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Effects of maturation on RNA transcription and protein expression of four MRP genes in human placenta and in BeWo cells

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Abstract

The placenta is a multifunctional organ that protects the fetus from toxic compounds and the MRPs contribute to this function. The expression of *MRP1*, *MRP2*, *MRP3*, and *MRP5* was compared in human placental tissue and in BeWo cells by real-time RT-PCR analysis; protein expression was assessed by Western blot. *MRP1* and *MRP3* were the most abundantly expressed genes in placenta but only *MRP1* was highly expressed in the BeWo cells. Expression of *MRP1* increased 4-fold in the third as compared with first trimester placental samples, and increased 20-fold with polarization of BeWo cells. *MRP2*, *MRP3*, and *MRP5* were weakly expressed both in placenta and BeWo cells. Protein expression followed mRNA quantification for *MRP1* and *MRP5* but not for *MRP2* and *MRP3*. These data indicated that *MRP1* and *MRP5* increase with trophoblast maturation, suggesting a particular role for these proteins in the organ functional development.

Keywords: MRP proteins; Placenta; Gene expression; BeWo; Maturation; Polarization; Real-time PCR

The immature renal and hepatic metabolic and transport systems of the fetus are inadequate to protect it from the many toxic compounds to which it might be exposed [1]. Instead, it is removal by the placenta that protects the fetus from harmful accumulation of toxic substances. The placenta starts to develop near the end of the first week of gestation, when the blastocyst becomes surrounded by multiple layers of primordial epithelial cells, the cytotrophoblasts. These ultimately differentiate into the endocrinologically active villous syncytiotrophoblasts and, to a lesser extent, into the junctional trophoblasts as well as the invasive intermediate trophoblasts that mediate the penetration of the anchoring villi into the maternal decidua. During the first trimester, the rapid growth of the placenta is driven mainly by multiplication of the cytotrophoblasts which predominate at this stage of development. As cytotrophoblasts mature, they lose mitotic activity and fuse to form the polynucleated, polarized syncytiotrophoblasts. During the second and third trimester the growth of the placenta is slower, but the ratio of syncytiotrophoblasts to cytotrophoblasts progressively increases.

The transplacental passage of nutrients from the mother to the fetus, as well as metabolites in the opposite direction, is finely regulated through concentration gradients and specific transporters of the syncytiotrophoblasts [2]. A wide variety of substrates crossing the placenta are transported by multiple carrier proteins that are specifically localized to either the basolateral (fetal facing) or apical (maternal facing) plasma membrane domains of the polarized trophoblasts [3–6]. Prominent among these are multidrug resistance proteins (MRPs) [7], with transport functions akin to those that are involved in the hepato-biliary elimination of organic anions, usually in the form of conjugates with glutathione, glucuronic acid, and sulphates [8–10]. The six known MRPs (MRP1–6) comprise a subfamily of the large class of ATP binding cassette (ABC) proteins, which include, among others, the Cystic Fibrosis Transmembrane

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conductance Regulator (CFTR). The MRPs are also abundantly expressed in the kidney, intestine, blood–brain barrier, and liver, where they have been shown to be involved in the resistance of these organs to chemotherapeutic agents [11]. In the liver, MRP2 is the canalicular multispecific organic anion transporter that uniquely accounts for secretion into bile of conjugated organic anions such as bilirubin glucuronides [12].

In the placenta, expression of mRNA for *MRP1* and *MRP5* [7] has been reported, as well as for *MRP2* and *MRP3* [13]. We have demonstrated previously that *MPR1* and, to a lesser extent, *MRP5* are expressed in a trophoblastic cell line (BeWo) derived from a choriocarcinoma of human placenta [14]. These cells display several functions of normal human trophoblasts, including hormone production [15,16]. They are mitotically active cytotrophoblast-like cells that, when grown on permeable supports, rapidly differentiate into polarized trophoblast monolayers.

