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Department of Pharmacology and Toxicology

Organic Cation Transporter 3 (OCT3/SLC22A3) and Multidrug and Toxin Extrusion 1 (MATE1/SLC47A1) Protein in the Placenta: Expression, Localization and Function

Doctoral Dissertation

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Study program: Pharmacy
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Mgr. Davoud Ahmadimoghaddam

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Abstract

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Department of Pharmacology and Toxicology
Candidate: **Mgr. Davoud Ahmadimoghaddam**
Supervisor: **Prof. PharmDr. František Štaud, Ph.D.**
Title of Doctoral Dissertation: **Organic Cation Transporter 3 (OCT3/SLC22A3) and Multidrug and Toxin Extrusion 1 (MATE1/SLC47A1) Protein in the Placenta: Expression, Localization and Function.**

The aim of the present study was to investigate the expression, localization, and function of organic cation transporter 3 (OCT3, *Slc22a3*) and multidrug and toxin extrusion protein 1 (MATE1, *Slc47a1*) in the rat placenta. Using qRT-PCR, Western blotting and immunohistochemical techniques, we demonstrated abundant expression of OCT3 on the basolateral, i.e., fetus-facing side of the placenta, and MATE1 on the apical, i.e., maternal side of the placenta. To investigate the role of these transporters in the transplacental pharmacokinetics, the *in situ* method of dually perfused rat term placenta was employed in open- and closed-circuit arrangements; 1-methyl-4-phenylpyridinium (MPP^+) was used as a model substrate of both OCT3 and MATE1. We provide evidence that OCT3 and MATE1 cause considerable asymmetry between maternal-to-fetal and fetal-to-maternal transport of MPP^+ in favor of fetomaternal direction. Using closed-circuit experimental setup, we further describe the capacity of OCT3 and MATE1 to transport their substrate from fetus to mother even against a concentration gradient. Additionally, employing a range of pH values (6.5, 7.3, and 8.5) on the maternal side of the placenta, we observed that the oppositely directed H^+-gradient can drive the secretion of MPP^+ from the placenta to mother, confirming MATE1 involvement in MPP^+ elimination from trophoblast cells to the maternal circulation.

In the following part of our study, we hypothesized that changes in placental levels of *Oct3*/OCT3 and *Mate1*/MATE1 throughout gestation might affect the fetal protection and detoxication. We were able to detect *Oct3*/OCT3 and *Mate1*/MATE1 expression in the rat placenta as early as on gestation day (gd) 12 with increasing tendency toward the end of pregnancy. In contrast, comparing the first vs. third trimester human placenta, we observed stable expression of *OCT1* and decreasing expression of *OCT2,3* isoforms. Contrary to current literature, we were able to detect also *MATE1,2* isoforms in the human placenta, however, with considerable inter- and intraindividual variability. Using infusion of MPP^+ into pregnant dams we observed that the highest amount of MPP^+ reached the fetus on gd 12 while from gd 15 onwards, maternal-to-fetal transport of MPP^+ decreased significantly.

In the final part of this study, we investigated the transplacental passage of metformin, which is a substrate of both OCT and MATE transporters; in addition, it is used during pregnancy to treat gestational diabetes mellitus. We observed concentration-dependent transplacental clearance of metformin in both maternal-to-fetal and fetal-to-maternal direction and also the capacity of OCT3 and MATE1 to transport this compound from the fetal to maternal compartment even against its concentration gradient. Furthermore, employing pH values from 6.5 to 8.5 on the maternal side, we observed that the oppositely directed H^+-gradient can drive the secretion of metformin from placenta to maternal circulation, confirming metformin elimination from trophoblast cells by MATE1.

We conclude that OCT3, in a concentration-dependent manner, takes up organic cations, such as MPP^+ or metformin, from the fetal circulation into the placenta, whereas MATE1, on the other side of the barrier, is responsible for efflux of these compounds from placenta to the maternal circulation. Furthermore, we propose that increasing expression of *Oct3*/OCT3 and *Mate1*/MATE1 in the rat placenta during gestation, along with general maturation of the placental tissues results in significantly lower transport of organic cations from mother to fetus. In contrast, decreasing expression of *OCT3* and *MATE1* in human placenta indicates these transporters may play a role in fetal protection preferentially at earlier stages of gestation. OCT3 and MATE1, thus, form an efficient transplacental eliminatory pathway and play an important role in the fetal protection and detoxication. This is the first time that OCT3/MATE1 vectorial pathway is described in the placenta.

Abstrakt

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Název dizertační práce: **Organic cation transporter 3 (OCT3) a Multidrug and toxin extrusion protein 1 (MATE1) v placentě: exprese, lokalizace a funkce.**

Cílem této studie bylo popsat expresi, lokalizaci a funkci dvou transportérů, organic cation transporter 3 (OCT3) a multidrug and toxin extrusion protein 1 (MATE1) v placentě potkana. Pomocí qRT-PCR, Western blotting a imunohistochemie jsme detekovali vysokou expresi OCT3 na fetální straně placentárního trofoblastu a expresi MATE1 na straně mateřské. Pro studium role těchto transportérů v transplacentární farmakokinetice jsme využili *in situ* metodu duálně perfundované potkaní placenty v otevřeném i uzavřeném systému a 1-methyl-4-phenylpyridinium (MPP^+) byl použit jako modelový substrát OCT3 a MATE1. Naše výsledky dokazují, že OCT3 a MATE1 způsobují asymetrii v transplacentárním přechodu MPP^+ s výraznou převahou transportu z plodu do matky. Pomocí uzavřeného systému duální perfúze potkaní placenty jsme dále popsali schopnost OCT3 a MATE1 transportovat MPP^+ z plodu do matky, a to i proti koncentračnímu gradientu. Dále jsme aplikovali různé hodnoty pH (6,5, 7,3, a 8,5) na mateřské straně placenty a zjistili, že opačně směrovaný H^+ gradient je hnací silou transportu MPP^+ z plodu do matky, což potvrzuje roli MATE1 v eliminaci MPP^+ z buněk trofoblastu do mateřského oběhu.

V další části naší studie jsme předpokládali, že změny v expresi placentárního Oct3/OCT3 a Mate1/MATE1 v průběhu březosti mohou ovlivnit ochranu plodu a jeho detoxikaci. V potkaní placentě jsme detekovali *Oct3*/OCT3 a *Mate1*/MATE1 již ve 12. dnu gestace (gd), přičemž exprese se v průběhu březosti dále zvyšovala. Exprese *OCT1* byla stabilní u lidských placent odebraných v prvním vs. třetím trimestru těhotenství, zatímco exprese izoforem *OCT2,3* klesala. Ačkoliv v dostupné literatuře jsou pouze negativní informace o přítomnosti transportérů *MATE1 a MATE2* v lidské placentě, nám se podařilo jejich expresi detekovat, ale s velkou intra- a interindividuální variabilitou. S využitím infuze MPP^+ do krevního oběhu březích samic jsme zjistili, že nejvyšší množství MPP^+ dosáhne fetální cirkulace v 12. gd, zatímco od 15. gd do konce březosti transport ve směru z matky do plodu výrazně klesá.

V závěrečné fázi naší studie jsme popsali transplacentární přechod metforminu, jenž je substrátem OCT i MATE transportérů a je užíván během těhotenství k léčbě gestačního

diabetu. Pozorovali jsme na koncentraci závislou eliminaci metforminu v materno-fetálním i feto-maternálním směru a zaznamenali jsme rovněž schopnost OCT3 a MATE1 transportovat toto léčivo z fetálního do mateřského kompartmentu i proti výraznému koncentračnímu gradientu. Dále jsme aplikovali různé hodnoty pH (6,5, 7,3, a 8,5) na mateřské straně placenty a zjistili jsme, že opačně směrovaný H^+ gradient je hnacím motorem transportu metforminu z plodu do matky, což potvrzuje roli MATE1 v eliminaci metforminu z buněk trofoblastu.

Z našich výsledků vyplývá, že OCT3, v závislosti na koncentraci, transportuje organické kationty, jako MPP^+ nebo metformin, z fetálního oběhu do placenty, zatímco na druhé straně bariéry je MATE1 odpovědný za eflux těchto kationtů z placenty do mateřského oběhu. Mimoto jsme došli k závěru, že zvýšená exprese *Oct3*/OCT3 a *Mate1*/MATE1 v potkaní placentě spolu s vyzráváním placentární tkáně má za následek výrazné snížení přechodu organických kationtů z matky do plodu. Snižování exprese *OCT3* a *MATE1* v lidské placentě v průběhu těhotenství naopak naznačuje, že tyto transportéry mohou hrát roli v ochraně plodu přednostně v dřívějších fázích těhotenství.

OCT3 a MATE1, tak tvoří účinnou transplacentární eliminační jednotku a hrají důležitou roli v ochraně a detoxikaci plodu. Toto je poprvé, kdy byl transport kationtů zprostředkovaný OCT3 a MATE1 popsán v placentě.

