P-glycoprotein expression and localization in the rat uterus throughout gestation and labor

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Abstract

Uterine tissues contain the efflux transporter P-glycoprotein (P-gp, encoded by *Abcb1a/1b* gene), but little is known about how it changes through gestation. Our aim was to investigate the expression profile and cellular localization of P-gp in the pregnant, laboring and post-partum (PP) rat uterus. We propose that during pregnancy the mechanical and hormonal stimuli play a role in regulating myometrial *Abcb1a/1b*/P-gp. Samples from bilaterally and unilaterally pregnant rats were collected throughout gestation, during labor, and PP ($n=4-6$ /gestational day). RNA and protein were isolated and subjected to quantitative PCR and immunoblotting; P-gp transcript and protein were localized by *in situ* hybridization and immunohistochemistry. Expression of *Abcb1a/1b* gene and membrane P-gp protein in uterine tissue (1) increased throughout gestation, peaked at term (GD19-21) and dropped during labor (GD23L); and (2) was upregulated only in gravid but not in empty horn of unilaterally pregnant rats. (3) The drop of *Abcb1a/1b* mRNA on GD23 was prevented by artificial maintenance of elevated progesterone (P4) levels in late gestation; (4) injection of the P4 receptor antagonist RU486 on GD19 caused a significant decrease in *Abcb1* mRNA levels. (5) *In situ* hybridization and immunohistochemistry indicated that *Abcb1*/P-gp is absent from myometrium throughout gestation; (6) was expressed exclusively by uterine microvascular endothelium (at early gestation) and luminal epithelium (at mid and late gestation), but was undetectable during labor. In conclusion, ABC transporter protein P-gp in pregnant uterus is hormonally and mechanically regulated. However, its substrate(s) and precise function in these tissues during pregnancy remains to be determined.

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Introduction

The ATP-binding cassette (ABC) transporter superfamily is among the largest and most broadly expressed groups of membrane proteins. The vast majority of its members are responsible for the active transport of a wide variety of compounds across biological membranes, including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, bile acids, as well as drugs and other molecules (Sharom 2008, Leslie *et al.* 2005). In tumor cell lines, multidrug resistance is often associated with an ATP-dependent decrease in cellular drug accumulation, which was originally attributed to overexpression of ABC drug transporter P-glycoprotein (P-gp), encoded by the *ABCB1* gene (Capella & Capella 2003). In addition to its role in drug resistance, P-gp is also expressed in nonmalignant tissues and is believed to be involved in protecting them from xenobiotic accumulation and the resulting toxicity (Yang *et al.*

1989, Schinkel *et al.* 1997). The ABC transporters have been studied extensively in the placenta, brain, liver and kidney (Hitchins *et al.* 1988, Tanabe *et al.* 2001). Earlier studies demonstrated that the level of *ABCB1* and P-gp was dramatically increased in the human endometrium (Axiotis *et al.* 1991) and secretory epithelium of the pseudo-pregnant and early pregnant murine uterus, regulated by the combination of estrogen and progesterone (P4), the major steroid hormones of pregnancy (Arceci *et al.* 1990). It was suggested that P-gp may be involved in utero-placental transport of substrates that are important in the implantation process, in early embryo–endometrial interactions or in the transport of P4 across the uterine epithelium during pregnancy (Axiotis *et al.* 1991). However, to date, little has been known about the distribution and expression profile of uterine P-gp throughout gestation. A microarray study found that *Abcb1* gene expression was dramatically (122-fold decrease) downregulated

in the uterine muscle (myometrium) right before term labor (Helguera *et al.* 2009). It is known that the uterus maintains a state of quiescence throughout most of pregnancy to protect the growing fetus, but during labor myometrial activation culminates in synchronous and powerful contractions that expel the fetus (Lye *et al.* 2001, Shynlova *et al.* 2009). Several hormonal and chemical signals have been shown to play important roles in this transition from the quiescent to the contractile uterine state (Lee *et al.* 2015, Shynlova *et al.* 2014). Therefore, we hypothesized that P-gp protein, which serves a barrier function in a variety of normal and diseased tissues, might be involved in maintaining the quiescent state of the pregnant uterus. The aim of the present study was to investigate, using the animal (rat) model of pregnancy, the expression profile and cellular/tissue localization of *Abcb1* gene and P-gp protein in the pregnant myometrium throughout gestation, during term labor, and post-partum involution period, as well as to examine potential mechanisms, which regulate its expression.