To investigate whether differentiation and/or polarization of the trophoblast may affect the expression of the above four MRP transporters, we compared the mRNA and protein expression for *MRP1*, *MRP2*, *MRP3*, and *MRP5* in total human placental tissue samples, and in both non-polarized and polarized BeWo cells in culture.

Materials and methods

Placental tissues and cell cultures. Normal human placentas were obtained at term from healthy pregnancies and in the first trimester (9–10 weeks of gestation) from therapeutic interruptions of pregnancy. These were kindly provided by Gynecology and Obstetric Department of "Ospedale Infantile Burlo Garofolo," Trieste, Italy, with a protocol approved by the Ethical Committee of the University of Trieste.

BeWo cells were obtained from Istituto Zooprofilattico Sperimentale (Brescia, Italy) and cell culture was performed under standard conditions in DMEM with 10% (vol/vol) FCS and 1% antibiotics (10,000 U/mL penicillin and 10 mg/mL streptomycin). As previously reported [14], the cells were routinely maintained in 75-cm^2 Falcon flasks for 3 days, then harvested by exposure to a solution of 0.25% trypsin and 0.02% EDTA, and transferred onto either petri dishes (100 mm diameter) or onto Transwell-clear polyester filters (24 mm diameter) (Costar, New York, NY, USA). The medium was replaced with fresh medium every other day. To assess the attainment of polarization, which occurred at 4 days, the transepithelial electrical resistance (TEER) across polarized BeWo cells grown on filter was measured serially, using the Millicell-ERS epithelial voltohmmeter and electrodes (Millipore, Bedford, MA, USA). Non-polarized cells were scraped from dishes at 2 days and polarized cells from Transwells at 5 days (TEER = 140 Ohm/cm^2), for extraction of total RNA and preparation of plasma membrane vesicles.

HOBIT, a human osteoblast-like cell line [17], used as an external reference, was kindly provided by Dr. M. Romanello of the Department of Biochemistry, University of Trieste, Italy.

The intestinal Caco-2 cell line was obtained from Istituto Zooprofilattico Sperimentale (Brescia, Italy) and the cells were grown similar to BeWo cells on Transwell filters.

Each experiment was performed on three separate placental samples and three different cell preparations.

Membrane vesicle preparation and Western blot analyses. Plasma membrane vesicles of BeWo and Caco-2 were obtained, as previously reported [18], by separation of a crude membrane fraction on a discontinuous sucrose gradient (38% w/v and 19% w/v) followed by a centrifugation at 200,000*g* at 4 °C for 120 min. The membrane fraction at the 19–38% sucrose interface was collected. Membrane preparations were stored in liquid nitrogen in 250 mM sucrose and 20 mM Hepes–Tris, pH 7.4, until use.

Crude membrane fractions from placenta at third trimester were obtained from frozen tissues with modification of the method previously reported [19]. Placenta samples were cut, washed in phosphate-buffered saline (PBS) solution, and further broken up by magnetic stirring overnight. All the passages were performed at 4 °C. The resulting material was homogenized, filtered, and centrifuged at 800*g* for 10 min at 4 °C. The supernatant was collected and centrifuged at 10,000*g* for 10 min, and subsequently at 30,000*g* for 60 min. The pellet was resuspended in the sucrose buffer (250 mM sucrose and 10 mM Hepes–Tris, pH 7.4), revesiculated in a Dounce homogenizer, and finally stored under liquid nitrogen. Protein concentration in membrane fractions was measured by BCA Protein Assay (Sigma–Aldrich, Milan, Italy).

