western Blotting in beiden Zelltypen bestätigt. Wie mit Immunfluoreszenzmikroskopie dargestellt wurde, internalisierten die kultivierten, CD31 positiven FECs IgG und die hFcRn α-Kette lokalisierte vor allem in intrazellulären Kompartimenten. Eine endosomale Lokalisation von hFcRn, wie sie auch in Trophoblasten-

artigen BeWo Zellen beobachtet wurde, entspricht der hFcRn Funktion, da dieser IgG hauptsächlich in diesen angesäuerten intrazellulären Organellen bindet.

Zusammenfassend kann gesagt werden, dass in dieser Studie erstmals die Expression der hFcRn α-Kette in beiden Zellschichten (STB, FEC) der reifen humanen Plazentabarriere *in situ* dargestellt wurde und die FEC-Expression in isolierten und *in vitro* kultivierten FECs bestätigt werden konnte. Diese Lokalisation des Rezeptors in sowohl STB wie auch FECs unterstützt das Konzept, dass hFcRn die IgG-Transzytose über die gesamte Plazentaschranke mediiert. Da jedoch auch die Expression von hFcγRIIb2 in FECs *in situ* und *in vitro* bestätigt wurde, müssen zukünftige Studien die individuelle Beteiligung von hFcRn und hFcγRIIb2 am IgG Transport über FECs untersuchen.

3. INTRODUCTION

3.1. Functions and structure of the human placenta

The human placenta is the first functional fetal organ and mediates a plethora of functions. It secretes many bioactive molecules such as hormones, growth factors or cytokines that maintain and support the pregnancy. At the interphase between maternal and fetal blood it shields the fetus from harm (e.g. infections) while at the same time it exchanges important nutrients and other molecules between mother and developing fetus. The placenta provides oxygen and essential nutrients to the fetus and returns waste products from the fetal to the maternal circulation. Importantly, the placenta transports maternal antibodies – immunoglobulin G (IgG) – to the offspring in order to protect the neonate against infections during the first months of postnatal life.

The human placenta at term is composed of a fetal side – fetal cells, as well as maternal side – a mixture of maternal and fetal cells. Fetal side is formed by the chorionic plate and maternal side by the decidua basalis *(see Fig 1A)*. The villous trees as well as the umbilical cord emerge from the chorionic plate. In the villous trees, stem villi branch out into intermediate and terminal villi. Terminal villi are characterized by their high degree of capillarization and the presence of sinusoids. Terminal villi are the main site for exchange processes between the maternal and the fetal circulation [Benirschke, 2006].

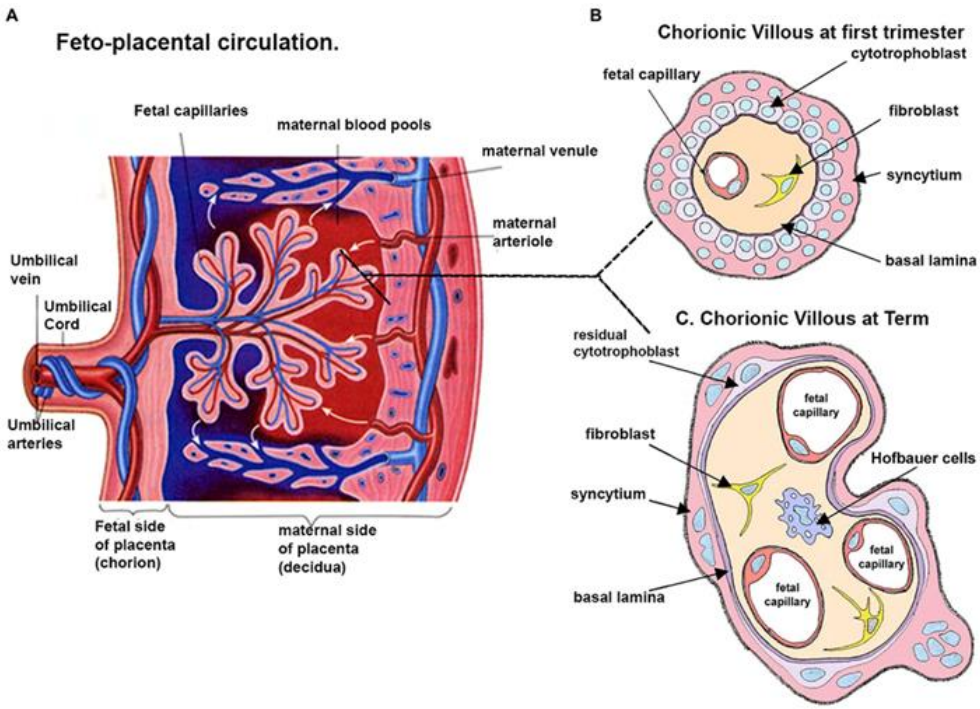

Figure 1. *The maternal-fetal interface [Murthi, 2014];*

A – feto-placental circulation; B – cross section of first trimester chorionic villous; C – cross section of chorionic villous at term

The chorionic villi project into the intervillous space (IVS) filled with maternal blood. Maternal blood is delivered to the IVS via the endometrial arteries. It floats around the villous trees and returns back into the maternal circulation via endometrial veins *(see Fig1A)*. Oxygen, nutrients and other substances present in maternal blood cross the placental barrier, reach the fetal blood and are then transported to the fetus via the umbilical vein. The oxygen-poor blood that contains fetal waste products is returned from the fetus to the placenta in two umbilical arteries [Benirschke, 2006].

Trophoblast cells form the surface of the villous trees and directly contact maternal blood in the IVS (*see Fig1, B and C*). The syncytiotrophoblast (STB) is a continuous, multinucleated epithelial cell layer without separating cell borders. During pregnancy, the thickness of the STB layer decreases, thereby promoting exchange processes between maternal and fetal blood [Mori, 2007], [Jones, 2008]. Subjacent to the STB are the epithelial cytotrophoblasts (CTB), which are proliferating precursor cells of the STB. CTBs differentiate and fuse into the STB, thereby enlarging and renewing the STB layer. During the first trimester of pregnancy the cuboidal CTBs form an almost continuous cell layer beneath the STB (*see Fig1B*). In the term placenta, CTB appear as flat cells with discontinuous distribution (*see Fig1C*), which are in contact via cytoplasmic extensions [Mori, 2007], [Jones, 2008].

The mesenchymal core of the villous tree contains cells types like fibroblasts, placental macrophages (Hofbauer cells) and fetal endothelial cells (FECs), embedded in extracellular matrix. FECs line the fetal vessels.

3.2. The placental barrier

The human placenta establishes a semipermeable barrier (*see Fig2*), which prevents intermingling of fetal and maternal blood. The selectivity of this placental barrier ensures that only certain substances are able to reach the fetal circulation.

Selective maternal-to-fetal transfer starts with uptake at the microvillous (apical) plasma membrane (MVM) of the STB that protrudes into the IVS filled with maternal blood (*see Fig2*). After uptake, molecules must cross the STB cytoplasm and the basal plasma membrane (BM) to exit the STB layer. Both STB plasma membrane domains, MVM as well as BM, contribute considerably to the selectivity of the placental barrier [Sideri, 1983]. The transported molecules then have to pass the villous stroma (at least the basal laminas of STB and FECs) and cross the FEC layer of fetal capillaries before entering the fetal blood circulation [Desforges, 2010], [Sibley, 2010].

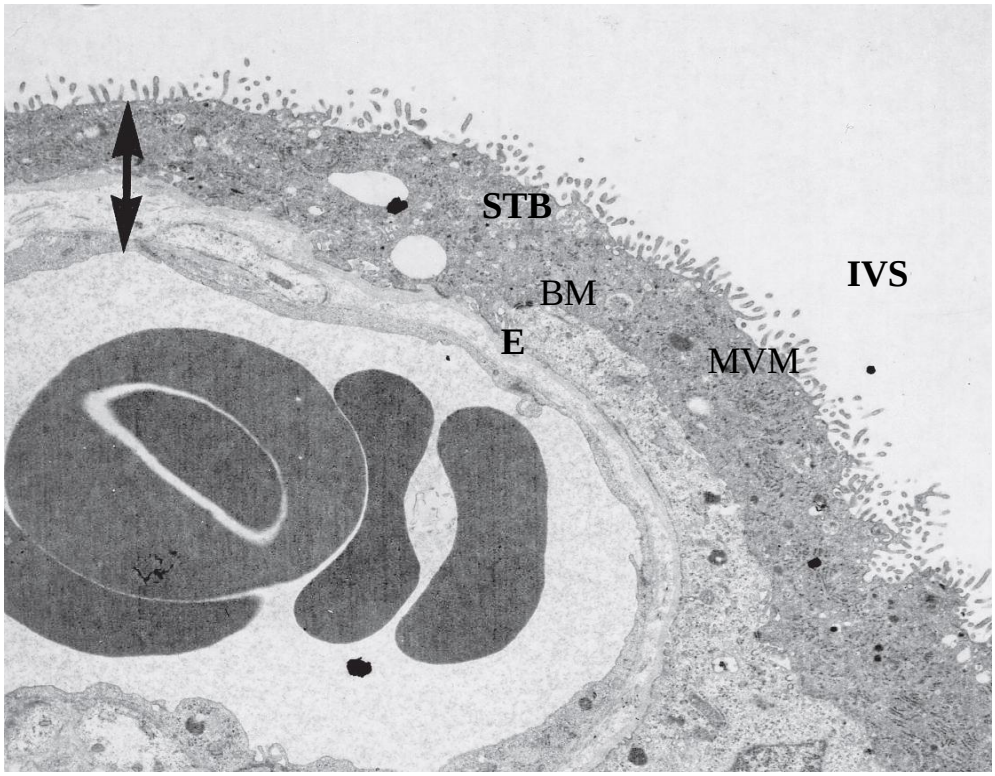

Figure 2. *Ultrastructure of the placental barrier (Electron Microscopic image) [Desforges, 2010];*

STB – syncytiotrophoblast; BM – basal plasma membrane of the STB (facing the basal lamina); MVM – microvillous plasma membrane (facing maternal blood); E – fetal capillary endothelium; IVS – intervillous space (filled with maternal blood); double headed arrow demonstrates the entire placental barrier at term

3.3. Model systems to study transport across the placental barrier

The multinucleated STB represents the first cell layer of the term placental barrier. For many years, research aiming at characterizing transplacental transport has focused on this cell type and various *in situ* and *in vitro* model systems have been established, including the isolated perfused placenta, placental villous explants as well as isolated and *in vitro* differentiated and cultured trophoblasts [Orendi, 2011], [Myllynen, 2013].

While the placental explant system offers the advantage to maintain the STB in its physiological tissue context, it limits the characterization of STB-specific transport processes [Miller, 2005], [Siman, 2001].

I*n vitro* STB cultures allow for more detailed studies of trophoblast-specific activities. Such cultures are obtained by immune-isolation and *in vitro* differentiation of CTB from of human placental villous tissue [Douglas, 1990], [Kliman, 1986]. A major disadvantage of primary trophoblast cultures is the lack of cell proliferation. As a result, the trophoblast culture is limited to 4-7 days. Moreover, to obtain a continuous and polarized STB monolayer enabling

transepithelial transport studies, multiple seedings of CTB and addition of specific growth factors are required [Hemmings, 2001].

Due to these limitations, immortalized cells, such as the BeWo cell line, are often used to study molecular or endocrine aspects of human trophoblasts. BeWo cells originate from a choriocarcinoma, which is a rare and highly malignant neoplasm [Pattillo, 1968]. Other choriocarcinoma cell lines also exist (e.g. JAR and JEG cells), but only BeWo cells have been demonstrated to form a tight monolayer *in vitro*. BeWo cells also exhibit trophoblast-like hormone secretion and, to some degree, syncytial fusion. Main disadvantages of BeWo cells are the long existence of the cell line and the existence of different strains. Furthermore, it is not clear, which stage of gestation BeWo cells represent. Consequently, results derived from choriocarcinoma cells should be interpreted carefully and confirmed in other placental systems [Orendi, 2011]. Overall, however, BeWo cells represent an important system for the investigation of transport processes across the STB [Ellinger, 1999], [Leitner, 2006].

FECs line the surface of fetal blood vessels, build up the second layer of the placental barrier and are in continuity with the fetal blood circulation. Recent years of research have demonstrated the importance of FEC function (and dysfunction) for the fetal development [Wadsack, 2012]. FECs can be isolated by enzymatic perfusion of vessels from (term) placentas and cultured *in vitro* (e.g. [Lang, 2003], [Lang, 2008]).

Placental FECs differ in morphology and functionality depending on the anatomic origin and the size of the vessels. Heterogeneity of FECs in the macro-circulation (i.e. endothelial cell of the umbilical cord) and microcirculation (FECs located in chorionic villi) was first demonstrated *in situ*, comparing their antigenic properties [Lang, 1993]. Morphological, but also functional differences were then demonstrated comparing isolated and cultured human full-term placental ECs (PLEC) derived from small vessels of chorionic villi with macro-vascular human umbilical vein ECs (HUVEC) and SV-40-transformed placental venous endothelial cell line (HPECA2) [Lang, 2003].

Later, microvascular human placental endothelial arterial (HPEAC) and venous (HPEVC) cells were isolated from term placentas and cultured [Lang, 2008]. The specification of endothelial cells (ECs) into arteries and veins is defined by distinct physiological factors like pressure of the blood flow, position and arrangement of smooth muscle cells etc., as well as by genetic mechanisms during development [Swift, 2009]. In contrast to other human organs, where arteries carry oxygen enriched blood, and possess tighter endothelial junctions, and veins carry deoxygenated blood and have looser endothelial junctions, the placental vascular system has arteries that deliver the oxygen-poor blood from the fetus and veins, which carry the oxygenated blood. Lang and coworkers demonstrated that HPEAC and HPEVC exhibit various differences. HPEAC have a polygonal shape with a smooth surface. They grow in loose arrangements and at confluency they build a monolayer with typical cobble-stone morphology (*see Fig3, C and D*). HPEVC have a spindle-like shape and feature numerous microvilli at their surface. These cells grow in immediate vicinity to each other and form swirling patterns (*see Fig3, A and B*) [Lang, 2008]. While HPEAC were described as mature and fully differentiated, HPEVC were found to be more immature. The placental venous FEC represent a juvenile phenotype with a high degree of plasticity. Both venous and arterial cells express classical endothelial markers, but they also demonstrate differences in expression of certain genes. Arterial endothelial cells express artery-related and endothelial-associated genes. In contrast, the venous endothelial cells show the overexpression of development-

associated genes and enhanced differentiation potential [Lang, 2008], [Murthi, 2008], [Murthi, 2014]. The two cell types also exhibit different functional characteristics [Scholler, 2012]. Most likely, the observed differences are the consequence of distinct gene expression mediated by promoter methylation differences. Such differences in global DNA methylation levels have been demonstrated recently with the HPVEC being hypomethylated relative to HPAEC [Joo, 2013].

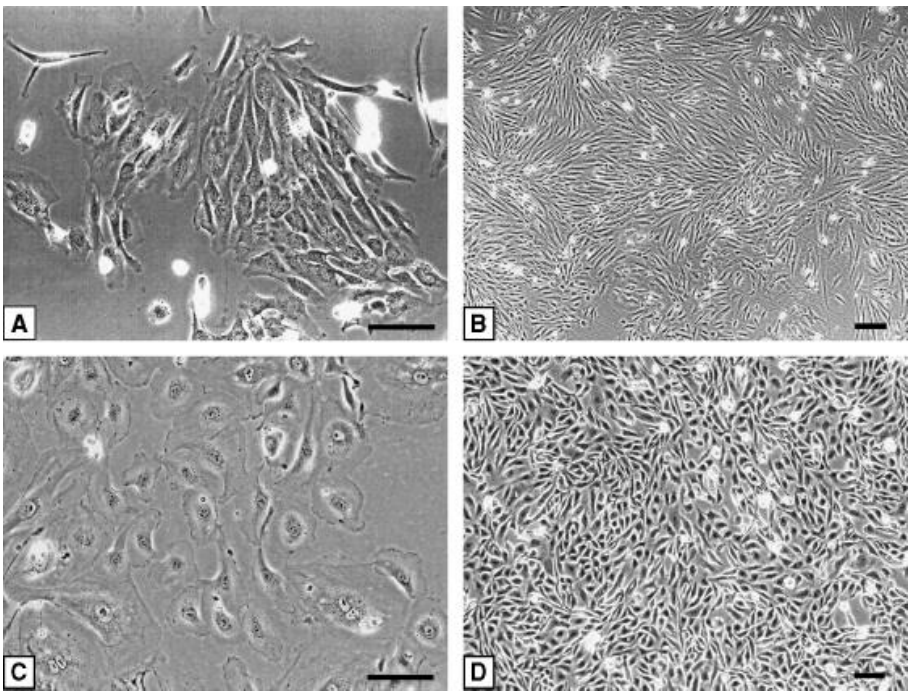

Figure 3. *The morphology of human placental endothelial arterial (HPEAC) and venous (HPEVC) cells [Lang, 2008];*

A – HPEVC grow attached to each other; B – HPEVC form typical swirling patterns at confluency; C – HPEAC show more scattered growth behavior; D – at confluency, HPEAC exhibit a cobble-stone like pattern

3.4. Passive immunization of the fetus with maternal IgG

The immune system of human newborn is immature and during the first month of life the baby is unable to produce the amount of antibodies needed for protection from infections and toxins. Therefore, already during pregnancy humoral immune-protection is provided by maternal IgG, which is transported across the placental barrier to the fetus.

Mainly monomeric IgG is transferred across the human placental barrier while IgG aggregates are trapped by stromal cells. In the fetal circulation the levels of monomeric IgG start to rise after the 17th week of pregnancy. Most of the IgG is transferred during the third trimester and reaches and finally exceeds the maternal concentration around the 35th week of pregnancy. This suggests an Fcγ-receptor-mediated transport mechanism. Despite the importance of IgG

transfer across the placental barrier under physiologic and pathologic conditions, the mechanism of IgG transport remains incompletely understood [Simister, 1998], [Palmeira, 2012], [Ellinger, 2012].

3.5. Immunoglobulin G (IgG)

IgG is the most frequent class of the five antibody classes (IgA, IgD, IgE, IgG and IgM) in the circulation and non-mucosal tissues and plays an important role in protective immunity against a wide range of pathogens and toxins.

The IgG molecule is known to be the only class of antibody that is actively transported from mother to the fetus across the human placenta [Kristoffersen, 1996], [Kristoffersen, 2000]. IgG has been localized at the entire placental barrier, including the STB microvillous plasma membrane, in coated and uncoated pits at both apical and basal sides of the STB, more diffused in the chorionic stroma and within the FECs [Leach, 1991].

IgG molecules consist of two identical heavy chains and two identical light chains, arranged in Y shape (*see Fig4*). Light chains have variable (VL) and constant (CL) regions, heavy chains are composed of one variable (VH) and three constant regions (CH1-CH3). The chains form two functional domains, the Fab- and the Fc-domain, respectively [eBioscience, 2000-2014].

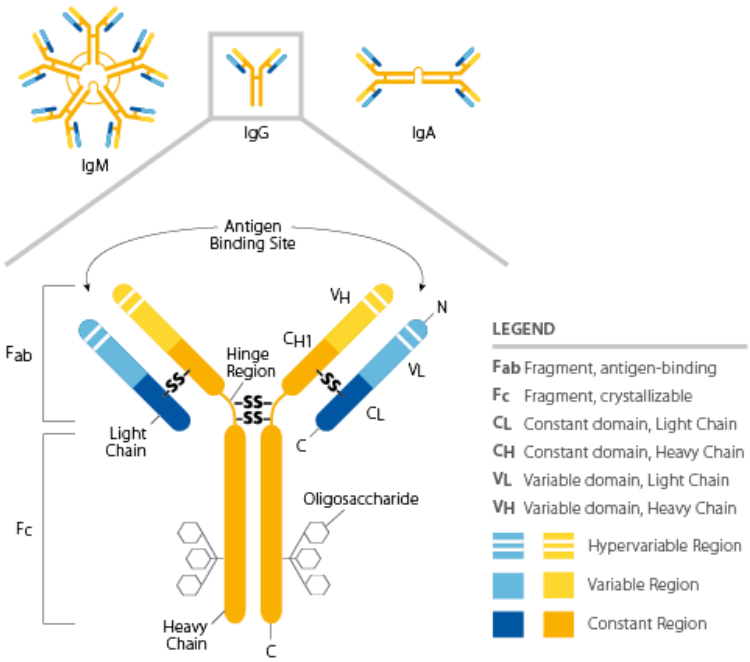

Figure 4. *The structure of Immunoglobulin G (IgG) [eBioscience, 2000-2014]*

The Fab-domain is responsible for specific antigen recognition via hyper-variable and variable regions. The Fc-domain (c from crystallizable) is the binding site for the Fcγ-

receptors and is involved in the immune effector function pathways against pathogens [West, 2000]. The Fc-domain is separated from the Fab-domain by a flexible hinge region.

The CH2 and CH3 domains within the Fc-region are known to bind to the human neonatal Fc receptor, hFcRn. Several histidine residues in the binding site are crucial for pH-dependent hFcRn/IgG interaction [Burmeister, 1994], [Mathur, 2013].

3.6. Fcγ-receptors

Fcγ-receptors are cell surface glycoproteins that permit the antibody-antigen interaction with immune cells, regulating both humoral and cellular immune responses. They function as regulators of several effector processes, including phagocytosis, degranulation, antibody-dependent cellular cytotoxicity, secretion of mediators, production of antibodies and immune complex clearance.

The Fcγ-receptor family consists of three receptor subclasses: FcγRI, FcγRII and FcγRIII, which exhibit structural homology (*see Fig5*). Each Fcγ-receptor has different binding affinities to the human IgG subclasses, caused by distinct interaction between extracellular Ig-like domains of the receptor and Fc-domain (interface between CH2 and CH3) of the IgG molecule [Li, 2009].

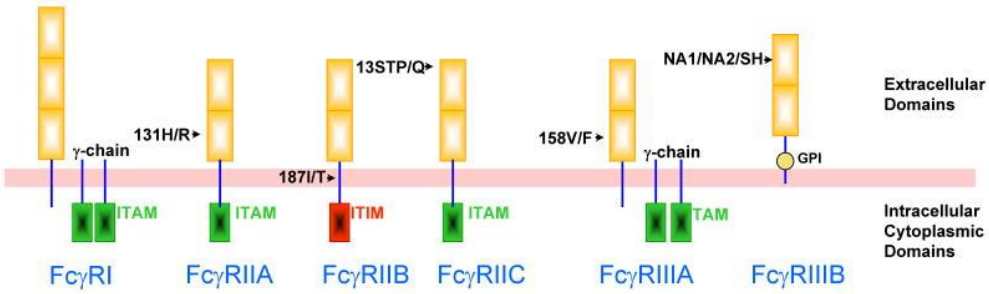

Figure 5. *Structural homology of Fcγ-receptor subclasses [Li, 2009]*

FcγRI is the only subclass of the Fcγ-receptors that has three extracellular Ig-like domains and binds IgG molecule with high affinity. FcγRI is expressed on monocytes, dendritic cells, macrophages and activated neutrophils [Li, 2009].

FcγRII is the most widely distributed Fcγ-receptor class. It has been found on monocytes, dendritic cells, neutrophils, B cells, platelets and natural killer cells. FcγRII contains only two extracellular Ig-like domains and exhibits a low binding affinity for IgG monomers, but shows high affinity for IgG aggregates. The FcγRII subclass is represented by three proteins, namely FcγRIIa, FcγRIIb and FcγRIIc. In contrast to other Fcγ-receptors, which contain an immunoreceptor tyrosin activation motif (ITAM) in their cytoplasmic domain, the FcγRIIb has an immunoreceptor tyrosin inhibitory motif (ITIM) and functions as the only inhibitory receptor of the Fcγ-receptor family [Li, 2009], [Sondermann, 1999].

FcγRIII subclass binds monomeric IgG with intermediate affinity. The subclass consists of two proteins FcγRIIIa and FcγRIIIb that differ in their extracellular domains. FcγRIIIb is a

GPI-anchored receptor and expressed on the surface of neutrophils and basophils. FcγRIIIa is expressed on natural killer cells, monocytes and macrophages. Both proteins can readily bind IgG complexes.

On the basis of the crystal structure of the human FcγRII the interaction between the Fcγ-receptor and IgG molecule was proposed to occur with a 2:1 stoichiometry [Sondermann, 1999]. In contrast, an NMR study of the Fcγ-receptor/IgG interaction showed a 1:1 stoichiometry as the physiologically most advantageous one [Kato, 2000].

All three subclasses of the Fcγ-receptors (FcγRI-III) were identified at the human materno-fetal interface [Mishima, 2007], [Takizawa, 2005], [Simister, 2003], [Simister, 1998], [Simister, 1997]. Expression of FcγRI and FcγRII was found in Hofbauer cells in the chorionic stroma. The FcγRIII receptors were identified on Hofbauer cells and on the STB [Lyden, 2001]. Due to their characteristics most of these receptors are however unlikely to contribute significantly to monomeric IgG transport as they do not bind monomeric IgG with high affinity. As mentioned above, these "classical" Fcγ-receptors receptors are usually involved in triggering a cellular response to bound IgG complexes through intracellular signaling pathways. In line with this, a mechanism for uptake, transcytosis and release of intact IgG has not been demonstrated for most of these receptors. The latter characteristics, however, are of relevance for receptor-mediated transfer of monomeric IgG across two cellular layers [Simister, 1998], [Simister, 1997], [Simister, 2003], [Li, 2009].

FcγRII was also identified on FECs, though not on the STB [Simister, 1998], [Simister, 1997], [Simister, 2003]. One specific FcγRII receptor, FcγRIIb2 was shown to be present within FECs *in situ* and to colocalize with IgG molecules in the term placenta in membrane-bounded organelles, which contain most of the intracellular IgG (~80%) [Lyden, 2001], [Mishima, 2007], [Takizawa, 2005]. In principle, hFcγRIIb2 is able to undergo endocytosis and transcytosis in polarized epithelial cells [Hunziker, 1989], however, a mechanism that would allow release of intact IgG after transcytosis has never been described. HFcγRIIb2 has nevertheless been claimed to be the receptor responsible for IgG transfer across human placental FECs [Takizawa, 2005].

3.7. Structural and functional characteristics of the human neonatal Fc receptor, hFcRn

HFcRn is an Fc-receptor, which is highly homologous to major histocompatibility complex (MHC) class I molecules. HFcRn is a heterodimeric glycoprotein and consists of a single transmembrane α-chain non-covalently bound to β2-microglobulin. The α-chain is composed of three extracellular domains (α1, α2, and α3), a transmembrane region and a short cytoplasmic tail. β2-microglobulin is a secretory, low molecular weight protein associated with the α3 domain of the α-chain and is necessary for the hFcRn transport out of the endoplasmic reticulum, IgG binding and transmission. As a part of MHC class I and class I-type proteins, β2-microglobulin is also a cell surface molecule [Burmeister, 1994].

The hFcRn receptor functions likely as a dimer of heterodimers (*see Fig6*) [Bowness, 2009].

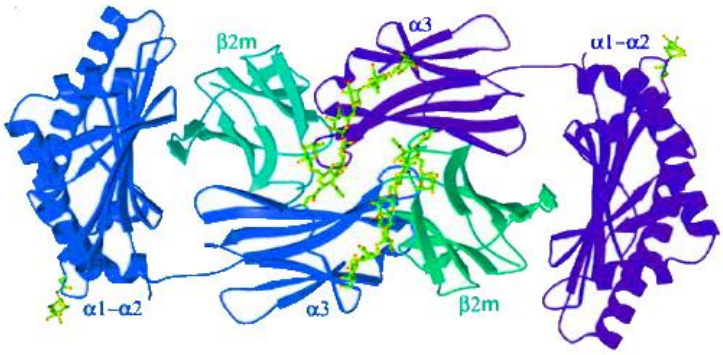

Figure 6. *The structure of the hFcRn dimer [Bowness, 2009]*

The dimerization of the hFcRn is induced by IgG binding and leads to formation of functionally active hFcRn dimer that can bind a single molecule of IgG antibody (*see Fig7*).