Materials and methods

Animals

Wistar rats (Charles River) were housed individually under standard environmental conditions (12h light:12h darkness cycle). Female virgin rats were mated and the day of vaginal plug detection was designated as gestational day (GD) 1. The average time of delivery under these conditions was in the morning of day 23 (GD23L). Our criteria for labor were based on delivery of at least one pup (Shynlova *et al.* 2004). All animal experiments were performed under ethics approval by the Toronto University Health Network Animal Care Committee.

Tissue collection

Animals were killed by carbon dioxide inhalation. For RNA and protein extraction, uterine horns were placed into ice-cold PBS, and dissected away from both pups and placentas. The myometrium was carefully dissected away from the decidua basalis (area where placenta was attached), flash-frozen in liquid nitrogen and stored at -80°C (Shynlova *et al.* 2004). For immunohistochemical studies, the whole uterine horn was cut into 5–10 mm segments and placed in 10% neutral buffered formalin (Harleco, Baltimore, MD, USA) or 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hartfield, PA, USA) for fixation. Samples were fixed for 24 h at 4°C . For each GD, tissue was collected from 4 to 6 different animals.

Experimental design

Normal pregnancy and term labor

Myometrial samples were collected from nonpregnant rats (NP), on GD 6, 8, 10, 12, 14, 15, 17, 19, 21, 23L, and 1 day postpartum (1 PP). Tissue was collected at 12:00 h on all days

with the exception of the labor sample (GD23L), which was collected once the animals had delivered at least one pup.

P4-delayed labor

To determine whether high plasma levels of P4 might modulate the expression of *Abcb1*, pregnant rats were randomized into two groups. Starting on GD20, they received daily s.c. (subcutaneous) injections of either vehicle (sterile saline) or P4 (medroxyprogesterone acetate, 16 mg/kg, Pharmacia Canada Inc., Mississauga, ON, Canada). For each time point and for each treatment, tissues from 4 animals were collected on GD21, GD22 and GD23L in the vehicle-treated group or GD21, GD22, GD23 and GD24 in the P4-treated group.

RU486-induced preterm labor

On GD19 two groups of rats were treated with either vehicle (corn oil containing 10% ethanol) or RU486 (10 mg/kg, s.c., Mifepristone; 17β -hydroxy-11 β -[4-dimethylaminophenyl]-17-[1-propynyl]-estra-4,10-dien-3-1; Biomol International, Plymouth, PA, USA) as described previously (Shynlova *et al.* 2004). Myometrial samples were collected from both groups of animals on GD20 when the RU486-treated animals had delivered at least one pup ($n=4/\text{group}$) or 24 h after injection (vehicle).

Unilaterally pregnant rats

Under general anesthesia, virgin female rats underwent tubal ligation through a flank incision to ensure that they subsequently became pregnant in only one horn (Shynlova *et al.* 2004). Animals were allowed to recover from surgery for at least 7 days before mating. Myometrial samples from empty (E) and gravid (G) horns were collected separately on GD 6, 12, 15, 17, 19, 21 and 23L ($n=4$).

Quantitative PCR analysis

RT and quantitative PCR (RT-qPCR) was performed to detect the mRNA expression of the *Abcb1* gene in rat myometrium as described previously (Shynlova *et al.* 2004). Briefly, total RNA was extracted from the frozen tissues using TRIzol (Life Technologies), RNA samples were column purified using RNeasy Mini Kit (Qiagen), and treated with DNase I to remove genomic DNA contamination. RNA concentration was measured by the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA quality was assessed using the Experion RNA StdSens Analysis Kit (Bio-Rad Laboratories). Samples with a RQI value greater than 7.5 and a clean separated gel image were accepted as intact RNA. cDNA was generated with iScript reverse transcription supermix (Bio-Rad). All PCR reactions were carried out in triplicates on the CFX384 System (Bio-Rad) with 5 ng cDNA, LuminoCt SYBR Green qPCR ReadyMix (Sigma-Aldrich), forward and reverse primers at a final concentration of 300 nM (Table 1). Gene expression values were analyzed using the $\Delta\Delta\text{C}_q$ mode on the CFX Manager software 3.0 (Bio-Rad Laboratories) and accounting for specific primer efficiencies in the analysis. The expression of both isoforms of *Abcb1a/1b* gene was normalized to three housekeeping

Table 1 Real-time PCR primer sequences of *Abcb1a/1b* and housekeeping genes.