Membrane proteins (50 μg) were solubilized in Laemmli buffer, fractionated on a 7.0% SDS–PAGE gel, and transferred to nitrocellulose membranes by electroblotting, using 25 mM Tris base, 192 mM glycine, and 20% methanol as transfer solution. The previously used [18] rabbit polyclonal antibody anti-Mrp1 (Mrp1-A23) was used to detect MRP1. Anti-MRP2 (M_2 III-6), anti-MRP3 (M_3 II-9), and anti-MRP5 (M_5 I-1) monoclonal antibodies were obtained from Alexis Italy (Vinci-Biochem, Vinci, Italy). Anti-MRP3 (M_3 II-9) was also obtained from Kamiya Biomedical (Seattle, USA). Blots were blocked in 4% (w/v) skimmed milk powder in TBS-T (0.2% Tween 20) for at least 1 h, incubated overnight at 4 °C with primary antibody, and diluted in blocking solution (Mrp1-A23 dilution 1:1.000; M_2 III-6 and M_3 II-9 dilution 1:30; and M_5 I-1 dilution 1:50). After washing three times for 10 min in blocking solution, horseradish peroxidase-conjugated antibodies (goat anti-rabbit IgG, dilution 1:10,000; goat anti-mouse IgG, dilution 1:2000; and goat anti-rat IgG, dilution 1:3000; Sigma–Aldrich, Milan, Italy) in blocking solution were added and blots were incubated for 1 h at room temperature. After washing five times for 5 min in blocking solution, immunoreactivity was visualized by enhanced chemiluminescence detection using ECL plus kit (Amersham Biosciences, Milan, Italy).

Quantitative PCR. Total RNA was extracted from placenta following standard guanidine–isothiocyanate protocols [20]. Total RNA was extracted from both polarized and non-polarized BeWo cells and from HOBIT cells by using the SV total RNA isolation System (Promega, Madison, WI), according to the manufacturer's instructions. After quantification of total RNA by spectrophotometer, RNA dilutions of 100, 10, 1 ng, 100, and 10 pg were prepared to create a quantitative reference standard. Two step quantitative RT-PCR was performed using the TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix. To obtain the maximum specificity in *MRP1*, *MRP2*, *MRP3*, and *MRP5* amplification, the primers for cDNA amplification were designed using the software Primer Express 1.5 (Applied Biosystems). The primers for *MRP1* were designed to amplify the exon 29–30 corresponding to the more conserved NBD2 domain of the protein: (5′-AACCTGGACCC ATTCAGCC-3′, 5′-GACTGGATGAGGTCGTCCGT-3′) as reported by Grant et al. [21]. Primers for *MRP2* (5′-ACAGAGGCTGGTGGC AACC-3′, 5′-ACCATTACCTTGTCACTGTCCATGA-3′), *MRP3* (5′-GGTCGACATTCTTTGCGGA-3′, 5′-CGCTCCAAGATCCTTT TAGCC-3′), and *MRP5* (5′-ACCAGCACCCAGTGGACAAT-3′, 5′-CGTCTGGCCCAACTTCATTC-3′) were chosen from specific gene regions.

Real-time PCR was performed on cDNA generated by RT reaction using the sequence detector 7700 ABI PRISM and SYBR Green I chemistry (Applied Biosystems). Thirty-five cycles were performed at the following temperatures: 95 °C for 10 min, then 95 °C for 30 s, and 60 °C for 1 min. TaqMan GAPDH Control Reagents (Applied Biosystems) were used to evaluate the transcription of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) housekeeping gene as an endogenous reference. As suggested by Applied Biosystems in the ABI 7700 Sequence Detection System User's Manual as well as in the Application Note (117MI02-02 Sequence Detection Systems Quantitative Assay Design and Optimization), a quality control of *MRP's* quantitative assays was performed by calibration against known dilutions of RNA extracted from HOBIT cells. From each known dilution, *MRPs* and *GAPDH* were amplified and the values (C_t) obtained were correlated with the different RNA dilutions, achieving similar correlation coefficients.

Primer concentrations were optimized for the two step RT-PCR to determine the minimum required to give the lowest threshold cycle (C_t) and the maximal signal (ΔR_n), while minimizing non-specific amplification. Four replicates of each RT-PCR for each total RNA dilution were run on ABI PRISM 7700. The fluorescent light emission was directly recorded in real time by the Gene Amp 7700 SDS Software. Two types of PCR controls were performed: (a) no template controls (NTC) with no target DNA and (b) no amplification control (NAC) with the addition of 1 μL SDS (0.5% w/v) in order to ensure the denaturation of the DNA polymerase. No increment in fluorescence was detected in either of the NTC after 50 cycles.