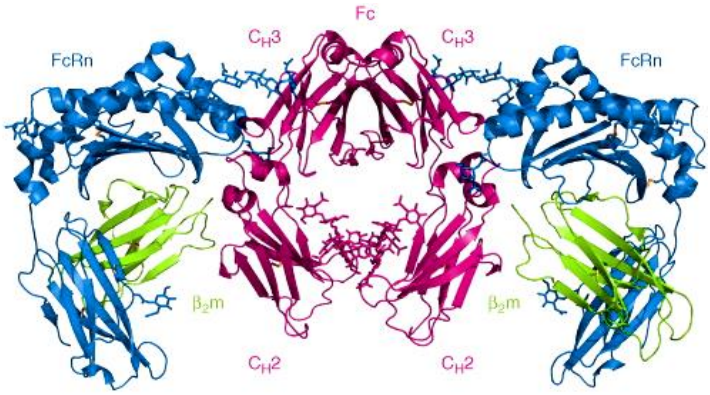

Figure 7. *The interaction of hFcRn dimer with IgG [Bowness, 2009]*

In solution, soluble hFcRn molecules are monomeric [Burmeister, 1994] (*see Fig8a*). The IgG binding site resides in the α2 domain (α-chain) of the hFcRn and interacts with the Fc region of IgG molecule by binding at an acidic pH (<6.5) but not at a physiological pH (7.4). The protonation of histidine residues in the Fc region and glutamic acid in the α2 domain at low pH leads to formation of salt bridges resulting in high affinity binding (*see Fig8b*). At physiological pH respective residues deprotonate and the hFcRn/IgG complex dissociates by release of IgG into the extracellular space [Ellinger, 2012].

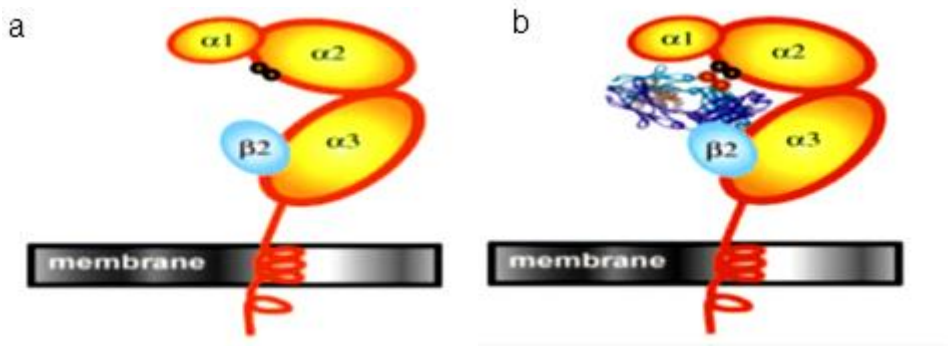

Figure 8. *Structure of monomeric hFcRn (a);*
IgG molecule bound to the hFcRn (b) [Ellinger, 2012]

This pH-dependency of IgG binding is of relevance for hFcRn-mediated trafficking of intact IgG. As one major function, hFcRn maintains the high concentration of IgG molecules in the circulation. It prolongates the half-life of IgG in the serum by binding the continuously internalized IgG molecules within acidic endosomes. HFcRn then recycles IgG back to the circulation, where it is released due to the physiologic pH of serum. HFcRn also binds albumin at acidic pH, but not at neutral pH and therefore likewise protects albumin from catabolism [Roopenian, 2010], [Kim, 2007].
HFcRn was initially isolated as the receptor transcytosing IgG from maternal milk across the neonatal rat intestine [Simister, 1989]. Later, Firan and coworkers demonstrated in an *ex vivo* placental model, that the human maternal-to-fetal transmission of monomeric IgG involves hFcRn by using an IgG mutant, which could not bind to hFcRn [Firan, 2001]. HFcRn is expressed in placental tissue [Leach, 1996], [Simister, 1996]. Using immunoelectron microscopy, hFcRn was localized to the apical but not to the basal membrane of the STB. In addition, the receptor was found in apical vesicles and in some vesicles in close proximity to the basal membrane. These vesicles are presumably transcytotic compartments [Fuchs, 2004]. In agreement with the *in situ* localization of the hFcRn in the STB, receptor expression was also demonstrated in isolated and *in vitro* cultured STB of full term human placenta [Szlauer, 2009].

3.8. HFcRn-mediated IgG transport across trophoblast cells

Endogenous expression of hFcRn, though at low levels, was also found in trophoblast-like BeWo cells [Ellinger, 1999]. Upon stable transfection of BeWo cells with hFcRn, [Leitner, 2006], a predominant intracellular steady state localization of hFcRn was demonstrated. Colocalization of hFcRn and IgG in Early Endosomal Antigen 1-positive endosomes suggested that this was the predominant localization where hFcRn can bind to IgG. Using BeWo cells as well as hFcRn overexpressing BeWo cells [Ellinger, 1999] [Leitner, 2006], a model for IgG transport across trophoblast cells was developed (*see Fig9*). The STB microvillous plasma membrane contacts maternal blood in the IVS. Although hFcRn is present at the plasma membrane, it cannot bind IgG with high affinity due to the neutral pH of serum. However, IgG is present at high concentrations (15 mg/ml) in serum and is continuously internalized by fluid phase endocytosis. The IgG-containing endocytic

compartment is then gradually acidified thereby allowing IgG to bind tightly to hFcRn present in the endosomal compartment. In the next step, the hFcRn/IgG complex is either recycled to the apical membrane or transcytosed to the basal side of the cell. After fusion of vesicles with the membrane, the physiological pH promotes the dissociation of IgG from hFcRn [Ellinger, 2012]. Intracellular trafficking of hFcRn is mediated by sorting signals contained in the cytoplasmic domain of the receptor [Stefaner, 1999] [Jerdeva, 2010], [Tesar, 2010], [Wang, 2013].

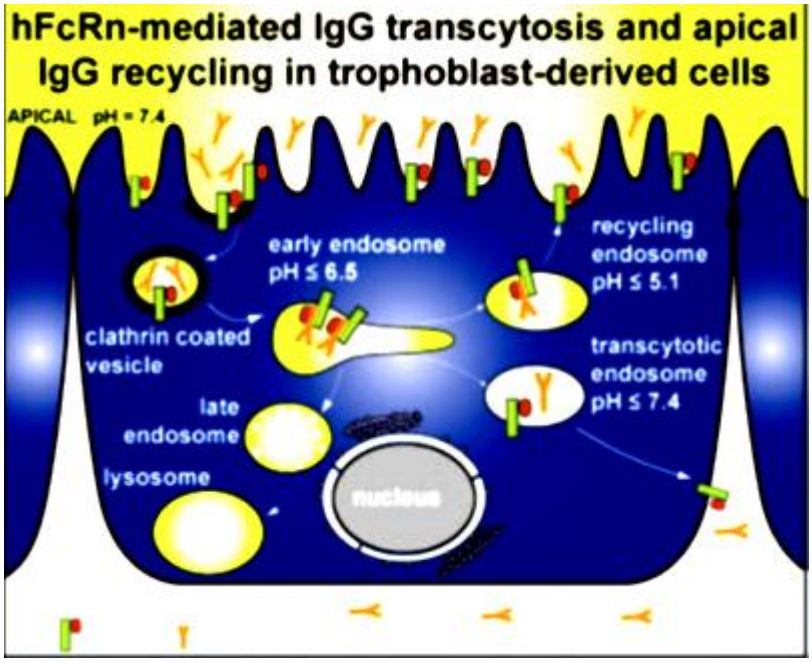

Figure 9. *HFcRn mediated transport of IgG [Ellinger, 2012]*

3.9. IgG transport across the FEC

HFcRn expression and hFcRn-mediated IgG transcytosis was also shown in primary human placental endothelial cells (HPEC) cultured on permeable membranes [Radulescu, 2004], [Antohe, 2001]. These studies proved that IgG transfer involved hFcRn and colocalization of IgG and hFcRn in endosomes was demonstrated. Bidirectional IgG transport was observed, but IgG was preferentially shuttled from the basolateral to the apical surface (directed towards the fetal circulation) [Radulescu, 2004], [Antohe, 2001]. These studies suggested, that hFcRn could be the receptor responsible for IgG transfer across the entire placental barrier consisting of STB and FEC.

However, neither localization of hFcRn nor colocalization of IgG with hFcRn was so far clearly demonstrated in FECs in terminal chorionic villi *in situ* [Leach, 1996], [Simister, 1996], [Kristoffersen, 1996] leading to the general assumption that hFcRn is responsible for IgG transfer across the STB layer, but not across FECs.

4. AIM

HFcRn is expressed in placental tissue [Leach, 1996], [Simister, 1996] and is involved in IgG transport across the human placenta [Firan, 2001]. The localization of hFcRn in the syncytiotrophoblast layer (STB) *in situ* has been clearly demonstrated [Kristoffersen, 1996] [Fuchs, 2004] and confirmed by expression in isolated and *in vitro* cultured STB [Szlauer, 2009] as well as in trophoblast-derived BeWo cells [Ellinger, 1999]. HFcRn can undergo transcytosis and can mediate transepithelial transfer of monomeric IgG by a pH-dependent mechanism [Ellinger, 2012].

In contrast, the expression of hFcRn in the endothelium of fetal vessel and its role in IgG transcytosis is still disputed. The majority of published data that investigated placental *in situ* localization of hFcRn could not demonstrate the receptor in FECs of terminal chorionic villi [Leach, 1996], [Simister, 1996], [Kristoffersen, 1996]. Other groups demonstrated expression of the IgG receptor hFcγRIIb2 and its colocalization with IgG in FECs [Lyden, 2001], [Mishima, 2007], [Takizawa, 2005] and consequently suggested hFcγRIIb2 to be responsible for IgG transcytosis across FECs. On the other hand, isolated and *in vitro* cultured HPEC were found to express hFcRn and exhibit hFcRn-dependent IgG-transcytosis. Unfortunately, placental *in situ* expression of the receptor was not shown in these studies [Radulescu, 2004], [Antohe, 2001].

IgG transport across the placental barrier is not only of physiologic importance for the offspring. Maternal autoantibodies can likewise cross the placenta and cause severe diseases of the offspring. Furthermore, biologicals (monoclonal antibodies), which are applied to treat maternal inflammatory diseases during pregnancy, can also cross the placenta. The search for new strategies to block the transfer of pathologic antibodies [Mathiesen, 2013] and the wish to design biologicals with maximal therapeutic effect for the mother and at the same time minimal transplacental transfer and consequently fetal exposure [Chaparro, 2011], are major drivers to clarify the mechanism of placental IgG transfer.

In this context, it is of great importance to clarify the localization of hFcRn at the human term placental barrier. The major aim of this master thesis was to investigate absence or presence of hFcRn in the FEC *in situ* and to confirm the result in isolated and *in vitro* cultured human placental FECs.

The following sub-aims were addressed:

1. Proof of specificity of available anti-hFcRn antibodies by Western Blotting and selection of the most appropriate antibody
2. Localization of hFcRn as well as hFcγRIIb2 in STB and/or FEC in human placental chorionic tissue *in situ* applying Immunofluorescence Microscopy
3. Demonstration of absence or presence of hFcRn mRNA in isolated FECs as well as the placental tissue by RT-PCR
4. Demonstration of absence or presence of hFcRn protein and ß2-microglobulin isolated FECs as well as the placental tissue by Western Blotting

5. Subcellular localization of hFcRn in trophoblast-derived BeWo cells overexpressing hFcRn by confocal Immunofluorescence Microscopy to establish double-labeling immunofluorescence protocols

6. Localization of hFcRn as well as FcγRIIb2 in isolated and *in vitro* cultured FECs applying Immunofluorescence Microscopy

7. Demonstration of human IgG internalization in isolated and *in vitro* cultured FECs in comparison to BeWo cells overexpressing hFcRn using Immunofluorescence Microscopy

5. MATERIALS and METHODS

5.1. Biological samples

5.1.1. Human placental chorionic tissue

Placental tissue samples were provided by Dr. Isabella Ellinger (Department of Pathophysiology and Allergy Research (IPA), Medical University Vienna). When indicated, samples were obtained from Dr. Christian Wadsack (Department of Obstetrics and Gynecology, Medical University Graz). Following elective cesarean sections, healthy term placentas were transferred to the laboratory at r.t. within 15 min and immediately processed to minimize destructive processes [Matsubara, 2000].

The placenta was placed on the fetal side (umbilical cord down). Chorionic tissue to be used for RNA and protein lysate preparation was cut out from the organ half way between the umbilical cord and the placental margins using scissors and forceps. The tissue (about 0.5 cm^3) was briefly washed in phosphate buffered saline (PBS, *for recipe see Chapter 5.1.4.*), the samples were frozen in liquid nitrogen and stored at -80°C. For Immunofluorescence Microscopy (IFM) see Chapter 5.4., tissue samples were processed as described in Chapters 5.4.1. and 5.4.2.

Preparation of human placental endothelial cells (HPEC) is described in Chapters 5.1.3.

5.1.2. Primary cells and immortalized cell lines

Primary cells – fetal endothelial cells derived from human placentas

Human placental arterial endothelial cells (HPAEC) as well as *human placental venous endothelial cells* (HPVEC) were isolated from healthy full term human placentas at the Department of Gynecology and Obstetrics, Medical University of Graz according to [Lang, 2008]. Following isolation, their endothelial identity was routinely confirmed by IFM using classical endothelial markers (e.g. von Willebrand factor [Lang, 2003]). Cells were maintained in flasks or chamber slides pre-coated with 1% gelatin at 37°C with 5% CO_2 and 95% humidity. The cells were used for RNA isolation, protein extraction or IFM approximately after they had reached confluency greater than 80%.

Immortalized cell lines

The BeWo cell line is derived from a rare malignant neoplasm, human choriocarcinoma [Pattillo, 1968] and exhibits various morphological and biochemical properties of placental trophoblast. Moreover, BeWo cells exhibit endogenous expression of hFcRn [Ellinger, 1999]. BeWo cells (clone 24 was used) were kindly provided by Dr. Ellinger (Department of Pathophysiology and Allergy Research/Medical University of Vienna). The cells were cultured at 37°C with 5% CO_2 and 95% humidity and used between passages 1 and 5.

BeWo + hFcRn(5) represent BeWo cells overexpressing hFcRn. In this study, these cells were used as positive control for hFcRn expression. The cell line was generated by stable transfection of BeWo cells with hFcRn cDNA and subsequent selection in the presence of G418 (Geneticin; [Ellinger, 2005]). Transfected cells were maintained in the presence of the antibiotic G418 under otherwise identical culture conditions as BeWo cells. The cells were used between passages 1 and 5.

5.1.3. Isolation of endothelial cells

The isolation protocol for HPAEC and HPVEC has been published by [Lang, 2008].The procedure is outlined in *Fig10* and described below. Isolation of placental endothelial cells was done in the laboratory of Dr. C.Wadsack. All subsequent experiments were performed at the IPA.

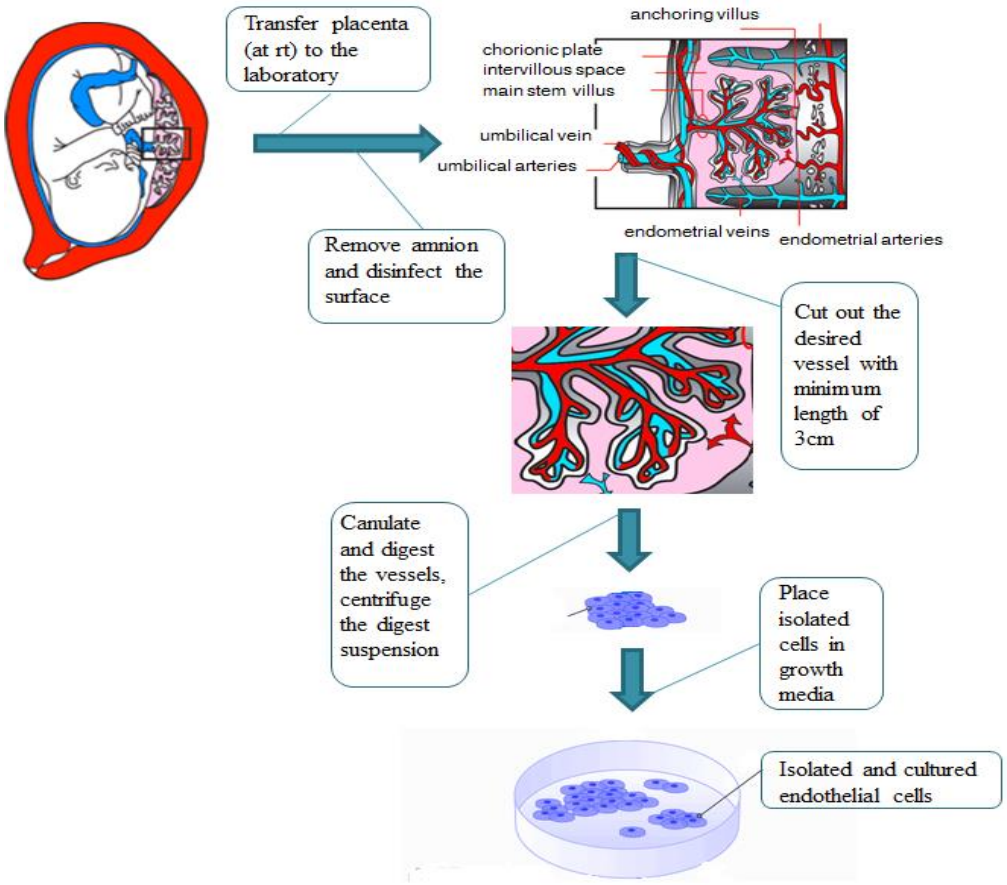

Figure 10. *The process of placental endothelial cell isolation according to [Lang, 2008]*

Materials

- Cannula (Optiva 2 I.V. Catheter Radiopaque)
- Centrifuge (Hettich Safe, Thermo Scientific)
- Centrifuge tubes, 50 ml (TPP)
- Laminar Flow (Hera Safe, Thermo Scientific)
- Perfusor syringe
- Sterile scissors and forceps
- Sterile glass plate
- Thread (Prolene)
- 4-well chamber slides, #1.5 borosilicate, sterile (Lab-Tek II, Nalge Nunc International)
- 12-well plate, sterile (Iwaki)
- Sterile glass pipettes, 5/10/25 ml (Iwaki)
- Sterile glass Pasteur pipettes (VWR International)
- Tissue culture flasks, 75cm^2/canted neck, sterile (Iwaki)

Reagents, buffers and solutions

- Betaisadona (1000 ml, chemical complex of polyvinylpyrrolidone, providone, PVP and elemental iodine, Mundipharma)
- Collagenase/Dispase enzyme solution (500 mg, Roche), dissolved in 1x HBSS (final concentration 0.5mg/ml, supplemented with 1% Penecillin/Streptomycin (Gibco)
- Endothelial Basal Medium (EBM), (500 ml, Lonza), stored at 4°C
- Gelatin (powder from porcine skin (100 g, Sigma)), dissolved in 1x HBSS (final concentration 1% (w/v), supplemented with 2% Gentamicin (Gibco)
- Hank's Balanced Salt Solution (HBSS), 1x, no calcium, no magnesium (500 ml, Gibco), stored at 4°C
- Pregnant serum, gained from maternal blood, inactivated at 53°C for 30 min, stored at -20°C

Method

- Disinfect the placental surface with Betaisadona
- Remove the amnion
- Dissect the desired vessels (arteries or veins, the minimum length should be 3 cm, diameter 1 to 3 mm)
- Transfer vessels to the wash tube containing HBSS, wash 2 times
- Transfer individual vessels on sterile glass plate and smooth them out with cannula to remove residual blood
- Canulate each vessel
- Remove the needle and connect the cannula to the syringe, which is filled with Collagenase/Dispase enzyme solution
- Start digestion process by slow perfusion with enzymatic solution (6-8 min)
- Collect cell suspension

- Centrifuge the suspension to collect cells at 900 rpm (200 x g) for 7 min at 4°C
- Aspirate the supernatant and resuspend the pellet in EBM culture medium containing 2% pregnant serum (prewarmed)
- Seed the cells into a 12-well plate pre-coated for 30-60 min with 1% gelatin solution
- Change the medium each day until day 4 after isolation, than 1-2 times per week
- After 2 weeks first attached cells are visible
- Transfer the cells (at approx. 80% confluency) to the 75 cm^2 culture flasks (for further culturing or RNA and protein isolation) or to 4-well chamber slides for indirect IFM. All isolated cells used in this study were confirmed to be endothelial cells by positive staining for the von Willebrand factor according to [Lang, 2008].

5.1.4. Cell culture

Materials

- Autoclave (Odelga)
- Centrifuge (Hettich Safe, Thermo Scientific)
- Centrifuge tubes, sterile, 15/50 ml (TPP)
- Cryovials, sterile, 1.2 ml (Sterilin)
- Glass Pasteur pipettes, sterile (VWR International)
- Glass pipettes, 5/10/25 ml, sterile
- Incubator (Heraeus Cytoperm 2, Thermo Electron Corporation)
- Laminar Flow (Hera Safe, Thermo Scientific)
- Osmometer (Osmomat 030-D, Gonotec)
- pH-Meter (827 pHLab, Metrohm)
- Tissue culture flasks, 25cm^2/75cm^2 canted neck, sterile (Iwaki)

Reagents

- DMEM (Dulbecco's Modified Eagle Medium) – high glucose (Sigma), with 4500 mg/l glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate, liquid, sterile-filtered, cell culture tested, stored at 4°C
- DMSO liquid, sterile (Sigma), stored at r.t.
- Endothelial Growth Medium-MicroVascular (EGM-MV) BulletKit Medium (Lonza), 500 ml EBM Basal Medium (stored at 4°C) plus SingleQuots Kit (contains human Endothelial Growth Factor (hEGF), 0.5 ml; Hydrocortisone, 0.5 ml; Bovine brain extract, 2.0 ml; Fetal bovine serum, 25.0 ml; Gentamicin sulfate, Amphotericin, 0.5 ml; stored at -20°C) to formulate EGM-MV Medium (growth medium)
- Fetal Bovine Serum Gold (FBS), sterile (PAA Laboratories), treated at 56°C for 30 min (performed to destroy complement, and to make sure that the cells will not be lysed by antibody binding), stored in ready-to-use aliquots at -20°C
- Gelatin, powder from porcine skin (100 g, Sigma)
- G418 disulfate salt solution (50 mg/ml), Geneticin Selective Antibiotic (Sigma, stored at 4°C)

- Glutamine (200mM), sterile (GlutaMAX-I, Gibco), stored in ready-to-use aliquots at -20°C
- HBSS, 1 x, no calcium, no magnesium (500 ml, Gibco, stored at 4°C)
- KCl (Potassium chloride, Merck)
- KH_2PO_4 (Potassium dihydrogen phosphate, Merck)
- Liquid Antibiotic Mixture (PSN), sterile, containing 5 mg/ml Penicillin, 10 mg/ml Streptomycin and 10 mg/ml Neomycin (Gibco), stored in ready-to-use aliquots at -20°C
- NaCl (Sodium chloride, Merck)
- NaOH (Merck)
- $Na_2HPO_4 \bullet 2H_2O$ (Disodium phosphate dihydrate, Merck)
- TrypLE (1x, Gibco), no phenol red, sterile, stored at r.t.
- Trypsin/EDTA solution, 10x, (0.5% Trypsin, 0.2% EDTA), sterile, (Gibco), stored at -20°C

Buffers and Solutions

- ***1x PBS***
    - Dissolve the following chemicals in 800 ml A. bidest.:
        - 8.0 g NaCl
        - 0.2 g KCl
        - 1.44 g Na_2HPO_4
        - 0.24 g KH_2PO_4
    - Adjust pH to 7.4
    - Adjust volume to 1000 ml with A. bidest.
    - Sterilize by autoclaving

- ***1x Trypsin/EDTA solution, sterile***
    - To 100 ml Trypsin/EDTA solution, 10x, sterile
    - Add 900 ml 1x PBS (sterile)
    - Prepare 50 ml aliquots and store at -20°C

- ***1% Gelatin solution***
    - dissolve 1 g in 100 ml 1x HBSS and supplement with 2% Gentamicin (Gibco)

Cell Culture Media

- ***Culture medium for BeWo cells***
    - 500 ml DMEM, high glucose, sterile
    - 50 ml FBS (10% v/v final concentration)
    - 5.5 ml Glutamine (2 mM final concentration)

- 5.5 ml Liquid Antibiotic Mixture (50 µg/ml Penicillin, 100 µg/ml Streptomycin, 100 µg/ml Neomycin final concentrations)

- ***Culture medium for BeWo + hFcRn(5) cells***
  - Per 50 ml culture medium for BeWo cells
  - Add 250 µl G418 (0.25 mg/ml final concentration)

- ***Culture medium for fetal endothelial cells, Endothelial Growth Medium (EGM)***
  - To 500 ml EBM Basal Medium add
    - 0.5 ml hEGF
    - 0.5 ml Hydrocortisone
    - 2.0 ml Bovine brain extract
    - 25.0 ml Fetal bovine serum
    - 0.5 ml Gentamicin sulfate, Amphotericin

- ***Freezing medium for BeWo cells and FEC***
  - 40 ml appropriate culture medium
  - 5 ml FBS (final concentration 20% (v/v))
  - 5 ml DMSO (final concentration 10% (v/v)), used as cryoprotective agent
  - Store at 4°C

Cell culture methods

The volumes described below are valid for 25 cm^2 flasks and must be scaled up by factor 3 for 75 cm^2 flasks.

- ***Feeding of BeWo cells and FEC***
  - Examine cells (cultured in flasks) using an inverted microscope (magnification 100 x– 200 x) to evaluate cell growth and detect possible infections
  - Transfer cells to laminar flow workbench
  - Aspirate and discard old medium
  - Wash cells once with PBS solution to remove dead cells and debris (5 ml PBS for 25 cm^2 flasks)
  - NOTE: No washing step for fetal endothelial cells!
  - Add appropriate new , pre-warmed medium: 5 ml for 25 cm^2 flask
  - Transfer cells to cell incubator providing 37°C, 5% CO_2 and 95% humidity

- ***Passaging of BeWo cells and FEC / Cell splitting***
  - Transfer cells to laminar flow workbench
  - Aspirate and discard old medium

- Wash cells once with PBS (BeWo) or HBSS solution (FEC) to remove debris and trypsin inhibitor contained in medium
- Add about 1 ml 1x Trypsin-EDTA solution (BeWo) or TrypLE solution (FEC)
- Incubate for about 5 min until cells round up and detach from support
- Evaluate process by light microscopy
- Add appropriate culture medium (4 ml per 25 cm^2 flask) and suspend cells by pipetting up and down a few times
- Add appropriate new medium to flasks (4 ml per 25 cm^2 flask)
- NOTE. For endothelial cells pre-coat the flasks for 30-60 min with 1% gelatin solution
- Transfer an aliquot of suspended cells into new flasks (1 ml per 25 cm^2 flask)
- Store cells in incubator

- ***Freezing of BeWo cells and FEC***
  - Grow cells in a 75 cm^2 flask to 80% confluency
  - Inspect cells and transfer healthy cells to laminar flow
  - Aspirate medium and wash cells twice with PBS (BeWo) or HBSS (FEC) to remove trypsin inhibitor contained in the medium
  - Add 1,5 ml per 75 cm^2 flask of 1 x Trypsin-EDTA solution (BeWo) or TrypLE solution (FEC)
  - Incubate for ~5min until cells round up an detach from the flask
  - Evaluate cell-mobility by light microscopy
  - Add appropriate culture medium (10 ml per 75 cm^2 flask) and resuspend cells by pipetting up and down a few times
  - Transfer cell suspension to centrifuge tube (15 ml)
  - Pellet cells by centrifugation at 200 x g (approx. 1000 rpm) for 5 min at 4°C
  - Discard supernatant, add 5 ml cell-freezing medium (cooled to 4°C), suspend cells by pipetting up and down a few times
  - Transfer cell suspension to cryo tubes (1 ml per tube)
  - Keep tubes on ice and transfer to freezer (-80°C)
  - After 24h, transfer to -196°C liquid nitrogen storage container

- ***Thawing of BeWo cells and transfected BeWo + hFcRn(5) cell line***
  - Retrieve tube with cells (containing 1ml cell suspension) from liquid nitrogen storage container (-196°C)
  - Thaw rapidly
  - Transfer to 15 ml centrifuge tube containing 4 ml culture medium (for BeWo + hFcRn5 without selection antibiotic (G418)
  - Pellet cells by centrifugation at 200 x g (approx. 1000 rpm) for 5 min at 4°C
  - Discard supernatant, add 5 ml culture medium (for BeWo + hFcRn(5) without selection antibiotic (G418)), resuspend cells by pipetting up and down a few times
  - Transfer to 25 cm^2 flask

- Place cells in incubator providing 37°C, 5% CO_2 and 95% humidity
- After attachment of cells, feed with appropriate medium

- ***Thawing of FEC***
  - Coat a 75 cm^2 flask with 1% gelatin solution, distribute it evenly to cover whole surface, remove the rest; incubate flasks for 30-60 min at 37°C
  - Add 10 ml EGM to the flask
  - Thaw tube with cells rapidly at r.t
  - Transfer to the flask gently without producing of air bubbles
  - Rinse the tube with 1 ml EGM, add to the flask
  - Place cells in incubator providing 37°C, 5% CO_2 and 95% humidity
  - Control the attachment of the cells on the next day
  - If the cells are attached change medium every day first three days, after 2 x a week

5.2. Detection of hFcRn transcripts (mRNA) in tissue and cells by Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

Following isolation of RNA from biological samples, RT-PCR combines reverse transcription and the polymerase chain reaction to generate and amplify complementary DNA (cDNA) from messenger RNA (mRNA) transcripts. The method was used in this thesis to study gene expression of hFcRn at the RNA level.