Target genes	Primer sequences	GenBank accession no.
<i>Abcb1a/1b</i>	Forward 5'-GTC CAG GAA GCG CTG GAC AAA-3' Reverse 5'-TCA TGA GCG CTT TGC TCC AGC-3'	NM_133401
<i>Hprt</i>	Forward 5'-CAGTCCCAGCGTCGTGAT-3' Reverse 5'-CAAGTCTTTCAGTCCTGTCCATAA-3'	NM_013556.2
<i>Ppia</i>	Forward 5'-CAC CGT GTT CTT CGA CAT CA-3' Reverse 5'-CCA GTG CTC AGA GCT CGA AAG-3'	NM_008907.1
<i>Igf1r</i>	Forward 5'-TCCCAAGCGATTTGCTGCAGTCATC-3' Reverse 5'-ACTCTTGCTCCTGTGCACACCA-3'	NM_013684

genes [insulin-like growth factor 1 receptor (*Igf1r*), peptidylprolyl isomerase A (*Ppia*) and hypoxanthine-guanine phosphoribosyltransferase (*Hprt*)] (Table 1), previously characterized in rodents' myometrium (Shynlova *et al.* 2014). A fold change was calculated relative to the expression of *Abcb1a/1b* in the GD23 sample. For unilaterally pregnant animals, the gene expression was shown as fold change relative to GD23 gravid horn mRNA level, whereas that of P4- and RU486-treated animals was shown as a fold change relative to the vehicle sample.

Isolation of plasma membrane fraction from pregnant rat myometrium

Myometrial tissue from NP and bilaterally pregnant rats (GD 6, 8, 12, 14, 19, 21, 23L, $n=4/\text{GD}$) as well as unilaterally pregnant rats (GD 6, 12, 15, 17, 19, 21, 23L, $n=3/\text{GD}$) was crushed under liquid nitrogen using a mortar and pestle. Crushed tissue was homogenized for 1 min using the TissueLyser II (Qiagen) in lysis buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.5) supplemented with 100 μM sodium orthovanadate and protease inhibitor cocktail tablets (Complete Mini, Roche). Samples were spun at 3000g for 5 min at 4°C, the supernatant was collected in 10 mL tubes (Beckman Coulter, Brea, CA, USA) and centrifuged at 100,000g for 1 h at 4°C. The supernatant (cytosolic fraction) were removed and the pellet was resuspended in 200 μL lysis buffer supplemented with 1% Triton X-100 and centrifuged again at 10,000g for 10 min at 4°C. The supernatant (enriched membrane fraction) was collected and stored at -80°C.

Western immunoblotting analysis

Protein concentrations were determined using BCA protein assay (Thermo Fisher Scientific). Equal amounts of membrane proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane using the Turbo Trans-Blot system (Bio-Rad). After blocking for one hour with 5% milk in TBS-T (Tris-buffered saline with 0.1% Tween 20), the membranes were incubated with primary antibody at 4°C overnight (ABCB1/P-gp, rabbit monoclonal, 1:1000, ab170904, Abcam). The membranes were subsequently probed with a horseradish peroxidase-conjugated secondary antibody (1:10,000; Santa Cruz Biotechnology). Signals were detected using Luminata HRP substrate (Millipore) and imaging was performed with a VersaDoc imaging system (Bio-Rad). Densitometric analysis was performed using ImageJ software

(NIH). To control for loading variations, PVDF membranes were stripped and re-probed with an antibody against the specific membrane housekeeping protein anti-caveolin 1 (1:500, Abcam).

In situ hybridization

Specific fragments for ABC transporters were amplified from rat uterus cDNA samples using primers: ABCB1-for 5'-CAG TGT GCT GGA ATT CAA ATC CAG CGG CAG AAC AGC-3' with ABCB1-rev 5'-GAT ATC TGC AGA ATT CCA CCA CGA TCAA GTC TGC GTT C-3' and PrimeSTAR GXL Polymerase (Clontech). PCR fragments that recognize both isoforms of rat *Abcb1* gene were purified, recombined into an EcoRI-linearized pCRII vector (Life Technologies), and transformed into competent stellar *E. coli* bacteria, using the In-Fusion HD cloning kit (Clontech) according to the manufacturer's protocol. Cloned inserts were verified by sequencing.