Table of Contents

1. List of Abbreviations

ABC	ATP-binding Cassette
BCRP	Breast Cancer Resistance Protein
gd	Gestation day
MATE	Multidrug and Toxin Extrusion Protein
MPP^+	1-methyl-4-phenylpyridinum
MPTP	1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine
MRP	Multidrug Resistance-associated Protein
OCT	Organic Cation Transporter
P-gp	P-glycoprotein
SLC	Solute Carrier Transporter
TEA	Tetraethylammonium
TGCs	Trophoblast giant cells

In the text, transporter symbols with all letters in uppercase and italic form (*OCT/MATE*) are used for human genes, whereas symbols with only the first letter in uppercase and the remaining in lowercase and italic form (*Oct/Mate*) are used for genes in other mammal's species. The transporter symbols with all letters in uppercase and non-italic form (OCT/MATE) are used for human and other mammal's species proteins.

2. Introduction
2.1. Placenta

The placenta is a temporary organ that maintains separation of the two blood circulation systems of the mother and fetus. It connects the developing fetus to the uterine wall and develops from the same sperm and egg cells. The placenta begins to develop upon implantation of the blastocyst into the endometrium and connects to the fetus by an umbilical cord that contains two arteries and one vein. During the pregnancy, it acts as a substitute lung, digestive tract and kidney for the fetus; it also provides endocrine and immunity functions that are necessary for the maintenance of a successful pregnancy (Boyd, 2013; Carter, 2012; Malek, 2013).

The human placenta is formed by both maternal (decidua basalis) and fetal tissues (chorionic plate and chorionic villi). The decidua basalis forms decidual septa which divide the organ into 20–40 compartments (cotyledons). These cotyledons represent the functional vascular units of the placenta. The villous tree, in each cotyledon, consists of chorionic villi, fetal capillary endothelium, and trophoblast layer (fig. 1). Maternal blood washes the chorionic villi, in which the fetal blood is circulating and nutrients and other substances are brought through the umbilical vein to the developing fetus. The mononucleated cytotrophoblasts are fused to form the multinucleated syncytiotrophoblast which covers the outer layer of each villous tree (Ji et al., 2012). The syncytiotrophoblast, which is polarized, forms a true syncytium and covers the surface of the placenta, thereby contributing to its barrier function.

The polarized syncytiotrophoblast layer consists of a basolateral, also called basal, fetal circulation-facing membrane and a brush-border, also called apical, maternal blood-facing membrane. It acts as the rate-limiting barrier for transplacental transfer of most endo- and exogenous compounds (Ceckova-Novotna et al., 2006; Ganapathy and Prasad, 2005; Staud et al., 2012). The basal and apical membranes of the syncytiotrophoblast are functionally and structurally distinct (fig. 1 and 2). The apical membrane possesses a microvillous structure that effectively amplifies the surface area, whereas the basal membrane lacks this structural organization (fig. 1). These two membranes are further differentiated from each other by their protein composition. Various enzymes, hormone receptors, and transporters are differentially distributed between the apical and basal membranes of the syncytiotrophoblast (Ganapathy

and Prasad, 2005; Ganapathy et al., 2000; Prouillac and Lecoeur, 2010; Staud et al., 2012; Vahakangas et al., 2011).
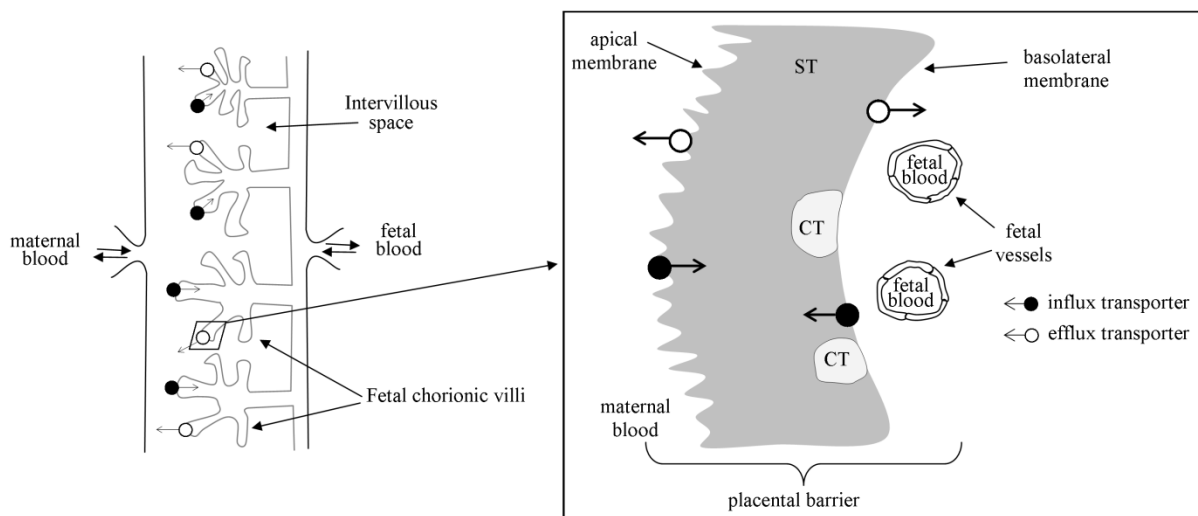

Figure 1. Schematic representation of the fetomaternal interface in the placenta. ST, syncytiotrophoblast; CT, cytotrophoblasts. (Adopted from Staud et al., 2012).

The Grosser classification (Grosser, 1927) recognizes different types of placenta, such as epitheliochorial, syndesmochorial, endotheliochorial, and hemochorial. In human, rats, mice, guinea pigs or rabbits the placenta is of hemochorial type in which maternal blood is in direct contact with the trophoblast (Furukawa et al., 2011). In human placenta one trophoblast layer (hemomonochorial) separates maternal and fetal endothelium whereas in the rat placenta maternal and fetal endothelium are separated by three trophoblast layers (hemotrichorial) (de Rijk et al., 2002). Interspecies differences in placental architecture as well as in transport protein expression must be taken into consideration when extrapolating animal data to human conditions.

In general, rats have an inverted yolk sac placenta with a discoid shape. In the hemotrichorial rat placenta, there are three principal regions. From the fetal surface, (1) the chorioallantoic connective tissue is followed by (2) the less differentiated trophoblast of the trophospongium (these trophospongium cells are presumably the precursor of more differentiated trophoblast cells) and, at the base, (3) the giant cell layer that interdigitates with the decidua (de Rijk et al., 2002; Schiebler and Knoop, 1959). The trophoblast cells of the labyrinth tissue are arranged in three distinct layers with respect to blood supply (Davies and Glasser, 1968; Martinek, 1970). Layer I is composed of fenestrated syncytiotrophoblast that

faces maternal blood. This layer is not thought to represent a barrier function of placenta due to its fenestrated structure. Layer II syncytiotrophoblast forms a contiguous epithelium, with their apical membrane facing the maternal blood. This apical domain is thought to be responsible for transport of most compounds to/from the maternal blood circulation and forms the main "barrier" for drugs and xenobiotics penetration. Layer III syncytiotrophoblast also forms contiguous syncytia, and faces the fetal blood. The basal membrane of layer III allows the transport of substances to/from the fetal compartment. Both functional membranes (apical and basal membrane) express transporters, which are necessary for nutrient (Matthews et al., 1998) as well as other endo- and exogenous transfer (Ganapathy and Prasad, 2005; Staud et al., 2012; Vahakangas and Myllynen, 2009).

Trophoblast giant cells (TGCs) arise directly from the trophectoderm and become the first terminally differentiated cells during embryogenesis (Furukawa et al., 2011; Hu and Cross, 2010; Terao et al., 2004). These cells are responsible for implantation (invasion into the uterine epithelium), and subsequent placental functions. They secrete a wide variety of substances including hormones (estrogen and progesterone), extracellular matrix, cell adhesion molecules, proteinases, and cytokines (Hu and Cross, 2010). Moreover, these cells were demonstrated to express glucose transporters in diabetic rats (Korgun et al., 2011), L-type amino acid transporter 1 participating in trophoblast invasion (Chrostowski et al., 2010), and Amino acid transport System A transporters such as sodium dependent neutral amino acid transporters (Novak et al., 2006). However, TGCs disappear around 17-19 days of pregnancy in the rats (Dorgan and Schultz, 1971).

For many years it had been thought that the placental barrier protects the fetus by restricting the passage of toxic substances and consequently provides absolute protection to the developing fetus. However, the thalidomide disaster in 1960s changed this opinion dramatically. It is now assumed that all drugs and xenobiotics, depending on their lipid solubility, molecular size, degree of ionization and plasma protein binding, cross the placenta to a certain extent (Audus, 1999; Pacifici and Nottoli, 1995).