Based on the construction of a standard curve (validation test for *GAPDH*), semi-quantitative assessment of *MRPs* expression was obtained by comparison between C_t values of the target and housekeeping genes. Results are referred to as transcription relative to *GAPDH*.

In order to normalize data obtained in BeWo cells and placenta, the quantitative results were also analyzed following the comparative C_t method and using HOBIT cells as external reference (User Bulletin 2 of the ABI Prism 7700 Sequence Detection System). The comparative quantitative results have been expressed as the result of $2^{-\Delta\Delta C_t}$ calculation.

As reported previously [22], after the quantitative evaluation of *MRPs* [23], a melting temperature assay (MTA) was performed on *MRPs* amplicons in order to verify the specificity of the amplification. The dissociation protocol included a slow cooling from 95 to 60 °C in 20 min at the end of PCR. The reaction was performed on the ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) equipped with the 7700 Sequence Detection System Software version 1.7. The melting profile of each amplicon was obtained by using appropriate software (ABI 7700 Dissociation Curves Software 1.0) which allowed identification of the point at which the re-association occurred (flexus point).

DNA sequencing. To confirm the specificity of the amplicons, both strands of amplified *MRP1* cDNAs were sequenced before the quantitative Real-time protocol, using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Amplicons were purified using exonuclease I and shrimp alkaline phosphatase (pre-sequencing kit Amersham Pharmacia Biotech, Cleveland, OH, USA) in order to remove from the PCR mixture residual single strand primers and excess dNTPs which could interfere with the sequencing reaction. DNA sequences were detected and analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using the software Sequence Navigator 1.01 (Applied Biosystems, Foster City, CA, USA).

Statistics. Data are expressed as means ± SD. Statistical analysis was performed by Student's "*t*" test and a p value less than 0.05 was considered statistically significant.

Results

Quantitative PCR

The melting dissociation profiles (data not shown), performed on *MRP1*, *MRP2*, *MRP3*, and *MRP5* cDNAs, allowed confirmation of the specificity of the amplifications. The melting temperatures of *MRP1*, *MRP2*, *MRP3*, and *MRP5* amplicons were 86.9 ± 0.2, 86.1 ± 0.1, 83.3 ± 0.4, and 84.7 ± 0.5 °C, respectively, whereas *GAPDH* had a melting temperature of 81.9 ± 0.2 °C. The specificity of the amplification was also confirmed by direct sequencing and sequence alignment with the *MRP1*, *MRP2*, *MRP3*, and *MRP5* sequences reported in GenBank (NM 004996; NM 000392; NM 003786; and NM 005688, respectively).

The relative expression of the four *MRP* genes in each of the different types of samples was calculated from a calibration curve (C_t vs. concentration) obtained by measuring *GAPDH* fragment amplification after serial dilution of the same RNA sample. The applied regression was $y = -2.95x + 30.9$ ($r^2 = 0.995$), where y represents the different average C_t values and x is the logarithm of the RNA concentration (ng).

The results of the relative quantification, normalized against *GAPDH* amplification, are shown in Fig. 1 which reports the expression of the four *MRP* genes in placenta (first and third trimester), BeWo cells (polarized and non-polarized) and HOBIT cells. In human placenta, *MRP3* was the most highly expressed gene in the first trimester (PI); *MRP1* was also expressed although almost 10 times lower as compared to *MRP3*. Both *MRP5* and *MRP2* were also detected but their expression was much lower. *MRP3* resulted in being the