5.2.1. RNA Isolation

Background

As a transient message, mRNA molecules carry the information from activated genes in the nuclei to ribosomes to enable protein formation. The first step to demonstrate the presence of specific mRNA molecules thereby confirming the expression of a certain gene, is to isolate RNA from the biological samples (tissue, cells, [Liu, 2013]). In this thesis, RNA isolation was done by an optimized guanidineisothiocyanate / phenol method according to the instructions of the manufacturer (PeqGOLD TriFast, PeqLab).

Materials

- Box with crushed ice
- Coolable Centrifuge (eg. Eppendorf, Centrifuge 5415R)
- Forceps
- Heating block (e.g. Eppendorf, Thermomixer 5436)
- Homogenizer (IKA Werke, T10 basic Ultra Turrax)
- Mortar + pistil
- Paper Towels

- Polystyrene Rack
- Pipette tips, 200 µl, 1250 µl, DNase, RNase, DNA free (e.g. Biozym, Safe seal tips professional) and appropriate pipettes
- Protective gear
    - Protective Glasses
    - Gloves for cryogenic work (handling of liquid N_2, e.g. Tempshield, Cryogloves)
    - Latex Gloves (e.g. Hartmann, Peha-Soft powderfree)
- Reaction tubes 1.6 ml (Biozyme, DNase, RNase free reaction tubes)
- Small vials (e.g. Greiner bio-one, scintillation vial 27.0/60.0 mm; saw off neck)
- 2 Styrofoam boxes with lids
- Vortexer (e.g. Labinco, L24)

Reagents and solutions

- Chloroform (Merck)
- DNase, RNase free distilled water, "PCR water" (Gibco, Invitrogen, Ultrapure)
- Ethanol 85% (diluted with PCR water from Ethanol absolute, VWR Prolabo)
- Isopropanol (Merck)
- Liquid N_2
- peqGOLD Trifast (peqLAB)
- PBS, 1x prepared as described in Chapter 5.1.4.

Methods

- ***RNA isolation from cells***
    - Cells (25 cm^2) are grown until they reached confluency of 70-100 %
    - Remove medium and wash cells 2 times with 10 ml PBS
    - Add 1 ml PBS and harvested cells with a cell scraper
    - Centrifuge cell suspension at 200 x g for 5 min at 4°C
    - Remove sup
    - All following RNA extraction steps are carried out on ice or at 4°C in the cold room
    - Add 1 ml TriFast Reagent and lyse pellet by repetitive pipetting
    - Incubate lysate for 5 min at r.t.
    - Add chloroform (200 µl/1 ml TriFast Reagent)
    - Shake tubes for 15 sec, and then incubate for 5 min at r.t.
    - Centrifuge for 15 min at 12.000 x g
    - Remove the upper aqueous phase carefully and transfer to the fresh tube containing 500 µl isopropanol
    - Incubate samples for 10 min at r.t.
    - Centrifuge for 10 min at 12.000 x g and remove (isopropanol) sup
    - Wash pellet with 1 ml ethanol (75%)
    - Centrifuge for 5 min at 12.000 x g and remove (ethanol) sup

- Dry pellets in a thermomixer at 55°C (for max. 3 min) with opened lid, do not dry completely, otherwise RNA becomes insoluble
- Add 20 µl PCR water and pipette up and down a few times until the RNA is dissolved
- Incubate lysate for 5 min at 55°C to dissolve RNA, centrifuge shortly
- Store RNA at -80°C

- ***RNA isolation from deep frozen tissue blocks***
  - Preparation
    - Fill the Styrofoam boxes with liquid N_2
    - Place the Styrofoam rack into the box and make sure it is surrounded with liquid N_2
    - Put the vials without neck in the Styrofoam rack
    - Put the sample into the Styrofoam box
    - Put the mortar into the second Styrofoam box and add some liquid N_2 to mortar and box
  - Sample preparation
    - Put 50 – 100 mg or about 5x5x1 mm of deep frozen tissue into the mortar filled with liquid N_2
    - Crush the tissue with the pistil and avoid complete evaporation of the N_2 in the mortar
    - Transfer the refined tissue and some liquid N_2 into a vial without neck
    - Keep the vial containing the sample in the Styrofoam box with liquid N_2 and avoid liquid N_2 drying out
  - TriFast utilization
    - After the tissue refinement, let the N_2 in the vial without neck evaporate and add 1 ml of TriFast reagent (per max. 100 mg of tissue)
    - Transfer the vial from the Styrofoam box on ice
    - Homogenize for 30 seconds with UltraTurrax (avoid excess heat generation)
    - Transfer the homogenated tissue into (per 1 ml Trifast) 1.6 ml reaction tube (RNase free)
    - Place the tube immediately on ice and incubate for 5 min
    - Incubate for 5 min at r.t. – from now on, work on a bench designated for RNA extraction. If necessary, samples could be stored here at -80 °C
  - RNA isolation
    - Add 200 µl chloroform
    - Vortex for 15 sec
    - Incubate for 5 min at r.t.
    - Centrifuge for 15 min at 12.000 x g / 4°C for Phase separation
    - Transfer the upper aqueous phase (containing RNA) to a fresh reaction tube
    - Centrifuge again for 15 min at 12.000 x g / 4°C
  - RNA precipitation

        - Fill fresh tube with 500 µl isopropanol
        - Transfer the aqueous phase (containing RNA) to the tube with isopropanol
        - Vortex for 10 sec
        - Incubate for 10 min on ice
        - Centrifuge for 15 min at 12.000 x g / 4 °C
        - Discard the isopropanol from the precipitate
    - RNA washing
        - Add 1ml ethanol 70 % to the pellet and vortex shortly
        - Centrifuge for 10 min at 12.000 x g / 4 °C
        - Remove the ethanol from the pellet
        - Repeat washing once
    - Solving and storing
        - Dry the pellet in a heating block at 55°C for 1 (max. 3) min with opened lid
        - Add 20 µl PCR water
        - Dissolve RNA by pipetting
        - Incubate at 55 °C for 5 min
        - Store RNA at -80 °C

5.2.2. Quantification and purity assessment of isolated RNA using spectrophotometry

Background

Quantity and purity of the isolated RNA can be determined by ultraviolet-visible (UV/Vis) spectrophotometry. The absorbance of samples is measured at 260 and 280 nm. An A_{260} reading of 1.0 is equivalent to ~40 µg/ml single-stranded RNA. The ratio A_{260} / A_{280} is used to estimate the purity of RNA samples. Values between 1.6 and 2.0 indicate a sufficiently high purity of RNA (according to PeqGOLD TriFast specifications).

Materials

- Box with ice (for transportation of samples)
- Paper towels
- Pipette tips, 10 µl, 20 µl, DNase, RNase, DNA free (e.g. Biozym, Safe seal tips professional) and appropriate pipettes
- Soft paper wipes
- Spectrophotometer (Thermo Fisher Scientific, NanoDrop (ND) 1000)
- Vortexer (e.g. Labinco, L24)

Reagents

- 10 µl solvent (here: DNase, RNase free distilled water, “PCR water” (Gibco, Invitrogen, Ultrapure) for blank measurements

Method

- Start the ND-1000 program
- Choose the button “Nucleic Acid”
- The software prompts to load a water sample – load 1 µl PCR water on the lower measurement pedestal, then click “OK”
- After initialization, clean upper and lower pedestals with a soft wipe
- Load a blank (1 µl PCR water)
- Click “Blank”
- Clean the pedestals
- Load 1 µl of sample
- Click “Measure”
- Take down the readings (e.g. concentration, 260/280 ratio)
- Clean the pedestals
- Repeat measurement for all samples
- Load a blank sample (1 µl PCR water)
- Click “Measure” – the spectrum should exhibit a relatively flat bottom line
- Clean the pedestals and close the sample arm
- Click “Exit”, then “Exit” again, to close the program

5.2.3. Quality control of isolated RNA with Experion RNA StdSens Analysis Kit

Background

A single-step RNA analysis can be performed with Experion RNA StdSens Analysis Kit from Bio-Rad. It is an automated electrophoresis system that provides detection, separation and analysis of the data within a single platform. The Experion RNA StdSens Analysis Kit is used to determine total RNA and mRNA integrity, purity, and concentration offering analysis at nanogram levels.

Materials

- Aluminum foil
- Bench top vortexer (Bio-Rad)
- Experion automated electrophoresis station (Bio-Rad)
- Experion priming station (Bio-Rad)
- Experion vortex station (Bio-Rad)
- Foam cleaning swabs (Bio-Rad)
- Latex Gloves (e.g. Hartmann, Peha-Soft powderfree)
- Microcentrifuge (1.000 – 10.000 x g, Bio-Rad)
- Pipette tips, 10 µl, 20 µl, 100µl, 200 µl, DNase, RNase, DNA free (e.g. Biozym, Safe seal tips professional) and appropriate pipettes
- Reaction tubes with flat lid, 0.2 ml, DNase, RNase, DNA free (Biozym)

- Reaction tubes with flat lid, 0.65 ml microcentrifuge tubes, DNase, RNase, DNA free(Bio-Rad)

Reagents and solutions

- DNase, RNase free distilled water, "PCR water" (Gibco, Invitrogen, Ultrapure)
- Experion electrode cleaner (Bio-Rad)
- Experion RNA StdSens Analysis Kit (Bio-Rad, for components and kit specifications (*see Table 1 and Table 2*)

Table 1. Components of the Experion RNA StdSens Analysis Kit

Item	Description
RNA StdSens	Microfluidic chips used for RNA separation
Cleaning chip	Chip used for cleaning electrodes
RNA gel	Proprietary polymeric sieving matrix
RNA StdSens stain	Proprietary fluorescent dye
RNA StdSens loading buffer	Buffer for sample preparation; contains lower marker for alignment of samples to the RNA ladder
RNA ladder	Standard containing 8 RNA fragments of 200–6,000 nt
Spin filters	Used for filtering reagents during sample preparation

Table 2. Experion RNA analysis kit specifications

Number of samples	Sample volume	Quantitation range		Limit of detection (total RNA)	Maximum salt concentration
		Total RNA	mRNA		
12	1 µl	25–500 ng/µl	25–250 ng/µl	5 ng/µl	TE buffer (10 mM Tris, 1 mM EDTA)

Method

- Prepare filtered gel (G): Pipet 600 µl RNA gel into a spin filter and centrifuge it at 1,500 x g for 10 min. Ensure that all of the gel has passed through the filter and then discard the filter.
- Prepare the Gel Stain (GS). Pipet 65 µl filtered gel into an RNase-free 0.65 ml microcentrifuge tube, add 1 µl RNA StdSens stain, and vortex for 10 sec. Wrap the tube of GS in aluminum foil to protect the stain from light.
- Once the RNA samples and RNA ladder have thawed on ice, vortex them briefly and spin down for a few sec in a microcentrifuge.
- Pipet ≥2 µl RNA sample into separate RNase-free microcentrifuge tubes. Keep all tubes on ice.

- Pipet RNA ladder into an RNase-free microcentrifuge tube: use 1 µl RNA ladder for one chip plus an extra 1 µl RNA ladder to accommodate variations in pipetting.
- Pipet 9 µl GS into the highlighted well labeled GS.
- Open the Experion priming station and put the chip onto the chip platform, matching the arrow on the chip with the alignment arrow on the chip platform.
- Close the priming station by pressing down on the lid and press "Start". A "Priming" message appears on the screen of the priming station, and the timer counts down. Priming requires ~30 sec. Do not open the priming station during the countdown.
- Open the priming station and take off the chip. Turn the chip over and inspect the microchannels for bubbles. If the chip is primed properly, the microchannels are difficult to see (compare a primed chip to a new, unused chip).
- Pipet 9 µl GS into the other well labeled GS.
- Pipet 9 µl filtered gel (G) into the well labeled G.
- Pipet 5 µl RNA loading buffer into each sample well (wells 1–12) and the ladder well, labeled L.
- Pipet 1 µl prepared RNA ladder into the well labeled L.
- Pipet 1 µl sample (or blanks, for example loading buffer, DEPC-treated water, or TE buffer) into the numbered sample wells.
- Inspect all wells for bubbles by holding the chip above a light-colored background and looking through the wells. Remove any bubbles at the bottom of a well with a clean pipet tip.
- Slide the chip into the Experion vortex station and start to vortex by pressing "Mix". Vortexing continues for 60 sec and then automatically shuts off. Remove the chip after vortexing.
- Inspect the wells again for excessive bubble formation from pipetting and that no liquid has spilled outside the wells during vortexing.
- Put the loaded chip into the Experion electrophoresis station and start the run within 5 min.
- After the run is completed remove the chip from the electrophoresis station. Insert the water chip as soon as the RNA chip is removed to prevent samples and/or buffers from drying on the electrodes.

5.2.4. Reverse transcription (RT) of isolated RNA

Background

As RNA cannot be amplified by PCR, it has to be transcribed into cDNA first [Spiegelmann, 1971]. The process requires the enzyme reverse transcriptase, specific primers for starting reverse transcription and deoxyribonucleotide triphosphates (dNTPs) for cDNA elongation. The addition of RNAse inhibitors prevents RNA degradation. At an ambient temperature of 37°C, the primers bind to RNA and induce the reverse transcription of complementary strands by the reverse transcriptase. Subsequent heating up to 85°C inactivates the enzymes. The obtained cDNA can be used for PCR.

Materials

- Box with ice
- Latex Gloves (e.g. Hartmann)
- Pipette tips, 10 µl, 20 µl, 100 µl, 200 µl, DNase, RNase, DNA free (e.g. Biozym, Safe seal tips professional) and appropriate pipettes
- Reaction tubes with flat lid, 0.2 ml, DNase, RNase, DNA free (Biozym)
- Storage box, - 20 °C (for reverse transcriptase and RNase inhibitor)
- Thermal cycler (Eppendorf Mastercycler personal)
- Vortexer (e.g. Labinco, L24)

Reagents

- DNase, RNase free distilled water, "PCR water" (Gibco, Invitrogen, Ultrapure)
- RT Kit (Applied Biosystems, "High capacity cDNA reverse transcription kit")
  - 10x RT buffer (A&B)
  - 10x RT random primers (A&B)
  - 25x dNTP Mix (100 mM)
  - Multiscribe Reverse Transcriptase, 50 U / µl
  - RNase inhibitor (20 U/µl)

Method

- Preparation of RT mastermix (10 µl per reaction)
  - Let components thaw slowly on ice (except Reverse transcriptase and RNase inhibitor)
  - Meanwhile, calculate the amounts of reagent according to the number of samples plus one aliquot in excess to accommodate variations in pipetting
  - Prepare the RT mastermix according to Table 3 adding the enzymes last (take them out of the refrigerator only in the -20 °C storage box and put them back in the refrigerator as soon as possible!)

Table 3. Recipe for 10 µl RT mastermix

Volume [µl]	Component
2.0	10x RT buffer
0.8	10x dNTP mix
2.0	10x RT random primers
3.2	PCR water
1.0	Reverse transcriptase
1.0	RNase inhibitor

    - Vortex short the RT mastermix and put it back on ice

- Preparation of the samples
    - Pipette 2 µg of RNA of each sample in a 200 µl tube
    - Add PCR water to a total volume of 10 µl
    - Add to each sample 10 µl of RT mastermix and mix by pipetting up and down
    - Store the prepared samples on ice

- Reverse transcription
    - Put all the samples in the thermal cycler
    - Set the reaction volume (thermal cycler) to 20 µl
    - Program the thermal cycler according to Table 4
    - Run the program

Table 4. Thermocycler program for RT

Step	Time	Temp [°C]
1	10 min	25
2	120 min	37
3	5 sec	85
4	Hold	4

- After the program is finished, store the transcription products (cDNA) at -20 °C.

5.2.5. Polymerase chain reaction (PCR)

Background

Polymerase chain reaction (PCR) is used for amplification of specific DNA sequences [Kramer, 2006]. This method relies on thermal cycling, which means repeated heating and cooling of the sample. The first step of the amplification is DNA melting, where two strands of the DNA double helix are physically separated at high temperature (95°C). The next step is annealing of sequence-specific primers. The temperature is lowered to 50-60°C to enable binding of primers to their target sequences. In the extension step the heat-stable DNA-Polymerase (Taq-Polymerase, derived from *Thermophilus Aquaticus)* synthetizes the complementary DNA (cDNA) strands at 72 °C so that two DNA double helices are built. These three steps compose one cycle, which is repeated several times.

Materials

- Box with ice
- Latex Gloves (e.g. Hartmann, Peha-Soft powderfree)
- Pipette tips, 10 µl, 20 µl, 100 µl, 200 µl, DNase, RNase, DNA free (e.g. Biozym, Safe seal tips professional) and appropriate pipettes
- Reaction tubes with flat lid, 0.2 ml, DNase, RNase, DNA free (Biozym) Storage box, -20 °C (for the Taq-polymerase)
- Thermal cycler (Eppendorf Mastercycler personal)
- Vortexer (e.g. Labinco, L24)

Reagents

- DNase, RNase free distilled water, "PCR water" (Gibco, Invitrogen, Ultrapure)
- dNTP Mix, 10 mM, (Fermentas, R0192)
- Forward and reverse primer as defined in Table 5 (Eurofins MWG Operons, 5 µM)
- Taq-Polymerase (Thermo Scientific, Taq DNA Polymerase (recombinant), 1U/µl, EP0404), supplied with:
  - 25 mM $MgCl_2$ (Fermentas)
  - 10x PCR buffer (Taq Buffer with KCl or Taq Buffer with $(NH_4)_2SO4$, Invitrogen)

Table 5. Primers (Eurofins MWG Operons)

Name	Sequence	Product
hFcRn forward	5'- CTCTCCCTCCTGTACCACCTTACC -3' (24bp)	457 bp
hFcRn reverse	5'- ATAGCAGGAAGGTGAGCTCCTTGT -3' (24bp)	

The α-chain gene sequence of hFcRn (*Homo sapiens (human) IgG Fc receptor hFcRn mRNA, complete cds, 1440 bp*), forward and reversed primer are indicated in blue, the resulting PCR product is shown in grey [Story, 1994]**:**

```
   1 cgggcgcaga agcccctcct cggcgtcctg gtcccggccg tgcccgcggt gtcccgggag
  61 gaaggggcgg gccgggggtc gggaggagtc acgtgccccc tcccgcccca ggtcgtcctc
 121 tcagcatggg ggtcccgcgg cctcagccct gggcgctggg gctcctgctc tttctccttc
 181 ctgggagcct gggcgcagaa agccacctct ccctcctgta ccaccttacc gcggtgtcct
 241 cgcctgcccc ggggactcct gccttctggg tgtccggctg gctgggcccg cagcagtacc
 301 tgagctacaa tagcctgcgg ggcgaggcgg agccctgtgg agcttgggtc tgggaaaacc
 361 aggtgtcctg gtattgggag aaagagacca cagatctgag gatcaaggag aagctctttc
 421 tggaagcttt caaagctttg gggggaaaag gtccctacac tctgcagggc ctgctgggct
 481 gtgaactggg ccctgacaac acctcggtgc ccaccgccaa gttcgccctg aacggcgagg
 541 agttcatgaa tttcgacctc aagcagggca cctggggtgg ggactggccc gaggccctgg
 601 ctatcagtca gcggtggcag cagcaggaca aggcggccaa caaggagctc accttcctgc
 661 tattctcctg cccgcaccgc ctgcgggagc acctggagag gggccgcgga aacctggagt
 721 ggaaggagcc cccctccatg cgcctgaagg cccgacccag cagccctggc ttttccgtgc
 781 ttacctgcag cgccttctcc ttctaccctc cggagctgca acttcggttc ctgcggaatg
 841 ggctggccgc tggcaccggc cagggtgact tcggccccaa cagtgacgga tccttccacg
 901 cctcgtcgtc actaacagtc aaaagtggcg atgagcacca ctactgctgc attgtgcagc
 961 acgcggggct ggcgcagccc ctcagggtgg agctggaatc tccagccaag tcctccgtgc
1021 tcgtggtggg aatcgtcatc ggtgtcttgc tactcacggc agcggctgta ggaggagctc
1081 tgttgtggag aaggatgagg agtgggctgc cagccccttg gatctccctt cgtggagacg
1141 acaccggggt cctcctgccc accccagggg aggcccagga tgctgatttg aaggatgtaa
1201 atgtgattcc agccaccgcc tgaccatccg ccattccgac tgctaaaagc gaatgtagtc
1261 aggccccttt catgctgtga gacctcctgg aacactggca tctctgagcc tccagaaggg
1321 gttctgggcc tagttgtcct ccctctggag ccccgtcctg tggtctgcct cagtttcccc
1381 tcctaataca tatggctgtt ttccacctcg ataatataac acgagtttgg gcccgaaaaa
```

Method

- Preparation of PCR mastermix
  - Let the components thaw slowly on ice (except TAQ-Polymerase enzyme)
  - Calculate the necessary amounts of reagent according to the number of samples plus one aliquot in excess to accommodate variations in pipetting
  - Prepare the PCR mastermix according to Table 6 adding the enzyme last (take it out of the refrigerator only in the -20 °C storage box and put them back in the refrigerator as soon as possible!)
  - Vortex short the PCR mastermix and put it on ice

Table 6. Recipe for PCR mastermix

Volume [µL]	Component
0.5	10 mM dNTP mix
0.5	forward primer
0.5	reverse primer
2.5	10x buffer
1.5	25 mM $MgCl_2$
6.0	PCR water
1,0	TAQ-Polymerase

- Preparation of the samples
  - Pipette 100 ng cDNA of each sample into a tube
  - Add PCR water to total volume of 12.5 µl
  - Prepare PCR negative control, 12.5 µl PCR water (blind, treat like a sample)
  - Add 12.5 µl PCR mastermix and pipette up and down a few times to mix
  - Store the prepared sample on ice

- Polymerase chain reaction
  - Put all the samples in the thermal cycler
  - Set the reaction volume (thermal cycler) to 25 µl
  - Program the thermal cycler according to Table 7
  - Run the program

- After the program is finished, store the PCR products at -20 °C

Table 7. Thermocycler program for hFcRn

Cycle	Step	Time	Temp [°C]
1	**1**	2 min	95
40	**2**	30 sec	95
	3	20 sec	60
	4	30 sec	72
1	**5**	5 min	72
1	**6**	Hold	4

5.2.6. Agarose Gel-Electrophoresis (GE)

Background

After PCR, the amplified cDNA of interest can be separated by size and identified by applying agarose gel electrophoresis (GE). The samples containing DNA are loaded into the pockets of the gel, which is submerged in Tris-acetic acid-EDTA (TAE) buffer solution. After the loading, a current is applied along the gel and DNA, due to its negative charge, moves to the positive charged anode. The gel delays the migration, so that larger nucleic acid molecules are more strongly retained than smaller ones. After separation procedure nucleic acids of different size appear under UV light as bands along the migration route.

To make these bands visible, the gel contains GelRed (Nucleic Acid Gel Stain), which intercalates with DNA molecules and results in fluorescence when the molecule complex is subjected to UV light. Therefore, while (trans-) illuminating the gel with UV light, nucleic acid bands can be seen and photographed.

Materials

- GE chamber (e.g. Peqlab)
- GE power supply (e.g. Peqlab)
- Latex Gloves (e.g. Hartmann, Peha-Soft powderfree)
- Microwave oven (e.g. Moulinex Micro-Chef Mo 55)
- Nitrile gloves (e.g. Meditrade Medicare, Senso Nitril)
- Pipette tips, 10 µl, DNase, RNase, DNA free (e.g. Biozym, Safe seal tips professional) and appropriate pipettes
- Plastic foil
- sterile 96 well plates (Greiner Labortechnik, PS-Mikrotiterplatte, 96K, kobaltsteril)
- UV transillumination imaging equipment (Biozym, ChemiImager 4400)

Reagents

- Agarose (Invitrogen)DNase, RNase free distilled water, “PCR water” (Gibco, Invitrogen, Ultrapure)
- Bromphenolblue (Amresco)
- EDTA, Na-Salt (Merck)
- GelRed (Nucleic Acid Stain, 0,5 ml, Biotium)
- Glacial acetic acid (Merck)
- Glycerol (Merck)
- 6x loading dye (Fermentas, 6x DNA Loading Dye)
- Ladder (Fermentas, GeneRuler 100 bp, SM0323)
- NaOH (Merck)
- Tris (Merck)
- Xylene cyanol FF (Amresco)

Preparation of buffers and solutions

- ***0.5 M EDTA stock solution***
    - Solve 93.05g EDTA Na-Salt in 400 ml A. bidest.
    - Adjust the pH to 8.0 with NaOH
    - Add A. bidest. to a final volume of 500 ml
    - Store at r.t.

- ***50x TAE stock solution***
    - Solve 242 g of Tris in 750 ml A. bidest.
    - Add 57.1 ml of glacial acetic acid
    - Add 100 ml of 0.5 M EDTA (*see above*)
    - Fill to 1000 ml with A. bidest.
    - Store at r.t.

- ***1x TAE buffer***
    - Dilute one part of 50x TAE stock solution with 49 parts A. bidest. (e.g. 20 ml + 980 ml for 1 l of 1x TAE buffer)
    - Store at r.t.

- ***6x loading dye***
    - Mix 0.25 % of Bromphenolblue
    - 0.25 % Xylene Cyanol FF
    - 30 % glycerol
    - and 69.5 % PCR water
    - Store in aliquots at -20 °C

Preparation of agarose gel

- Prepare and clean the GE chamber with combs and walls
- According to the desired concentration of the gel, mix the respective amount of agarose with 1x TAE buffer (e.g. 4 g agarose in 200 ml 1x TAE buffer for a 2,0% gel)
- Microwave the solution until the first boiling appears
- Shake the solution and let it cool down to about 60 °C
- Add 20 µl GelRed solution
- Pour the gel into the prepared GE chamber and let the gel solidify
- If not used immediately, remove the gel(s) and store them wrapped in plastic foil at 4°C

Method

- ***Gel-electrophoresis***
    - Mix 10 µl PCR-sample with 2 µl of loading dye in a well

- Repeat for all samples
- Load the sample wells and the ladder into the gel with the prepared mixtures
- Ensure correct alignment of the gel in the GE rack and close the lid
- Connect the GE electrodes to the GE power supply
- Run the gel at 150 V for 90 min (inspect the propagation of the bands during the run)

- ***Analysis of the gel***
  - Open the hatch of the ChemiImager and place the gel on the surface for UV illumination
  - Start the Chemi-Imager 4400 program
  - Click on "Acquire"
  - Activate epi-white, ensure that the aperture is not set too low
  - Align the gel and magnification so that it fills the preview completely
  - Click on "Exposure Preview"
  - Close the hatch, deactivate epi-white and activate UV
  - Change the exposure time and aperture so that the gel is correctly exposed
  - Click on "Capture Image"
  - After imaging, deactivate UV and click "Print"
  - Exit the program
  - Open the hatch, remove the gel, clean the UV surface with A. bidest. and close the hatch again
  - Scan the printed image (.tif) and process with Photoshop

5.3. Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE) and Western Blotting

Background

SDS-PAGE separates proteins derived from cell- or tissue lysates according to their electrophoretic mobility [Laemmli, 1970]. Electrophoretic mobility is a function of molecular weight, higher order protein folding, posttranslational modification, protein charge, and other characteristics.

