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Expression of the neonatal Fc-receptor in placental-fetal endothelium and in cells of the placental immune system

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ABSTRACT

Introduction: Starting from the second trimester of pregnancy, passive immunity is provided to the human fetus by transplacental transfer of maternal IgG. IgG transfer depends on the neonatal Fc receptor, FcRn. While FcRn localization in the placental syncytiotrophoblast (STB) has been demonstrated unequivocally, FcRn expression in placental-fetal endothelial cells (pFECs), which are part of the materno-fetal barrier, is still unclear. Therefore, this study aimed to elucidate the spatio-specific expression pattern of FcRn in placental tissue.

Methods: FcRn expression was investigated by western blotting in term placentas and in isolated human placental arterial and venous endothelial cells (HPAEC, HPVEC) using a validated affinity-purified polyclonal anti-peptide antibody against the cytoplasmic tail of FcRn α-chain. *In situ* localization of FcRn and IgG was studied by immunofluorescence microscopy on tissue sections of healthy term placentas.

Results: FcRn expression was demonstrated in placental vasculature particularly, in HPAEC, and HPVEC. FcRn was localized in cytokeratin 7^+ STB and in $CD31^+$ pFECs in terminal as well as stem villi *in situ*. Additionally, $CD68^+$ placental macrophages exhibited FcRn expression *in situ*. Endogenous IgG partially co-localized with FcRn in STB, pFECs, and in placental macrophages.

Discussion: Placental FcRn expression in endothelial cells and macrophages is analogous to the expression pattern in other organs. FcRn expression in pFECs suggests an involvement of FcRn in IgG transcytosis and/or participation in recycling/salvaging of maternal IgG present in the fetal circulation. FcRn expression in placental macrophages may account for recycling of monomeric IgG and/or processing and presentation of immune complexes.

1. Introduction

The neonatal Fc receptor, FcRn, appears as heterodimer consisting of an α-chain and β2-microglobulin. FcRn is expressed in many human tissues [1,2] including the placenta [3,4], where it serves several functions. First, it extends the serum half-life of its two ligands, IgG and albumin, by protecting them from degradation, mainly in endothelial and hematopoietic cells. In addition, FcRn mediates IgG-based immune responses at mucosal sites. This includes transcytosis of IgG and immune complexes (IC) across epithelial cells, MHC class II-restricted antigen presentation and MHC class I-restricted cross-presentation of IgG-complexed antigens in antigen-presenting cells [5]. FcRn is increasingly exploited in drug delivery applications [1,6–9].

Placental transfer of maternal IgG provides humoral immunity to the fetus. In humans, IgG transfer occurs *in utero* predominantly during the third trimester [10]. Placental IgG transfer was shown to depend on

Abbreviations: Human neonatal Fc receptor, FcRn; Syncytiotrophoblast, STB; Placental-fetal endothelial cells, pFEC; Human placental arterial endothelial cells, HPAEC; Human placental venous endothelial cells, HPVEC

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its interaction with FcRn by using an IgG mutant unable to bind to FcRn in *ex vivo* placental perfusion experiments [11]. Over recent years, placental IgG transfer has gained attention for several reasons. First, vaccination of the mother has become an important strategy to increase early life immunity [12]. Second, transplacental transport of therapeutic molecules allotted to the fetus is considered a possibility for prenatal disease treatment [13]. Some therapeutic antibodies, which are only approved for the treatment of maternal diseases, in contrast, may compromise the fetus upon placental transfer. Third, maternal auto- and alloimmune antibodies can reach the fetus and cause severe diseases in newborns [14,15]. Finally, microbial antigens and allergens may be transferred across the placenta, most likely as IgG-ICs [16]. Exploiting or influencing placental IgG transport requires a thorough understanding of the underlying cellular transport mechanism. This is also important for the functionalization of IgG-subtypes for transplacental transfer, which is a promising prophylactic and therapeutic approach [17–19].

Uptake of maternal IgG is mediated by the syncytiotrophoblast (STB). In agreement with the *in situ* localization of FcRn in the STB [3,4,20,21], receptor expression was confirmed in isolated and *in vitro* cultured term STB [22]. Due to the high affinity of FcRn to IgG at low pH (< 6.5), binding of IgG to FcRn occurs after fluid phase endocytosis in acidified endosomes. Subsequently, FcRn mediates either IgG transcytosis or recycling in STB [23–25].

Placental tissue also contains an endothelium, the role of which has been neglected in placental transport [26–28]. Endothelial cells present in the placental macro- or microcirculation differ with respect to phenotype and physiology [29,30]. In addition, venous and arterial placental-fetal endothelial cells (pFECs) with different phenotypic, genotypic, and functional characteristics have been described [31].

The mechanism of placental IgG transcytosis across pFECs is not understood. While FcRn expression in endothelial cells of various organs has been shown [32,33], FcRn *in situ* localization in pFECs remains contradictory. The majority of data reported an absence of FcRn from pFECs [3,4,20]. One publication [34] mentioned FcRn expression in pFECs, but without presenting images, while a recent publication showed FcRn expression in pFECs and STB [2]. In contrast to these *in situ* data, *in vitro* cultured primary human placental endothelial cells were found to express FcRn and exhibit FcRn-dependent IgG-transcytosis and recycling. In these studies, *in situ* expression of the receptor has not been investigated [35,36].