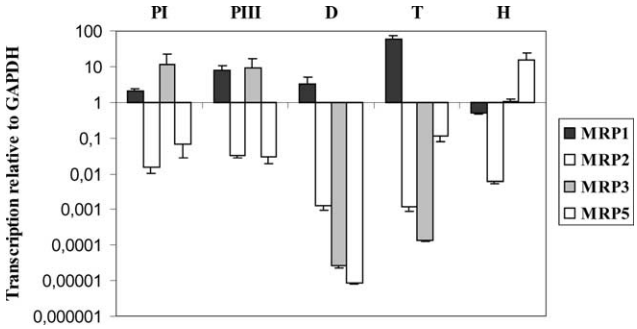

Fig. 1. Transcription of MRPs relative to GAPDH. *MRP1*, *MRP2*, *MRP3*, and *MRP5* transcription in BeWo cells (T and D), placental tissues (PI and PIII), and HOBIT cells (H). First-strand cDNA was prepared from three different placental tissues at early (PI) and 3 at late gestational age (PIII). Three different RNA samples of polarized (T), three of dish grown (D) BeWo, and one preparation of HOBIT cells were also used to obtain other first-strand cDNA. Real-time PCR was used to quantitate transcription of the different *MRPs* relative to the housekeeping transcript *GAPDH*. Values are reported as *MRP* genes relative to the housekeeping transcript *GAPDH*. Note the logarithmic scale of *y*-axis.

most abundantly expressed gene also in samples of full term placenta (third trimester, PIII); *MRP1* was also highly expressed, while the expression of *MRP2* and *MRP5* remained marginal. In the BeWo cells grown on dishes (non-polarized, D) *MRP1* was the most expressed gene while other *MRP* genes were marginally transcripted, particularly *MRP5*. When cells were polarized (T), *MRP1* was still the highest expressed gene followed by appreciable levels of *MRP5*. Differently, low to extremely low levels were found for both *MRP2* and *MRP3*. The analysis of *MRP* gene expression in HOBIT cells (H), derived from human osteoblasts [17], revealed a different expression profile. *MRP5* was the highest expressed gene, followed by quite comparable expression of *MRP3* and *MRP1*; the presence of *MRP2* was minimal.

Since all the *MRP* genes resulted were expressed in HOBIT cells, these cells were chosen to serve as an external calibrator. The analyses were performed by following the comparative C_t method (see Materials and methods) which allows a more accurate evaluation of the specific presence of the different genes. The results are expressed in terms of relative quantitation with respect to HOBIT cells, maintaining *GAPDH* as endogenous reference for the normalization.

The expression profile of *MRP1* (Fig. 2, panel A) showed that this gene is maximally expressed in polarized, Transwell grown BeWo cells (T) with a $2^{-\Delta\Delta C_t}$ value (154 ± 17), about 20 times higher than that found in non-polarized, petri-grown cells (D) (7.51 ± 0.81, $p < 0.001$) and more than 30 times the value found in placenta at an early gestational age (PI) (4.59 ± 0.52, $p < 0.006$ vs. petri-grown cells). The expression level in full term placenta (PIII) (19.95 ± 2.12) resulted in being four times higher than that in the early gestational age ($p < 0.0001$), but still remaining 7.5 times lower as compared to polarized BeWo cells.

The expression of *MRP2* (Fig. 2, panel B) varied slightly in the different models, with values ranging from 0.41 ± 0.06 in polarized BeWo to 4.9 ± 0.6 in placenta at third trimester. Of notice, a 2 times increase was found in the late vs. early gestational stage placenta (4.9 ± 0.6 vs. 2.65 ± 0.3, $p < 0.004$).

When *MRP3* was analyzed (Fig. 2, panel C), a great difference was found between BeWo cells and placental tissue. The non-polarized BeWo cells showed a marginal gene expression, while polarization increased the mRNA levels by around 3 times ($5.2 \times 10^{-4} \pm 6.1 \times 10^{-5}$ vs. $1.42 \times 10^{-4} \pm 3.0 \times 10^{-5}$ T and D, respectively, $p < 0.006$). Differently, the expression of *MRP3* in placenta was almost 10,000 times greater and no significant difference was detectable between the two gestational stages (12.38 ± 1.5 vs. 11.55 ± 1.2, PI and PIII, respectively, NS).