The addition of the anionic detergent SDS to the protein samples results in denaturation of secondary and non-disulfide-linked tertiary structures. Moreover, it adds a negative charge to each protein in proportion to its mass. The addition of a reducing agent to the sample buffer (e.g. ß-mercaptoethanol) and boiling ensures disulfide bond destruction and destruction of tertiary and quaternary structure (reducing SDS-PAGE). Consequently, proteins are linearized and separated strictly by their molecular weight. Polyacrylamide (PA) gels are synthetic gels that are thermostable, strong and relatively chemically inert. Discontinuous electrophoresis combines two gels of different pore sizes and pH. The stacking gel is a large-pore polyacrylamide gel containing 4 % acrylamide and acts as a starting zone. Due to the use of Tris buffer, pH 6.8, proteins concentrate in there, which is known as Kohlrausch reaction. The

subsequent separating gel is a polyacrylamide gel of smaller pore size containing higher acrylamide concentrations (e.g. 5-30 %) and Tris, pH 8.8.

Western Blotting (Immunoblotting; [Towbin, 1979]) allows for detection of a specific protein based on antibody-antigen interaction. Following SDS-PAGE (to separate denatured proteins by molecular size), proteins are transferred to a membrane like polyvinylidene difluoride (PVDF) or nitrocellulose, where they are probed using antibodies specific to the target proteins. One method for transferring proteins from gel to membrane is electroblotting; Using electrocurrent to pull proteins from the gel into the PVDF/nitrocellulose membrane. As the result of the blotting process, the proteins are finally exposed on the surface of the membrane maintaining the organization they had within the gel. PVDF and nitrocellulose membrane both bind protein non-specifically by hydrophobic as well as charged interactions. Nitrocellulose is more fragile, but cheaper than PVDF membranes.

The uniformity and effectiveness of protein transfer from the gel to the membrane can be checked by staining all proteins transferred to the membrane non-specifically with Ponceau S. Ponceau S is a sodium salt of a diazo dye that is used for a rapid and reversible detection of protein bands on PVDF and nitrocellulose membranes. The stain is easily removed with washes at neutral pH (e.g. with PBS), facilitating subsequent immunological detection [Salinovich, 1986].

Enhanced chemoluminescence is used for detecting immobilized primary antibodies bound to specific target proteins by horseradish-peroxidase-enzyme-labeled (HRP-labeled) secondary antibodies. The latter induce enzymatic generation of light from a substrate. Emitted light is recorded on autoradiography films.
Amido black stain is applied for rapid staining of protein bands on nitrocellulose/PVDF membranes. It can detect proteins in the lower microgram range with a clear background and can serve as a loading control [Lanoix, 2012].

5.3.1. Preparation of cell and tissue lysates

Materials

- Cell scraper (IWAKI)
- Coolable Centrifuge (eg. Eppendorf, Centrifuge 5415R)
- Forceps
- Glass Pasteur pipettes (VWR)
- Homogenizer (IKA Werke, T10 basic Ultra Turrax)
- Ice box with ice
- Mortar + pistil
- Paper Towels Pipette tips (Thermo Scientific, Finntip Flex 200),
- Pipette (ThermoScientific, Finnpipette F2)
- Polystyrene Rack
- Reaction tubes, 1.6 ml, 2 ml, transparent (Biozym)
- Small vials (eg. Greiner bio-one, scintillation vial 27.0/60.0 mm, 619301; saw off neck)

- 2 Styrofoam boxes with lids
- Thermomixer Comfort (Eppendorf AG, DE)
- Vacuum pump
- Vortexer (e.g. Labinco, L24)

Reagents and buffers

- Halt Protease Inhibitor Cocktail (Pierce Biotechnology B7786)
    - EDTA Solution
    - Protease Inhibitor Cocktail
- Liquid N_2
- PBS, 1x ice-cold (for preparation, see Chapter 5.1.4.)
- T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology)

Method

- ***Protein extraction from cells***
    - Transfer cells at about 80% confluency in the culture flasks (e.g. 75 cm^2) from incubator to the lab. From now on, work only on ice!
    - Aspirate medium and wash cells 2 times with 10 ml of ice-cold PBS
    - Add 1 ml of ice-cold PBS and use cell scraper to detach cells and obtain a cell suspension
    - Transfer suspension into a 2 ml reaction tube.
    - Centrifuge cell suspension at 200 x g at 4°C for 5 min
    - Remove and discard the supernatant
    - Add ca. 500 µl (for 75 cm^2 cell flask) of T-PER Tissue Protein Extraction Reagent and add 5 µl Protease Inhibitor Cocktail (ratio 1:100)
    - Shake the suspension for 10 min at 4°C in the Thermomixer at 1400 rpm
    - Centrifuge suspension for 15 min at 4°C and at 16000 x g
    - Carefully transfer the supernatant to the new tube. The supernatant contains total proteins
    - Aliquot and store protein solutions at -20°C or immediately determine the protein concentrations in the samples

- ***Protein extraction from deep frozen tissue blocks***
    - Fill the Styrofoam boxes with liquid N_2
    - Put the Styrofoam rack into the box (make sure it is surrounded with liquid N_2)
    - Put the small vials in the Styrofoam rack
    - Put the sample (from -80 °C cooling unit) in the Styrofoam box
    - Put the mortar into the second Styrofoam box and fill mortar and box with some liquid N_2

- Place 50 – 100 mg or about 5x5x1 mm of tissue (break if necessary) into the mortar (with liquid N_2)
- Crush the tissue with the pistil and avoid complete evaporation of N_2 in the mortar
- Transfer the crushed tissue and some liquid N_2 into a vial
- Keep the vial containing the sample in the Styrofoam box with liquid N_2 and avoid drying out of the liquid N_2 in the flask
- Homogenize minced tissue with a hand-held Polytron, using a low setting to prevent foaming. Transfer homogenate to a new 1.6 ml reaction tube.
- When all samples are homogenized, let the N_2 in the vial evaporate and add 500µl of PBS to tissue, centrifuge at 1000 × g for 5 minutes at 4°C.
- Remove and discard the supernatant
- Add ca. 500 µl of T-PER Tissue Protein Extraction Reagent per tube and add 5 µl Protease Inhibitor Cocktail (ratio 1:100)
- Shake the suspension for 10 min at 4°C in the Thermomixer at 1400 rpm
- Centrifuge suspension for 15 min at 4°C and at 16000 x g
- Carefully transfer the supernatant to the new tube. The supernatant contains total proteins
- Aliquot and store protein solutions at -20°C or immediately determine the protein concentrations in the samples

5.3.2. Determination of the protein concentration using the bicinchoninic acid (BCA) assay

Background

With the BCA assay, the total concentration of proteins in a solution can be determined [Walker, 1994]. The concentration of protein is indicated by a colour change of the sample solution from green to purple in proportion to protein concentration measured by a colorimeter. Bicinchoninic acid and several other components such as cupric sulphate are present in the assay solution, which is highly alkaline. As a first step, Cu^{2+} ions from the cupric sulphate in the BCA solution are reduced to Cu^{+} by the peptide bonds of the proteins. This temperature-dependent reaction should take place at an ambient temperature of 37°C. Then, two molecules of bicinchoninic acid bind to each Cu^{+} ion forming a purple product that has a peak absorbance at a wavelength of 562 nm. The amount of protein in the sample is quantified by measuring and comparing their absorption values with absorption values of protein standards with known concentration.

Materials

- Ice bath
- Incubator, set to 37°C
- Infinite M200 PRO multimode microplate reader device
- Paper Towels
- Pipette tips (Thermo Scientific, Finntip Flex 200),
- Pipette (ThermoScientific, Finnpipette F2)
- Reaction tubes, 2 ml, transparent (Biozym)

- 96-well plate, flat bottom (Greiner Bio-One)

Reagents

- BCA Protein Assay Kit (Pierce Biotechnology)
  - Reagent A (500 ml)
  - Reagent B (25 ml)
  - Albumin standard (bovine serum albumin, 10 x 1 ml)

Method

- Pipette 25 µl of the previously prepared cell or tissue lysates into an empty well (2 repetitions per sample)
- If protein concentration in the samples is very high, dilute samples with T-PER buffer (e.g. use 5 µl of the lysates and add 20 µl of the T-PER buffer)
- The Standard included in the BCA-Assay is diluted with T-PER buffer: 1:1 (1+1), 1:5 (1+4), 1:10 (1+9), 1:25 (1+24), 1:50 (1+49) and 1:100 (1+99). These dilutions can be stored at -20°C and be reused.
- Apply 3 x 25 µl of each BSA-Standard dilution as well as of pure T-PER buffer (blank) to the plates.
- Mix reagent A (BCA Protein Assay Kit) with Reagent B (BCA Protein Assay Kit) immediately before use in a ratio of 50:1. Add 200 µl to each well containing a sample/standard/blank. Calculate the appropriate amount of reagent mixture (e.g. 26 wells filled, round up to 30 wells. Multiply with x 200 µl = 6.000 µl Reagent A; 120 µl Reagent B).
- Shake plate for 10 sec in microplate reader Infinite M200 PRO and incubate for 30 min at 37°C
- Read out samples in microplate reader Infinite M200 PRO at 562 nm
- Determine the protein concentration (µg / µl) of the samples by comparing the measured absorbance of the samples with the measured absorbance of the albumin-standards correlated with their given protein concentration using linear regression (e.g. Excel/Microsoft Office)

5.3.3. SDS-PAGE and Western Blotting

Materials

- Blotting apparatus (PeqLab, PerfectBlue Semi-Dry Electroblotter)
- Exposure cassette (Sigma, Du Pont Cronex Lightning Plus)
- Centrifuge tubes, 15 ml (TPP)
- Chemiluminescence film (GE Healthcare, Amersham Hyperfilm ECL)
- Extra Thick Blot Paper, Miniblot size 7 x 8.4 cm (Bio-Rad)
- Film-Developing machine (Agfa GP 1000)
- Gel electrophoresis apparatus (PeqLab PerfectBlue Twin S)
- Heat-block, adjusted to 95°C

- Glass Pasteur pipettes, 230 mm, sterile (VWR)
- Glass pipettes,10 ml, sterile
- Griffin beakers, 5000 ml, 500 ml, 200 ml (Vitlab)
- Incubation shaker (Scientific Industries, Roto-Shake Genie)
- Laboratory bottles, 2000 ml, 1000 ml (Schott, Duran)
- Latex examination gloves (Meditrade Medicare, Senso Skin Plus)
- Magnetic stirrer (Framo-Gerätetechnik, M21/1)
- Microtubes, 1.5 ml (Plasticbrand)
- Pipette, 100-1000 µl (Acura 825), 10-100 µl, 2-20 µl (Peqlab, Peqpette)
- Pipette tips, 1000 µl, 100 µl, 20 µl (Biozym, Premium Tips)
- Pipettor (Labnet, Fastpipette)
- pH-Meter (Metrohm, pHLab 827)
- Plastic freezer bags
- Power supply for electrophoresis apparatus (PeqLab EV231)
- Power supply for blotting apparatus (PeqLab Consort EV231)
- PVDF transfer membranes, 0.45 µm (Pall Corp. Pall BioTrac P/N 66543)
- Saran wrap
- Scale (Mettler, PJ3000)
- Scanner (Hp Scanjet 5470c)
- Thermo mixer (Eppendorf, ThermoMixer Comfort 1,5 ml)
- Vacuum aspiration system
- Vacuum bag sealer (AudioTon)
- Vortex mixer (NeoLab, 7-2020)

Reagents

- Acetic acid (Merck)
- Acetone (Merck)
- Acrylamide Bis solution 40%, 37.5:1 (Bio - Rad) for separation gel
- Acrylamide Bis solution 40%, 19:1 (Bio - Rad) for stacking gel
- Amersham Hyperfilm ECL (GE Healthcare)
- Amido black staining solution (Sigma, A8181)
- Ammonium persulfate (AMPS; Sigma)
- Bromophenol blue (LKB)
- Chemiluminescent substrate for detection of HRP (Thermo Scientific, SuperSignal West Pico Chemiluminescent Substrate 34080)
- Dry milk powder (Fixmilch Instant, Maresi)
- Film processor chemicals (Agfa, G153 Developer and G353/G354 Fixer)
- Glycerol; about 87% (Merck)
- Glycine (Merck)
- Hydrochloric acid, fuming 37% (Merck)
- Methanol (Fisher Scientific M/4000/17)

- Isopropanol (Merck)
- ß-Mercaptoethanol (Sigma)
- Ponceau S, practical grade (Sigma)
- Precision Plus Protein Western C Standards (BioRad; 250/150/100/75/50/37/25/20/15/10)
- Primary and secondary (HRP-conjugated) antibodies (*see Table 8*)
- Saran wrap
- Sodium dodecyl sulfate (SDS; Sigma)
- Tetramethylethylendiamin (TEMED; Bio-Rad)
- Tris(hydroxymethyl)aminomethane (Merck)
- Tween 20 for electrophoresis (Sigma)
- Western Blot Stripping Buffer (ThermoScientific)

Table 8. Antibodies used for Western Blotting

Primary	**Dilution**	**Secondary**	**Dilution**
Rabbit anti-human hFcRn Ab (antibody #4, directed against a peptide in the cytoplasmic tail of the receptor), [Leitner, 2006 #92]	1:100	Goat anti-rabbit IgG-HRP conjugated (sc-2004 Santa Cruz Biotechnology Inc.)	1:2000
Rabbit anti-human β2-microglobulin Ab (M8523, Sigma)	1:100	Goat anti-rabbit IgG-HRP conjugated (sc-2004 Santa Cruz Biotechnology Inc.)	1:2000
rabbit anti-human CD31/PECAM-1 Ab (M-20)-R, (sc-1506-R, Santa Cruz Biotechnology Inc.)	1:200	Goat anti-rabbit IgG-HRP conjugated (sc-2004 Santa Cruz Biotechnology Inc.)	1:2000
Goat anti-mouse serum albumin Ab (ab19194, Abcam)	1:1000	Donkey anti-goat IgG-HRP conjugated (sc-2020, Santa Cruz Biotechnology Inc.)	1:2000
Monoclonal mouse anti-α-Tubulin (T5168, Sigma Aldrich)	1:8000	Goat anti-mouse IgG-HRP conjugated (sc-2005, Santa Cruz Biotech. Inc.)	1:2000

Preparation of buffers and solutions

- ***10 % (w/v) AMPS solution***
    - Dissolve 1 g AMPS in a final volume of 10 ml A.bidest.
    - Aliquote (1 ml) and store at -20°C

- ***10 % (w/v) SDS solution***
    - Dissolve 10 g SDS in a final volume of 100 ml A.bidest.
    - Store at r.t.

- ***3 M Tris-HCl, pH 8.8 (500 ml)***
    - Dissolve 181, 7 g Tris in A.bidest. (~450 ml)
    - Adjust pH 8.8 with HCl
    - Adjust to a final volume of 500 ml

- ***0.5 M Tris-HCl, pH 6.8 (100 ml)***
    - Dissolve 6, 1 g Tris in A.bidest. (~80 ml)
    - Adjust pH 6.8 with HCl
    - Adjust to a final volume of 100 ml

- ***4x SDS-PAGE sample buffer (stock solution)***
    - Mix and dissolve by stirring
        - 5 ml 0.5 M Tris-HCl, pH 6.8
        - 3 ml Glycerol
        - 2 ml β-mercaptoethanol
        - 0.8 g SDS
        - 5 mg Bromphenol blue
    - filter and store in aliquots at -20°C

- ***1x SDS-PAGE sample buffer (working solution)***
    - Mix 1 vol. 4x stock solution and 3 vol. A.bidest.
    - Final concentration
        - Tris 62.5 mM
        - Glycerol 6.5 % (v/v)
        - SDS 2% (w/v)
        - β-mercaptoethanol 5 % (v/v)

- ***10x running buffer (stock solution)***
    - 30 g Tris (0.25 M)
    - 144 g Glycine (1.92 M)
    - 10 g SDS (1% w/v)
    - Dissolve and bring to a final volume of 1000 ml A.bidest.
    - Store at r.t.

- ***1x running buffer***
    - Mix 1 vol. 10x stock solution and 9 vol. A.bidest. (GFL Bi-Dest).
    - Store at r.t.
    - Final concentration
        - Tris 25 mM
        - Glycine 192 mM
        - SDS 0.1% (w/v)

- ***Transfer buffer***
    - 5.8 g Tris (48 mM)
    - 2.93 g Glycin (39 mM)
    - 0.375 g SDS (0.0375 % (w/v))
    - 200 ml MeOH (20% (v/v))
    - Dissolve and bring to a final volume of 1000 ml A.bidest.
    - Store at r.t.

- ***Wash buffer***
    - PBS (1x (see Chapter 5.1.4.)
    - 0.1% (v/v) Tween 20 (i.e. 1 ml Tween 20 + 999 ml PBS)

- ***Ponceau red staining buffer***
    - 0.5 g ponceau red (0.1% (w/v))
    - 25 ml acetic acid (5% (v/v))
    - Dissolve and bring to a final volume of 500 ml A.bidest.

- ***Ponceau red destaining solution***
    - 5 ml acetic acid (1% (v/v))
    - Dissolve and bring to a final volume of 500 ml A.bidest.

- ***Blocking buffer (=Blotto)***
    - 25 g dry milk powder (5% (w/v))
    - Dissolve and bring to a final volume of 500 ml PBS containing 0.1% (v/v) Tween 20

- ***Amidoblack destaining solution***
    - 25 % (v/v) isopropanol
    - 10 % (v/v) acetic acid
    - 65% A.bidest.

Methods

- ***Sample preparation***
    - To load equal amounts of proteins per sample. (e.g. 40 µg / lane), the protein concentration in each sample should be determined (by e.g. BCA-assay). The volume per sample containing the desired protein amount must be calculated
    - Preferable, the protein concentration in the sample is high enough to mix the calculated sample volume directly with a 4x SDS sample buffer. The final volume loaded on the gel depends on the type of spacers used and the number of slots / gel (the manual of the electrophoresis apparatus details volume sizes for each gel size, spacer size and combs).
    - Assuming a maximum loading volume of 20 µl, one could therefore either mix the sample (volume containing e.g. 40 µg should be < 15 µl) with 5 µl of 4x SDS-PAGE sample buffer and if necessary - fill up to 20 µl with A.bidest.
    - If the concentration of the proteins in the sample is too low to have the desired protein amount in 15 µl, the required sample volume (e.g. 80 µl) must be mixed with ice-cold acetone (best 10x of sample volume, i.e. 800 µl, at least it should be 5x vol.) and incubated on ice for 30 min. The precipitated proteins are now collected by centrifugation for 15 min at 4°C at 14.000 x g. The supernatant is removed (invert tube for 3-5 min) and the pellet is dissolved in 20 µl of 1 x sample buffer (place in thermoshaker for 10 min and shake to dissolve.
    - Incubate samples for 3 min at 95°C, spin briefly
    - If not used immediately for SDS-PAGE, store samples at -20°C
    - Before use, heat again, collect fluid by centrifugation and load on gel

- ***Preparation of separation gels***
    - All materials used (plates, combs, spacers) must be clean (washed with detergent and water, followed by 70% ethanol) and dry
    - Assemble Aluminium-Hydroxid plates, spacers (0.75 mm) and glass plates, respectively, to build the casting stand

- Mix first 4 ingredients listed in table 9 and add AMPS and TEMED immediately before pouring the gel, as they induce the gel polymerization
- Pour gel-solution into casting stand and leave place for stacking gel (~2 cm)
- Overlay each gel with isopropanol to ensure vertical surfaces
- Allow gels to polymerize for at least 1 h at r.t.
- Gels not used immediately can be wrapped in saran wrap, placed in a wet-chamber and stored for a maximum 2 weeks at 4°C

Table 9. Recipe for separation gels

%	Acrylamide Bis solution (40%) 37.5:1	Tris-HCl (3 M, pH 8.8)	SDS (10%)	A.bidest.	AMPS (10%)	TEMED
12	15 ml	5 ml	0.4 ml	27.8 ml	0.4 ml	40 µl
15	16.9 ml	5 ml	0.4 ml	21.6 ml	0.4 ml	40 µl

- ***Preparation of stacking gel***
  - Fix the number of gels required for the experiment in the gel electrophoresis apparatus
  - Remove any remaining fluid on the surface of the separation gel using e.g. filter paper
  - To prepare the stacking gel, mix required buffers and solutions as indicated in Table 10, again add AMPS and TEMED immediately before gel pouring
  - Overlay the separation gel with stacking gel solution and insert a cleaned comb (15 wells, 0.75 mm) carefully preventing inclusion of air bubbles
  - Allow gel to polymerize for at least 30 min at r.t.
  - Remove combs
  - Proceed with electrophoresis

Table 10. Recipe for stacking gel

%	Acrylamide Bis solution (40 %) 19:1	Tris-HCl (0.5 M, pH 6.8)	SDS (10%)	A.bidest.	AMPS (10%)	TEMED
4	1.5 ml	1.9 ml	150 µl	11.6 ml	150 µl	20 µl

- ***Electrophoresis***
  - Add 1x running buffer to the upper and lower buffer reservoirs of the electrophoresis apparatus
  - Load samples and markers into the sample wells of the gels

- Fill empty sample wells with 1x SDS-PAGE sample buffer and run the gel at 150 V until the blue dye (Bromphenol blue) in the sample buffer has reached the bottom of the gel
- Turn off the power supply and disassemble the electrophoresis apparatus

- ***Transfer of proteins to PVDF- membranes using a Semi-dry - Electro blotter***
  - While running the electrophoresis, prepare 2 pieces of extra-thick blotting paper per gel and 1 piece of PVDF membrane per gel with the same size as the gel
  - Submerge the PVDF membrane first in methanol (for 1 min), then in A.bidest, and finally in blotting buffer (~10 min). As the PVDF membrane is extremely hydrophobic will not wet in aqueous solutions until pre-wetted with methanol
  - Submerge the filter papers in blotting buffer
  - After electrophoresis, remove stacking gel from separation gel and submerge gel in transfer blotter for 5 min
  - Assemble blotting apparatus in the following way: Anode - filter paper - PVDF membrane - separation gel - filter paper - Cathode
  - The components of the stack should be wet but there should not be excess fluid
  - Avoid inclusion of air bubbles when building the stack!
  - Transfer proteins for 90 min at 3 mA / cm^2 of gel

- ***Ponceau staining of membranes***
  - After transfer, disassemble blotting apparatus (clean all components)
  - Stain proteins on PVDF membrane by incubating the membrane in Ponceau S solution for 5 min (shaking)
  - Remove excess dye by using Ponceau S destaining solution for 5 min at r.t. (shaking)
  - Wrap in saran wrap and scan blot to preserve the protein pattern; save as .tif-file
  - Thereafter, wash membrane with PBS for 5 to 10 min at r.t. to completely remove Ponceau S staining from blot

- ***Blocking of unspecific binding sites on the membrane***
  - Incubate blots with blocking buffer for 1 h at r.t. (alternatively incubate at 4° overnight/o.n.) to prevent unspecific binding of Abs to PVDF membranes

- ***Antibody binding***
  - For each incubation step, 2 ml Ab-solution per blot are prepared at the dilutions indicated in Table 8. Ab-dilutions are prepared in blocking buffer
  - For incubation, each membrane (Blot) is sealed in a plastic bag to minimize Ab consumption
  - Blots are incubated on a shaker with first antibody o.n. at 4°C (or for 1 h r.t.)

- Wash blots at r.t. with wash buffer, change buffer at least 3 times and do each washing step for at least 15 min
- Incubate blots for 1 h at r.t. (or at 4° o.n.) with HRP-conjugated, secondary antibodies. For dilutions see Table 8. Ab-dilutions are prepared in blocking buffer
- Wash blots at r.t. with wash buffer, change buffer at least 3 times and do each washing step for at least 15 min

- ***Detection of HRP-labelled antibody-complexes bound to membranes***
  - Mix solution A + B of the chemoluminescence reagent according to instructions of the manufacturer (final volume 0.1 ml / cm^2 or 2 ml / blot)
  - Drain the excess wash buffer from the washed membranes
  - Incubate surface of each membrane with detection reagent for the time indicated
  - Drain off excess detection reagent by holding blot against a paper towel
  - Place the blot in a fresh piece of saran wrap and gently smooth out any air bubbles
  - Place the wrapped blots (protein side up) in an X-ray film cassette
  - Place a sheet of Hyperfilm ECL (adjust size!) on top of the membrane
  - Close the cassette and expose for a certain time (e.g. 5 sec to several minutes)
  - Remove and develop the first piece of the film immediately and on the basis of its appearance estimate how long to continue the exposure of the second piece of film (this can vary from a few seconds to 1 h)
  - Scan the developed films and store data as .tif-files

- ***Stripping of membranes***
  - Wash blot with PBS
  - Incubate 5-15 min at r.t. with restore Western Blot Stripping Buffer
  - Wash with PBS
  - Start new antibody incubation with blocking of unspecific binding sites (Blocking buffer!)

- ***Amido black staining (perform at the end as a loading control)***
  - Blots are stained for 1 min in amido black staining solution
  - Blots are destained for 30 min in amido black destaining solution
  - Scan the stained blots and store data as .tif-files

5.4. Immunofluorescence microscopy (IFM)

Background

IF staining is a method employed to study the occurrence and location of proteins in tissues *in situ* or in cultured cells, using a fluorescence-labeled Ab, which is visualized by a fluorescence microscope [Robertson, 2011].

Staining of samples is done using the antigen-Ab technique. To enhance signals, often indirect IFM is applied. A primary, unconjugated Ab recognizes a target antigen and binds to it. Primary Ab is raised in a host species different from the target tissue and can either be poly- or monoclonal. After primary Ab has bound to its target, a secondary Ab is applied. Secondary Ab specifically binds to the primary Ab based on the primary Ab's host species. Secondary Ab is conjugated to a fluorescent dye, which emits light of specific wavelength after excitation and thereby allows the detection of a target antigen.

In each IFM experiment a negative control is of great importance inspecting that the fluorescence comes only from specific binding of the secondary Ab. For the negative control either primary Ab is omitted, or it is replaced by IgG or the whole serum (originating from primary Ab's host species) that was not stimulated previously with the antigen of interest.

Before staining, tissues and cells in culture need to be pre-processed accordingly. Fixation is important to ensure that the structure is maintained. Fixation can be done using a variety of chemicals, such as formaldehyde, methanol or HOPE-solution. Fixed tissue samples, which are usually much thicker than cell layers, must then be sectioned prior to staining to guarantee access of Abs to the proteins of interest. To enable sectioning, the tissue needs to be either frozen in liquid N_2 (cryo-section) or embedded in paraffin (paraffin sections). Afterwards depending on the sectioning method the preparation of the tissue for subsequent staining is different. With paraffin sections the procedure is more complex: the paraffin has to be removed and the tissue has to be rehydrated using descending alcohol series. Moreover, the rehydration procedure depends on the chemicals used for fixation.

For a tissue embedded in paraffin, antigen retrieval has to be done, as paraffin in association with chemicals can mask target antigens. This is done by boiling the sections in special buffers such as citrate buffer at pH 6 or citraconic anhydride 0.05% or Tris-EDTA at pH 9.

After rehydration step, the tissue has to be permeabilized using detergents (e.g. Triton X-100 or saponin). This ensures that primary Ab can access its target and is not hindered by the membranes. The unspecific binding of primary Ab is prevented by incubation of samples with blocking serum from the animal the secondary Ab was derived from or with a protein dissolved in buffer such as bovine serum albumin (BSA) to reduce the background fluorescence.