Additionally, *in situ* expression of another IgG receptor, FcγRIIb2, and its co-localization with IgG in pFECs suggested FcγRIIb2 involvement in placental IgG transfer [34,37,38]. Increased IgG endocytosis and transcytosis has indeed been shown in FcγRIIb2-transfected human umbilical vein endothelial cells compared to non-transfected cells *in vitro* [39], but the *in vivo* contribution of FcγRIIb2 to IgG transcytosis across pFECs remains still unclear. FcγRIIb2 has higher affinity to aggregated IgG compared to monoclonal IgG. It was thus originally suggested to play a role in placental immunosurveillance [40]. At least in mouse yolk sac, IgG transfer to the fetus was found to be independent of FcγRIIb2 [41].

Knowledge about IgG receptors expressed in the individual placental cell types is essential to further explore the interaction of antibodies with cells at the placental barrier and design appropriate therapeutic antibodies targeting the offspring. Due to the contradictory and incomplete available data, we re-investigated the expression of FcRn in human pFECs *in situ,* as well as in isolated and *in vitro* cultured placental endothelial cells using a characterized *anti*-FcRn α-chain antibody (*anti*-$FcRn_{tail}$) [25,42]. Our results not only confirm FcRn expression in pFECs, but also, for the first time, report FcRn expression in human placental macrophages.

2. Materials and methods

2.1. Antibodies

If not stated otherwise, FcRn α-chain was detected by Western blotting (WB) and immunofluorescence microscopy (IFM) using an affinity-purified rabbit antiserum (*anti*-$FcRn_{tail}$; ≈ 0.3 mg/mL; WB: 1:500 and 1:100; IFM: 1:50). *Anti*-$FcRn_{tail}$ was prepared against the sequence PGEAQDADLKDVNVI in the cytoplasmic tail of human FcRn α-chain. Absence of homologies with any protein other than FcRn α-chain was confirmed by NCBI/BLASTP search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *Anti*-$FcRn_{tail}$ detects a ≈ 45 kDa protein by WB in total placental lysates and cell lines transfected with FcRn α-chain cDNA, but does not react with any protein in HL60 lysates. HL60 cells do not express FcRn [3,43], but express FcγRI and II [44].

Other primary antibodies used were rabbit anti-human β2-microglobulin (Sigma, M8523, WB: 1:100), rabbit anti-human CD31/PECAM-1 (Santa Cruz, SC-1506-R (M20), WB: 1:200), mouse anti-human CD31 (Dako, M0823, clone JC70A, IFM: 1:100); mouse anti-human Cytokeratin 7, (Dako, M7018, clone OV-TL 12/30, IFM: 1:100), mouse anti-human CD68 (Dako, M0876, clone PG-M1, IFM: 1:100), mouse *anti*-γ smooth muscle (γsm)-actin (MP Biomedicals, 69133, clone B4, IFM: 1:250), and Alexa Fluor® 488 goat anti-human IgG (Life Technologies-Molecular Probes, A-11013, IFM: 1:2000). Secondary antibodies used were Alexa Fluor 488 (A-11008) or Alexa Fluor 568 (A-11011) conjugated goat anti rabbit IgG, Alexa Fluor 488 (A-11011), Alexa Fluor 568 (A-11004) or Alexa Fluor 647 (A-21235)-conjugated goat anti-mouse IgG (all Life Technologies-Molecular Probes, IFM: 1:2000) and goat anti-rabbit IgG-HRP-conjugated antibodies (Santa Cruz, SC-2004, WB: 1:2000).

2.2. Human placental chorionic tissue

Human placental tissue (n = 5) was obtained within 15 min after caesarian sections of healthy pregnancies at 38–40 weeks of gestation (Department of Obstetrics and Gynecology, Medical University Vienna; EK 724/2010). The tissues were transferred to the laboratory at room temperature within 15 min. For IFM, chorionic tissue from 3 areas per placenta was immediately processed by HOPE-fixation (DCS Innovative Diagnostik-Systeme) and paraffin-embedding [45]. Chorionic tissue of one placenta was additionally formaldehyde-fixed [45].

For isolation of pFECs, human healthy term placentas (n = 3) were obtained after given informed consent of the mothers. Approval of the ethical committee of the Medical University of Graz was granted (24–529 ex 11/12).

2.3. Cell cultures

Isolation of human placental arterial endothelial cells (HPAEC) as well as human placental venous endothelial cells (HPVEC) was done according to Ref. [31] with some modifications. Briefly, after vaginal delivery of the placenta, amnion was removed and corresponding arterial and venous chorionic blood vessels at the apical surface of the chorionic plate were resected. HPEAC and HPEVC were isolated by separate perfusion of chorionic arteries and veins with HBSS containing 0.1 U/ml collagenase, 0.8 U/ml dispase (Roche), and antibiotics (Gibco), pre-warmed to 37 °C. The obtained cell suspension was centrifuged (200 × *g* for 5 min), the pellet was re-suspended with endothelial cell media (PromoCell) and plated on culture plates precoated with 1% gelatin (Sigma). Immunocytochemical characterization was performed on cells cultured up to passage 15 as described in detail elsewhere [30]. In general, the percentage of endothelial cell marker-positive cells in the primary culture was about 99%.