In the case of *MRP5* (Fig. 2, panel D), the highest expression was observed in the polarized BeWo cells (T), where the value ($6 \times 10^{-3} \pm 6.2 \times 10^{-4}$) was higher than that found in placenta of the first trimester ($3.3 \times 10^{-3} \pm 8.1 \times 10^{-4}$, $p < 0.01$) and more than 5 times higher than that in full term placenta ($1.1 \times 10^{-3} \pm 4.5 \times 10^{-4}$). A great difference between non-polarized (D) and polarized (T) choriocarcinoma cells was also found, with values 1500 times lower in non-polarized cells ($4.1 \times 10^{-6} \pm 5.0 \times 10^{-7}$ vs. $6 \times 10^{-3} \pm 6.2 \times 10^{-4}$).

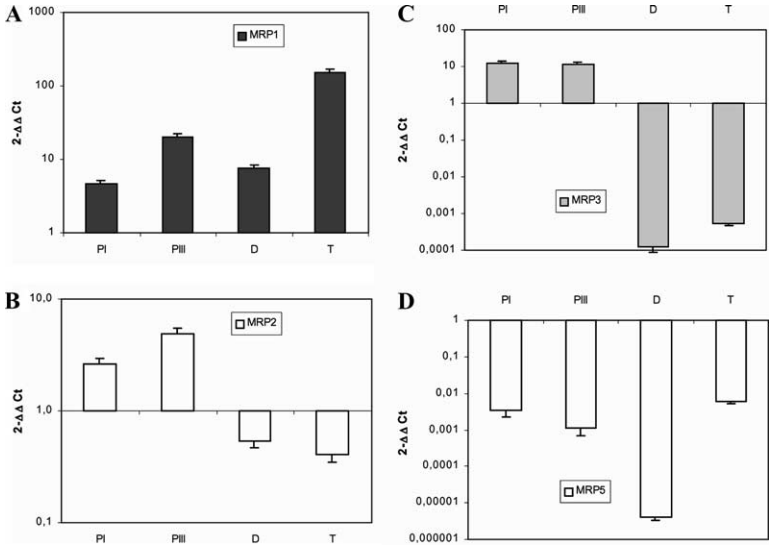

Fig. 2. Comparative quantitative evaluation of MRPs in placental and BeWo samples. Evaluation of *MRP1*, *MRP2*, *MRP3*, and *MRP5* expression in placenta and BeWo cells by using HOBIT cells as an external calibrator. Real-time PCR analyses were performed normalizing *MRP* expression values to endogenous *GAPDH* transcription and relatively to target gene expression evaluated in HOBIT cells. Panel A: *MRP1* transcription levels in polarized (T) and non-polarized (D) BeWo cells and in placenta from first (PI) and third (PIII) trimester. Panels B–D: *MRP2*, *MRP3*, and *MRP5* relative transcription in the same samples. Note the logarithmic scale of *y*-axis.