Current knowledge indicates that the placental barrier is formed by two components: (i) mechanical barrier, passively protecting the fetus by membranes separating the maternal and fetal compartments (i.e., syncytiotrophoblast, thin layer of connective tissue, and vascular fetal endothelium) and (ii) functional components in the syncytiotrophoblast that actively protect the fetus against xenobiotics from the mother; these are metabolizing enzymes and

drug-efflux transporters (Hahnova-Cygalova et al., 2011). While the clinical importance of drug-metabolizing enzymes in the placenta remains questionable, placental drug transporters have been confirmed to substantially modulate transplacental pharmacokinetics, fetal protection, and detoxification (Ceckova-Novotna et al., 2006; Hahnova-Cygalova et al., 2011).

Many drugs and xenobiotics, to which the mother is exposed during pregnancy, may affect the placenta as well as the developing fetus; understanding the role of transporters involved in transplacental pharmacokinetics is, therefore, of pharmacological, toxicological, and therapeutic importance. If the mother is the patient, for instance in gestational diabetes mellitus, epilepsy, or infections, transfer of the drugs into the placenta and fetus may cause harmful effects to both the fetus and mother. On the other hand, if the fetus is the patient, for example in fetal cardiac arrhythmias, transfer of the drugs from the mother to the fetus is an important factor in the pharmacotherapy.
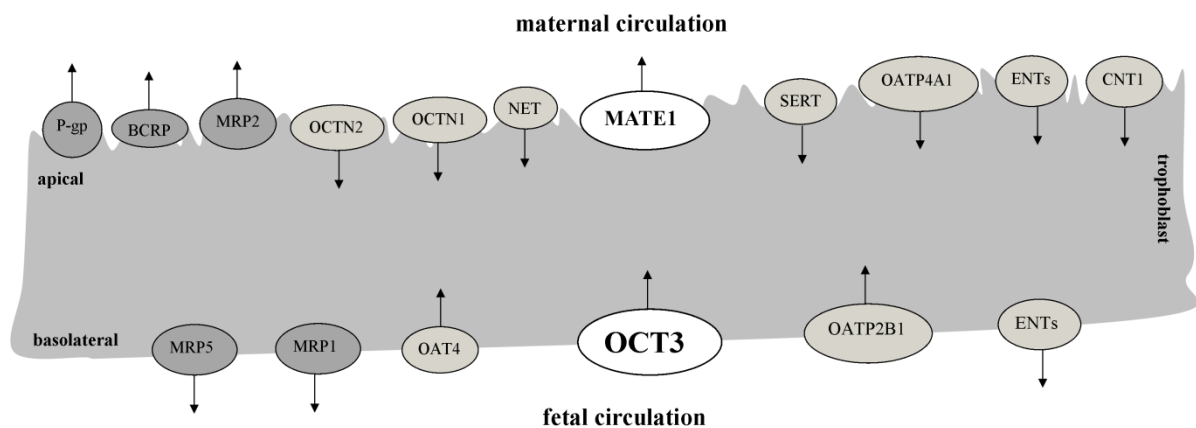
Figure 2. Schematic depiction of the main transporter proteins expressed in the placental barrier and their localization within the trophoblast. BCRP, breast cancer resistance protein; CNT1, concentrative nucleoside transporter 1; ENTs, equilibrative nucleoside transporters; MATE1, multidrug and toxin extrusion protein 1; MRP, multidrug resistance-associated protein (1, 2, and 5); NET, noradrenalin transporter; OAT4, organic anion transporter 4; OATP, organic anion-transporting polypeptide (4A1 and 2B1); OCT3, organic cation transporter 3; OCTN, carnitine transporter (1 and 2); Pgp, P-glycoprotein; SERT, serotonin transporter. (Based on Vahakangas and Myllynen, 2009 and Staud et al., 2012).

A large number of drug transporters of both ATP-binding cassette and solute carrier families are expressed in the placenta. In the following part, the most important ABC and SLC transporters in the placenta are briefly introduced.

2.2. ABC transporters in the placenta

The ATP-binding cassette (ABC) superfamily has been extensively studied in many tissues including placenta. They utilize the energy of adenosine triphosphate (ATP) hydrolysis to efflux their substrates across biological membranes. In general, they play a significant role in drug absorption, elimination and distribution (Bodo et al., 2003; Leslie et al., 2005); however, they are initially known for their role in the multidrug resistance in cancer. Three main subfamilies of ABC transporters such as P-gp, BCRP and multidrug resistance-associated proteins (MRPs) are of particular importance in transplacental pharmacokinetics. Most of the identified ABC transporters in the placenta are expressed on the apical membrane of placental syncytiotrophoblast. Therefore, they play an important role in fetal protection against harmful substances present in the maternal circulation (Behravan and Piquette-Miller, 2007; Staud et al., 2012).

2.2.1. P-glycoprotein (P-gp)

P-gp is encoded by *ABCB1* (MDR1) gene in the human, whereas in the rodents two closely located genes *Abcb1a* and *Abcb1b* encode for P-gp. Apart from tumor cells, it is functionally expressed in many physiological tissues such as the liver, intestine, kidney, brain and placenta (Staud et al., 2010). In the human placenta, the expression of P-gp decreases during the pregnancy (Gil et al., 2005); in contrast, in the rat placenta an increase in P-gp expression toward the end of gestation has been reported (Novotna et al., 2004). P-gp is localized in the apical membrane of placental syncytiotrophoblast (fig. 2) where it effluxes its substrates to the maternal circulation (Ceckova-Novotna et al., 2006; Molsa et al., 2005). Therefore, P-gp is believed to provide a fetoprotective role by active back-transport of xenobiotics and drugs to the maternal circulation. P-gp transports a large variety of hydrophobic compounds, including many therapeutic drugs. Some clinically relevant substrates/inhibitors of P-gp include verapamil, digoxin, rosiglitazone, abacavir, tenofovir disoproxil fumarate, efavirenz, nevirapine, ritonavir, delavirdine, atazanavir, lopinavir, indinavir, saquinavir, nelfinavir, phenytoin, phenobarbital, lamotrigine, levetiracetam, carbamazepine, and ondansetron (Staud et al., 2012).

2.2.2. Breast cancer resistance protein (BCRP)

BCRP was first cloned from the doxorubicin-resistant MCF7 breast cancer cell line (MCF-7/AdrVp) and called breast cancer resistance protein (Doyle et al., 1998). It is also named placental ABC transporter (*ABCP*) (Allikmets et al., 1998) and mitoxanterone resistance protein (Miyake et al., 1999). BCRP (encoded by *ABCG2*) is expressed in the placenta, brain, intestine, kidney, liver, heart, pancreas, and in venous and capillary endothelial of almost all tissues (Agarwal and Elmquist, 2012; Hahnova-Cygalova et al., 2011; Staud and Pavek, 2005). Different expression patterns of *BCRP*/BCRP at mRNA and protein levels have been reported in the human placenta. Mathias et al stated no gestational age dependence of *BCRP*/BCRP at mRNA and protein levels in the human placenta (Mathias et al., 2005). On the other hand, other studies have reported an increase (Yeboah et al., 2006) or decrease (Meyer zu Schwabedissen et al., 2006) in BCRP protein expression at term compared to preterm human placenta. The expression of *Bcrp* mRNA in the rat was reported to decrease from mid-gestation to the end of gestation (Cygalova et al., 2008). BCRP is expressed in the apical membrane of syncytiotrophoblast (fig. 2) where it pumps its substrates to the maternal circulation (Hahnova-Cygalova et al., 2011; Mao, 2008). Additionally, BCRP plays a role as survival factor during the formation of the placental syncytium and protects the trophoblast against cytokine-induced apoptosis (Evseenko et al., 2007). Furthermore, BCRP cooperates with OATP2B1 in vectorial transport of conjugated sulfate from the fetus to the maternal circulation (Grube et al., 2007). BCRP, similar to P-gp, has a fetoprotecive role in the placenta. It is able to transport a broad spectrum of substances. Many drugs that may be used during pregnancy such as glyburide, zidovudine, lamivudine, abacavir, efavirenz, delavirdine, lopinavir, ritonavir, nelfinavir, saquinavir, and atazanavir are substrates/inhibitors of BCRP (Staud et al., 2012).

2.2.3. Multidrug resistance-associated proteins (MRPs)

Among the MRPs family members, two members, MRP1 (encoded by ABCC1) and MRP2 (ABCC2), are functionally expressed in the placenta; however with different localization in the syncytiotrophoblast (fig. 2). It must be noted that other placental MRPs members such as MRP3 (*ABCC3*), MRP5 (*ABCC5*), and MRP8 (*ABCC11*) in human and MRP4 (*Abcc4*), MRP5 (*Abcc5*), MRP6 (*Abcc6*), and MRP7 (*Abcc10*) in rodents need further studies to evaluate their roles in transplacental pharmacokinetics. MPRs are involved in efflux transport of many substances including organic anion drugs, anionic conjugates, and

nucleotides in mammalian cells (Ceckova-Novotna et al., 2006). They are expressed in many tissues including the placenta, brain, liver, kidney, heart, intestine, lung and testes (Borst et al., 2000; Klaassen and Aleksunes, 2010). Although P-gp and BCRP have been well documented in the transplacental pharmacokinetics much less is known about the role of MRPs in the placenta.