BeWo cells (clone 24) and two clones of BeWo cells stably transfected with FcRn α-chain cDNA, were cultured as described [25]. The cells were used for protein extraction after they had reached > 80%

confluence.

2.4. Protein extraction

For WB, chorionic placental tissue samples were briefly washed in phosphate buffered saline, pH 7.4 (PBS), frozen in liquid nitrogen and stored at −80 °C.

Total lysates from human term placentas and cell lines were prepared using Tissue Protein Extraction Reagent (Pierce Biotechnology). A protease inhibitor cocktail (Pierce Biotechnology) was added to all samples. Protein concentrations were determined using a BCA assay (Pierce Biotechnology).

2.5. Western blotting (WB)

Proteins in lysates were concentrated by acetone precipitation, dissolved in reducing SDS sample buffer, subjected to 12% reducing SDS-PAGE and transferred onto PVDF membranes. Blots were blocked with 5% dry milk powder in PBS containing 0.1% Tween 20 (Blotto) for 1h and then incubated with the respective primary antibody diluted in Blotto at 4 °C overnight. Blots were washed and incubated with matching secondary HRP-conjugated antibody. Bound antibodies were detected by chemoluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Amido Black or Ponceau S (both Sigma) staining was performed to visualize total proteins [46].

2.6. Immunofluorescence microscopy (IFM)

4 µm tissue sections were de-waxed and rehydrated according to the fixation used [47]. Antigen retrieval was done with 0.05% (v/v) citraconic anhydride solution, pH 7.4, for 20 min [48]. Sections were incubated with 5% (v/v) goat serum (Jackson ImmunoResearch Laboratories) in PBS containing 0.05% (w/v) saponin (Sigma) for 1h at room temperature (blocking buffer). Primary antibodies and corresponding Alexa-Fluor®-conjugated secondary antibodies, diluted in blocking buffer, were applied overnight at 4 °C or for 2h at room temperature, respectively. In negative control incubations, primary antibodies were omitted. Nuclei were stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Roche Diagnostics GmbH, 50 µg/mL in PBS). DRAQ5 (Thermo Scientific; 25 µM in PBS) labeling of nuclei was applied when images were acquired by confocal microscopy. After each incubation step, sections were washed intensively with PBS. In co-localization studies, antibodies were added consecutively. Fluoromount-G (SouthernBiotech) was used as mounting medium. Images were acquired using an automated wide-field fluorescence microscope (Axio Imager Z1, Zeiss), equipped with an EC Plan-Neofluar 20x/0.5 objective (Plan-Neofluar, Zeiss) or 63×/1.4 oil objective (Plan-Apochromat, Zeiss) and the following filter sets (Chroma Technology Corp.): 49000 ET-DAPI, 49002 ET-FITC/Cy2, 49008 ET-mCherry, TxRed and 49006 ET-Cy5 in combination with TissueFAXS Image Acquisition and Management Software (Version 4.2; TissueGnostics GmbH). Using a monochrome camera (Hamamatsu), grayscale images of individual fluorescence channels were acquired. Acquired regions were composed of at least 5 x 5 single images that were stitched by the software when required. When appropriate, pseudocolors were assigned to the individual images. Alternatively, confocal images (10 images per section) were acquired using an UltraVIEW ERS Rapid Confocal Imager (Perkin-Elmer) connected to a Zeiss Axiovert 200 microscope fitted with a 63x/1.4 oil objective lens (Plan-Apochromat, Zeiss). Alexa Fluor-488 and -568 fluorophores as well as DRAQ5 were excited at 488, 568 or 647 nm, respectively, using a 488/548/647 multiline argon/krypton laser. Pictures were digitalized and processed by Volocity software (Version 5.5, Perkin Elmer). Individual representative images were further processed with Adobe Photoshop using identical conditions for positive and negative controls.