For generation of digoxigenin (DIG)-labeled RNA probes, vectors were linearized (antisense, BamHI; sense, EcoRV), purified using phenol chloroform, and *in vitro* transcribed using the T7 High Yield Transcription Kit (Fermentas, Thermo Fisher Scientific), or the SP6 DIG RNA labeling kit (Roche). Labeled RNA probes were purified using the RNeasy Micro Kit (Qiagen), and labeling efficiency was analyzed by dot blot analysis. *In situ* hybridizations were performed on 8 μm sections of 4% PFA-fixed, paraffin-embedded rat tissues with optimized amounts of labeled probes tested for each preparation as described by Kibschull *et al.* (2014). For each day of gestation and each treatment, tissues from three different animals were analyzed. DIG-labeled RNA probes were detected by an alkaline phosphatase conjugated anti-DIG antibody; BCIP/NBT was used as substrate. Positive blue staining revealed the detection of *Abcb1* transcript in rat gestational tissues. Pink counterstaining was performed using Nuclear Fast Red (Sigma).

Immunohistochemistry

PFA-fixed uterine tissues were sectioned at 5 μm thickness, collected on Superfrost Plus slides (Thermo Fisher) and processed as described previously (Nuessler *et al.* 1997). Briefly, paraffin sections were dewaxed in xylene and rehydrated in ethanol in descending gradients. Endogenous peroxidase activity was quenched during a rehydration step with 3% hydrogen peroxide solution in absolute methanol (Sigma) for 30 min at room temperature. For anti-P-gp

Figure 1 *Abcb1* gene expression and regulation in the pregnant rat uterus. (A) *Abcb1* mRNA levels in the rat uterus throughout gestation; (B) the effects of RU486 and (C) progesterone (P4) on uterine *Abcb1* mRNA expression; (D) Expression of *Abcb1* mRNA in uterine tissue from unilaterally pregnant rats throughout gestation. Quantitative RT-PCR analysis of *Abcb1* mRNA levels were normalized to three housekeeping genes (see "Methods" section) and presented in fold changes relative to GD23 (Labor) gravid samples. Gravid or vehicle-treated (black bars), P4-treated (striped bars), RU486-treated (diagonal bar), and empty horn (white bars) samples are shown. Values represent mean $\pm$ s.d. ($n=4$ at each GD and for each treatment). Data labeled with different letters are significantly different from each other ($P<0.05$). A significant difference between two groups is indicated by # $P<0.05$.

antibody, antigen retrieval was performed by 2 microwave heating cycles in 10mM sodium citrate solution (pH 6) for 5 min. For anti-CD31 antibody, antigen retrieval was performed by a microwave heating in 10mM EDTA (pH 9) for 5 min. Sections were washed in PBS, blocked in protein blocking solution (Dako) for 1 h, and incubated overnight with the primary antibodies: anti-P-gp rabbit (1:250, EPR-25, Abcam). For negative controls, ChromPure nonspecific rabbit IgGs (Jackson ImmunoResearch) were used instead of primary antibodies at the same concentration (1:250). In addition, sections were incubated with secondary antibodies in the absence of primary antibodies. Slides were washed in PBS, incubated with secondary antibody

(1:300, 1 h, room temperature; goat anti-rabbit, Dako), and then washed in PBS and incubated with streptavidin-HRP (Dako); staining was detected with the peroxidase substrate kit DAB (Dako) resulting in brown deposition. Slides were counterstained with hematoxylin (blue), dehydrated in ethanol and cover-slipped. The representative images of each sample were captured using an Olympus BX61 upright microscope with an Olympus DP72 digital color camera run by CellSens Standard proprietary acquisition software (Olympus). For each day of gestation and each treatment, tissue from three different animals was analyzed.

Statistical analysis

Gestational profiles were subjected to one-way ANOVA followed by pairwise multiple comparison procedures (Student–Newman–Keuls) to determine differences between groups. P4-delayed labor model (GD21, 22, and 23) and tubal ligation data were analyzed by two-way ANOVA followed by pairwise multiple comparison procedures as described

Figure 2 Localization of *Abcb1* mRNA in the luminal epithelium of rat uterus. *In situ* hybridization performed on uterine sections from non-pregnant (NP), early pregnant (D6), mid pregnant (D15), late pregnant (D19, D21) and laboring (D23) rats. *Abcb1* transcripts (blue) were detected in luminal epithelium (arrowheads) (original magnification 400 $\times$). For each day of gestation, tissues were collected from three animals ($n=3$). Note lack of staining after incubation with sense (S) probe (*in situ* negative controls).