MRP1 is expressed in the basal membrane of syncytiothrophoblast (fig. 2) and also in fetal capillaries (Atkinson et al., 2003; Nagashige et al., 2003). MRP1 has a broad substrate specificity including many drugs such as antiretrovirals, antineoplastics, and antibiotics (Deeley and Cole, 2006). Clinically relevant substrates/inhibitors of MRP1 include emtricitabine, abacavir, tenofovir, lamivudine, delavirdine, efavirenz, nevirapine, indinavir, ritonavir, lopinavir, atazanavir, methotrexate and folate analogs, glutathione, glucuronide and sulfate conjugates as well as heavy metal anionic complexes (Staud et al., 2012). In the placenta, MRP1 transports endogenous substrates such as leukotrienes and reduced glutathione to the fetus. It also works in concert action with OATP4A1 to make a vectorial transport of conjugated compounds from mother to fetus (Nishikawa et al., 2010). MRP2 is localized to the apical membrane of the syncytiotrophoblast (fig. 2) similar to P-gp and BCRP (Meyer zu Schwabedissen et al., 2005; St-Pierre et al., 2000), thus, assumed to have a fetoprotective role by efflux of its substrates from syncytiotrophoblast to the mother. MRP2 has shown similar substrate specificity with MRP1 (Staud et al., 2012).

2.3. Solute carrier (SLC) transporters in the placenta

The solute carrier (SLC) superfamily is the largest family of transporters, consisting of over 300 members. The members of the SLCs are expressed in a variety of tissues, especially in kidney, liver, placenta, brain, intestine, lung, and testis (Damme et al., 2011; Klaassen and Aleksunes, 2010; Koepsell et al., 2007). They translocate many substances either into, i.e. uptake, or out, i.e. efflux, of cell. They are substantial in transport of organic substances and ions across plasma membranes. SLCs have been shown to mediate transport of various compounds with different molecular structures and dimensions. Therefore, they are so called "polyspecific" transporters. They play a major role in transport of endogenous compounds, such as nucleosides, sugars, amino acids, hormones, leukotrienes, and prostaglandins (Damme et al., 2011; Nies et al., 2011). Furthermore, they are important in the transport of many clinically used drugs (Damme et al., 2011; Koepsell et al., 2007; Nies et al., 2011). Interestingly, some members of the SLC family such as OCTs and MATEs work in concert

manner to mediate vectorial transport of organic cations in the major excretory organs, i.e. the kidney and liver, where OCTs are responsible for uptake and MATEs take responsibility for efflux of organic cations (Giacomini et al., 2010; Nies et al., 2011; Otsuka et al., 2005; Yonezawa and Inui, 2011). This type of vectorial transport has not yet been described in the placenta.

In the placenta, SLCs facilitate the transport of hydrophilic or charged compounds in the energy-independent manner (Staud et al., 2012). Whereas other transporter family such as ABC drug efflux in the placenta was extensively studied in terms of expression, localization, transport function, and substrate specificity, much less attention has been paid to the role of SLCs in transplacental pharmacokinetics.

2.3.1. Organic cation transporters (OCTs/*SLC22A*)

Generally, organic cation transporters (OCTs) transport small hydrophilic compounds, ranging from 60 to 350 Da, in the bidirectional manner (Jonker and Schinkel, 2004). They translocate organic cations, weak bases that are positively charged at physiological pH, and some non-charged compounds (Klaassen and Aleksunes, 2010; Koepsell et al., 2007). The data suggest that the OCTs contain substrate binding regions that are accessible from extracellular and/or intracellular space and exhibit similar but not identical substrate specificity from both sides (Koepsell et al., 2007). They are facilitative diffusion systems that transport cations in both directions and operate independently of H^+ and Na^+ gradients (Ciarimboli, 2008). The driving forces that determine the direction of the translocation is provided jointly by the concentration gradient of the transported substrate and by the membrane potential (Koepsell and Endou, 2004; Nies et al., 2011).

OCT1 (encoded by *SLC22A1*), OCT2 (*SLC22A2*), and OCT3 (*SLC22A3*) are three subtypes of OCTs family. They play an important role in the pharmacokinetics of substrate drugs, mediating hepatic uptake and direct intestinal secretion (OCT1), renal secretion (OCT1 and OCT2) and uptake into the heart, liver and trophoblast cells (OCT3) (Ciarimboli, 2008; Jonker and Schinkel, 2004; Koepsell et al., 2007; Nies et al., 2009; Staud et al., 2012). OCT1 and OCT2 are more expressed in the liver and kidney but OCT3 is more predominant in the placenta (Bottalico et al., 2004; Ciarimboli, 2008; Koepsell et al., 2007; Leazer and Klaassen, 2003; Nies et al., 2011). In humans the strongest expression of *OCT3*/OCT3 was found in the placenta, heart, brain, skeletal muscle and liver; however, it was also detected in other organs such as kidney and lung as well as some cancer cells (Klaassen and Aleksunes, 2010; Nies et

al., 2011). In rodents, *Oct3*/OCT3 is the most abundantly expressed in the placenta (Ganapathy and Prasad, 2005; Kekuda et al., 1998; Klaassen and Aleksunes, 2010; Leazer and Klaassen, 2003), and its expression was also reported in a variety of other tissues including kidney, liver, testis, skeletal muscle, blood vessels, ovary, heart, spleen, intestine, brain and lung (Koepsell et al., 2007).

Several studies have investigated the expression and function of OCT3 in the placenta (Ganapathy and Prasad, 2005; Kekuda et al., 1998; Lee et al., 2009b; Sata et al., 2005); however, the outcomes of these studies were often inconsistent and contradictory. With respect to the placental OCT3, Kekuda et al. (1998) suggested that OCT3 may be responsible for the uptake of cationic substances from the fetal circulation into the placenta. Other studies proposed inconsistent viewpoints of OCT3 role in the placenta suggesting that "OCT3 may transfer organic cation from placenta into fetus" (Ganapathy and Prasad, 2005) and "OCT3 constitutes a leak pathway for fetal exposure" (Lee et al., 2009b). In 2005, Sata et al. suggested that OCT3 is expressed on the basal membrane of human trophoblast cells and plays an important role in the placental transport of cationic compounds.

It must be remembered that OCT3 cannot mediate transcellular transfer of organic cations by itself and other transporter(s) is/are required for complete transfer of organic cations. For instance, in the transcelluar transport of organic cations across the biological membrane in the kidney and liver, OCTs on the basolateral membrane take up organic cations and then on the apical membrane other transport protein(s) such as P-gp, MATE1, MATE2-K or MATE2 is/are responsible for the efflux of organic cations (Giacomini et al., 2010; Koepsell et al., 2007; Komatsu et al., 2011; Nies et al., 2011; Yonezawa and Inui, 2011). In the placenta, it has not been systematically explored whether OCT3 cooperates with other transporter(s) to translocate organic cations.

OCT3 recognizes many endo- and exogenous compounds, as substrates or inhibitors, such as the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), tetraethylammonium (TEA), agmantine, cimetidine, prazosin, metformin, phenformin, dopamine, atropine, dopamine, epinephrine, norepinephrine, etilefrine, guanidine, histamine, guanidine, corticosterone, desipramine, imipramine, lidocaine, oxaliplatin, azidothymidine, tenofovir, and lamivudine (Klaassen and Aleksunes, 2010; Koepsell et al., 2007; Nies et al., 2011). Abundant placental expression and wide substrate/inhibitor specificity indicate the importance of OCT3 on the fetomaternal interface; however, its role in transplacental pharmacokinetics is still not fully

understood. Thus, in this study, we focused on its placental expression, localization, and function. Additionally, since OCT3 shares many substrates/inhibitors with MATE1 (table 1) we hypothesized that the MATE1 in the placenta might cooperate with OCT3 to translocate organic cations.

Table 1. Common substrates and inhibitors of OCT3 and MATE1.