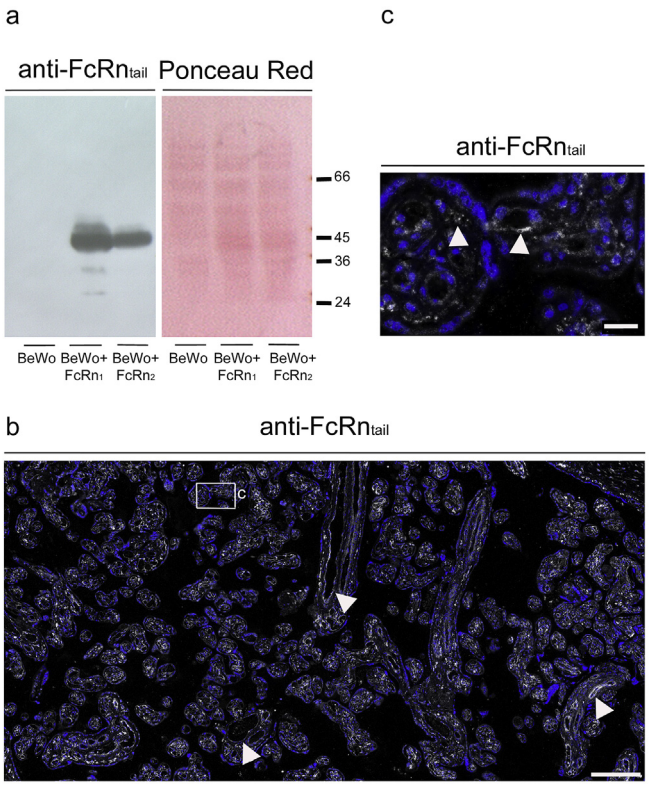

Fig. 1. (A) Detection of FcRn α-chain by WB using an affinity purified polyclonal antibody (*anti*-$FcRn_{tail}$) against the cytoplasmic tail of FcRn α-chain ([25,42]). Lysates of BeWo cells and two different clones of BeWo cells transfected with hFcRn α-chain cDNA (50 µg) were separated on reducing 12% SDS-gels, transferred onto PVDF membranes and probed with *anti*-$FcRn_{tail}$ (dilution 1:500) followed by an HRP-labeled secondary antibody. Binding of antibodies was detected using enhanced chemoluminescence, total proteins were detected with Ponceau S staining. Arrowhead indicates the 45 kDa FcRn α-chain. Molecular weight markers are shown on the right. (b) Localization of FcRn α-chain (red) by IFM in HOPE-fixed and paraffin-embedded 4 µm sections of term chorionic villi. *Anti*-$FcRn_{tail}$ (1:50) in combination with a fluorochrome-labeled secondary antibody was used. Nuclei were labeled with DAPI (blue). Arrowheads indicate cells in the villi stroma detected by *anti*-$FcRn_{tail}$. The bar in (b) represents 200 µm. The area within the white square is displayed enlarged in (c), where the bar represents 20 µm. Images in (b) and (c) were acquired with a 20× objective.

To evaluate FcRn expression in γsm-actin-positive and γsm-actin-negative villi, term chorionic tissue was triple-labeled by consecutive incubation with *anti*-$FcRn_{tail}$, goat anti-rabbit IgG Alexa Fluor 568, mouse *anti*-CK7, goat anti-mouse IgG Alexa Fluor 647, mouse *anti*-γsm-actin and goat anti-mouse IgG Alexa Fluor 488. Nuclei were stained with DAPI. Images were acquired, digitized and stitched with the automated wide-field fluorescence microscope.

3. Results

3.1. FcRn α-chain expression in STB and stromal cells of chorionic villi in situ

To study FcRn α-chain expression in human placental chorionic tissue, we employed a previously characterized *anti*-$FcRn_{tail}$ antibody [25,42]. In WB, the *anti*-$FcRn_{tail}$ antibody detects a ≈45 kDa protein in total lysates of FcRn α-chain cDNA transfected BeWo cells (Figs. 1a and 5 [25]) as well as in 293T + cells (Supplemental Fig. 2a [42]), but not in mock-transfected cells. The additional high molecular weight bands seen in FcRn-transfected 293T + probably correspond to hFcRn dimers [49,50] that may form due to insufficient β2-microglobulin expression.

Importantly, in total placental tissue lysates, this antibody displayed a single protein band of ≈45 kDa (Supplemental Fig. 2a, left panel and Fig. 5 [25,42]).

IFM with *anti*-FcRn$_{tail}$ antibody on HOPE-fixed chorionic tissue sections (Fig. 1b and c) revealed *in situ* FcRn α-chain localization in the STB as well as stromal cells of chorionic villi (Fig. 1b and c, arrowheads).

In order to confirm this placental expression pattern of FcRn α-chain, we tested two other *anti*-FcRn antibodies (see supplemental data for antibody description). *Anti*-FcRnα$_2$ antibody targets the α2-domain of the FcRn α-chain. It detected a ≈45 kDa protein in placental lysates [42] and revealed a ≈45 kDa protein in MDCK cell lysates transfected with either hFcRn cDNA or hFcRn-flag-tagged cDNA (Supplemental Fig. 1a). In IFM, *anti*-FcRnα$_2$ produced a staining pattern similar to *anti*-FcRn$_{tail}$ antibody: FcRn was loclized in the STB and stromal cells (Supplemental Figs. 1b and c). Supplemental Fig. 2 displays a comparison of a commercial *anti*-FcRn antibody (Santa Cruz, H-274) and *anti*-FcRn$_{tail}$. In WB experiments, the commercial antibody and *anti*-FcRn$_{tail}$ exhibited a similar reaction with a ≈45 kDa protein in lysates of FcRn overexpressing (293T+) cells, but not in lysates of mock-transfected (293T) cells. On total placental lysates, however, the commercial antibody, in contrast to *anti*-FcRn$_{tail}$ reacted with several proteins (Supplemental Fig. 2a, right panel), questioning the specificity of this antibody in placental tissue. In IFM, the commercial antibody also stained stromal cells as observed for *anti*-FcRn$_{tail}$ (Supplemental Fig. 2b, arrows), but exhibited a more pronounced staining of the apical microvillus membrane of the STB (Supplemental Fig. 2b, right image, arrows).

Labeling of the STB as well as stromal cells with *anti*-FcRn$_{tail}$ was confirmed in a total number of five placentas and three different regions per placenta. Exemplary images of three placentas are displayed in Supplemental Fig. 3.

Alternative fixation with formaldehyde and immunolabeling with *anti*-FcRn$_{tail}$ also resulted in detection of FcRn α-chain in STB and stromal cells (Supplemental Fig. 4a). Under identical staining, acquisition and image-processing conditions, formaldehyde-fixed tissue exhibited weaker reactivity with *anti*-FcRn$_{tail}$ and was more dependent on the type of antigen retrieval used than HOPE-fixed tissue (Supplemental Fig. 4b), which was expected from previously published results [45,48].