Figure 3 Localization of *Abcb1* mRNA in rat uterine vascular endothelium. *In situ* hybridization performed on uterine sections from non-pregnant (NP), early pregnant (D6), mid pregnant (D15), late pregnant (D19, D21) and laboring (D23L) rats. *Abcb1* transcripts were detected in uterine vascular endothelium (original magnification 400×). Arrows mark specific *Abcb1* staining (blue). For each day of gestation, tissues were collected from three animals ($n=3$). S1 and S2 denote negative controls (sense probe).

previously. The GD24 in P4-treated group was compared with the GD23 from vehicle group using a *t*-test. RU486 results were compared with vehicle using *t*-test, and where required the data were log transformed to obtain normal distribution. Statistical analysis was carried out using SigmaStat version 2.01 (qPCR data, Jandel, San Rafael, CA, USA) and GraphPad Prism 5.0 (Western immunoblotting, GraphPad Software) with the level of significance set at $P<0.05$.

Results

Abcb1 gene expression in rat myometrium during pregnancy and spontaneous term labor, and its regulation by progesterone and gravidity

The transcript levels of both isoforms of *Abcb1a/1b* mRNA in the pregnant rat uterine tissues were low at early to mid-gestation (GD6–GD12), increased starting from GD15, and peaked at GD19. Most interestingly, we observed a dramatic decrease during term labor (23.6 ± 8.9 fold decrease on GD23L vs GD19, $P<0.05$),

which was maintained at 1PP (Fig. 1A). This modulation of *Abcb1a/1b* expression suggested an involvement of the steroid hormone progesterone (P4). To investigate this possibility, we experimentally modulated P4 exposure, either by administration of RU486 on GD19 to induce premature blockage of P4-signalling, or by administration of exogenous P4 to prevent the normal decline in this hormone at term. Treatment of pregnant rats with the P4 receptor antagonist, RU486, on GD19 caused the onset of preterm labor within 24 h and a significant decrease in gene expression of *Abcb1a/1b* ($P<0.05$; Fig. 1B). Administration of exogenous P4 delayed the onset of labor by 24 h and prevented the fall of *Abcb1a/1b* mRNA levels on GD23 compared with vehicle-treated animals delivering at term ($P<0.05$; Fig. 1C). Moreover, the mRNA levels of *Abcb1a/1b* in P4-treated rat myometrium remained high on GD24 (24 h after the normal delivery time) compared with vehicle-treated animals on GD23L (laboring sample; $P<0.05$).

To assess the effect of uterine occupancy or mechanical stretch of the uterine walls by the growing fetus, we compared *Abcb1a/1b* transcript levels in myometrial tissue collected from the empty and gravid uterine horn of unilaterally pregnant rats. The mRNA profile in the gravid horn was similar to those of normal pregnant animals (Fig. 1D), while expression remained at very low levels in the empty horn throughout gestation. Importantly, in the gravid horn we observed that during term labor (GD23L) there was a rapid decrease in *Abcb1a/1b* expression to a level similar to the empty horn.

Figure 4 Modulation of *Abcb1* transcripts in the luminal epithelium of pregnant rats on day 20 or during RU486-induced preterm labor (RU486) and on day 23 during term labor (Vehicle) or in progesterone-treated animals (P4, not in labor). *In situ* hybridization performed on uterine sections from late pregnant (D19) and laboring (D23L) rats. *Abcb1* transcripts were detected in luminal epithelium. Arrowheads mark specific *Abcb1* staining (blue). For each day of gestation, tissues were collected from three animals ($n=3$).

Spatial and temporal distribution of *Abcb1* in rat uterus throughout gestation and labor

In order to study the temporal expression and spatial distribution of *Abcb1* mRNA, *in situ* hybridization was performed on tissue sections from nonpregnant and pregnant rat uteri. In the nonpregnant rat uterus, *Abcb1a/1b* mRNA was almost undetectable (Fig. 2). In contrast, in pregnant rat uterine tissue samples, *Abcb1a/1b* transcript was detected typically in endometrial luminal epithelial cells (Fig. 2), as well as in vascular endothelial cells of the myometrial blood vessels (Fig. 3). *Abcb1a/1b* transcript was not detected in myometrial smooth muscle cells throughout gestation (Supplementary Fig. 1). As demonstrated in Fig. 2, *Abcb1a/1b* mRNA was expressed at low levels in luminal epithelial cells in early gestation (GD6), but highly upregulated at mid-gestation (GD15), reaching a peak at GD19, followed by a decrease to low level in late gestation (GD21), and during labor (GD23). This expression in luminal epithelium was clearly regulated by P4: blocking P4 signaling by RU486 on GD19 prematurely decreased *Abcb1a/1b* mRNA, while P4 treatment on GD20–GD23 prevented a physiologic

decrease in *Abcb1a/1b* mRNA on GD23 (Fig. 4). The gravidity had a major influence on the luminal epithelium expression: *Abcb1a/1b* transcripts were highly upregulated in the gravid horn on GD15–GD19, but remained low in the empty horn (Fig. 5).