Common substrates/inhibitors of OCT3 and MATE1	References
Amantadine	(Amphoux et al., 2006; Tsuda et al., 2009b)
Cimetidine	(Lee et al., 2009a; Otsuka et al., 2005; Tanihara et al., 2007; Tsuda et al., 2009b)
Cisplatin	(Yonezawa et al., 2006)
Corticosterone	(Hayer-Zillgen et al., 2002; Otsuka et al., 2005)
Desipramine	(Tsuda et al., 2009b; Wu et al., 2000)
Diltiazem	(Tsuda et al., 2009b; Umehara et al., 2008)
Diphenylhydramine	(Muller et al., 2005; Tsuda et al., 2009b)
Disopyramide	(Hasannejad et al., 2004; Tsuda et al., 2009b)
Famotidine	(Sata et al., 2005; Tsuda et al., 2009b)
Guanidine	(Tanihara et al., 2007; Wu et al., 2000)
Imipramine	(Tsuda et al., 2009b; Wu et al., 2000)
Metformin	(Kimura et al., 2009; Tanihara et al., 2007; Tsuda et al., 2009b)
1-methyl-4-phenylpyridinium (MPP$^+$)	(Han et al., 2010; Matsumoto et al., 2008; Sata et al., 2005; Tanihara et al., 2007; Terada et al., 2006; Tsuda et al., 2007; Wu et al., 2000)
Nicotine	(Lips et al., 2005; Otsuka et al., 2005)
Oxaliplatin	(Yonezawa et al., 2006)
Procainamide	(Hasannejad et al., 2004; Tanihara et al., 2007; Tsuda et al., 2009b; Wu et al., 2000)
Quinidine	(Bourdet et al., 2005; Hasannejad et al., 2004; Ming et al., 2009; Tsuda et al., 2009b; Umehara et al., 2008)
Quinine	(Muller et al., 2005; Tanihara et al., 2007)
Ranitidine	(Muller et al., 2005; Tsuda et al., 2009b)
Serotonin	(Amphoux et al., 2006; Otsuka et al., 2005)
Tenofovir	(Minuesa et al., 2009; Tanihara et al., 2007)
Tetraethylammonium (TEA)	(Bourdet et al., 2005; Dresser et al., 2002; Chen et al., 2007; Ming et al., 2009; Tanihara et al., 2007)

2.3.2. Multidrug and toxin extrusion (MATEs/*SLC47A*) proteins

Multidrug and toxin extrusion (MATE) protein transporter was first recognized in bacteria and named NorM and YdhE (Morita et al., 1998). In 2005, the first human orthologue was identified as MATE1 (Otsuka et al., 2005). Recent studies revealed that two genes (*MATE1/SLC47A1* and *MATE2/SLC47A2*) are responsible for encoding human orthologues of MATE protein; i.e. MATE1 and MATE2 proteins (Kajiwara et al., 2009; Otsuka et al., 2005).

The MATE2-K and MATE2-B were identified as additional MATE2 isoforms; but only MATE2-K shows about 94% amino acid similarity with MATE2 (Masuda et al., 2006). Additionally, MATE2 and MATE2-K are reported to be functional and MATE2-B is nonfunctional (Komatsu et al., 2011; Masuda et al., 2006; Tanihara et al., 2007). In 2007, the MATE family was classified as SLC47 family by Human genome organization (HUGO) Gene Nomenclature Committee (HGNC). MATEs function as efflux transporter proteins, even though they are categorized to the SLC transporter families (Aleksunes et al., 2008). They have been identified in humans (Masuda et al., 2006; Otsuka et al., 2005), rats (Ohta et al., 2006), mice (Otsuka et al., 2005; Shuster et al., 2012), and rabbits (Zhang et al., 2007). MATEs appear to work in concert with other OCTs in the kidney and liver (Klaassen and Aleksunes, 2010; Koepsell et al., 2007; Nies et al., 2011). They mediate the excretion and/or secretion of structurally diverse array of many endogenous and exogenous compounds including many clinically used drugs, toxins, and endogenous metabolites (Damme et al., 2011; Nies et al., 2011; Tanihara et al., 2007; Yonezawa and Inui, 2011).

Several studies, using Real-time PCR, northern and western blot analyses revealed that MATE1 mRNA/protein is highly expressed in the kidney and liver; additionally, it is also expressed in other tissues such as the adrenal gland, skeletal muscle, and testis. MATE2 and MATE2-K were recognized as kidney-specific transporter proteins (Komatsu et al., 2011; Masuda et al., 2006; Otsuka et al., 2005). Rat *Mate1* (*Mate1*) mRNA is highly expressed in the kidney and placenta, and slightly expressed in pancreas, spleen, bladder and lung (Nishihara et al., 2007; Terada et al., 2006). Immunohistochemical analyses revealed that MATE1 protein is abundantly expressed on the apical region of the proximal (Masuda et al., 2006; Otsuka et al., 2005) and distal convoluted tubules of the kidney (Otsuka et al., 2005), and in the liver it is present on the apical canalicular membrane of hepatocytes (Otsuka et al., 2005).

Many in vivo and in vitro studies revealed that MATE transporters' driving force is supplied by an oppositely directed proton gradient for translocation of various polyspecific organic cations across the plasma membrane (Otsuka et al., 2005; Tanihara et al., 2007; Tsuda et al., 2007; Tsuda et al., 2009a; Tsuda et al., 2009b). Additionally, MATE1 has been shown to transport zwitterionic β-lactam antibiotics such as cephalexin and cephradine (Tanihara et al., 2007; Watanabe et al., 2010). The renal and biliary secretory process of many endo- and exogenous organic cations including many therapeutic drugs is an essential physiological function of MATE transporters (Moriyama et al., 2008; Terada and Inui, 2008; Yonezawa and

Inui, 2011). This function is performed by the synchronized activity of MATE and OCT transporters in the kidney and liver.

Endogenous substrates of MATEs include thiamine, guanidine, creatinine, and estrone sulfate (Damme et al., 2011). MATEs translocate also many exogenous compounds such as MPP^+, TEA, cimetidine, metformin, quinidine, quinine, corticosterone, creatinine, thiamin, serotonin, oxaliplatin, fexofenadine, procainamide, levofloxacin, desipramine, imipramine, acyclovir, ganciclovir, and tenofovir. More than 30 therapeutic drugs have been demonstrated to interact with MATE transporters (Damme et al., 2011; Nies et al., 2011). Many of these clinically used drugs showed to be common substrates and/or inhibitors of MATE and OCT transporters (Klaassen and Aleksunes, 2010; Nies et al., 2011). In table 1, some shared substrates/inhibitors of MATE1 and OCT3 are shown. The significant overlap in substrate/inhibitor specificity of MATE1 and OCT3 transporters are key points that support their coordinated function as well as clinically important drug-drug interaction.

Although MATE transporters are expressed in many tissues and play a pivotal role in pharmacokinetics, their expression and function in the placenta is not fully known to date. Therefore, in this study we investigated its placental expression, localization, and function.

2.4. Compounds used for functional studies

In this study, to test the function of placental OCT3 and MATE1 transporters 1-methyl-4-phenylpyridinium (MPP^+), as a model substrate, and metformin, as a clinically used drug during pregnancy, were used.

2.4.1. 1-methyl-4-phenylpyridinium (MPP^+)

MPP^+ is a positively charged molecule with chemical formula $C_{12}H_{12}N^+$ (fig.3). It is an active metabolite of dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is converted to the toxic cation MPP^+ by the enzyme monoamine oxidase B (MAO-B) of glial cells (Heikkila et al., 1984; Langston et al., 1984; Markey et al., 1984). MPP^+ is a toxin that acts by interfering with oxidative phosphorylation in mitochondria, causing depletion of ATP and death (Mizuno et al., 1987; Obata, 2006; Poltl et al., 2012). The compound reduces dopamine levels, inhibits the biosynthesis of catecholamines, depletes cardiac norepinephrine and inactivates tyrosine hydroxylase (Dohi et al., 2004; Feuerstein et

al., 1988; Hemrick-Luecke et al., 1990; Chagkutip et al., 2003; Ozaki et al., 1988) and has been shown to cause symptoms that mimic Parkinson disease (Wang et al., 2011).
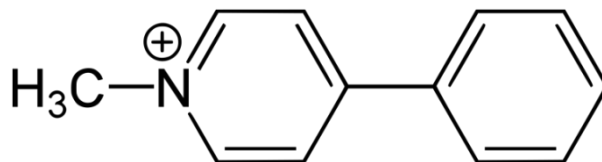
Figure 3. Structure of MPP^+.

Since MPP^+ is a charged molecule, it is not able to cross placental barrier by passive diffusion; therefore, it is an ideal candidate model for our studies as it also is a well-established substrate of OCT3 (Sata et al., 2005; Wu et al., 1998) and MATE1 (Tanihara et al., 2007; Tsuda et al., 2007). Furthermore, it is not subjected to metabolic degradation (Sayre, 1989) which makes it a suitable model compound for functional analysis of OCT and MATE transporters. In addition, little is known regarding its transplacental passage.