Among the different types of chorionic villi, terminal villi represent the main sites of materno-fetal exchange, including IgG transport. In mature placenta, they comprise about 40% of the villous volume. Stem villi, on the other hand, serve as mechanical support of the villous trees. Their participation in exchange processes is presumably low [51]. Only stromal cells in stem villi express γsm-actin [52,53]. To explore the presence of FcRn α-chain throughout the chorionic tissue from stem to terminal villi, we performed a consecutive triple staining of tissue sections employing *anti*-FcRn$_{tail}$ to label FcRn α-chain (red in Fig. 2a), followed by immunodetection of the STB marker CK7 [47] (white in Fig. 2c) to outline all villi and finally an *anti*-γsm-actin antibody to identify stem villi (green perivascular labeling in Fig. 2b and d). Visual inspection of the resulting images confirmed FcRn α-chain presence in cells of the stroma of all villi (Fig. 2a), those positive for γsm-actin (Fig. 2b, arrows) as well as those negative for this marker (Fig. 2b, arrowheads). For quantitative comparison of *in situ* FcRn α-chain expression in γsm-actin-positive and -negative villi, we employed automated image analysis (Supplemental Figs. 5a–c). The mean intensities of FcRn α-chain associated fluorescence in γsm-actin-positive stem villi and in γsm-actin-negative villi were found to be identical (Supplemental Fig. 5d). In summary, these results demonstrated FcRn α-chain expression in terminal villi and stem villi.

3.2. FcRn α-chain expression in pFECs and macrophages in situ

To clarify the identity of FcRn α-chain-positive stromal cells, we performed co-immunolabeling. Tissue sections were stained for FcRn α-chain using *anti*-FcRn$_{tail}$ in combination with either *anti*-CK7 or anti-CD31 (pFEC marker [54]) and analyzed by confocal microscopy (Fig. 3). FcRn is shown in CK7-positive STB (Fig. 3a–c) as well as in CD31-positive pFECs (Fig. 3e–g). Fig. 3d and h, respectively, are the corresponding negative controls where primary antibodies were omitted. Additionally, CD31-negative cells in the stromal core exhibiting high FcRn α-chain expression, were observed (asterisks in Fig. 3 g). Triple staining of tissue sections for simultaneous detection of FcRn α-chain, CD31, and CK7 was also carried out (Supplemental Fig. 6) and the samples were analyzed by wide-field fluorescence microscopy. FcRn α-chain (Supplemental Fig. 6a) was present in the CK7-positive STB (Supplemental Fig. 6c, arrowhead) as well as in CD31-positive pFECs (Supplemental Fig. 6b, arrow). Again, CD31-negative stromal cells with strong staining of FcRn α-chain were observed (Supplemental Figs. 6a and b, asterisk). By triple staining of tissue sections with *anti*-FcRn$_{tail}$ (Supplemental Fig. 7a), *anti*-CK7 (Supplemental Fig. 7c) and anti-CD68, a macrophage marker [55] (Supplemental Fig. 7b), the stromal cells with intense FcRn α-chain expression were identified to be macrophages (Supplemental Figs. 7a and 7b, asterisks). FcRn α-chain-positive, but CD68-negative cells are STB (arrowhead) and pFECs (arrow). Nuclei of the cells are shown in Supplemental Figs. 6d and 7d. Supplemental Figs. 6e–h and 7e-h show the corresponding negative controls.

We also performed co-immunostaining for FcRn α-chain (Fig. 4a, d) and endogenous IgG (Fig. 4b and e) and visualized the staining by confocal microscopy. FcRn α-chain showed expression in the STB, in pFECs (identified by the vessel lumen, arrows in Fig. 4c and f) and other stromal cells (presumably macrophages, asterisks, in Fig. 4c and f). IgG was also found in all these cells, but exhibited comparable staining intensities in the STB and in pFEC. In some cells an intermediate color (yellow in Fig. 4c and f) was observed, which is indicative for co-localization of FcRn α-chain and IgG. However, such an intermediate