In addition, *Abcb1a/1b* mRNA was detected in vascular endothelial cells at early (GD6) gestation, was evident at mid-gestation (GD15–GD19), but was not found in late gestation (GD21) or during labor (GD23) (Fig. 3). *In situ* negative controls show no staining after incubation of uterine tissues with a sense (S) probe (Figs 2 and 3).

Expression of P-gp protein in rat myometrium

We applied immunoblotting technique to analyze whether P-gp protein expression correlated with gene expression in nonpregnant and pregnant rat myometrium. Plasma membrane fraction was isolated

Figure 5 Localization of *Abcb1* mRNA in the luminal epithelium of unilaterally pregnant rats throughout gestation (original magnification 100 $\times$). Arrowheads mark specific *Abcb1* staining (blue). For each day of gestation, tissues were collected from three animals ($n=3$).

Figure 6 P-gp protein expression in pregnant rat myometrium. Representative Western blotting and densitometric analysis illustrating myometrial P-gp expression levels in bilaterally pregnant (A) and unilaterally pregnant (B) rats. P-gp protein levels were normalized to caveolin 1 and expressed in ROD (Relative Optical Density). (A) Bar graphs showing the mean $\pm$ S.E.M. ($n=4$ at each gestational point). Data labeled with different letters are significantly different from each other ($P<0.05$). (B) Bar graphs showing the mean $\pm$ S.E.M. ($n=3$ for each GD). A significant difference between the empty (E) and the gravid (G) uterine horn from the same gestational day is indicated by * ($P<0.05$).

Figure 7 Immunohistochemical localization of P-gp protein in rat luminal epithelium of non-pregnant and pregnant rats. Labeling was performed on uterine tissue sections from non-pregnant (NP), early pregnant (D6), mid pregnant (D15), late pregnant (D19, D21) and laboring (D23L) rats. Note the lack of staining after incubation with nonspecific rabbit IgGs (NC1) or omitting the primary antibody (NC2). For each day of gestation, tissues were collected from three animals ($n=3$). Original magnification 400 $\times$.

by differential centrifugation and analyzed by immunoblotting with antibody against P-gp protein. There was no difference between the membrane expression of protein in nonpregnant and early pregnant myometrium. Similar to *Abcb1a/1b* transcripts, P-gp protein levels in myometrial plasma membrane fraction was low at early to mid-gestation (GD6–14), increased significantly at GD19–21 and was reduced during term labor (GD23L) (Fig. 6A).

The effect of gravidity on P-gp protein was examined in the plasma membrane fraction isolated from unilaterally pregnant rat myometrium. In the empty horn, the expression of P-gp protein was low throughout gestation. In the first half of gestation (GD6–GD15) protein levels of P-gp in the gravid horn were similar to those of the empty horns. However, at GD19 a significant increase in the P-gp protein expression ($P<0.05$) was observed only in the gravid horn as compared to the corresponding empty uterine horn of unilaterally pregnant rats with a decrease by term labor (Fig. 6B).

Spatial and temporal distribution of P-gp in pregnant rat uterus

Immunostaining using specific anti-P-gp antibody fully confirmed mRNA *in situ* results, demonstrating no expression in uterine smooth muscle cells over gestation (Supplementary Fig. 2). P-gp protein was highly upregulated in luminal epithelium between GD15 and GD19, but disappeared during term labor (Fig. 7). Also in correspondence with *in situ* hybridization results, P-gp protein was expressed by vascular endothelial cells from early to mid-gestation (GD6–GD19), but only weak endothelial immunostaining was detected in late gestation and during labor (GD21–GD23) (Fig. 8). P-gp immunostaining was also detected throughout gestation in the microvascular epithelial cells of the empty horn in unilaterally pregnant rats (Supplementary Fig. 3). We observed that the number of vessels increased in both empty and gravid uterine horns throughout

Figure 8 Immunohistochemical localization of P-gp protein in microvascular endothelium of non-pregnant and pregnant rats. Labeling was performed on uterine tissue sections from non-pregnant (NP), early pregnant (D6), mid pregnant (D15), late pregnant (D19, D21) and laboring (D23L) rats. Note the lack of staining after incubation with nonspecific rabbit IgGs (NC1) or omitting the primary antibody (NC2). For each day of gestation, tissues were collected from three animals ($n=3$). Original magnification 400 $\times$.

gestation. Starting from mid-gestation, visible increase in vessels' diameter was detected only in the gravid uterine horn, while in the empty horn their size was not changed. As for the glandular epithelial cells, positive P-gp immunostaining was detected in the gravid horn only in early and mid-gestation and in the empty horn throughout gestation (Supplementary Fig. 4). Secretory endometrial glands were not found at late gestation in the gravid horn.