2.4.2. Metformin

Metformin is an oral anti hyperglycemic agent of the biguanide class (fig. 4). Its pKa of 11.5 indicates metformin will exist almost entirely in the ionized form at physiological pH (Graham et al., 2011). Metformin does not bind to plasma proteins and is eliminated by the kidneys without significant metabolism in urine (Graham et al., 2011). In the major excretory organs such as the kidney and liver, metformin is transported by vectorial transport mediated by the cooperative action of OCTs and MATEs (Chen et al., 2010; Tsuda et al., 2009a); OCTs facilitate the uptake of metformin, and MATEs are responsible for its efflux.
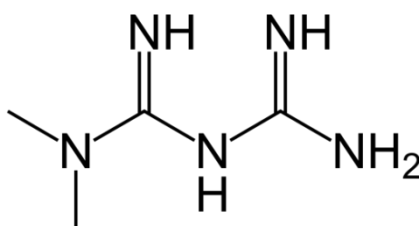
Figure 4. Structure of metformin.

Metformin has been recommended as an alternative to insulin for the treatment of gestational diabetes mellitus (Goh et al., 2011; Rowan et al., 2011). It is also frequently used in pregnant women with polycystic ovary syndrome (Ghazeeri et al., 2012; Kumar and Khan, 2012; Lord et al., 2003; Morin-Papunen et al., 2012). Additionally, the risk of abortions was reduced during the first trimester of pregnancy when metformin was administered (Glueck et al., 2004; Glueck et al., 2001). Although metformin has been considered to be non-teratogenic for a long time (Coetzee and Jackson, 1979; Coetzee and Jackson, 1984; Coetzee and Jackson, 1985; Goh et al., 2011; Rowan et al., 2011), its recommendation for the use during pregnancy was introduced without proper knowledge of its transplacental passage.

Several research groups have investigated metformin transport across the placenta (Kovo et al., 2008a; Kovo et al., 2008b; Nanovskaya et al., 2006; Tertti et al., 2010; Tertti et al., 2008); however, the outcomes of these studies are unclear and the exact mechanism(s) of the transplacental transfer of metformin remain(s) unknown.

3. Aims of the study

Earlier in the text, it was explained that placental transporters play an important role in maternofetal disposition of many endo- and exogenous compounds. Consequently, good knowledge of placental transporters and their interactions with xenobiotics is important for optimizing pharmacotherapy during gestation and predicting the risks thereof. The main aim of this study was to investigate the expression, localization and function of OCT3 and MATE1 transporters in the rat placenta. In detail, partial goals were to investigate:

1) the expression of *Oct*/OCT and *Mate*/MATE isoforms in the rat placenta and fetal tissues using qRT-PCR and Western blot analysis

2) the localization of OCT3 and MATE1 in the rat placenta at different stages of pregnancy using immunohistochemical analysis

3) the function of OCT3 and MATE1 using the technique of dually perfused rat term placenta and employing MPP^+ as a common substrate of OCT3 and MATE1

4) the fetal exposure to organic cations at different stages of pregnancy using infusion of MPP^+ in pregnant rats

5) the role of placental OCT3 and MATE1 in vectorial transport of metformin across the perfused rat term placenta.

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5. Author's contribution to the publications

The author is the first author of three research articles and a coauthor of an invited review.

In publication I, where the expression, localization, and function of OCT3 and MATE1 in the rat term placenta are presented, the author performed the *in situ* method of dually perfused rat term placenta and sample processing. The qRT-PCR and Western blot analyses were performed by the author at the Institute of Pharmacology, Faculty of Medicine in Hradec Kralove with the help of Assoc. Prof. MUDr. Stanislav Micuda, Ph.D. and PharmDr. Eva Dolezelova, Ph.D. The immunohistochemical analysis was performed at the Department of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Kralove with the help of Assoc. Prof. PharmDr. Petr Nachtigal Ph.D. and Mgr. Lenka Zemankova. The author has contributed to data processing, analysis, and publication preparation.

In publication II, which presents the expression profile and fetus protective role of OCT3 and MATE1 in the placenta and fetal tissues at different stages of gestation, the author performed the *in vivo* infusion method and sample processing. The qRT-PCR and Western blot analysis were performed by the author at the Institute of Pharmacology, Faculty of Medicine in Hradec Kralove with the help of Assoc. Prof. MUDr. Stanislav Micuda, Ph.D. and PharmDr. Eva Dolezelova, Ph.D. The human placenta qRT-PCR analysis was performed by PharmDr. Lukas Cerveny, Ph.D. The immunohistochemical analysis was performed at the Department of Biological and Medical Sciences, Faculty of Pharmacy in Hradec Kralove with the help of Assoc. Prof. PharmDr. Petr Nachtigal Ph.D. and Mgr. Lenka Zemankova. The author has contributed to data processing, analysis, and publication preparation.

In publication III, which describes metformin transfer across the rat placenta by OCT3 and MATE1, the author performed the *in situ* method of dually perfused rat term placenta and sample processing. The author has contributed to data processing, analysis, and publication preparation.

In publication IV, presenting the review of MATE transporters, the author was involved in literature research and analysis, figure drawing, and publication preparation.

6. Published articles

I. Synchronized activity of organic cation transporter 3 (Oct3/SLC22A3) and multidrug and toxin extrusion 1 (Mate 1/SLC47A1) transporter in transplacental passage of MPP$^+$ in rat.

Davoud Ahmadimoghaddam, Jakub Hofman, Lenka Zemankova, Petr Nachtigal, Eva Dolezelova, Lukas Cerveny, Martina Ceckova, Stanislav Micuda, and Frantisek Staud.

Synchronized Activity of Organic Cation Transporter 3 (Oct3/Slc22a3) and Multidrug and Toxin Extrusion 1 (Mate1/Slc47a1) Transporter in Transplacental Passage of MPP+ in Rat

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The aim of the present study was to investigate the expression, localization, and function of organic cation transporter 3 (Oct3, Slc22a3) and multidrug and toxin extrusion protein 1 (Mate1, Slc47a1) in the rat placenta. Using qRT-PCR and Western blotting techniques, we demonstrated abundant Oct3 and Mate1 mRNA and protein expression achieving significantly higher levels than those in the maternal kidney (positive control). Immunohistochemical visualization revealed preferential localization of Oct3 on the basolateral, i.e., fetus facing side of the placenta, whereas Mate1 positivity was located in the labyrinth area predominantly on the apical, i.e., maternal side of the placenta. To investigate the role of these transporters in the transplacental pharmacokinetics, the *in situ* method of dually perfused rat term placenta was employed in open- and closed-circuit arrangements; 1-methyl-4-phenylpyridinium (MPP+) was used as a model substrate of both Oct3 and Mate1. We provide evidence that Oct3 and Mate1 cause considerable asymmetry between maternal-to-fetal and fetal-to-maternal transport of MPP+ in favor of fetomaternal direction. Using closed-circuit experimental setup, we further describe the capacity of Oct3 and Mate1 to transport their substrate from fetus to mother even against a concentration gradient. We conclude that Oct3, in a concentration-dependent manner, takes up MPP+ from the fetal circulation into the placenta, whereas Mate1, on the other side of the barrier, is responsible for MPP+ efflux from placenta to the maternal circulation. These two transport proteins, thus, form an efficient transplacental eliminatory pathway and play an important role in fetal protection and detoxication.

Key Words: organic cation transporter 3; multidrug and toxin extrusion transporter 1; placenta; pregnancy; pharmacokinetics; MPP+.

The placenta is a crucial organ for proper fetus development, enabling communication between the maternal and fetal circulations. Equipped with various transport and biotransformation proteins, placenta, on one hand, facilitates transport of nutrients to the developing fetus and, on the other hand, ensures its protection against harmful compounds from the maternal circulation. It has been widely accepted that placenta is not only a passive barrier but it can also actively defend the fetus against maternal toxins. This active protective role of the placenta has mainly been attributed to ATP-binding cassette (ABC) drug efflux transporters that are functionally expressed on the apical, maternal-facing membrane, such as P-glycoprotein (Ceckova-Novotna *et al.*, 2006) and breast cancer resistance protein (Hahnova-Cygalova *et al.*, 2011). To date, much less attention has been paid to the role of the solute carrier (SLC) family of transporters in transplacental pharmacokinetics.

In this study, we focus on placental expression, localization, and function of organic cation transporter 3 (Oct3; in the text, transporter symbols with all letters in uppercase (OCT/MATE) are used for human genes and proteins, whereas symbols with only the first letter in uppercase and the remaining in lowercase (Oct/Mate) are used for genes/proteins in other mammal species). This protein was first cloned from the rat placenta (Kekuda *et al.*, 1998), and its expression was consequently reported in a variety of tissues, including the brain, lung, intestine, heart, spleen, skeletal muscle, blood vessels, kidney, and liver (Lee *et al.*, 2009). Of all organic cation transporters, OCT3 is the one most abundantly expressed in the placenta (Ganapathy and Prasad, 2005; Kekuda *et al.*, 1998) recognizing many endo- and exogenous compounds such as the neurotoxin 1-methyl-4-phenylpyridinium (MPP+), tetraethylammonium, agmantine, cimetidine, prazosin, metformin, dopamine, and norepinephrine (Nies *et al.*, 2011). Abundant placental expression and wide substrate specificity indicate the importance of OCT3 on the maternofetal interface; however, its role in transplacental pharmacokinetics is still not fully understood.