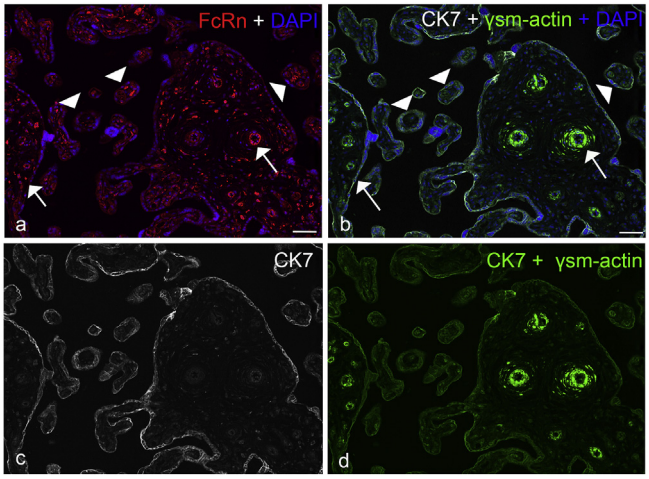

Fig. 2. Localization of FcRn α-chain by IFM in terminal and stem villi of HOPE-fixed and paraffin-embedded 4 μm sections of term chorionic villi. (a) FcRn α-chain was labeled with *anti*-FcRn$_{tail}$ in combination with an Alexa Fluor 568-conjugated secondary anti-rabbit antibody (red). Thereafter, the sections were consecutively incubated with a mouse antibody against CK7 combined with an Alexa Fluor 647-conjugated secondary antibody (white) to identify the STB (c), followed by a mouse antibody against γsm-actin combined with an Alexa Fluor 488-conjugated secondary antibody (green) to identify the perivascular γsm-actin-positive cells in stem villi (d). (b) Shows an overlay of images (c) and (d). Since the primary antibodies against CK7 and γsm-actin were both generated in mouse, the Alexa Fluor 488-conjugated secondary antibody added in the last step did not only detect γsm-actin (displayed in green), but also CK7 (white), resulting in a white-green labeled STB area in (b). Nuclei were stained with DAPI (blue). FcRn α-chain expression is present in γsm-actin-positive (arrows) as well as γsm-actin-negative villi (arrowheads). Images were acquired with a 20× objective. Bars represent 50 μm.

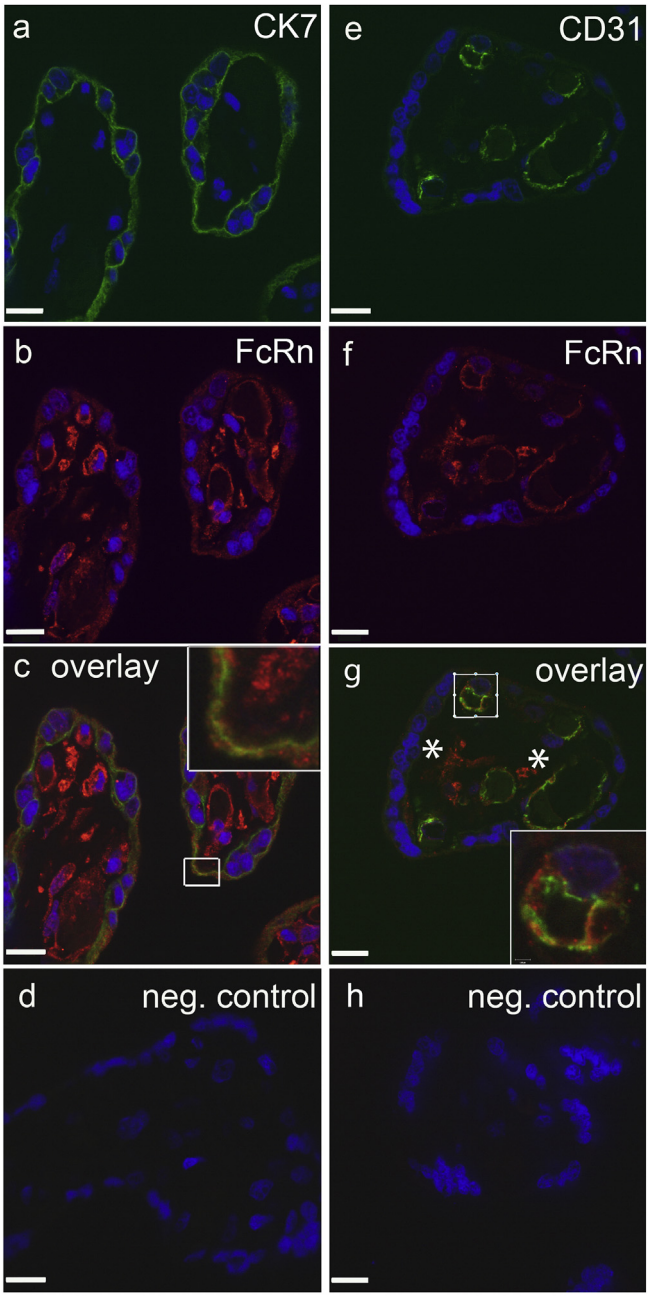

Fig. 3. Localization of FcRn α-chain (red in b, c, f, g) by IFM in HOPE-fixed and paraffin-embedded 4 μm sections of term chorionic villi analyzed by confocal microscopy. The sections were co-labeled with antibodies against CK7 (green in (a) and (c)) to identify the STB as well as CD31 (green in (e) and (g)) to identify pFECs. Nuclei were stained with DRAQ5 (blue). (d) and (h) show the corresponding negative controls where primary antibodies were omitted. Inserts in (c) and (g) show FcRn α-chain expression in the STB (CK7 +) and the pFEC ($CD31^{+}$), respectively, in more detail. FcRn α-chain expression is present in CK7-positive STB (c) as well as in CD31-positive-pFECs (g). Images were acquired with a 63× objective. Bars represent 12 μm.

color is obtained only if the intensities of the two fluorescence probes are similar [56]. Since the expression levels of FcRn vary significantly among the placental cells and, likewise, the concentration of IgG among and within individual cells was not homogenously distributed, the question to which extent FcRn and IgG interact in the different placental cell types cannot be answered by this approach.