Multiple staining are performed if more than one protein has to be detected in the same sample. In this case primary Abs against the different proteins have to be derived from different species.

After the staining procedure for the proteins is completed, to make the nuclei of the cells visible the tissue is usually incubated with "nucleic acid dye" like 4',6-diamidino-2-phenylindole (DAPI).

Finally, the tissue sections or cells are embedded in a mounting medium specific for fluorescence dyes such as Fluoromount or Mowiol.

5.4.1. Sampling, fixation and embedding of placental tissue for applications in IFM

Background

The Hepes Glutamic Acid Buffer Mediated Organic Solvent Protection Effect (HOPE) technique provides an exquisite preservation of protein antigenicity together with well-presented morphological details in paraffin embedded tissues [Blaschitz, 2008].

Materials

- Electrically heated forceps ECEP (Medite)
- Embedding cassettes (e.g. Medite)
- Embedding molds (e.g. Engelbrecht 24 x 24 x 5 mm)
- Ice bath
- Incubator up to 60 °C
- Microtome blade S 35 Feather 35 °C (cutting of tissue)
- Permanent marker (labeling for embedding cassettes, e. g. Medite)
- Pipette, 100-1000 µl (Acura 825)
- Pipette tips, 1000 µl, 100 µl, (Biozym, Premium Tips)
- Scissors
- Tissue Embedding unit TES 99 consisting of dispenser heating and cooling unit (Medite)
- Trimming handle (cutting of tissue)
- Tubes (acetone resistant) 100 pcs
- Tweezers

Reagents

- HOPE I (ready-protective solution) 500 ml (DCS)
- HOPE II (concentrated solution) 1 ml (DCS)
- Low melting paraffin (52-54 °C) 1 x 2 kg (DCS)
- Pure acetone (Fisher Chemical)

Method

- Place placenta on the fetal side (cord), maternal side up. Cut out samples using scissors
- Cut these pieces into 1 cm thick slices (if sample are too thick, fixation will only occur at the outside, but not in the center of the sample)

- Place pieces of tissue separately into the tubes containing HOPE I solution at 0 to 4 °C (ratio: tissue 1,5 x 1 x 1 cm tissue / about 5 ml HOPE I). Fixation duration is minimal 24h, but the tissue can stay up to several days in the HOPE I solution.
- Cut the tissue to 1,5 x 1 x 1 cm blocks (embedding size)
- Remove HOPE I solution and add the ice-cold HOPE II/acetone solution (mix 100 ml of pure acetone with 100 µl of HOPE II, ratio: 1,5 x 1 x 1 cm tissue / about 5 ml HOPE II) to the tissues and transfer immediately back into refrigerator or ice bath. Incubate for 2h at 0 to 2 °C
- Drain tubes and add pure ice-cold acetone, incubate for 2h at 0 to 2 °C, repeat this step twice
- Remove acetone and add immediately pre-warmed low-melting paraffin. Incubate o.n. at exactly 54-55 °C (incubator), avoid trapping of air bubbles in the paraffin (no cap on the tube)
- Pour paraffin blocks with the addition of fresh paraffin in the usual way (use TES 99)
- Store finished blocks and cuts at 4 °C
- Table 11 gives an overview on the HOPE fixation procedure

Table 11. HOPE fixation procedure

Reagent	Incubation time
HOPE I solution	Minimal 24 hours
HOPE II/acetone solution	2 hours
Pure acetone	2 hours
Pure acetone	2 hours
Pure acetone	2 hours

5.4.2. Preparation of paraffin sections from embedded tissue samples

Materials

- Brush (e.g. LIBRO Aquarell-Hairbrush)
- Cooling plate (Medite TES 99)
- Feather blade microtomes
- S 35 Standard Blade for routine biopsis
- Glas slides, Superfrost Ultra Plus (VWR) for immunohistochemistry, immunofluorescence (coated)
- Kleenex
- Microtome (Microm HM 400)
- Pencil to marking of slides (Medite)
- Preparation needle (AKH)
- Rotilabo-slide boxes (Cari Roth)
- Slide rack (metal bracker slides AKH, Bracket AKH)
- Water bath (Histo-line Laboratories, TEC-2601)

Method

- The paraffin tissue blocks are precooled (4°C) for approx. 30 min on the cooling plate
- After fixation in the microtome, 2-4 µm thick sections are cut and transferred (shining side downward) with a brush to the water bath (adjusted to approx. 40 °C)
- If necessary, the paraffin block must be cooled down again between individual cuts for a couple of seconds
- After the cut has stretched out on the water bath, it is transferred to a pre-marked slide with the preparing needle (note the correct orientation of the cut)
- The slides are subsequently dried on side of the water bath (minimal 10 min)
- Slides are transferred to slide rack and dried overnight at 37 °C
- Slides are stored at 4 °C (HOPE-fixed) in a slide box until IFM staining

5.4.3. IFM on placental tissue sections

Materials

- Cool-able Centrifuge (Eppendorf 5415R)
- Cover glasses (VWR International)
- Dako Pen (DAKO)
- Food Steamer (Multigourmet FS10, Braun)
- Glass cover slips (Menzel Gläser)
- Heating Block (Thermo mixer 5436, Eppendorf)
- Ice Machine (Ziega)
- Latex gloves (Hartmann, Peha-Soft powder free)
- Microwave (AKL 520, IGNIS)
- pH-Meter (Metrohm)
- Pipettes (Biozym)
- Pipette tips: 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl (Biozym)
- Reaction tubes 1,6 mL (Biozym)
- Soft paper wipes (Kleenex)
- Vortexer (Labinco L24)

Reagents

- Animal sera (e.g. goat; Jackson Immuno Research)
- Antibodies (*see Table 12*)
- Citric acid monohydrate (Merck)
- Citraconic anhydride (Sigma)
- 4,6-diamidino-2-phenylindole (DAPI) (Roche, diluted 1:5000 in PBS)
- Disodium phosphate dihydrate (Na_2HPO_4 x $2H_2O$) (Merck)
- Ethylenediaminetetraacetic acid (EDTA) (Merck)
- Ethanol absolute (VWR Prolabo)
- Fluoromont G (Southern Botech)

- Saponin (Sigma)
- Sodium chloride (NaCl) (Merck)
- Tri-sodium citrate dehydrate (Sigma)
- Tris Base (Merck)

Table 12. Applied antibodies for IFM on tissues

Primary	Dilution	Secondary	Dilution
Rabbit anti-human hFcRn Ab (antibody #4, directed against a peptide in the cytoplasmic tail of the receptor), [Leitner, 2006]	1:50	Alexa Fluor 568 goat anti-rabbit IgG (A-11011, Molecular Probes/Invitrogen)	1:1000
Mouse anti-human CD31/PECAM-1, Clone JC70A, (M0823, Dako)	1:100	Alexa Fluor 647 goat anti-mouse IgG, (A-21235, Molecular Probes/Invitrogen)	1:1000
Rabbit monoclonal anti-human FcgammaRIIb2 (C-Term) Ab (Epitomics/Senova)	1:50	Alexa Fluor 568 goat anti rabbit IgG, (A-11011, Molecular Probes/Invitrogen)	1:1000
Monoclonal mouse anti-human Cytokeratin 7 (Clone OV-TL12/31, Dako)	1:100	Alexa Fluor 647 goat anti mouse IgG, (A-21235, Molecular Probes/Invitrogen)	1:1000

Solutions

- ***PBS, 1x sterile*** (see Chapter 5.1.4.)

- ***Blocking buffer***
  - PBS with 0,05% (w/v) saponin and 5% (v/v) animal (e.g. goat) serum

- ***Retrival solution***
  - 0,5 g citraconic anhydride dissolve in 1000 ml A.bidest.
  - Adjust pH to 7.4
  - Store at 4 °C

Method

- ***General remarks***
  - From the beginning of rehydration until embedding of the paraffin sections, the tissue sections must be kept wet

- To avoid drying of sections during incubation in small volumes all incubation steps are performed in a wet chamber
- Slides should be handled very carefully to avoid detaching
- All solutions used for incubations are applied directly with a pipette

- ***Single staining***
  - Paraffin melting at 60 ºC for 25 min
  - Rehydration of the tissue in:
    - Isopropanol I pre-warmed at 60 ºC for 10 min
    - Isopropanol II pre-warmed at r.t., washing
    - Acetone I (cool) at +4 ºC for 10 min
    - Acetone II (cool) at +4 ºC for 10 min
    - A.bidest. 2 x 5 min
  - For antigen retrieval, the slides are placed in hot retrieval solution and boiled for 15 min. Then, they are cooled to r.t. for 20 min, and washed 2 x 5 min in PBS at r.t.
  - Tissue sections are encircled with Dako Pen to minimize the volume of solutions to be applied
  - For tissue permeabilization and blocking of non-specific Ab-binding, the tissue is incubated with appropriate blocking buffer with Saponin for 30 min at room temperature
  - For incubation with primary Ab, the primary Ab is applied after dilution in blocking buffer o.n. at 4 ºC in a humidified chamber
  - Sections are washed with PBS 3 x 5 min at r.t.
  - For incubation with secondary Ab, the secondary Ab is applied for 2 h at r.t. in a humidified chamber in the dark
  - Sections are washed in PBS 3 x 5 min at r.t.
  - For staining of nuclei, specimens are incubated with DAPI for 10 min in the dark at r.t.
  - Slides are washed 3 x 10 min in PBS, and there after rinsed briefly in A.bidest.
  - Finally, slides are mounted with Fluoromount G and cover-slipped.

- ***Multiple staining***
  - If primary Abs against different cellular proteins are derived from different animal species, they can be applied in the same tissue. In this case, the first protein is detected with the respective primary and secondary Abs as described in the chapter *single staining.* Before application of the DAPI stain, the staining for the second protein starts with the application of a respective blocking buffer.
  - For all specimens, control tissue sections must be processed identically and at the same time, replacing primary Ab with blocking buffer or non-reactive IgG.

5.4.4. IFM on cultured cells

Materials

- Autoclave (Odelga)
- Automated cell counter (Bio-Rad)
- 4-well chamberslides , #1,5 borosilicate, sterile (Lab-Tek II, Nalge Nune International)
- Glass cover slips (Menzel Gläser)
- Glass pipettes, sterile (5/10/25 ml)
- Glass Pasteur pipettes (VWR International)
- Incubator (Heraeus Cytoperm 2, Thermo Electron Corporation)
- Laminar Flow (Hera Safe, Thermo Scientific)
- Osmometer (Osmomat 030-D, Gonotec)
- pH-Meter (827 pHLab, Metrohm)
- 24-well plates, sterile (Iwaki)
- Tissue culture flasks, 25cm^2/canted neck, sterile (Iwaki)
- Vacuum pump

Reagents

- Ammoniumchloride (NH_4Cl, Sigma)
- Animal sera (e.g. goat; Jackson Immuno Research)
- Antibodies (*see Table 13*)
- 4,6-diamidino-2-phenylindole (DAPI) (Roche, diluted 1:5000 in PBS)
- Disodium phosphate dihydrate (Na_2HPO_4 x $2H_2O$) (Merck)
- Ethylenediaminetetraacetic acid (EDTA) (Merck)
- Ethanol absolute (VWR Prolabo)
- Fluoromont G (Southern Biotech)
- Human IgG Endobulin S/D IgG anti-Endobulin (Baxter)
- Paraformaldehyde (PFA, Merck)
- Potassium dihydrogen phosphate (KH_2PO_4) (Merck)
- Potassium chloride (KCl) (Merck)
- Saponin (Sigma)
- Sodium hydroxide (NaOH) (Merck)
- Tri-sodium citrate dihydrate (Sigma)
- Tris Base (Merck)

Table 13. Applied antibodies for IFM on cultured cells

Primary	Dilution	Secondary	Dilution
Human IgG Endobulin S/D IgG anti-Endobulin (Baxter)	1:400	Goat anti-human IgG-488 (Invitrogen Molecular Probes)	1:1000
LAMP purified mouse anti-human CD107b (Pharmingen)	1:200	Alexa Fluor 647 goat anti mouse IgG (A-21235, Molecular Probes/Invitrogen)	1:1000
EEA1 monoclonal mouse anti-human Ab (Abcam)	1:50	Alexa Fluor 647 goat anti mouse IgG (A-21235, Molecular Probes/Invitrogen)	1:1000

Buffers and solutions

- ***PBS, 1x sterile*** (*see Chapter 5.1.4.)*
- ***HBSS, sterile***
- ***TrypLE, sterile***
- ***Culture medium for BeWo cells*** *(see Chapter 5.1.4.)*
- ***Culture medium for BeWo + hFcRn5 cells*** *(see Chapter 5.1.4.)*
- ***Culture medium for fetal endothelial cells, Endothelial Growth Medium (EGM)*** *(see Chapter 5.1.4.)*
- ***1x Trypsin/EDTA solution, sterile*** *(see Chapter 5.1.4.)*
- ***1% Gelatin solution*** *(see Chapter 5.1.4.)*

- ***4% PFA in PBS***
    - Heat 800 ml of 1x PBS to approximately 60 °C (avoid boiling)
    - Add 40 g of PFA powder to the heated PBS solution
    - Add dropwise 10 mM NaOH until the solution clears
    - Cool and filter the solution
    - Adjust the volume to 1 l with 1x PBS
    - Adjust the pH to approximately 7.4 with small amounts of diluted HCl
    - Aliquote and store at -20°C

- ***1M NH_4Cl in PBS (stock solution)***
    - Add 5,35 g of NH_4Cl to 80 ml 1x PBS
    - Mix until solution is clear, add PBS up to 100 ml
    - Store at 4°C.

- ***50 mM NH_4Cl in PBS***
    - Dilute 500 µl of NH_4Cl stock solution in 9,5 ml 1x PBS
    - Store at 4°C

- ***1M HEPES***
    - Solve 2,383 g in 8 ml A.bidest.
    - Adjust pH to 7,4 with 10 mM NaOH
    - Store at 4°C

- ***0,5M MES***
    - Dissolve 0,976 g in 8 ml A.bidest.
    - Adjust pH to 6,0 with 10 mM NaOH
    - Store at 4°C

- ***Cell culture medium, pH 7.4, 20mM HEPES***
    - Mix 49 ml of respective cell culture medium with 1 ml of 1M HEPES
    - Adjust pH to 7,4
    - Store at 4°C

- ***Cell culture medium, pH 6.0, 20mM MES***
    - Mix 48 ml of respective cell culture medium with 2 ml of 0,5M MES
    - Adjust pH to 6.0
    - Store at 4°C

Method

- ***Seeding of BeWo cells for IFM***
    - Add 13 mm glass coverslips (sterile) to wells of sterile 24-well plate
    - Detach cells with trypsin/EDTA treatment and add detached cells to wells:
        - Aspirate and discard old medium
        - Wash cells once with PBS solution to remove debris and trypsin inhibitor contained in medium
        - Add 500 µl of Trypsin/EDTA solution

        - Incubate for about 5min/until cells detach
        - Add 4,5 ml culture medium and mix by pipetting up and down a few times
        - Add 2 ml cell suspension to the 15 ml tube containing 12 ml culture medium, mix by pipetting up and down a few times
        - Transfer 1 ml to each well
    - Keep 24-well plate in laminar flow for 30 min
    - Grow cells for 1 day in incubator at 37°C
    - From then on, cells can be handled at normal lab.

- ***Seeding of FEC (HPEC) cells for IFM***
    - At confluency, wash cells once with HBSS to remove debris and trypsin inhibitor contained in medium
    - Add 1,5ml TrypLE per 75 cm^2 culture flask
    - Incubate for about 5 min/until cells are properly detached
    - Add 4,5 ml culture medium and mix by pipetting up and down a few times
    - Determine cell number in cell suspension with automated cell counter (cells/ml)
    - Seed out 1x 10^5 cells/ 4-well chamber slide
    - Keep 4-well chamber slides in laminar flow for 30 min before returning to incubator
    - Cells are grown for 1-2 days in incubator at 37°C
    - For IFM staining, cells can be handled at normal lab.

- ***Staining protocol***
    - Wash cells with 1 ml 1x PBS (2 times)
    - Fix with 1 ml 4% (w/v) PFA/PBS for 20-30 min at r.t. .
    - Wash with PBS
    - Wash and quench aldehyde groups with 1 ml 50 mM NH_4Cl in PBS for 10 min at r.t.
    - Wash with PBS
    - Block and permeabilize cells with 0.5 ml PBS containing 5% goat serum and 0,05% saponin (blocking solution) for 30 min at r.t.
    - Incubate 1 h at r.t. with 50 µl primary antibody diluted in blocking solution according to Table 13
    - Wash 3 times with 1 ml PBS (10 min each step slightly shaking)
    - Incubate 1 h at r.t. with 50 µl secondary antibody in blocking solution according to Table 13 in the dark
    - Wash cells with PBS
    - Incubate with DAPI solution for 10 min
    - Wash 3 times with 1 ml PBS
    - Dip coverslip in A.bidest., remove excess water (paper towel) and embed in Mowiol-solution on glass slides, store in box in the dark at 4°C

- ***Internalization of human IgG at different pH***
    - Seed cells as described above
    - Wash the cells 2 times with 1x PBS
    - Incubate cells with respective culture medium pH 7.4 or pH 6.0 containing hIgG (100/1000 ng/µl) for 30 min at 37°C in water bath
    - Wash cells with cold 3 x PBS to stop the internalization
    - Start with fixation step of the staining protocol as detailed above, if necessary apply two different primaries and two different corresponding secondary Abs as detailed for tissue section labeling
    - Controls are processed identically and at the same time, except that they are not exposed to primary Ab (in case of internalization, they are not exposed to hIgG Ab).

5.4.5. Image acquisition

The stained sections were viewed and images were digitized using two systems: the TissueFAXS system (TissueGnostics GmbH, Vienna, Austria) and a confocal scanning microscope (Carl Zeiss Microscopy GmbH).

TissueFAXS system

The TissueFAXS system is a fully automated microscopic system, where a ZEISS Axio Imager Z1 microscope is driven by the TissueFAXS software (TissueGnostics GmbH, Vienna, Austria). Objectives used were the EC-Plan Neofluar 2.5 x / 0.07 (for Preview) and the EC-Plan Neofluar 20 x / 0.5 (for final image acquisition). The camera was a PCO Pixel fly which acquires black/white images from every single fluorescence channel used. Images of different channels can be labeled with false colours and merged, if desired. Images (.png) were processed with Adobe Photoshop.

Confocal scanning microscope

The confocal microscope is used to obtain optical images with high contrast as well as high resolution in three dimensions x, y and z by scanning a sample sequentially point by point, or multiple points at once, and assembling the pixel information to one image. Therefore, confocal microscopy is often applied in life sciences as well as for topographic tasks on materials surfaces. Confocal microscope was used to investigate the localization of hFcRn in single cells. Images were taken with a ZEISS Axiovert 200M microscope (inverted) driven by Volocity software (PerkinElmer Vertriebs GmbH). A Plan-Neofluar 63 x / 0,95 NA Korr. Objective was used for acquisition. Images of different channels were merged, if desired. Images (.png) were processed with Adobe Photoshop.

6. RESULTS and DISCUSSIONS

6.1. Localization of the hFcRn at the human placental barrier

6.1.1. Determination of anti-hFcRn antibody (Ab) specificity by Western Blotting (WB)

Human placental chorionic tissue expresses hFcRn α-chain [Leach, 1996], [Simister, 1996]. To investigate hFcRn α-chain localization, the specificity of several antibodies (Abs), directed against hFcRn α-chain was compared by WB method on total placental lysates using lysates of hFcRn α-chain overexpressing 293T cells (293T + hFcRn) as positive controls and lysates of 293T cells as negative controls. Three affinity-purified rabbit anti-hFcRn α-chain antisera raised against different peptides sequences of hFcRn α-chain, previously produced by Dr. I. Ellinger (partly published: [Leitner, 2006]), as well as a commercially available Ab (H-274; Santa Cruz Biotechnology) were tested.

As demonstrated in *Fig11*, the three affinity-purified Abs directed either against the extracellular domain of hFcRn α-chain (Ab #1 and #3) or against the cytoplasmic domain (Ab #4) recognised predominantly a 45 kDa protein in placental lysates, which was also detected in 293T + hFcRn lysates but not in 293T lysates. Among these Abs, Ab #4 exhibited the highest specificity for hFcRn α-chain [Leitner, 2006] and was therefore used in the subsequent experiments for hFcRn α-chain detection. In contrast, a commercial Ab (H-274, Santa Cruz) directed against the N-terminal extracellular domain, demonstrated crossreaction with several proteins in placental lysates.

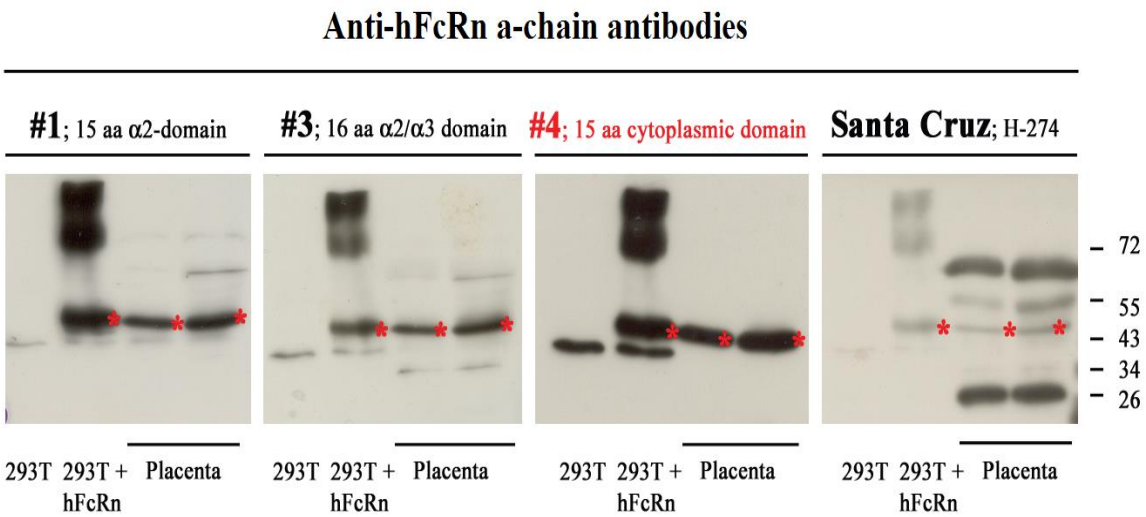

Figure 11. *Detection of anti-hFcRn α-chain Abs specificity by WB;*

293T cell lysates as negative control and hFcRn overexpressing 293T cell lysates as positive control (both Santa Cruz Biotechnology, 7.5 µg per lane) together with human placental lysates (30, 60 µg per lane) were separated by 12% SDS-PAGE and transferred onto PVDF membrane; blots were incubated with anti-hFcRn Abs and secondary HRP-conjugated Abs (Santa Cruz Biotechnology) and developed using a chemoluminescence substrate (Thermo Scientific); red stars indicate hFcRn α-chain protein (~ 45 kDa)

6.1.2. Localization of hFcRn α-chain in human placental chorionic tissue

Localization of hFcRn α-chain in placental chorionic tissue was first investigated by indirect Immunofluorescence Microscopy (IFM) using rabbit anti-hFcRn α-chain Ab #4 and a fluorophore (Alexa Fluor 568)-conjugated goat anti-rabbit IgG Ab. The origin and processing of placental tissue samples is described in Chapters 5.1.1., 5.4.1. and 5.4.2. The staining protocol is detailed in Chapter 5.4.3. The resulting fluorescence signals were detected by confocal scanning microscopy (*see Chapter 5.4.5.).* The representative images are shown in *Fig12*.

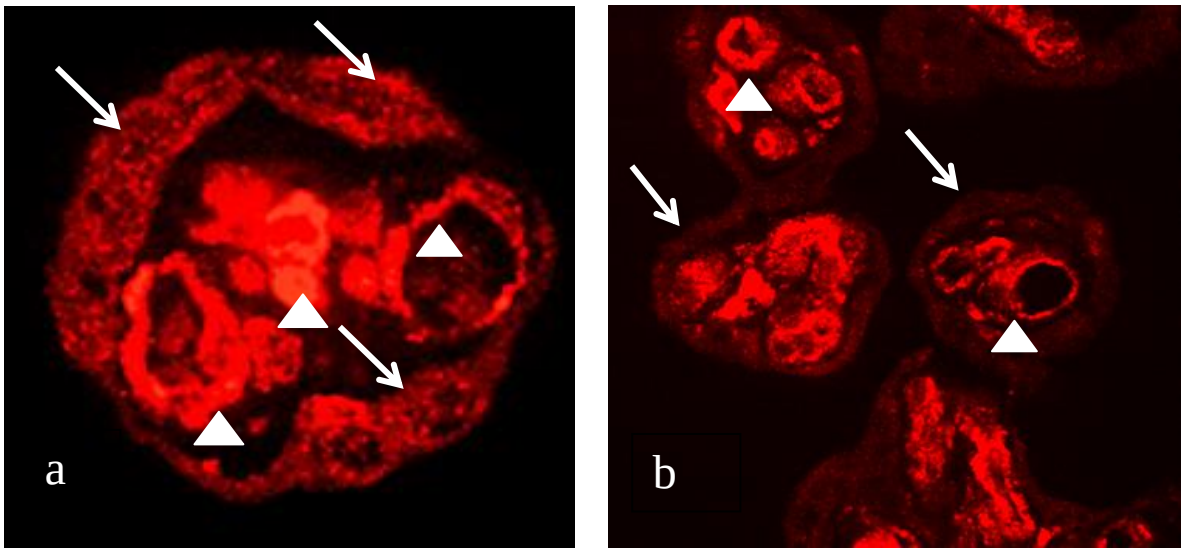

Figure 12. *Immunolocalisation of hFcRn α-chain (red) in human placental chorionic tissue; arrows – expression of hFcRn α-chain in the syncytiotrophoblast (periphery), arrowheads – hFcRn α-chain in stromal cells*

IFM confirmed expression of hFcRn α-chain in human placental chorionic tissue (*see Fig12*). Vesicular localization of hFcRn α-chain was observed in the syncytiotrophoblast (STB, *see arrows in Fig12, a and b*). This finding is in agreement with published data [Kristoffersen, 1996], [Leach, 1996], [Simister, 1996]. Interestingly, more intense staining was observed in

stromal cells of the villous core (*see arrowheads in Fig12, a and b*). This is in contrast to the published data, where hFcRn α-chain staining was not [Simister, 1996], occasional [Kristoffersen, 1996] or with very low intensity [Leach, 1996] detected in stromal cells. However, in agreement with our data hFcRn α-chain had been detected in isolated human placental endothelial cells (HPEC) [Antohe, 2001], [Radulescu, 2004].

The differences in the *in situ* staining patterns may be partly explained by the differences in specificity of the Ab used in the various studies. Unfortunately, specificity of the Ab used on placental samples had not been detailed in the published data. Comparing the Ab #4 and the Ab from Santa Cruz Biotechnology (SC) by IFM (*see Fig13*) we observed a clear deference in their staining pattern. The anti-hFcRn α-chain Ab from SC labeled predominantly the STB layer, while the more specific Ab #4 rather labeled the stromal cells.