Several researchers have employed various *in vitro*, *in situ*, and *in vivo* models to investigate the expression and function

of OCT3 in the placenta; these include JAR human placental choriocarcinoma cell line (Martel and Keating, 2003), human placental basal membrane vesicles (Sata *et al.*, 2005), *in situ* perfused human term placenta (Tertti *et al.*, 2010), and Oct3 knockout mice (Zwart *et al.*, 2001). However, this research has provided inconsistent and often contradictory outcomes. In their original work, Kekuda *et al.* (1998) suggested that OCT3 may be responsible for placental uptake of cationic xenobiotics from the fetal circulation and "may hence be a key player in the barrier function of the placenta to protect the developing fetus from possible deleterious effects of xenobiotics that may be present in the maternal circulation." Contradictory views of OCT3 role in the placenta were later presented by other authors suggesting that "OCT3 may transfer MPP$^+$ from placenta into fetus" (Ganapathy and Prasad 2005) and "OCT3 constitutes a leak pathway for fetal exposure" (Lee *et al.* 2009).

To understand the role of OCT3 in drug transport across biological membranes, it must be remembered that OCT3 cannot mediate transcellular passage (i.e., transport across both basolateral and apical membranes) of organic cations on its own. In excretory organs, such as kidney and liver, OCTs have been shown to facilitate the first step of cation excretion, i.e., uptake of organic cations across the basolateral membrane into the cell. The second step, active efflux of the cationic compounds from the cell across the apical membrane, is accomplished by other transport protein(s) such as P-glycoprotein (MDR1, ABCB1) or multidrug and toxin extrusion protein (MATE1, SLC47A1) (Giacomini *et al.*, 2010; Koepsell *et al.*, 2007; Nies *et al.*, 2011; Yonezawa and Inui, 2011). In the placenta, such a vectorial transfer of cationic endo- and xenobiotics has not been systematically explored to date.

The aim of this study was to investigate the expression and localization and clarify the function of Oct3 in the rat placenta. We also aimed to search for a "collaborating" transporter on the apical membrane of the placenta that would efflux organic cations from the trophoblast cell into the maternal circulation. We used MPP$^+$ as a model toxin and prototypical organic cation substrate of OCT3 (Masuda *et al.*, 2006; Sata *et al.*, 2005; Wu *et al.*, 2000), MATE1 (Tanihara *et al.*, 2007), and P-glycoprotein (Bleasby *et al.*, 2000; Martel *et al.*, 1996). Using the technique of *in situ* dually perfused rat term placenta, we show that Oct3, in a concentration-dependent manner, takes up MPP$^+$ from the fetal circulation into the placenta, whereas Mate1 is responsible for MPP$^+$ efflux to the maternal circulation. Our results suggest that these two transport proteins control the transplacental pharmacokinetics of organic cations and play an important role in fetal protection and detoxication.

MATERIALS AND METHODS

Reagents and chemicals. 1-methyl-4-phenylpyridinium iodide (MPP$^+$), corticosterone, metformin hydrochloride, and cimetidine were obtained from Sigma-Aldrich (St Louis, MO). The radiolabeled [^{3}H]MPP$^+$ was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Rabbit polyclonal

antibodies anti-Oct3, directed to the Oct3 (catalog no. BS3359) (70 kDa) and anti-Mate1 directed to Mate1 (catalog no. sc-138983) (65 kDa) were obtained from Bioworld Technology, Inc. (MN) and Santa Cruz Biotechnology, Inc. (CA), respectively. The loading control for Western blot, rabbit polyclonal anti-β-actin antibody (42–45 kDa) was purchased from Sigma-Aldrich. Horseradish peroxidase–linked donkey anti-rabbit immunoglobulin G, F(ab')$_2$ fragment was purchased from GE Healthcare (Prague, Czech Republic). GF120918, dual P-glycoprotein and BCRP inhibitor, was from GlaxoSmithKline (Greenford, UK). All other chemicals were of analytical grade.

Cell cultures. HRP-1 rat trophoblast cells, a generous gift from Dr Michael Soares (University of Kansas City, KS) (Soares *et al.*, 1987), were used in our studies as a potential *in vitro* model of rat placental barrier (Staud *et al.*, 2006). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100μM streptomycin, 1mM sodium pyruvate, and 50μM β-mercaptoethanol. Cells from passage 17 were used for qRT-PCR and Western blot analysis.

Animals. Pregnant Wistar rats were purchased from Biotest Ltd (Konárovice, Czech Republic) and were maintained in 12-/12-h day/night standard conditions with water and pellets *ad libitum*. Experiments were performed on 21st gestation day. Fasted rats were anesthetized with 40 mg/kg of pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL) administered into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986).

RNA isolation and reverse transcription-polymerase chain reaction analysis. Placentas and maternal kidneys, as a positive control, from four different animals of 21st gestation day were collected. The organs were frozen in liquid nitrogen immediately after dissection and stored at −70°C until analysis. Expression of Oct1, Oct2, Oct3, Mate1, and Mate2 mRNA was analyzed using qRT-PCR on 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) as described previously (Brcakova *et al.*, 2009). Total RNA was isolated from the term placenta, maternal kidney, and HRP-1 rat trophoblast cells using Qiagen RNeasy Mini Kit (Bio-Consult Laboratories spol. s r. o., Czech Republic) and converted into cDNA via High Capacity cDNA reverse transcription kit (Life Technologies, Foster City, CA). Reaction mixture contained 30 ng of analyzed cDNA. The amplification of each sample was performed in triplicate using TaqMan Fast Universal PCR Master Mix and predesigned TaqMan Gene Expression Assay for Oct1 (Slc22a1, Rn00562250_m1), Oct2 (Slc22a2, Rn00580893_m1), Oct3 (Slc22a3, Rn00570264_m1), Mate1 (Slc47a1, Rn01460731_m1), and Mate2 (Slc47a2, Rn02601013_m1) provided by Life Technologies. The time-temperature profile used in the "fast" mode was 95°C for 3 min; 40 cycles: 95°C for 7 s, 60°C for 25 s. For greater precision of the mRNA quantification results, two housekeeping genes were selected by the geNorm algorithm (Vandesompele *et al.*, 2002): Ywhaz (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) (Ywhaz_Q1, NM_013011, exon5/exon6; GENERI BIOTECH Ltd, Hradec Kralove, Czech Republic) was used because of its stable expression in the placenta (Meller *et al.*, 2005), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (4352338E; Life Technologies) was used because of its stable expression in the kidney (Seidel *et al.*, 2006). Expression values of each sample were obtained as described previously (Radilova *et al.*, 2009; Vandesompele *et al.*, 2002). The expression data were normalized by the geometric mean of GAPDH and Ywhaz expressions; the relative expression between reference (maternal kidney) and term placenta and HRP-1 rat trophoblast cells was determined by comparison of normalized data.

Membrane preparation. A modified method of Chen *et al.* (2005) was used for membrane preparation. Briefly, maternal kidney, rat term placenta, and HRP-1 rat trophoblast cells were minced in ice-cold Ripa buffer (Sigma-Aldrich), containing 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 2 μg/ml aprotinin, and 50 μg/ml benzamidine, and then maternal kidney and term placenta were homogenized using a Magna Lyser Instrument (Roche Diagnostics, Prague,

Czech Republic) at 6500 rpm for 2 × 30 s; HRP-1 rat trophoblast cells were homogenized using Ultrasonic processor UP100H (Hielscher-Ultrasound Technology, Teltow, Germany). The supernatants were obtained after a 3000 g centrifugation at 4°C for 10 min. The protein concentration was determined with the BCA assay (Pierce, Rockford, IL), and the samples were stored at –80°C.

Western blot analysis. Crude membrane–containing homogenates of rat term placenta, maternal kidney, and HRP-1 cells proteins (20 µg) were incubated with sample buffer at room temperature for 30 min and separated by SDS-PAGE on 7.5 and 10% polyacrylamide gel for Oct3 and Mate1, respectively. After the proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA), the membrane was blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). The membranes were then incubated with primary antibodies for 1 h at concentrations of 1:1000 and 1:100 for Oct3 and Mate1, respectively. Then the membranes were washed four times with TBST and incubated for 1 h with a horseradish peroxidase–linked donkey anti-rabbit IgG antibody at concentrations 1:2000 and 1:200 for Oct3 and Mate1, respectively. After washing with TBST buffer, the membranes were developed using enhanced chemiluminescent reagent (GE Healthcare) and subjected to autoluminography for 1–5 min. The immunoreactive bands on the exposed films were quantified as described previously (Brcakova *et al.*, 2009). Equal loading of proteins onto the gel was confirmed by immunodetection of β-actin.