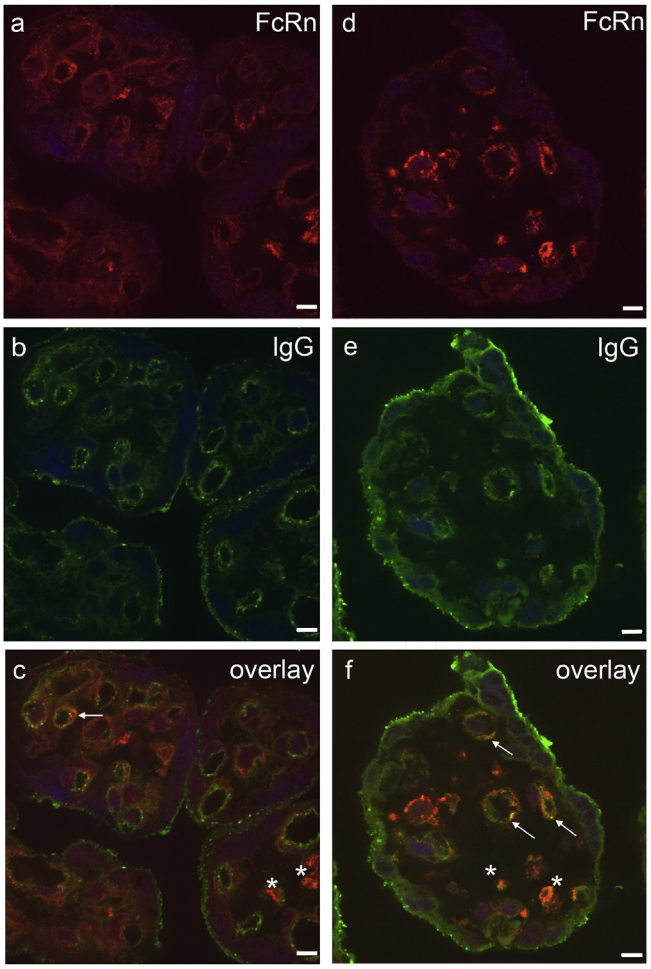

Fig. 4. Localization of FcRn α-chain (red in a, c and d, f) by IFM in HOPE-fixed and paraffin-embedded 4 μm sections of term chorionic villi analyzed by confocal microscopy. The sections were co-stained with a fluorescent-labeled antibody against endogenous IgG (green in b, c and e, f). Nuclei were stained with DRAQ5 (blue). FcRn α-chain and IgG were found in the STB layer, in pFECs, which were identified by vessel lumens (arrows) and in macrophages (asterisks). Images were acquired with a 63× objective. Bars represent 12 μm.

3.3. FcRn α-chain is expressed in isolated and cultured human placental endothelial arterial (HPEAC) and endothelial venous (HPEVC) cells

Lysates of HPEACs and HPEVCs and of the corresponding total placental tissue samples from three independent preparations were analyzed for expression of FcRn α-chain using *anti*-$FcRn_{tail}$ and its small subunit, β2-microglobulin by WB. Lysates of BeWo cells and BeWo cells overexpressing FcRn α-chain were included (Fig. 5). A strong signal for FcRn α-chain was observed in BeWo cells overexpressing FcRn α–chain (B +), while endogenous FcRn expression in BeWo cells (B) is low and requires affinity purification for detection by WB [23]. All placental tissue samples as well as HPEVCs and HPEACs with the exception of one sample showed expression of FcRn α-chain. The membrane was stripped and re-probed with an *anti*-β2-microglobulin antibody. β2-microglobulin was detected in all samples. All samples with the exception of the epithelial BeWo cells expressed the pFEC-marker CD31.

4. Discussion

In contrast to FcRn expression in the STB, expression of FcRn in pFECs remained contradictory [2–4,20,34–36]. In this work, we demonstrated FcRn expression both in the STB and in pFECs in term chorionic villi *in situ,* as well as in isolated and cultured venous and

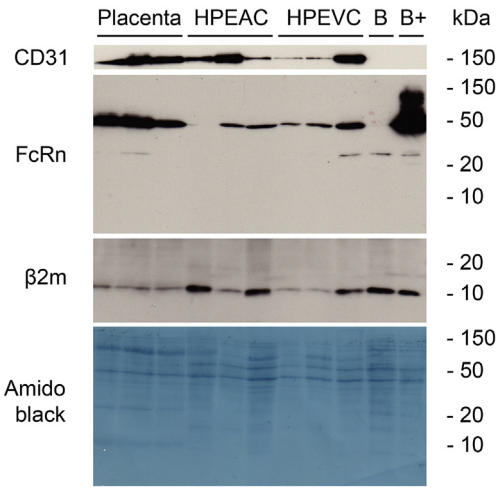

Fig. 5. Detection of CD31, FcRn α-chain and β2-microglobulin by WB in isolated and cultured placental endothelial cells. Lysates of 3 term placentas and 3 corresponding preparations of HPEAC and HPEVC, BeWo as well as FcRn α-chain cDNA transfected BeWo cells (40 μg/lane) were separated on reducing 12% SDS-gels and transferred onto PVDF membranes. Blots were probed with *anti*-FcRn$_{tail}$ antibody diluted 1:100, followed by an HRP-labeled secondary antibody. Binding of antibodies was detected using enhanced chemoluminescence. Following stripping, the same blot was probed with *anti*-β2 microglobulin antibody diluted 1:100 followed by an HRP-labeled secondary antibody. Amido black staining was applied to visualize the amount of proteins loaded. A separate blot was produced and incubated with anti-CD31 antibody (1:200) followed by an HRP-labeled secondary antibody. Molecular weight markers are shown on the right.

arterial pFECs. For the first time, we show FcRn expression in placental macrophages *in situ*.