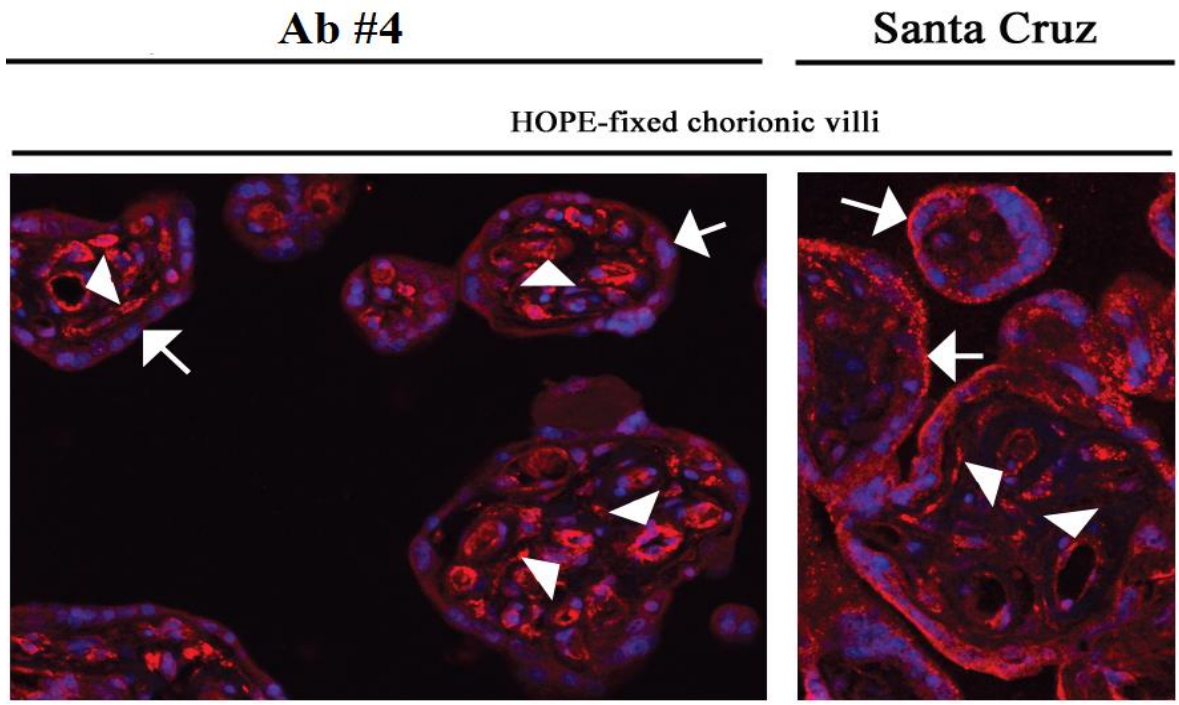

Figure 13. *Immunolocalisation of hFcRn α-chain (red) in human placental chorionic tissue by use of different antibodies;*

arrows – expression of hFcRn α-chain in the syncytiotrophoblast (periphery), arrowheads – hFcRn α-chain in stromal cells

6.1.3. Identification of stromal cells expressing hFcRn α-chain using IFM

The core region of the placental villous tree consists of mesenchymal tissue (fibroblasts), placental macrophages and endothelial cells (arterial and venous) that line fetal capillaries (*see Fig14*).

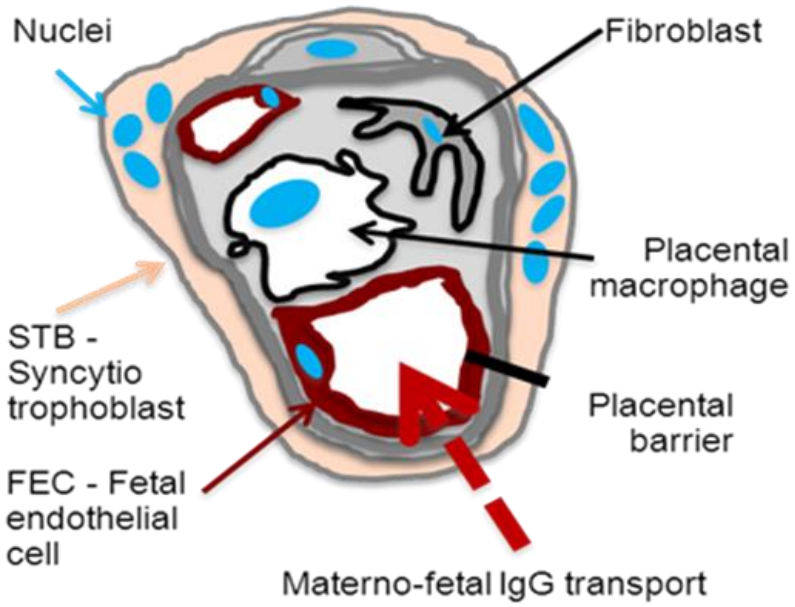

Figure 14. *Schematic depiction of cell types in placental chorionic tissue;*

large arrow indicates the route of materno-fetal IgG transport across the placental barrier formed by STB and FEC

This study is focused on the investigation of hFcRn expression in placental fetal endothelial cells (FECs), which together with the STB form the placental barrier. To investigate the presence of hFcRn α-chain in the FEC, placental chorionic tissue sections were double labeled by IFM as detailed in Chapter 5.4.3. The primary Ab #4 in combination with an Alexa Fluor 568 goat anti-rabbit IgG Ab was applied to detect hFcRn α-chain, while mouse anti-human CD31 and Alexa Fluor 647 goat anti-mouse IgG Ab were used to label FECs. CD31, also known as PECAM-1 antigen (Platelet Endothelial Cell Adhesion Molecule, CD31, endoCAM. gpIIa, hec7) is specifically expressed in FECs [Dye, 2001], [Newman, 1994]. Nuclear counterstaining was done with DAPI. Single black and white fluorescence channel images were acquired using the TissueFAXS system and two or three channels were displayed in overlays in artificial colours (*see Fig15*). In *Fig15a* and *15c* hFcRn α-chain expression (red) is combined with nuclei staining (blue). Among the predominantly labeled stromal cells, a large percentage also displayed expression of CD31 (*white in Fig15b*), demonstrating for the first time hFcRn α-chain expression in placental FECs *in situ*. Expression of hFcRn α-chain in the STB was significantly lower than in stromal cells (*see Fig15c*). The STB was identified by colabeling of hFcRn α-chain with Cytokeratin7 (CK7)

[Maldonado-Estrada, 2004]. A similar pattern of high placental FEC expression has been recently observed in the rabbit placenta [Catunda Lemos, 2012].

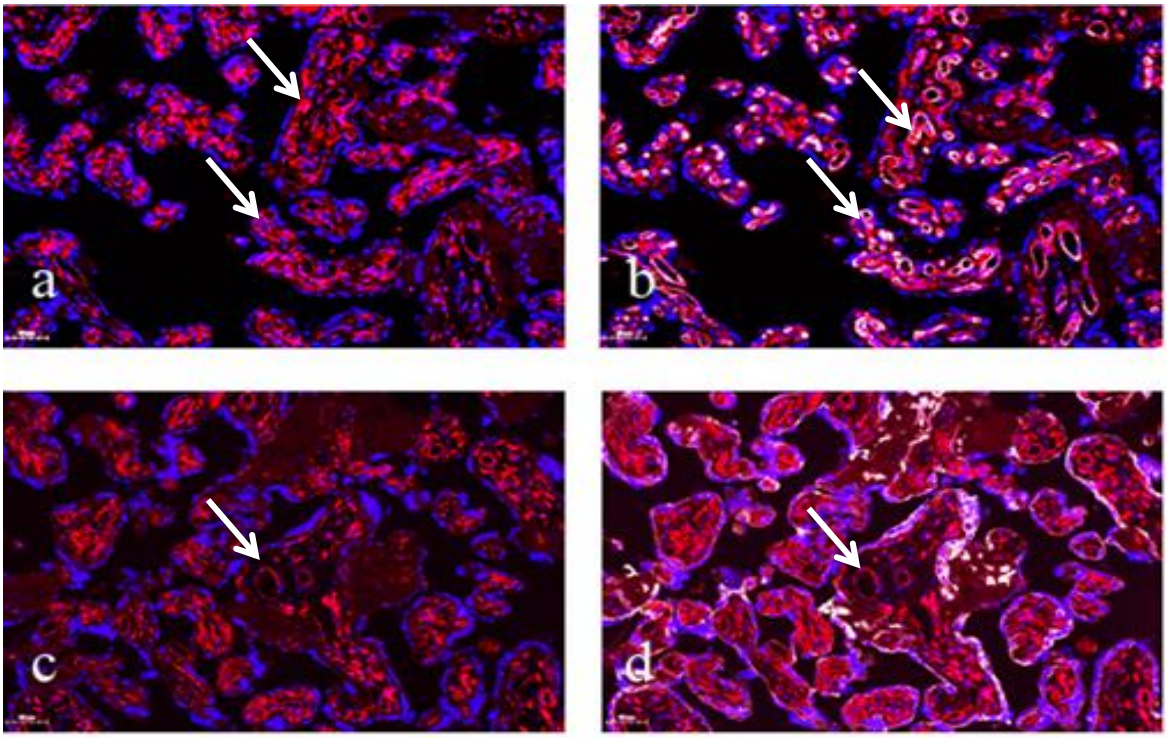

Figure 15. *Immunolocalisation of the hFcRn α-chain in FEC (a, b) and STB (c, d) of placental tissue;*

hFcRn α-chain expression is shown in red, nuclei are labeled by DAPI (blue); in b endothelial cells are labeled by CD31 (white); in d STB is labeled by CK7 (white); the expression of hFcRn α-chain in FEC is pointed out with arrows

Double immunofluorescence staining proved presence of hFcRn in placental FEC and STB (*see Fig15*) and this expression of hFcRn in the STB as well as the FEC suggests an involvement of the receptor in IgG transport across the entire placental barrier. Of interest, in addition to the CD31 positive FECs in the chorionic stroma, placental macrophages (CD68 positive) were found to be positive for hFcRn (I. Ellinger, unpublished results).

6.1.4. Localization of hFcγRIIb2 in human placental chorionic tissue

While hFcRn α-chain has not been/hardly detected in human placental FECs *in situ* so far, the expression of hFcγRIIb2 (~ 35kDa) in this cell type has been published [Takizawa, 2005], [Lyden, 2001]. By using the rabbit anti-human FcγRIIb2 Ab [Takizawa, 2005] in combination with an Alexa Fluor 568 goat anti-rabbit IgG Ab and colabeling with either mouse anti-human CD31 Ab (FEC cells, *see Fig16b*) or mouse anti-human CK7 (STB cells, *see Fig16d*) according to Chapter 5.4.3., we could confirm expression of hFcγRIIb2 in FECs. In *Fig16a* and *16c* hFcγRIIb2 expression (red) is combined with DAPI nuclei staining (blue).

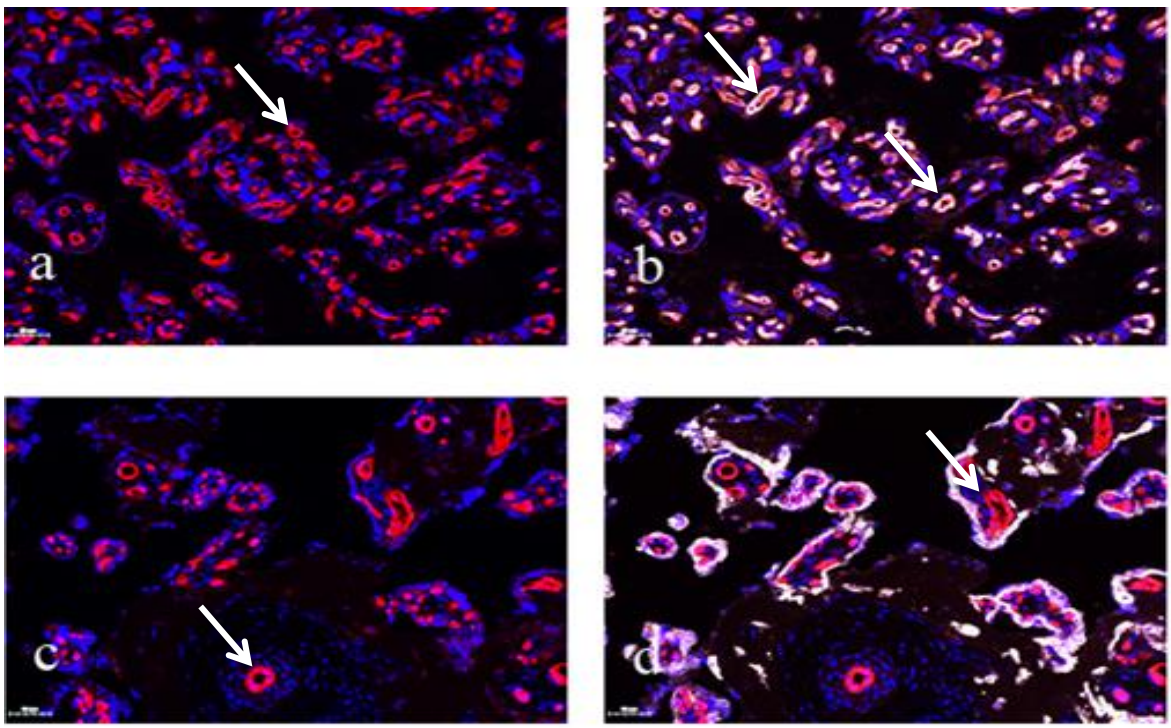

Figure 16. *Immunolocalisation of hFcγRIIb2 in FEC (a, b) and STB (c, d) of placental tissue; hFcγRIIb2 expression is shown in red, nuclei are labeled by DAPI (blue); in b endothelial cells are labeled by CD31 (white); in d STB is labeled by CK7 (white); the expression of hFcγRIIb2 in FEC is pointed out with arrows*

Our data demonstrating the presence of hFcγRIIb2 in FECs are in agreement with literature [Takizawa, 2005], [Lyden, 2001]. In summary, we observed the presence of two distinct IgG receptors in FEC cells *in situ*, namely hFcRn and hFcγRIIb2. To confirm these *in situ* findings, which were based on IFM studies, we aimed to demonstrate the expression of hFcRn in isolated and *in vitro* cultured human placental endothelial cells (HPEC). This subsequent part of the project was done in cooperation with Dr. Christian Wadsack from the Medical University of Graz (MUG)/Austria.

6.2. Expression of hFcRn α-chain in the placental fetal endothelial cells (FEC)

6.2.1. Expression of hFcRn α-chain mRNA in isolated and in vitro cultured human placental endothelial cells (HPEC)

To analyze mRNA expression of hFcRn α-chain in the placental fetal endothelial cells (FEC), mRNA of three preparations of human placental endothelial arterial cells (HPEAC) and human placental endothelial venous cells (HPEVC) was obtained from the group of Dr. C. Wadsack from the MUG. These cells had been isolated according to [Lang, 2008]. Moreover, mRNA from three term placentas (MUG) and one term placenta from MUV was provided. As a positive control, mRNA was isolated from the choriocarcinoma cell line BeWo exhibiting low endogenous expression of hFcRn α-chain as well as hFcRn α-chain overexpressing BeWo cells (BeWo + hFcRn(5)) [Leitner, 2006] (*see Table 14*). Information on all cell lines used in this study is provided in Chapter 5.1.2. RNA isolation was done according to the protocol detailed in Chapter 5.2.1.

Table 14. mRNA samples used to demonstrate hFcRn α-chain expression

Biological material	Number of preparations	Origin
Placental term tissue	3/1	Wadsack/Ellinger
HPEAC	3	Wadsack
HPEVC	3	Wadsack
BeWo cells	1	Ellinger
BeWo + hFcRn(5)	1	Ellinger

The quality/integrity and quantity of the isolated RNA was determined by two different methods, spectroscopic (Thermo Fisher Scientific, NanoDrop 1000) and by the Experion automated electrophoresis system (*see Chapters 5.2.2. and 5.2.3* .).

Using the NanoDrop Spectrophotometer the purity of all RNA samples was analyzed by determination of the 260nm/280nm ratio, which revealed acceptable values in the range from 1.6 to 1.8. The concentrations measured at 260 nm are given in *Table 15*:

Table 15. The concentrations of RNA samples measured by NanoDrop at 260 nm

Sample	Concentration, ng/µl	Purity, 260/280
HPEAC 1	643,2	1,79
HPEAC 2	1164,6	1,78
HPEAC 3	1196,2	1,81
HPEVC 1	573,9	1,79
HPEVC 2	534,6	1,77
HPEVC 3	952,5	1,62
Term tissue 1	387,2	1,65
Term tissue 2	584,4	1,68
Term tissue 3	630,7	1,63
Term tissue 4	1127,3	1,61
BeWo	1599,2	1,62
BeWo + hFcRn(5)	3101,1	1,68

Additionally, using the Experion system, determination of the integrity of all RNA samples except for BeWo cell lines was repeated and reported visually as electropherogram (*see Fig17*) and as RNA quality indicator (RQI) number. RQI characterizes the integrity of the sample on the scale from 1 to 10, where 1 is highly degraded and 10 totally intact RNA. All samples showed RQI numbers from 6 to 10 (10 including for RNA isolated from HPEC) indicating high integrity of isolated RNA. The RNA concentrations in the samples displayed in *Table 15* were confirmed by the Experion system.

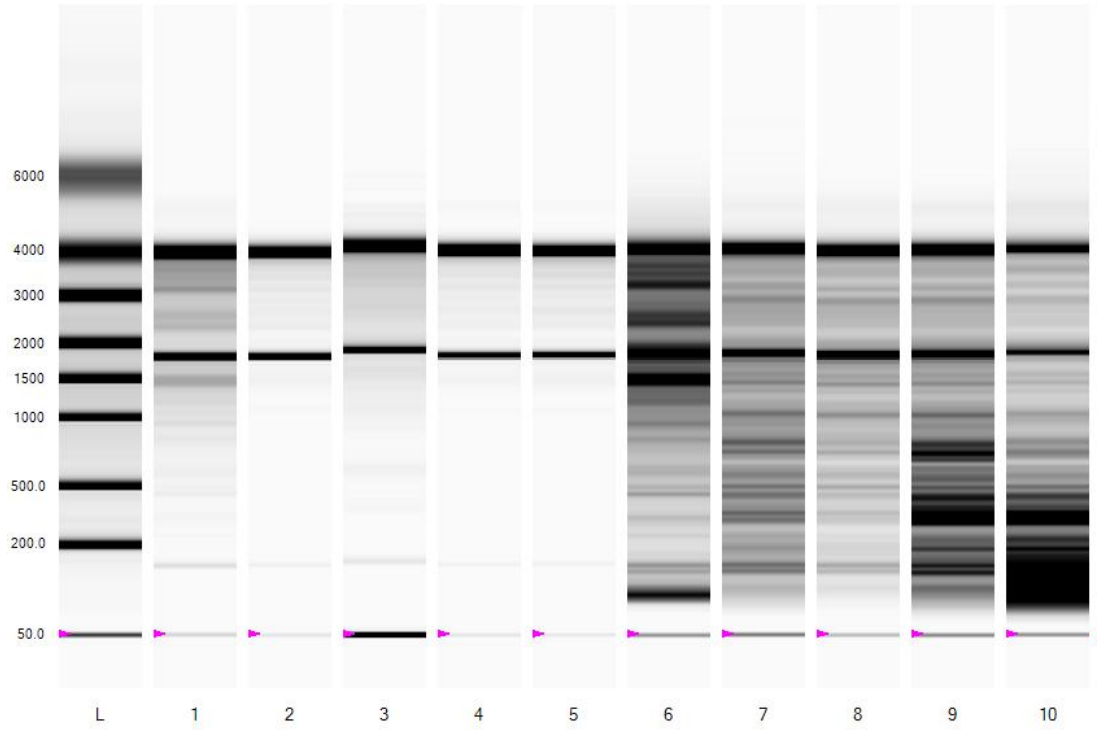

Figure 17*. Electropherogram of RNA samples;*

L – RNA ladder, 1-3 – HPEAC, 4-6 – HPEVC, 7-10 – placental tissue, pink arrow – fitting size marker

Reverse transcription (RT) of isolated RNA was performed according to the protocol described in Chapter 5.2.4. Per sample 2 µg total RNA were reversely transcribed to obtain 2 µg cDNA in 20 µl (100 ng/µl). Per sample one RT-negative control was processed, which contained all compounds of RT mastermix except the enzyme Reverse Transcriptase.

To detect hFcRn α-chain mRNA in the samples Polymerase Chain Reaction (PCR) was performed. 100 ng cDNA were used per reaction; primers and all other experimental conditions are described in Chapter 5.2.5. RT-negative samples were also subjected to PCR to guarantee that mRNA and not genomic DNA was amplified by PCR. A sample containing only water instead of cDNA was also processed to proof the absence of PCR products in all ingredients of PCR mastermix. After PCR amplification, 10 µl of each sample were mixed with 2 µl of loading dye and were loaded and separated on 2% agarose gel containing GelRed Nucleic Acid Stain (*see Chapter 5.2.6.*). The PCR products were visualized under UV light.

A PCR product of 457 bp was expected (*see Chapter 5.2.5.*). As depicted in *Fig18*, a band of ~ 500 bp was indeed amplified in the positive control samples (BeWo and BeWo + hFcRn(5)) as well as in all HPEC (HPEAC and HPEVC) samples, while RT-negative samples were always free of the product.

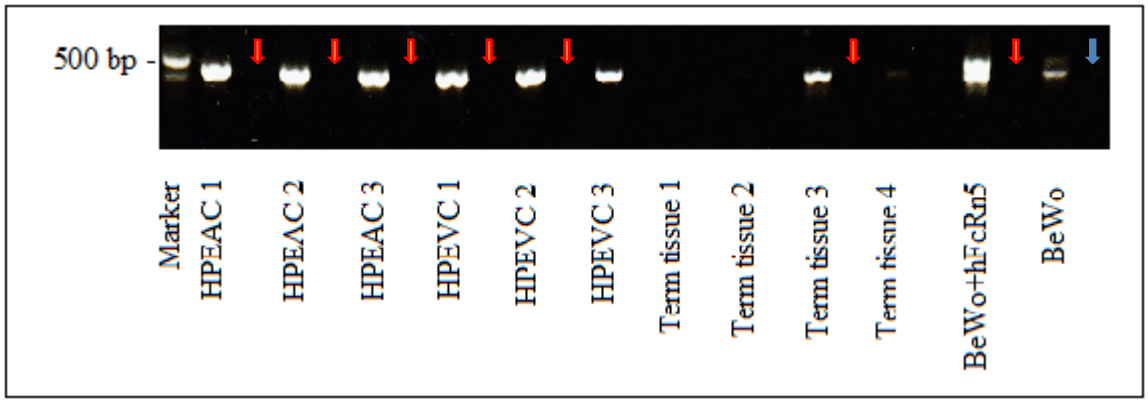

Figure 18. *HFcRn mRNA expression analyzed by RT-PCR (1);*

next to each positive sample the RT-negative sample was loaded, pointed out with red arrows, the last one contains only water (blue arrow)

As amplification of a PCR product failed in three of four placental tissue samples, for all placental tissue samples and HPEAC1 sample together with their RT-negative controls the PCR reaction was repeated under identical conditions. The result is displayed in *Fig19* demonstrating amplification of the PCR product of expected size.

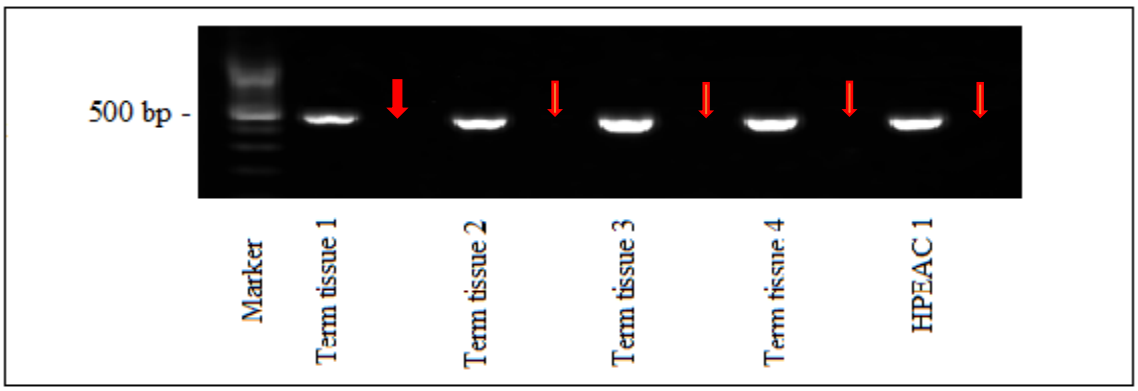

Figure 19. *HFcRn mRNA expression analyzed by RT-PCR (2);*

next to each positive sample the RT-negative sample was loaded, pointed out with red arrows

In summary, hFcRn α-chain mRNA expression was investigated and confirmed by RT-PCR in human placental chorionic tissue samples in agreement with literature [Leach, 1996], [Simister, 1996]. Moreover, expression of hFcRn α-chain mRNA was demonstrated in isolated HPEC cells of arterial and venous type obtained by the method of [Lang, 2008]. This is in agreement with published data [Antohe, 2001], demonstrating hFcRn α-chain mRNA expression in placental FEC (HPEC) isolated by the protocol described by [Jinga, 2000].

6.2.2. Expression of hFcRn α-chain protein and β2-microglobulin in isolated HPEC

To analyze hFcRn α-chain protein expression, Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) and Western blotting (WB) was performed as described in Chapter 5.3. Placental tissue samples, HPEAC, HPEVC as well as the positive control samples BeWo and BeWo + hFcRn(5) were used. Total protein was extracted from the samples and protein concentration was determined by a BCA assay using the microplate reader M200 PRO at 562 nm (*see Chapter 5.3.2.*). Protein concentration in the samples is given in *Table 16.*

Table 16. Protein concentration

#	Sample	Concentration, µg/µl
1	Term tissue 1	2,48
2	Term tissue 2	4,0
3	Term tissue 3	4,0
4	Term tissue 4	4,0
5	HPEAC 1	2,2
6	HPEAC 2	2,12
7	HPEAC 3	2,18
8	HPEVC 1	3,14
9	HPEVC 2	2,09
10	HPEVC 3	2,45
11	BeWo	1,72
12	BeWo + hFcRn(5)	2,38

40 µg protein per sample were then precipitated with acetone and the pellet was dissolved in 20 µl 1x reducing SDS sample buffer. In addition to the tissue and cell samples described in Table 16, 5 µg human serum albumin (HSA) was loaded on the gels as often antibodies exhibit cross reaction with HSA.

Proteins were separated by 15% SDS PAGE and transferred onto the PVDF membrane. To detect hFcRn α-chain, the blocked membranes were incubated with rabbit anti-human Ab #4 (diluted 1:100) and a secondary HRP-conjugated goat anti-rabbit IgG Ab. The procedure for Ab incubation and signal detection is given in Chapter 5.3.3.

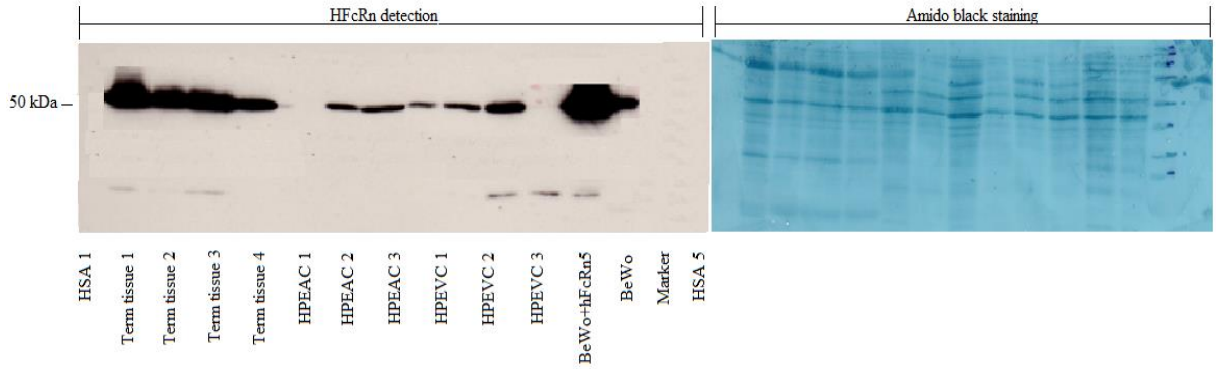

Figure 20. *Detection of the hFcRn α-chain in protein lysates (40 µg) of placental tissue, fetal endothelial cells (HPEAC and HPEVC) and BeWo cells*

As shown in *Fig20*, hFcRn α-chain protein (~ 45-50 kDa) was detected in the positive control cell lines BeWo [Ellinger, 1999]. A strong band was observed in BeWo cells overexpressing hFcRn α-chain (BeWo + hFcRn(5)) [Leitner, 2006]. Likewise, all placental tissue samples showed presence of hFcRn α-chain, which is in agreement with literature [Leach, 1996], [Simister, 1996]. Almost all HPEC preparations exhibited presence of hFcRn α-chain. Ab #4 did not crossreact with HSA.