Discussion

The multidrug resistance protein P-gp (also known as MDR1), encoded by the *ABCB1* gene, is a plasma membrane protein that can actively extrude a wide variety of substances from different cells (Hitchins *et al.* 1988, Yang *et al.* 1989, Leslie *et al.* 2005). Multiple studies have shown that the P-gp can protect normal tissues from a wide spectrum of potentially toxic drugs and xenobiotics (Hitchins *et al.* 1988, Yang *et al.* 1989, van Kalken *et al.* 1991, Baello *et al.* 2014). Rats and mice share two genes encoding for P-gp, *Abcb1a* and *Abcb1b* (also known as *mdr1a* and *mdr1b*), which are similarly expressed between the two species (Croom *et al.* 1989, Kwan *et al.* 2003). Earlier, Arceci *et al.* observed that in the mouse uterus there was an extremely high level of *Abcb1* transcripts in the secretory glandular and luminal epithelium, which was associated with pregnancy (Arceci *et al.* 1988). Another study had examined *ABCB1*/P-gp endometrial expression and regulation during early gestation in human (Arceci *et al.* 1990, Axiotis *et al.* 1991). A recent study found that *Abcb1* gene levels in rat uterus decreased dramatically during a short time window from late pregnancy to parturition (Helguera *et al.* 2009). Our study is the first comprehensive examination of both the *Abcb1a/1b* gene and P-gp protein expressions as well as distribution patterns in uterine tissue from nonpregnant state, throughout pregnancy, labor and postpartum. However, we did not discriminate between two rat isoforms of *Abcb1*/P-gp. Instead we wanted to analyze the spatial and temporal expression of *Abcb1* gene and P-gp protein in the pregnant uterus during gestation. In agreement with previous studies (Axiotis *et al.* 1991, Helguera *et al.* 2009), we showed here that in rat uterus *Abcb1a/1b* gene was actually induced by gestation, increased at mid-gestation, peaked at late gestation and displayed a dramatic decrease during term labor. It was in contrast to the high expression of both *Abcb1* isoforms (1a and 1b) in rat placenta, where P-gp plays the protective barrier role. Interestingly, the expression of two *Abcb1* isoforms was markedly higher in the labyrinth zone compared with the junctional zone, providing a molecular basis for efflux of excess glucocorticoids from syncytiotrophoblasts (Mark *et al.* 2009). The expression of *Abcb1a* in the rodent placenta is very low compared with expression of *Abcb1b* (Kalabis *et al.* 2005, Mark *et al.* 2009), which allows us to speculate that changes

detected in the luminal epithelium and vascular endothelium reflect increase in the *Abcb1b* gene.

A number of studies have reported that *Abcb1* gene expression can be regulated by various factors such as steroid hormones (Nuessler *et al.* 1997, Nakayama *et al.* 1999), cellular membrane stretch (Sardini *et al.* 1994, Wang *et al.* 2013), and inflammation (Salkeni *et al.* 2009, Poller *et al.* 2010, Gibson *et al.* 2012). P4 is the major regulatory hormone of pregnancy, essential for the maintenance of pregnancy and uterine growth (Thijssen 2005). Previous studies demonstrated that P-gp is induced in the uterine secretory epithelium by the combination of estrogen and P4 (Arceci *et al.* 1990, Axiotis *et al.* 1991). It was also suggested that P-gp may be involved in the transport of P4 across the uterine epithelium during pregnancy (Arceci *et al.* 1990). The gestational profile of *Abcb1a/1b* mRNA described in this study (increase at late gestation and dramatic decrease during labor) is mirrored by the changes in plasma P4 levels in the rat (Wiest 1970), indicating that it might be directly regulated by this ovarian hormone. We further show that a blockade of P4 signaling by RU486 causes a rapid decrease, while the maintenance of high P4 levels prevents the drop in *Abcb1a/1b* expression during term labor. These data support the hormonal regulation of *Abcb1* mRNA by P4.