Validation of antibody specificity is crucial for reliability of obtained results [57–61]. We predominantly used *anti*-FcRn$_{tail}$ antibody characterized previously [25,42] and in this work. We tested the specificity of the antibody in WB experiments using lysates from various FcRn α-chain-overexpressing cell lines as positive controls and lysates from the corresponding mock-transfected cell lines and FcRn α-chain-negative HL60 cells (see also [25,42]) as negative controls. In all FcRn α-chain expressing samples, the antibody detected one major protein of ≈45 kDa, which is equivalent to the molecular weight of FcRn α-chain purified from human placental tissue [3]. Most importantly, in total placental lysates, *anti*-FcRn$_{tail}$ antibody revealed only a single band of 45 kDa. We confirmed FcRn expression in STB, pFECs and placental macrophages *in situ* using a second affinity-purified *anti*-FcRn antibody, *anti*-FcRnα$_2$, directed against the extracellular domain of the receptor. In the majority of IFM experiments, we used HOPE-fixation, which is known for excellent preservation of antigenicity [45]. Binding of antibodies in HOPE-fixed placentas to endogenous Fc-receptors known to be expressed in pFECs and macrophages [62] seems unlikely, since various antibodies directed against other antigens demonstrated the expected specific localization in this study as well as others [45]. Although formaldehyde-fixation reduced staining intensities, FcRn expression in stromal cells was still detected.

Several previous studies failed to detect FcRn expression in pFECs and macrophages in terminal chorionic villi *in situ* [3,4,20]. Various antibodies were used in these studies, such as polyclonal anti-peptide antibodies targeting amino acid sequences in the extracellular domain of human FcRn [3,4], or monoclonal antibodies against the extracellular domain of rat FcRn [20,63]. While total placental tissue is used for immunolocalization studies, not all authors validated these antibodies for total placental tissue. Leach and coworkers investigated the specificity of their antibody via WB and found a single protein band corresponding to FcRn α-chain. But the proteins subjected to WB were enriched from placental lysates by low pH-dependent IgG binding [3]. Simister and coworkers on the other hand used STB brush-border-enriched fractions [4], obfuscating whether these antibodies would recognize additional proteins in total placental tissue lysates. As shown in our study (Supplemental Fig. 2) and those of others [57,60], not all antibodies are specific for the protein of interest in the target tissue. The commercial *anti*-FcRn antibody employed in comparison to *anti*-FcRn$_{tail}$ antibody recognized several proteins in addition to a 45 kDa protein (FcRn) in total placental protein lysates. In immunolocalization experiments, the most intense signal obtained with the commercial antibody was found in the STB. In contrast, upon staining with *anti*-FcRn$_{tail}$ antibody, staining intensity in the STB was often lower as compared to expression in pFEC and macrophages.

There are several publications in line with FcRn expression in pFECs. Lyden et al. [34] reported FcRn expression in pFECs using the antibody described in Ref. [3]. Isolated and *in vitro* cultured human pFECs showed FcRn expression [36] and exhibited FcRn-dependent IgG-transcytosis and recycling [35]. Recently, FcRn expression was investigated throughout many tissues using an antibody developed against a recombinant human protein corresponding to amino acids Lys175 to Ser294 of hFcRn. FcRn expression in human placental STB and pFEC was shown in immunolocalization experiments [2].

FcRn expression in placental macrophages corresponds to the reported FcRn expression in macrophages of other organs and cynomolgus monkey placentas [2,33,64].

FcRn functions in epithelial, endothelial and antigen-presenting cells have been intensively studied [1,8,19]. FcRn can transcytose IgG across epithelia [64] and endothelia [35,36], suggesting a role of FcRn in overall transplacental passage of IgG. Presence of FcRn in pFECs in terminal villi of the placenta that provide the largest materno-fetal exchange surface [51,65] and are thought to mediate transplacental passage of molecules, would support this function. Endothelial [35] as well as immune cells [33] can recycle (monomeric) IgG. Thus, macrophages might play a role in recycling monomeric IgG within the placental stroma, while pFECs may recycle IgG (or albumin) at their luminal side thus preventing the degradation of IgG (or albumin) present in the fetal circulation. Localization of FcRn in all vessels of the chorionic villi would be in line with a protective FcRn function. Finally, FcRn can bind both monomeric IgG molecules as well as IgG-ICs. Binding of IgG-ICs results in increased sorting to lysosomes and results in their degradation [64]. Expression of FcRn in different placental cell types may thus be important to reduce placental transfer of IgG-ICs. Indeed, IgG-ICs are not as efficiently transferred across the placenta as monomeric IgG; a significant portion of IgG-ICs is trapped in the placenta [40,66].

In conclusion, using a specific antibody, we demonstrated FcRn expression in STB, macrophages, and pFECs of all chorionic villi in human term placenta. Although the functional roles of FcRn in the different placental cells remain to be deciphered in future studies, the partial co-localization of endogenous IgG and FcRn suggests a functional relationship not only in STB, but also pFECs and macrophages.

Declaration of interest

None.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.02.012.

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