As described in the introduction, hFcRn is a heterodimer of hFcRn α-chain and β2-microglobulin. To verify the expression of β2-microglobulin in the FEC – HPEC cell lysates, the blot was stripped and reproved with a rabbit anti-human β2-microglobulin Ab followed by incubation with a goat anti-rabbit IgG HRP-conjugated secondary Ab.

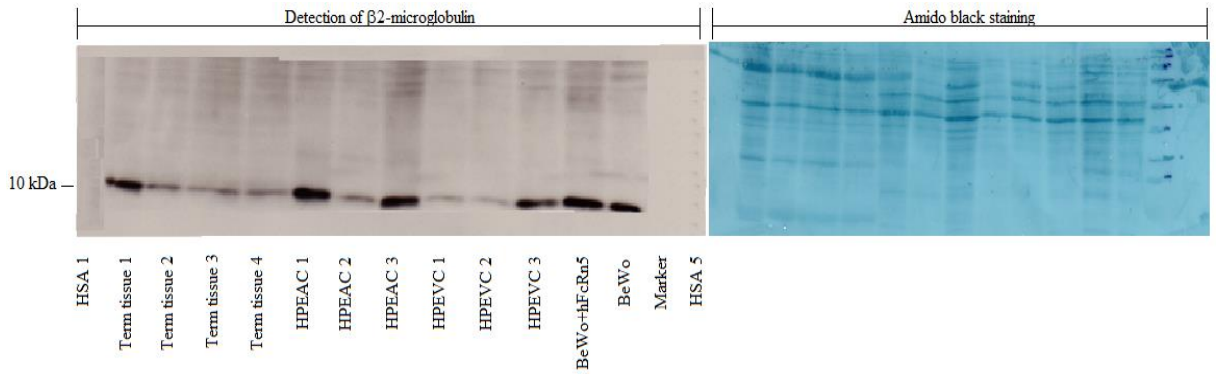

Figure 21. *Detection of β2-microglobulin in protein lysates (40 µg) of placental tissue, fetal endothelial cells (HPEAC and HPEVC) and BeWo cells*

As displayed in *Fig21*, all samples including HPEC express β2-microglobulin. This has been demonstrated for placenta [Leach, 1996] and BeWo cells [Ellinger, 2005]. The result was expected as β2-microglobulin is not only the small unit of hFcRn but also of MHC class I molecules expressed in almost all cell types [Solheim, 1999].

In summary, isolated HPEC of venous and arterial type express both subunits of hFcRn.

To verify the identity of two types of HPEC the stripped membrane was reincubated with an Ab against the endothelial marker protein CD31, which should be found in placental tissue as well as endothelial cell lysates, but not in the epithelial cell line BeWo. CD31 or PECAM-1 has a molecular size of ~ 130 kDa. For this incubation rabbit anti-human CD31 Ab was combined with an HRP-conjugated goat anti-rabbit IgG Ab.

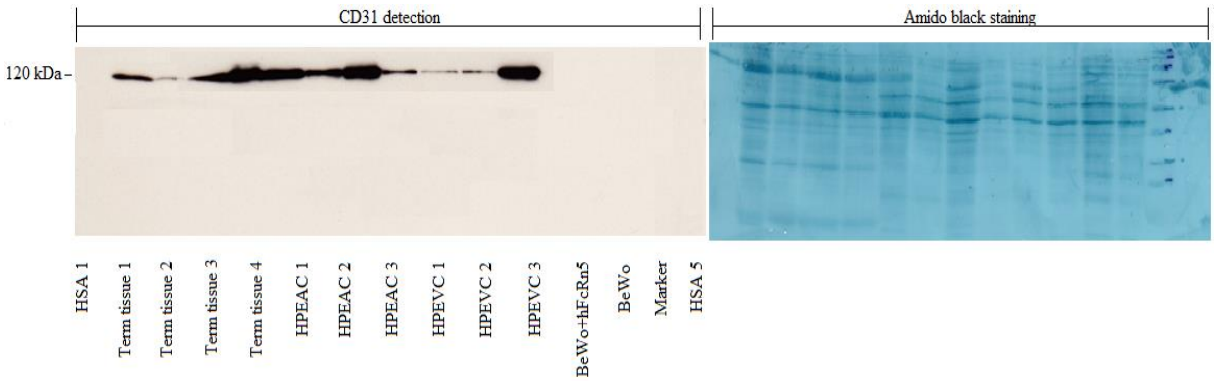

Figure 22. *Detection of CD31 in protein lysates (40 µg) of placental tissue, fetal endothelial cells (HPEAC and HPEVC) and BeWo cells*

As shown in *Fig22*, the expression of endothelial marker was indeed found in isolated HPEAC and HPEVC.

None of the applied antibodies crossreacted with albumin that was also loaded on the gel. Serum albumin (~ 67 kDa), however, is internalized by placental STB and is therefore present in placental lysates [Lambot, 2006]. According to [Lambot, 2006], it is recycled by the STB back to maternal blood and does not enter the FEC. Isolated HPEC cells should therefore be devoid of albumin. Indeed, when the PVDF membrane was reincubated with a goat Ab directed against HSA combined with a secondary HRP-conjugated donkey anti-goat Ab, only HSA positive controls and placental tissue lysates, but not HPEC reacted with Ab (*see Fig23*).

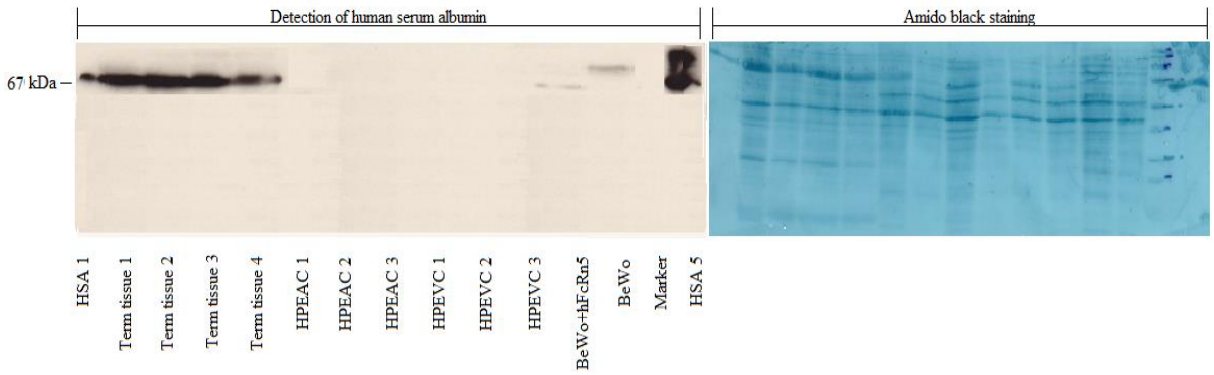

Figure 23. *Detection of human serum albumin in protein lysates (40 µg) of placental tissue, fetal endothelial cells (HPEAC and HPEVC) and BeWo cells*

Finally, membranes were incubated (after stripping) with mouse Ab against the cytoskeletal protein α-tubulin, which was detected via a goat anti-mouse IgG HRP-conjugated Ab (*see Fig24*). α-Tubulin, which is expressed in FECs [Solder, 2012] and BeWo cells [Ockleford, 1984], served as loading control to proof that similar protein amounts of all lysates had been loaded. α-Tubulin has a molecular weight of ~ 55 kDa. The expression level of α-tubulin was much lower in total placental lysates, most likely due to the presence of tubulin-free extracellular matrix. Afterwards, the membrane was stained with Amido black, which in the placenta has been proven to serve as the most stable reference protein marker [Lanoix, 2012].

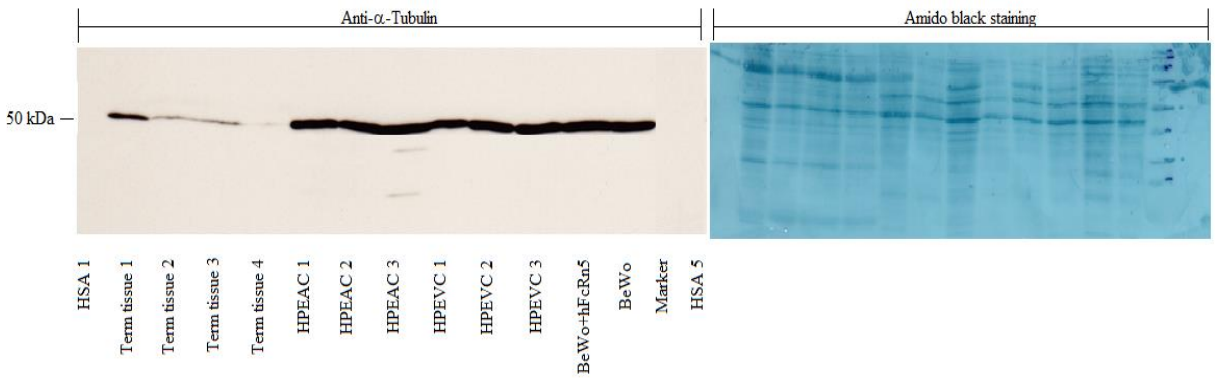

Figure 24. *Detection of anti-α-Tubulin in protein lysates (40 µg) of placental tissue, fetal endothelial cells (HPEAC and HPEVC) and BeWo cells*

In summary, it was demonstrated that isolated HPEC (CD31 positive, albumin negative) of arterial (HPEAC) as well as venous (HPEVC) phenotype express hFcRn α-chain subunit mRNA and protein as well as β2-microglobulin. These data are in line with the results from [Antohe, 2001], who demonstrated hFcRn mRNA and protein expression in FECs prepared by a different protocol than the one used in this study [Jinga, 2000].

6.3. Localization of hFcRn α-chain in trophoblast-derived BeWo + hFcRn(5) cells and isolated and cultured HPEAC

6.3.1. Localization of hFcRn α-chain in BeWo cells overexpressing hFcRn α-chain

To establish the IFM staining protocol for cells in culture and confirm usability of the applied anti-hFcRn Ab, BeWo cells stably transfected with hFcRn α-chain cDNA (BeWo + hFcRn(5) cell line) were analyzed. Cell culture parameters and IFM protocols are detailed in Chapter 5.4.4. These cells express high levels of hFcRn α-chain protein, which can be detected by anti-hFcRn α-chain Ab #4 [Leitner, 2006]. Cells were seeded on glass coverslips and after 48h were processed by IFM. The Ab #4 was combined with an Alexa Fluor 568 goat anti-rabbit IgG Ab and nuclei were labelled with DAPI. To control for unspecific binding of the secondary Ab, primary Ab was omitted. Cells were analysed with TissueFAXS system.

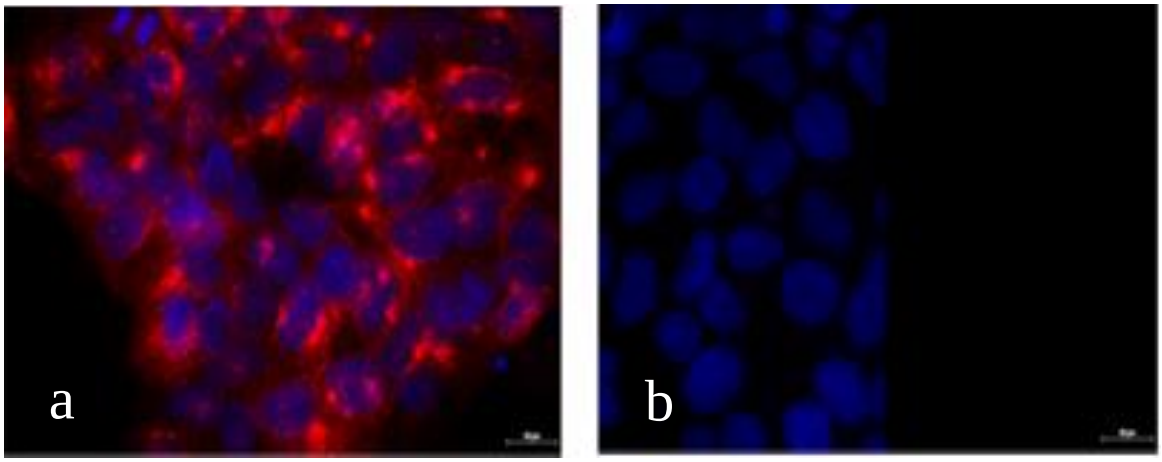

Figure 25. *IFM analysis of hFcRn α-chain expression in BeWo+hFcRn(5) cells; strong red fluorescence signal indicates high expression of the hFcRn in the cells (a – overlay of TexaRed + DAPI channels); no signal is observed when primary Ab is omitted (b – overlay of TexaRed + DAPI channels)*

In analogy to [Leitner, 2006], bright, mainly vesicular staining of the BeWo + hFcRn(5) cells was observed (*see Fig25a*), which was not seen in negative control (*see Fig25b*).

To verify intracellular localization and to establish double staining IFM protocols for cells in culture, colocalization of hFcRn α-chain with early endosomal antigen, EEA1 as well as lysosomal marker LAMP2 was investigated, combining the anti-hFcRn α-chain Ab #4 and Alexa Fluor 568 goat anti-rabbit IgG Ab with either monoclonal mouse anti-human EEA1 or LAMP2 Ab and Alexa Fluor 647 goat anti-mouse IgG Ab. Nuclei were stained with DAPI.

Double labeling was performed as described in Chapter 5.4.4., and cells were investigated by confocal microscopy as described in Chapter 5.4.5. Due to the editing of microscopic images, the colours of fluorescence signals do not correspond to the colours of respective emission spectrum.

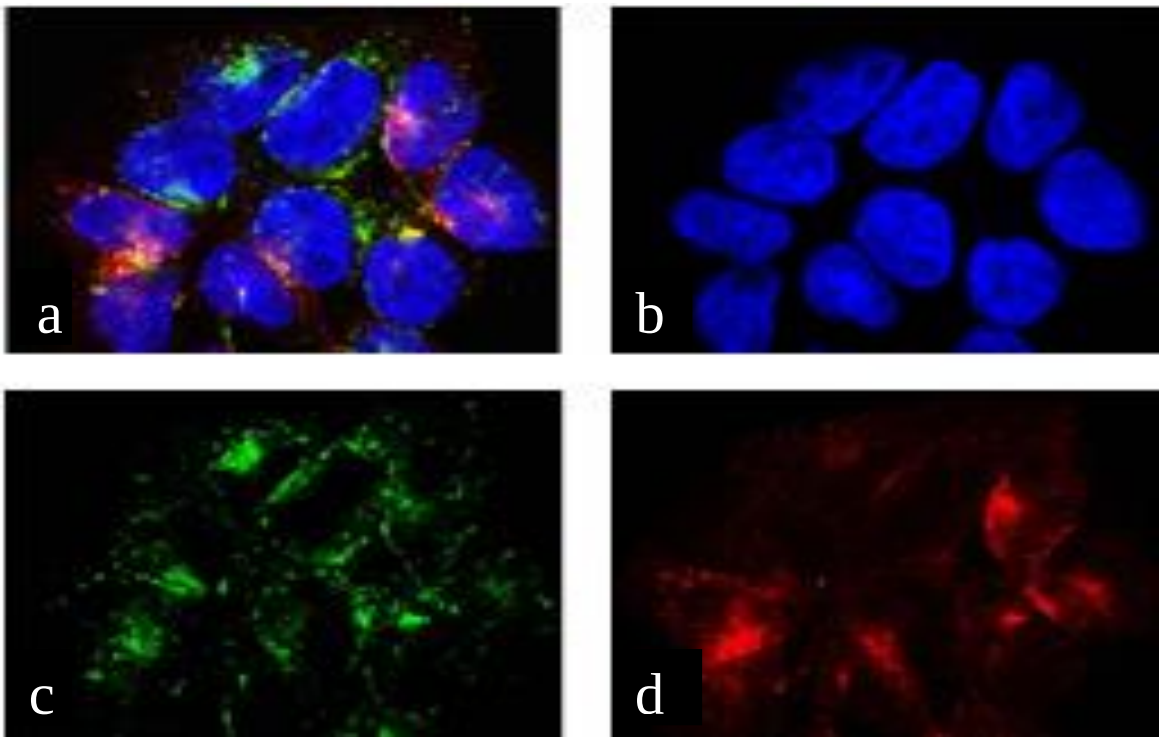

Figure 26. *IFM analyses of endosomal localization of hFcRn α-chain in BeWo+hFcRn(5) cells; red-yellow fluorescence indicates the colocalization of the anti-hFcRn Ab with endosomal marker EEA1 (a – overlay of TexaRed, Cy5 and DAPI channels); blue fluorescence – nuclei staining (b – DAPI channel); green fluorescence – punctured staining pattern of EEA1 (c – Cy5 channel); red fluorescence indicates hFcRn expression pattern (d – TexaRed channel)*

Colocalization of hFcRn α-chain with early endosomal marker EEA1 in intracellular vesicles was confirmed in agreement with published data [Leitner, 2006]. The results are displayed in *Fig26*. DAPI (*see Fig26b*), EEA1 (*see Fig26c*) and hFcRn (*see Fig26d*) are displayed separately. In *Fig26a* yellow colour indicates the colocalization of hFcRn α-chain and EEA1.

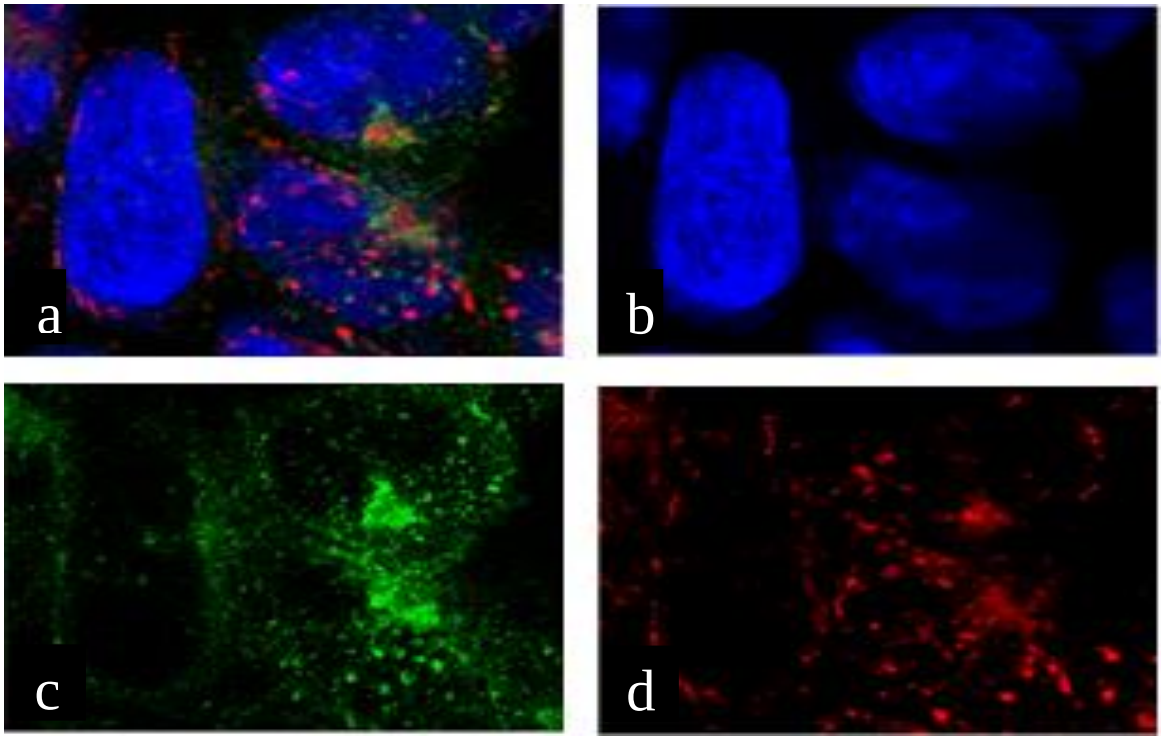

Figure 27. *IFM analyses of lysosomal localization of hFcRn α-chain in BeWo+hFcRn(5) cells; red-yellow fluorescence indicates the colocalization of the anti-hFcRn Ab with lysosomal marker LAMP2 (a – overlay of TexaRed, Cy5 and DAPI channels); blue fluorescence – nuclei staining (b – DAPI channel); green fluorescence indicates hFcRn expression pattern (d – TexaRed channel); red fluorescence – punctured staining of LAMP2 (c – Cy5 channel)*

Double staining of BeWo + hFcRn(5) cells for hFcRn α-chain (*see Fig27d*) and LAMP2 (*see Fig27c*) is displayed in *Fig27*. Colocalization of the proteins is shown in *Fig27a* by yellow colour, but was rarely observed, which is in good agreement with [Leitner, 2006].

6.3.2. Localization of hFcRn α-chain and hFcγRIIb2 in isolated and cultured human placental endothelial arterial cells (HPEAC)

HPEAC were isolated form healthy term placentas according to Chapter 5.1.3. Cells were cultured for about one week and after they reached confluency were seeded and processed by IFM as described in Chapter 5.4.4.

Using anti-hFcRn α-chain Ab #4 and Alexa Fluor 568 goat anti-rabbit IgG Ab, the expression pattern of hFcRn α-chain was visualized (*see Fig28a*). Nuclei were stained with DAPI. Negative control, generated by omittance of primary Ab is displayed in *Fig28b*. Strong, mainly vesicular (*see arrows in Fig28a*) localisation of hFcRn α-chain was observed in HPEAC. A similar pattern of localization was also observed by [Antohe, 2001].

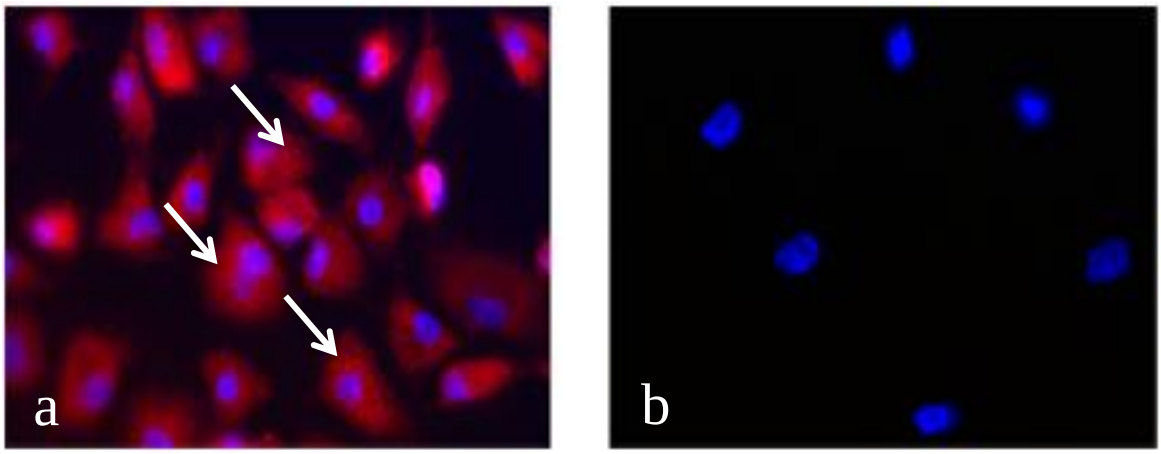

Figure 28. *Immunolocalization of hFcRn α-chain in HPEAC by single staining; red bright fluorescence indicates high presence of the hFcRn α-chain (a – TexaRed and DAPI channels); no signal is observed when primary Ab is omitted (b – TexaRed and DAPI channels)*

To verify that cells expressing hFcRn α-chain were indeed endothelial cells, colabeling of cells with anti-hFcRn α-chain Ab and anti-CD31 Ab was performed. HFcRn α-chain was visualized with an Alexa Fluor 568 conjugated secondary Ab (*see Fig29b*), while CD31 was visualized with an Alexa Fluor 647 conjugated secondary Ab (*see Fig29c*). Nuclei were stained with DAPI.

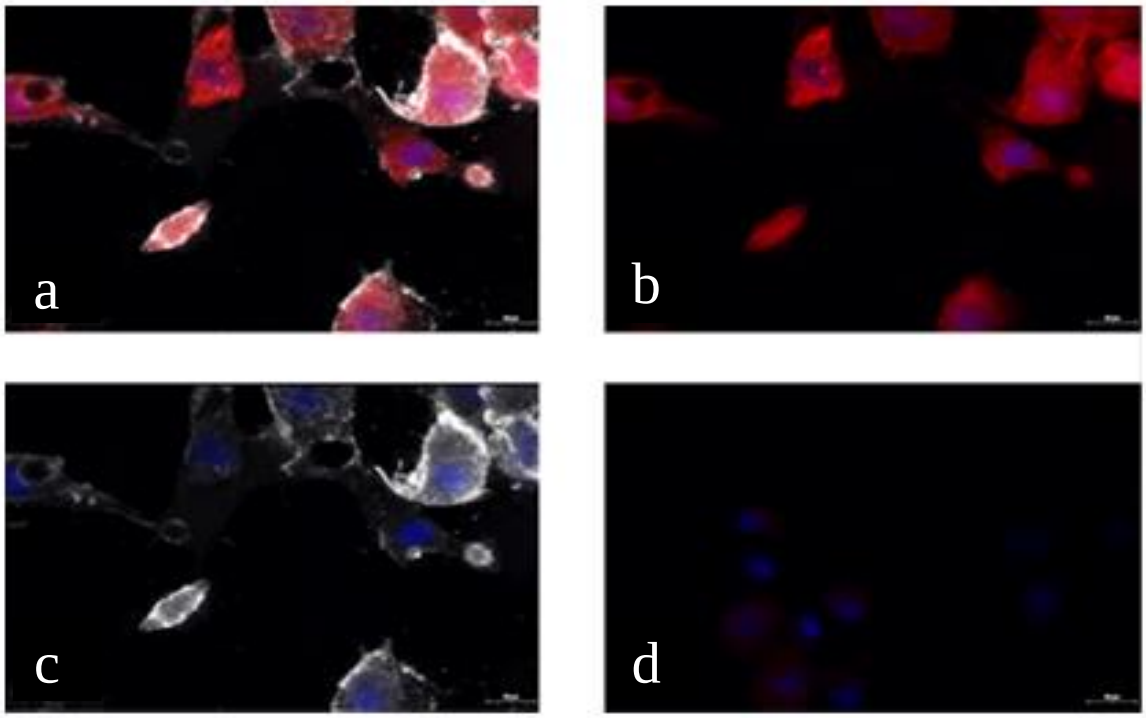

Figure 29. *Colocalization of hFcRn α-chain and CD31 in HPEAC by double staining; white-pink fluorescence indicates colocalization of hFcRn with endothelial marker, CD31 (a – overlay TexaRed, Cy5 and DAPI channels); bright red fluorescence – hFcRn expression (b – TexaRed and DAPI channels); white fluorescence – CD31 expression (c – Cy5 and DAPI channels), no signal is observed when primary Ab is omitted (d - overlay TexaRed, Cy5 and DAPI channels)*

The majority of cultured cells displayed CD31 expression and showed high expression levels of hFcRn α-chain. Thus, isolated and cultured placental cells are endothelial cells that express hFcRn α-chain.

In agreement with [Lyden, 2001] we demonstrated in FECs *in situ* not only the expression of hFcRn but also the presence of hFcγRIIb2. To verify that isolated and *in vitro* cultured cells likewise express not only hFcRn α-chain, but also hFcγRIIb2, cells were processed by IFM using rabbit anti-hFcγRIIb2 Ab and secondary Alexa Fluor 568 goat anti-rabbit IgG Ab. Nuclei were stained with DAPI.