In addition, we utilized a rat model of unilateral pregnancy to examine the effect of gravidity (presence of the fetal-placental unit) on the expression of *Abcb1a/1b*/P-gp. This model allows for the separation of the effect of ovarian hormones (in the empty uterine horn) from the combined effect of ovarian hormones and biological mechanical stretch caused by the growing fetuses (in the gravid uterine horn). Our data clearly indicate that in addition to the endocrine factors there was an effect of mechanical stretch, manifested by much higher levels of *Abcb1a/1b* mRNA in the gravid horn as compared to the empty horn of late pregnant rats. Interestingly, at term (GD21), the level of *Abcb1a/1b* mRNA induction in the gravid uterine horn of unilaterally pregnant animals was higher than that in the gravid uterus of bilaterally pregnant rats, while the P-gp protein levels were similar between empty and a gravid horn. One possible explanation of this phenomenon is the different degree of mechanical distention experienced by unilaterally pregnant animals due to different numbers of pups in the gravid horn (from 4 to 14) as compared to normal gestation (~8 in averages). In summary, we speculate that the drop in *Abcb1a/1b* and plasma membrane P-gp expression at term and during labor may result from a combination of physiologic P4 withdrawal and change in mechanical distension of the uterine walls by fetus(es).

As mentioned earlier, the cellular localization of P-gp has the potential to influence the absorption, distribution and excretion and thereby modify the toxicity of drugs and environmental toxins. It can transport numerous physiological substrates, including pro-/anti-inflammatory

cytokines and ovarian hormones (reviewed in Bloise *et al.* 2016). P-gp expressed in endothelium and/or epithelium during early gestation makes a significant contribution to the tissue defense mechanism. For instance, P-gp expression in the luminal plasma membrane of the vascular endothelium was shown to prevent the passage of drugs and toxins across the capillary membrane into the fetal brain (Iqbal *et al.* 2012). It has also been suggested that P-gp can prevent the accumulation of inflammation-inducing bacteria and bacterial products (Neudeck *et al.* 2004). *Abcb1* knockout mice are more susceptible to developing inflammatory disease (Kooij *et al.* 2009, Staley *et al.* 2009). A growing body of evidence suggests that a variety of chemical exposures, especially during the third trimester of pregnancy, are associated with increased risk of preterm birth (Yang *et al.* 2002, Villanueva *et al.* 2005). Therefore, we interpret our data to suggest that the decreased expression of P-gp in the uterine endothelium in late gestation may no longer limit the passage of certain exogenous and/or endogenous factors from the maternal circulation (i.e. cytokines, uterotonic agents, xenobiotics and their metabolites) into the myometrial tissue, which might enable activation of the uterine smooth muscle and induction of labor.

In addition, in some animals, it is thought that the signal for initiation of parturition arises from the fetus (Challis *et al.* 2001), but the source of this signal remains unclear. Several studies found a significant difference in the concentration of hepatic and other metabolites in amniotic fluid collected during spontaneous preterm and term birth in women as compared to nonlaboring samples (Romero *et al.* 2010, Menon *et al.* 2014). In this study, we observed an increasing level of P-gp protein expression in the uterine luminal epithelium at late gestation. Therefore, we proposed that during early to mid-gestation uterine luminal epithelial P-gp protein might serve as a barrier preventing fetal metabolites, cytokines or toxins from entering into the (maternal) uterine tissues and thus preventing preterm labor. In support of this idea, recent studies have demonstrated that P-gp located at the apical surface of the epithelial cells lining the intestine was able to prevent and/or modulate the passage of certain xenobiotics or their metabolites from the gut into the circulation (Haslam *et al.* 2008). At term, P-gp expression decreased, which may promote myometrial activation by fetal secreted products (i.e. surfactants (Condon *et al.* 2004)) and uterine contractions to expel the fetus(es) during labor.

In conclusion, upregulation of P-gp in the pregnant uterus (vascular endothelium and luminal epithelium) at specific phases of gestation suggest a potential barrier function, which first limits the entry of cytokines or endogenous metabolites from the maternal circulation, and next from the amniotic fluid, therefore maintaining a quiescence and protecting the uterus from activating stimuli. The reduction in P-gp at term suggests that this protective barrier is abolished, allowing substrate transfer and supporting labor

induction. The exact physiological role of P-gp in different compartments of the pregnant uterus and the specific substrates transported warrant further investigation.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0161>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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