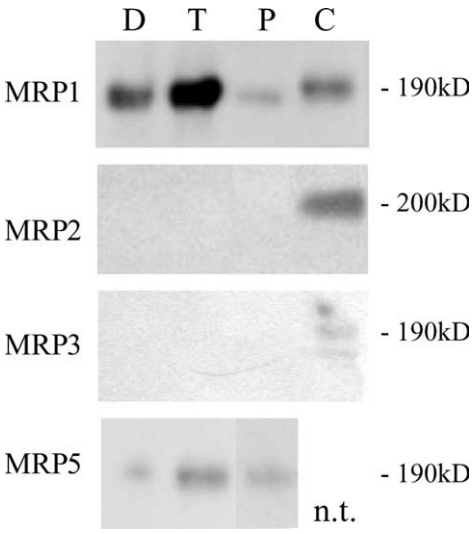

Fig. 3. Western blot analysis of MRP proteins in placenta and BeWo cells. Evaluation of *MRP1*, *MRP2*, *MRP3*, and *MRP5* expression in placenta (P), BeWo cells both non-polarized (D), and polarized (T). Caco-2 cells (C) were used as positive control, but not tested (n.t.) for MRP5. MRPs were detected by specific antibodies (see Materials and methods) and showed an apparent molecular weight of around 190–200 kDa. The double band found for MRP3 in Caco-2 cells is in line with that previously reported for this ABC protein [34].

Western blot analyses

Fig. 3 shows the results obtained by Western blot in placenta and BeWo cells; Caco-2 cells were used as positive control of the presence of different MRPs [24,25] with the exception of MRP5. No sample of the placenta of the first trimester was analyzed due to the intrinsic difficult to obtain enough material for RNA and protein analyses of the same donor. MRP1 was present both in the placenta and in BeWo cells grown in polarized (T) and non-polarized (D) fashion. The protein expression was higher when cells were polarized in line with the higher *MRP1* mRNA presence. The lack of MRP2 protein in placenta and BeWo cells was also in line with the transcription of the gene. On the contrary, in spite of a robust genomic expression, MRP3 was not detected both in placenta and in BeWo cells; Caco-2 showed a faint positivity. Similar to MRP1 and in line with RNA content, the expression of MRP5 was particularly high in polarized BeWo cells as compared to non-polarized cells.

Discussion

During gestation, it is essential that the development of the placenta is synchronized with that of the embryo. The placental barrier, which interfaces between the fetus and the mother, must cope with the gradually increasing requirements of the fetus for supply of nutrients and removal of toxic metabolites. At the different stages of fetal development, these functions must be served by a constantly evolving differential expression of the various genes involved in the relevant transplacental exchanges. In the present work, therefore, the expression of the four *MRP* genes reported to be present in placenta was assessed in human placental samples obtained early and late in gestation. The results obtained in the whole placenta, both in the 1st and 3rd trimester, were compared to the expression of the same four *MRP* genes in BeWo cells, a cell line that is derived from a well-differentiated human choriocarcinoma [26] shown to have many functions of normal human placenta [15,27]. Since placental samples include cytotrophoblasts and endothelial cells, as well as highly differentiated syncytiotrophoblast, the BeWo cells were used as a model for the maturation of trophoblasts in the placenta from the non-polarized cytotrophoblasts of early gestation into the polarized syncytiotrophoblasts of late gestation. It has been shown earlier that BeWo cells mimic the expression of transporters which need a polarized cell structure to be functional (e.g., the transferrin receptor [28,29] and the Cu-ATPases [30]). The polarization of filter-grown BeWo cells at confluence was proven by both the development of transepithelial electric resistance [14] and by the high density of microvilli on the apical surface of the monolayer [15].

By using the expression of the four *MRP* genes in HOBIT cells as an external reference for a more accurate comparative quantitative PCR analysis, the relative expression of the four *MRP* genes could be compared among the placental samples from early and late gestation and the analogous non-polarized and polarized BeWo cells, respectively. As shown in Figs. 1 and 2, the most important findings were that, of the four *MRP* genes studied, *MRP1* was the most prominently expressed, with comparable levels in placenta at the first trimester and non-polarized BeWo cells. The expression increased 4× in the placenta at the later gestational age and 20× in the BeWo cells with polarization. These data suggest that, in the placenta, *MRP1* is highly expressed in both cytotrophoblast and syncytiotrophoblasts, but that *MRP1* expression levels in the whole organ reflect also the presence of this protein in placental cells other than trophoblasts, such as vascular endothelial cells [13,31]. These results also support a major role for MRP1 in the syncytial barrier, contributing to the protection of the fetus from potentially toxic metabolites, such as unconjugated bilirubin. We previously reported that the cellular efflux of unconjugated bilirubin was higher in polarized BeWo cells and that this activity correlated with the development of an apical membrane domain (polarization) and a higher expression of *MRP1* [14], suggesting the involvement of MRP1 in the placental transport of unconjugated bilirubin [32]. The present results further support transport of unconjugated bilirubin by MRP1 and, at the same time,

essentially exclude a role for MRP2, MRP3, and MRP5 in this process.