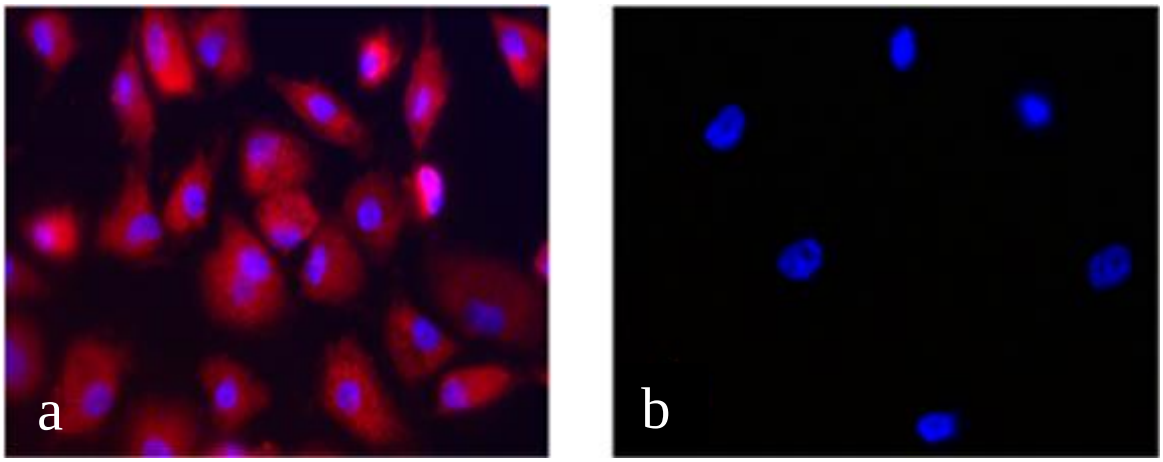

Figure 30. *Immunolocalization of the hFcγRIIb2 in HPEAC by single staining; red bright fluorescence indicates high presence of the hFcγRIIb2 (a – TexaRed and DAPI channels); no signal is observed when primary Ab is omitted (b – TexaRed and DAPI channels)*

In analogy to the *in situ* results, isolated cells exhibited bright staining when incubated with primary and secondary Abs (*see Fig30a*). No staining was seen when primary Ab was omitted (*see Fig30b*). We used an Ab for detection that should recognize a 50-55 kDa protein (hFcγRIIb2, anti-CD32B). [Gafencu, 2003] could not demonstrate expression of FcγRIIB receptor mRNA by RT-PCR, however they described an ~ 55 kDa IgG receptor (in addition to hFcRn) in their publication. In contrast, [Lyden, 2001] by applying various anti-FcγRII antibodies, described expression of hFcγRIIb2 in placental endothelial cells *in situ*, but claim absence of hFcRn. Expression of hFcγRIIb2 in FECs *in situ* was later confirmed by [Takizawa, 2005].

In summary, our results demonstrated expression of both, hFcRn α-chain and hFcγRIIb2, in the FEC layer, in isolated and *in vitro* cultured HPEAC.

6.4. Internalization of human IgG by BeWo + hFcRn(5) cell line and isolated and cultured HPEAC

6.4.1. Internalization of human IgG by BeWo + hFcRn(5) cells

BeWo cells exhibit endogenous expression of hFcRn [Ellinger, 1999]. Although, the cells express hFcRn predominantly in endosomes, a small fraction of hFcRn is present at the plasma membrane. This is probably responsible for the observed higher binding of IgG to the plasma membrane at pH 6,0 than at pH 7,4 [Ellinger, 1999].

BeWo + hFcRn(5) cells were grown on coverslips and labeled for 30 minutes with human IgG (Endobulin) at pH 7,4 (HEPES buffer) and pH 6,0 (MES buffer) in accordance with the protocol detailed in Chapter 5.4.4. For internalization of human IgG, two different concentrations (100, 1000 µg/ml) of hIgG were used. For the detection of hIgG, fixed cells were incubated with goat anti-human Ab and Alexa Fluor 647 donkey anti-goat IgG Ab. Nuclei were stained with DAPI. For detailed information on the Abs, their dilution and origin see Chapter 5.4.4., Materials. Images were taken with TissueFAXS system and are displayed in *Fig31* for pH 7.4 internalization and *Fig32* for pH 6.0 internalization.

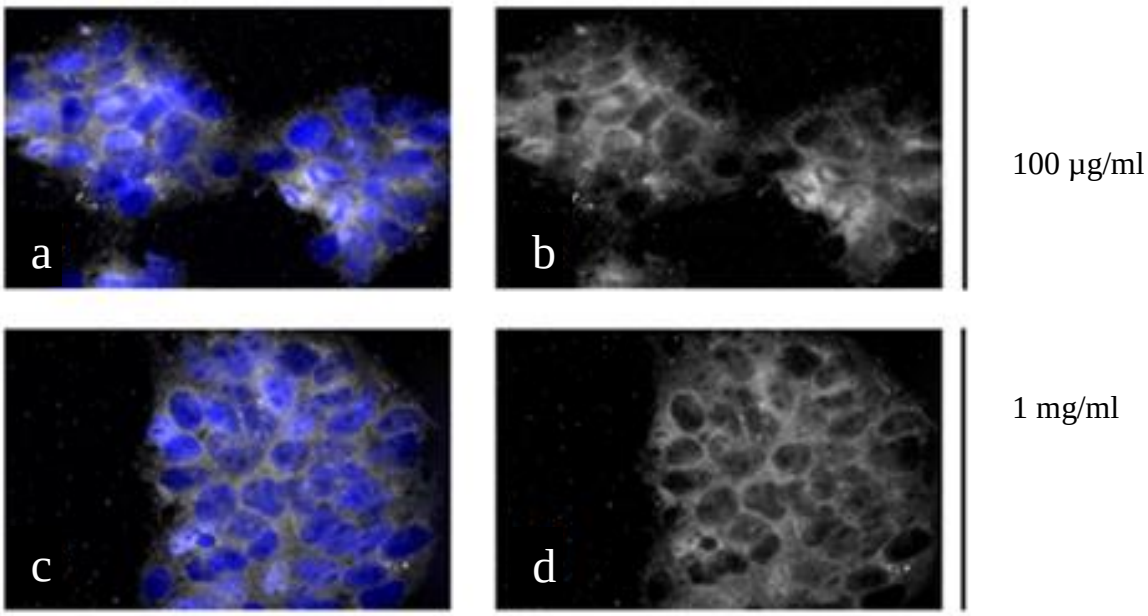

Figure 31. *IFM analysis of hIgG internalization into BeWo+hFcRn(5) cell line at physiological pH; white fluorescence signal indicates presence of hIgG within the cells (a – Cy5 and DAPI channels, b – Cy5 channel – IgG concentration 100 µg/ml; c - Cy5 and DAPI channels, d – Cy5 channel – IgG concentration 1000 µg/ml)*

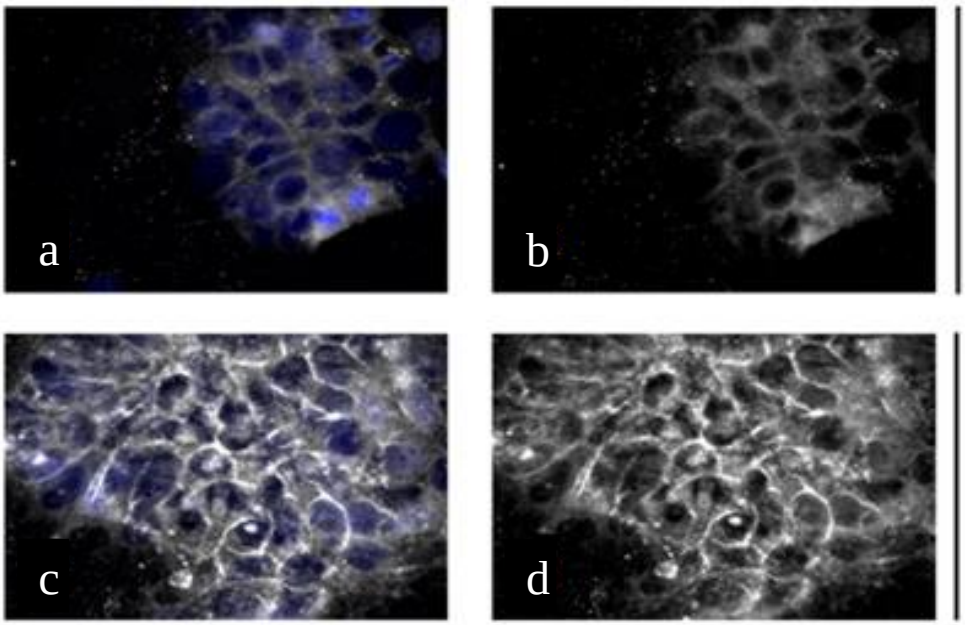

Figure 32. *IFM analysis of hIgG internalization into BeWo+hFcRn(5) cell line at acidic pH; white fluorescence signal indicates presence of hIgG within the cells (a – Cy5 and DAPI channels, b – Cy5 channel – IgG concentration 100 µg/ml; c - Cy5 and DAPI channels, d – Cy5 channel – IgG concentration 1000 µg/ml)*

Two types of negatives samples were prepared to control for the specificity of either goat anti-human (by applying of both goat anti-human Ab and Alexa Fluor 647 -conjugated donkey anti-goat IgG Ab) or Alexa Fluor 647 donkey anti-goat Ab (by applying donkey anti-goat Ab only).

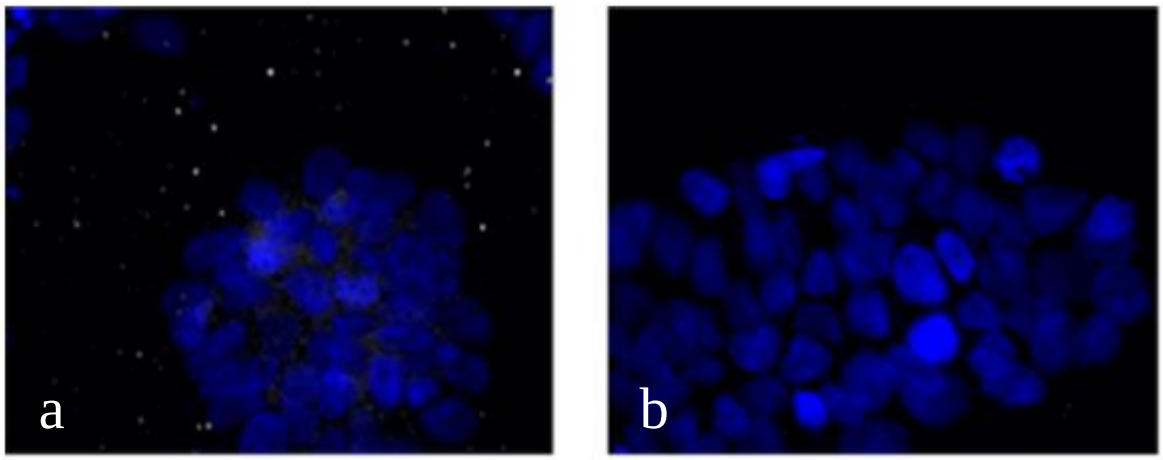

Figure 33. *The negative control staining using goat anti-human and donkey anti-goat Abs (a – Cy5 and DAPI channels); or Alexa Fluor 647 donkey anti-goat Ab only (b – Cy5 and DAPI channels)*

Internalization of hIgG occurred at both IgG concentrations, but was much more pronounced at higher IgG concentration. Internalization at pH 6.0 resulted in a stronger staining of cells, which is in line with binding to hFcRn already at the plasma membrane.

Absence of staining in the negative controls (absence of hIgG) confirmed good specificity of secondary Abs (*see Fig33*).

6.4.2. Internalization of human IgG by isolated and cultured HPEAC

IgG internalization was next repeated under identical conditions in isolated and cultured HPEAC. For identification of the cells mouse anti-human CD31 was used and visualized by Alexa Fluor 647 goat anti-mouse IgG (*see Chapter 5.4.4.*). The preparation of the cells and performance of the experiment was done according to the protocol described in Chapter 5.4.4.

Results for pH 7.4 internalization are displayed in *Fig34*, for pH 6.0 internalization in *Fig35*. Negative controls are shown in *Fig36*.

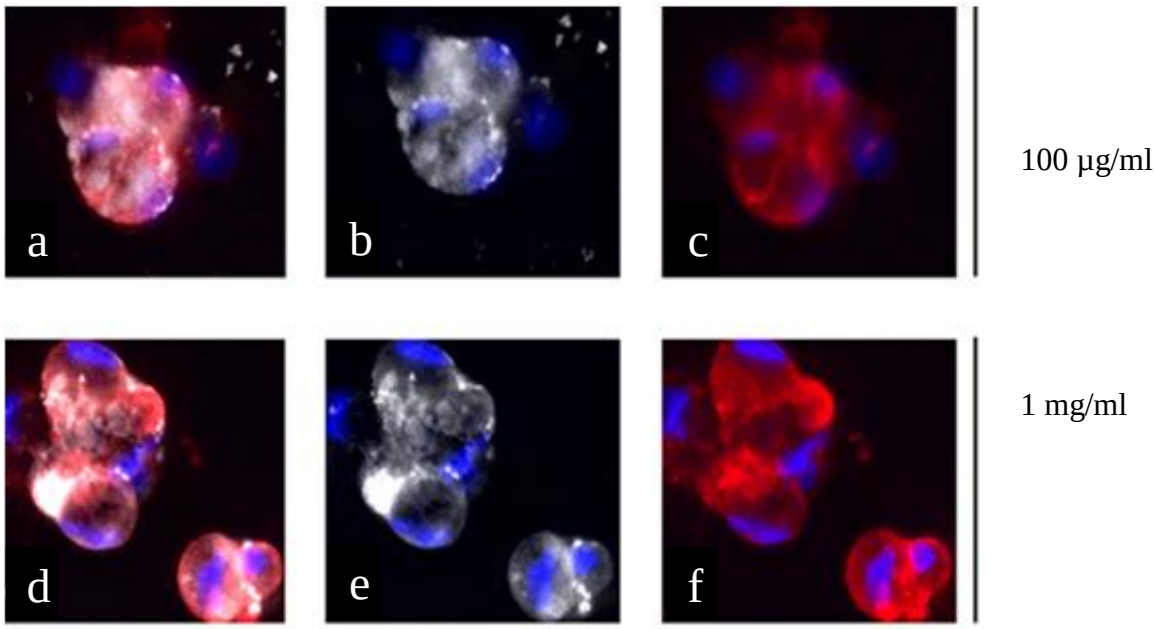

Figure 34. *IF analysis of hIgG internalization into HPEC at physiological pH; pink-white fluorescence indicates colocalization of IgG detecting Ab with endothelial marker CD31 (a – overlay of TexaRed, Cy5 and DAPI channels (100 µg/ml IgG), d - overlay of TexaRed, Cy5 and DAPI channels (1 mg/ml IgG)); white fluorescence signal indicates presence of hIgG within the cells (b – Cy5 and DAPI channels (100 µg/ml IgG), e - Cy5 and DAPI channels (1 mg/ml IgG)); red fluorescence – expression of CD31(c – TexaRed and DAPI channels (100 µg/ml IgG), f - TexaRed and DAPI channels (1 mg/ml IgG))*

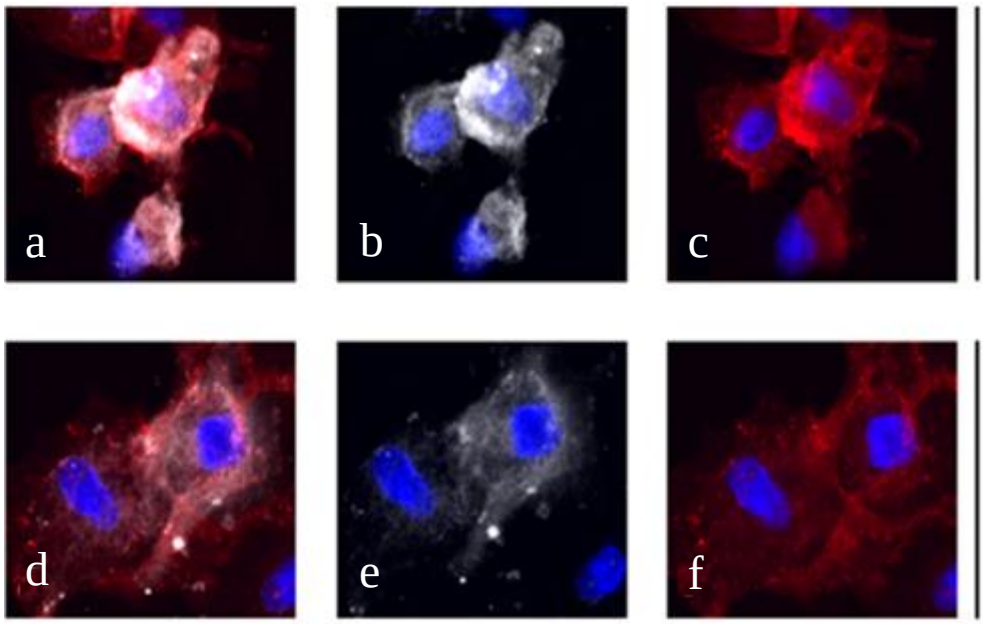

Figure 35. *IF analysis of hIgG internalization into HPEC at acidic pH; pink-white fluorescence indicates colocalisation of IgG detecting Ab with endothelial marker CD31 (a – overlay of TexaRed, Cy5 and DAPI channels (100 µg/ml IgG), d - overlay of TexaRed, Cy5 and DAPI channels (1 mg/ml IgG)); white fluorescence signal indicates presence of hIgG within the cells (b – Cy5 and DAPI channels (100 µg/ml IgG), e - Cy5 and DAPI channels (1 mg/ml IgG)); red fluorescence – expression of CD31(c – TexaRed and DAPI channels (100 µg/ml IgG), f - TexaRed and DAPI channels (1 mg/ml IgG))*

The negative controls were prepared at the same time and identically except for omittance of hIgG and CD31 Ab, thereby controlling for the specificity of either goat anti-human (by applying of both goat anti-human and fluorescence labeled donkey anti-goat Abs) or Alexa Fluor 647 donkey anti-goat Ab (by applying donkey anti-goat Ab only).

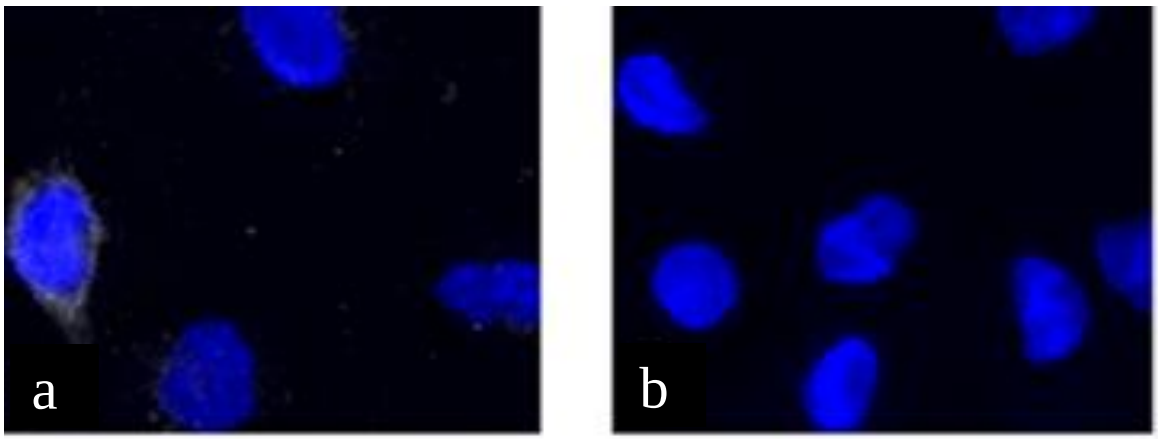

Figure 36. *The negative control stining using goat anti-human IgG and fluorescence labeled Abs (a – Cy5 and DAPI channels) or fluorescence labeled Abs only (b – Cy5 and DAPI channels)*

Cells expressing CD31 and therefore representing placental FECs under all conditions internalized human IgG and IgG was transferred to vesicular compounds within the cells. However, no clear difference was observed for different amounts of IgG internalized at pH 6.0 or pH 7.4 or at different IgG concentrations, respectively. However, in contrast to BeWo cells, where the only IgG receptor expressed is hFcRn, FECs express at least two different IgG receptors, hFcRn and hFcγRIIb2, respectively. This might influence the internalization of IgG in FECs.

Clearly, more experiments are required to characterize the mechanism of endocytosis and the participation of the individual receptors in these cells. Previous work in placental FECs isolated as described by [Jinga, 2000], strongly argued for involvement of hFcRn in bipolar transcytosis across polarized grown FEC cells [Radulescu, 2004]. Based on the presence of a 55 kDa Fcγ receptor at the surface of the cell, [Gafencu, 2003] suggested a contribution of this second receptor to the internalization of IgG. Though [Gafencu, 2003] did not identify this protein, hFcγRIIb2 would in principle be capable of bipolar transcytosis [Hunziker, 1989] or internalization of at least IgG complexes [Miettinen, 1989]. However, hFcγRIIb2 has low affinity for monomeric IgG, which instead of immune complexes should be transferred to the offspring [Karas, 1982].

7. SUMMARY

In this work, for the first time, we demonstrated the presence of hFcRn (α-chain) not only in the first cell type of the placental barrier (STB), but also in FECs *in situ*. [Firan, 2001] had clearly shown the involvement of hFcRn in materno-fetal transfer of IgG, but due to contradictory data in literature, expression of hFcRn in the FEC remained doubtful.

Using a very specific anti-hFcRn α-chain antibody we clearly showed by IFM *in situ* and by RT-PCR, Western blotting and IFM *in vitro* that hFcRn is expressed in the placental endothelial cells. Furthermore, expression of β2-microglobulin was demonstrated in placental endothelial cells. A protocol was established to study the subcellular localization of hFcRn by IFM and confocal microscopy. The localization of hFcRn was investigated in trophoblast-derived BeWo cells overexpressing hFcRn. Compartments positive for hFcRn were colocalized with a marker for early endosomes (EEA1), which is in line with the IgG transporting function of the receptor. We speculate that problems to detect hFcRn α-chain *in situ* so far were related to antibody specificity.

We also demonstrated the presence of a second Fc receptor, hFcγRIIb2 in FECs *in situ* and *in vitro*. Although, we were able to show internalization of human IgG into these cells, we could not clarify the individual contribution of hFcRn or hFcγRIIb2 to IgG transport in these cells.

8. OUTLOOK

Understanding the mechanism of IgG transport across the placental barrier is of great importance not only with respect to the physiologic delivery of antibodies to the offspring. Pathogenic antibodies are also delivered across the placenta and research aims to find new strategies to block this transfer [Mathiesen, 2013]. To design these strategies the receptor(s) involved in the transfer must be known. Moreover, biologicals (monoclonal antibodies) used to treat e.g. maternal inflammatory diseases during pregnancy, also cross the placenta [Chaparro, 2011], [Andersen, 2009]. Detailed knowledge of the placental IgG receptors will help to design biologicals with maximum effect for the mother but minimal transplacental passage.

The proof of hFcRn expression in placental FEC is an important step in the characterization of transplacental IgG transfer. Future work has to clarify the individual contribution of hFcRn and hFcγRIIb2 to monomeric IgG transport across this cell type.

9. ABBREVIATIONS

A.bidest.	Aqua bidestillata
Ab	Antibody
AMPS	Ammonium Persulfate Solution
BCA	Bicinchoninic Acid Assay
BM	Basal plasma membrane
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CH1-CH3	Constant regions of IgG heavy chain
CK7	Cytokeratin7
CL	Constant region of IgG light chain
CTB	Cytotrophoblast
DAPI	4',6-diamidino-2-phenylindole (nuclear stain)
DC	Dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EBM	Endothelial Cell Basal Medium
EC	Endothelial cells
ECL	Enhanced chemoluminescence
EDTA	Ethylenediaminetetraacetic acid
EGM-MV	Endothelial Cell Growth Medium – Microvascular
FCS	Fetal Calf Serum
FEC	Fetal endothelial cells
GE	Gel Electrophoresis
HBSS	Hank's Balanced Salt Solution
HEGF	Human Epidermal Growth Factor
HFcRn	Human neonatal Fc receptor
HOPE	Hepes Glutamic Acid Buffer Mediated Organic Solvent
HPEAC	Human placental endothelial arterial cells
HPEC	Human placental endothelial cells
HPEVC	Human placental endothelial venous cells
HRP	Horseradish Peroxidase
IF	Immunofluorescence
IFM	Immunofluorescence microscopy
IgG	Immunoglobulin G
IPA	Department of Pathophysiology and Allergy Research
ITAM	Immunoreceptor tyrosin activation motif
ITIM	Immunoreceptor tyrosin inhibitory motif
IVS	Intervillous space
KCl	Potassium chloride
KH_2PO4	Potassium dihydrogen phosphate
MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter
MVM	Microvillous plasma membrane
Na_2HPO_4	Disodium hydrogen phosphate
Na_2HPO_4 x $2H_2O$	Disodium hydrogen phosphate dihydrate

NaCl	Sodium chloride
ND	NanoDrop
NH_4Cl	Ammonium chloride
NK	Natural killers
nt	Nucleotide
PA	Polyacrylamide
PBS	Phosphate buffered saline
PCR	Polymerae Chain Reaction
PECAM-1	Platelet Endothelial Cell Adhesion Molecule, CD31
PFA	Paraformaldehyde
PVDF	Polyvinylidene fluoride
r.t.	Room temperature
rpm	Revolutions per minute
RQI	RNA Quality Indicator
RT	Reverse Transcription
SDS PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
STB	Syncytiotrophoblast
TAE	Tris-acetic acid-EDTA
TEMED	Teramethylethylendiamin
T-PER	Tissue Proten Extraction Reagent
VH	Variable region of IgG heavy chain
VL	Variable region of IgG light chain
WB	Western blotting
%	Percent
µl	Microliter

10. REFERENCES

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2012	Bachelor thesis at the Department of Pathophysiology and Allergy Research (MedUniWien, General Hospital, Vienna). Title: "In situ analysis of vitamin D_3-synthesizing enzyme 1alpha-hydroxylase (CYP27B1) expression in immune cells within colorectal cancer"
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04/04/2014	Participation at the Long Night of Research ("Lange Nacht der Forschung") at the Medical University of Vienna 2014 *http://www.meduniwien.ac.at/hp/ipa/news/singleview/?tx_ttnews[tt_news]=2535&cHash=649a1cf63264b582bace6622ea87c64e*
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Languages

Ukrainian	native language
German	fluent
English	fluent
Russian	fluent

IT

- Microsoft Office (Word, Excel, PowerPoint), Adobe Photoshop
- Operating systems (LINUX, Windows 2000, XP, Vista, 8)

Special skills

- Structured principle of operation
- Organizing ability
- Teamplayer
- Creative ideas
- Practical knowledge of various analytical methods

Hobbies

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2003-2005	Humane Medizin Studium an der Staatlichen Medizinischen Universität der Stadt Ivano-Frankivsk, Ukraine
2005-2007	VWU Vorstudienlehrgang der Wiener Universitäten, Österreich
2007-2012	Biologie Studium an der Universität Wien, Österreich
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04/04/2014	Teilnahme an „Lange Nacht der Forschung“ / Medizinische Universität Wien 2014 *http://www.meduniwien.ac.at/hp/ipa/news/singleview/?tx_ttnews[tt_news]=2535&cHash=649a1cf63264b582bace6622ea87c64e*
15/07/2014	Tutor bei Workshop “Was tut Daniel Düsentrieb? So werkt ein Forscher” an KinderuniWien / Medizinische Universität Wien (team of Dr. I.Ellinger) *http://www.meduniwien.ac.at/expatho/placenta/ellinger/Home.html*

Sprachen

Ukrainisch	Muttersprache
Deutsch	fliessend
Englisch	fliessend
Russisch	fliessend

Spezielle Fähigkeiten

- Analytische Denkweise und schnelle Auffassungsgabe
- Strukturierte Arbeitsweise
- Teamplayer
- Praktische Kenntnis von verschiedenen analytische Methoden
- Hohe Eigenmotivation

IT

- Microsoft Office (Word, Excel, PowerPoint), Adobe Photoshop
- Operating systems (LINUX, Windows 2000, XP, Vista, 8)

Freizeit

Schwimmen, Spazieren, Kochen, Tanzen, Musik, Kino