Placental *MRP3* was expressed at similar levels in the first trimester placenta, but did not increase in later gestation, and was only marginally detectable in the BeWo cells, despite some up-regulation with polarization. The high expression of *MRP3* in whole placenta, but not in BeWo cells, is compatible with a study that demonstrated that MRP3 protein in term placenta is maximally expressed in blood vessel endothelial cells, with a rare presence at the apical surface of the syncytotrophoblasts [13]. However, protein analysis did not find detectable expression either in placental or BeWo derived membrane samples. The negative result in protein detection might be due to a low reactivity of the antibodies used (purchased from two different companies), as also indicated by the rather low signal found in Caco-2 cells, an intestinal cell line reported to have a high expression of MRP3 [33]. The availability of a more reactive antibody(s) may allow one to define the discrepancy between transcription and translation of *MRP3* in placenta as already reported for other organs [34].

The transcription of *MRP2* was very low both in placenta and BeWo cells, suggesting that maturation or polarization was not accompanied by a significant increase in expression of *MRP2* in either system. The lack of MRP2 protein expression in BeWo cells points to the absence of this ABC transporter in human trophoblasts. This is partially contrary to what was reported in immunohistochemical studies where MRP2 was observed on the maternal domain of trophoblasts [13]. Although this may be due to different experimental models and/or antibodies used, the lack in increase of *MRP2* mRNA after cellular polarization speaks in favor of the absence of gene regulation since MRP2 is the only member of the six MRP proteins found to be localized at the apical membrane domain in specialized epithelia other than placenta [11,31].

MRP5 mRNA was quite marginally detectable in both placental samples and BeWo cells, but seemed to be highly sensitive to growth conditions, since its expression increased dramatically (around 1500 times) in polarized BeWo cells. In contrast, no increase in *MRP5* was observed in placental samples obtained at the third, as compared with the first, trimester. The explanation for this discrepancy may reside in the physiological function of this protein that has been shown to transport nucleotide analogues, particularly GMPc [35]. The higher expression of *MRP5* in placenta of the first trimester and the suggested role of this protein in GMPc cellular function [36] may indicate a specific need of MRP5 by the early placenta to prevent GMPc accumulation. A similar need could be present in BeWo only when they reach polarization. Western blot analysis on BeWo cells supports a transcriptional and post transcriptional regulation of *MRP5* which is peculiar as compared to that of the other MRP genes. MRP5 protein is, in fact, effectively translated in BeWo cells grown on dishes in spite of marginal mRNA levels, while the fundamental role of differentiation/polarization on protein expression has been confirmed by the data obtained with the cells grown on Transwell filters.

The present results with *MRP1* and *MRP5* in BeWo cells confirm and quantify our earlier observations [14]. By contrast, our findings are in poor agreement with immunohistochemical studies of term placenta [13] that reported low expression of MRP1, localized primarily to vascular endothelial cells, and high expression of MRP2 at the apical level of syncytotrophoblasts. These discrepancies may be due, at least in part, to the different experimental techniques used, particularly different antibodies.

In conclusion, these data indicate that mRNA for at least four different *MRP* genes is expressed in human placenta and human choriocarcinoma derived trophoblastic cells, and that these genes are differentially regulated by gestational maturity and cellular organization (polarization). This also applies to protein expression as far as MRP1 and MRP5 are involved. This suggests that these placental transporters, especially MRP1, participate in protection of the fetus from endogenous and exogenous toxic metabolites. The specific role of each MRP and the mechanisms regulating their differential expression in the placenta remain to be elucidated.

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