Immunohistochemistry. Preparations of the rat placental tissue were performed as described previously (Pavek *et al.*, 2003). Specimens of the placenta were fixed in 4% paraformaldehyde and then paraffin embedded for immunohistochemistry. Sections of the placenta (thickness, 7 µm) were rehydrated through a series from xylene to ethanol solutions. The antigen Oct3 was unmasked by heating the specimens in sodium citrate buffer (pH 6.0) in a microwave oven at 750 W. Blocking of nonspecific background staining was performed with 10% normal goat serum (Sigma-Aldrich) in PBS (pH 7.4) for 30 min. Slides were incubated with polyclonal primary antibody Rabbit anti-rat OCT3 (Alpha Diagnostic, San Antonio), diluted 1:50 in bovine serum albumin (BSA), 1 h at room temperature. Endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS solution for 15 min. Subsequently, the slides were developed with a secondary antibody, goat anti-rabbit IgG conjugated to peroxidase-labeled polymer (DAKO EnVision⁺, Carpinteria), in the presence of rat IgG (Sigma-Aldrich) for 30 min. The reaction was visualized using diaminobenzidine (DAB substrate-chromogen solution; DAKO), and the sections were counterstained by hematoxylin. The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins. The Mate1 immunohistochemistry was performed in the same manner. The primary antibody Rabbit anti-rat MATE1 (Santa Cruz Biotechnology) was incubated 1 h at room temperature in dilution 1:50 in BSA.

Photo documentation and image digitizing were performed using Olympus AX 70 with a digital firewire camera Pixelink PL-A642 (Vitana Corp., Ottawa, Canada) and image analysis software NIS (Laboratory Imaging, Czech Republic).

Dual perfusion of the rat placenta. The method of dually perfused rat term placenta was used in our study, as described previously (Staud *et al.*, 2006). In brief, one uterine horn was excised and submerged in heated Ringer's saline. A catheter was inserted into the uterine artery proximal to the blood vessel supplying a selected placenta and connected with the peristaltic pump. Krebs perfusion liquid containing 1% dextran was brought from the maternal reservoir at a rate of 1 ml/min. The uterine vein, including the anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighboring fetuses by ligatures. The umbilical artery was catheterized by use of a 24-gauge catheter connected to the fetal reservoir and perfused at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner, and the selected fetus was removed. Before the start of each experiment, the fetal vein effluent was collected into preweighed glass vial to check for a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was terminated. Maternal and fetal perfusion pressures were maintained at levels close to physiological values and monitored continuously throughout the perfusion experiments; pH values in the perfusion

reservoirs were maintained by controlled oxygenation with carbogen mixture (5% CO_2/95% O_2) as described previously (Pavek *et al.*, 2001). At the end of experiment, placenta was perfused with radioactive-free buffer for 10 min, excised from the uterine tissue, and dissolved in tissue solubilizer (Solvable; PerkinElmer Life and Analytical Sciences), and its radioactivity was measured to detect tissue-bound MPP⁺ (Tri-Carb 2900TR; PerkinElmer).

Two types of perfusion experiments were used in this study, i.e., open-circuit and closed-circuit (recirculation) perfusion systems:

Open-circuit perfusion system was employed to study maternal-to-fetal and fetal-to-maternal clearances of MPP⁺ at various concentrations. In this experimental setup, MPP⁺ was added to either maternal (maternal-to-fetal studies) or fetal (fetal-to-maternal studies) reservoir immediately after successful surgery. After 5 min stabilization period, the sample collection started (time 0). Fetal effluent was sampled into preweighed vials in 5 min interval, radioactivity was measured, and transplacental clearance was calculated.

Closed-circuit (recirculation) perfusion system was employed to investigate the potential of Oct3/Mate1 to remove MPP⁺ from the fetal circulation. In this experimental setup, both maternal and fetal sides of the placenta were infused with equal concentrations of MPP⁺ and after 5 min stabilization, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (250 µl) were collected every 10 min from the maternal and fetal reservoirs, and [³H]MPP⁺ concentration was measured. This experimental setup ensures steady MPP⁺ concentration on the maternal side of the placenta and enables investigations of maternal/fetal concentration ratio at equilibrium; any net transfer of the substrate implies transport against a concentration gradient and is evidence for active transport.

Effect of substrate concentration on transplacental clearance. Open-circuit perfusion system was employed to investigate the effect of various MPP⁺ concentrations on maternal-to-fetal and fetal-to-maternal clearances. MPP⁺ with a trace amount of [³H]MPP⁺ was added to the maternal or fetal reservoir in the following concentrations: 0.001, 0.01, 0.1, 1, 10, 100, or 1000µM. The inflowing MPP⁺ concentration was maintained constant for the duration of the experiment; transplacental clearances of MPP⁺ were calculated as described below.

Effect of inhibitors on transplacental passage of MPP⁺. To study the effect of inhibitors on fetomaternal MPP⁺ concentration ratio at equilibrium, both maternal and fetal sides of the rat term placenta were perfused with low, nonsaturating concentration (0.001µM) of [³H]MPP⁺ in closed-circuit perfusion system. Effect of several compounds was investigated; MPP⁺ (1000µM), corticosterone (100µM), metformin (100µM), cimetidine (100µM), or GF120918 (2µM) was added to both maternal and fetal reservoirs to inhibit the transporters and to demonstrate their effect on fetomaternal equilibrium of MPP⁺. The inhibitor concentrations were based on previous studies: corticosterone (Wu *et al.*, 1998), GF120918 (Cygalova *et al.*, 2009), cimetidine (Ito *et al.*, 2011), and metformin (Tsuda *et al.*, 2009).

Effect of pH on transplacental passage of MPP⁺. To study the effect of pH on fetomaternal MPP⁺ concentration ratio at equilibrium, both maternal and fetal sides of the placenta were perfused with low, nonsaturating concentration (0.001µM) of [³H]MPP⁺ in closed-circuit perfusion system. pH in the maternal reservoir was adjusted to 6.5, 7.3, or 8.5. In the fetal reservoir, pH 7.3 was used in all experiments.

Pharmacokinetic analysis of transport activity in the placenta. Organ clearance concept was applied to mathematically describe maternal-to-fetal and fetal-to-maternal transport of MPP⁺ in open-circuit perfusion system (Staud *et al.*, 2006). Average data from the intervals of 25 to 35 min of placenta perfusion were used for the following calculations. Maternal-to-fetal transplacental clearance (Cl_{mf}) was calculated according to Equation 1.

$$Cl_{mf} = \frac{C_{fv} \cdot Q_f}{C_{ma} \cdot W_p} \qquad (1),$$

where C_{fv} is MPP⁺ concentration in the umbilical vein effluent, Q_f is the umbilical flow rate, C_{ma} is MPP⁺ concentration in the maternal reservoir, and W_p is the wet weight of the placenta. Fetal-to-maternal transplacental clearance (Cl_{fm}) was calculated according to Equation 2.

$$Cl_{fm} = \frac{(C_{fa} - C_{fv})Q_f}{C_{fa} \cdot W_p} \qquad (2),$$

where C_{fa} is MPP$^+$ concentration in the fetal reservoir entering the perfused placenta via the umbilical artery.

Statistical analysis. For each group of placental perfusion experiments, the number of animals was $n \geq 3$. For the qRT-PCR and Western blot analysis, the number of samples was $n \geq 4$. Data are presented as means ± SD. Statistical significance was examined by unpaired Student's *t*-test or one-way ANOVA followed by Bonferroni test using Graphpad Prism 5.0 software (Graphpad Software, Inc., San Diego, CA); the same software was used for curve fitting. A difference of $p < 0.05$ was considered statistically significant.

RESULTS

Real-time PCR Analysis

The mRNA levels of Oct and Mate isoforms in the rat term placenta, maternal kidney, and HRP-1 rat trophoblast cells as evaluated by qRT-PCR are shown in Fig. 1. Of all Oct isoforms tested, only Oct3 mRNA was detected in the placenta in significant amount (345-fold expression compared with that in maternal kidney), whereas mRNA expressions of Oct1 and Oct2 isoforms achieved less than 3 and 1% of those in maternal kidney, respectively (Fig. 1A). Similarly, we observed abundant expression of Mate1 mRNA in the placenta (13 times higher when compared with maternal kidney), whereas Mate2 mRNA expression was approximately 6 times higher compared with kidney (Fig. 1B). In the HRP-1 rat trophoblast cells, only Oct1 mRNA expression was detected (Figs. 1A and B).

Western Blot Analysis

Protein quantification of Oct3 and Mate1 was performed in homogenates of the placenta, maternal kidney, and HRP-1 rat trophoblast cells. The relative amount of Oct3 and Mate1 protein was expressed as a percentage of the maternal kidney values. The Oct3 and Mate1 protein expressions in placenta were 1.6- and 1.4-fold higher compared with that in maternal kidney, respectively (Fig. 2). The HRP-1 rat trophoblast cells showed very low protein expression of Oct3 and Mate1 compared with maternal kidney (Fig. 2).

FIG. 1. qRT-PCR analyses of (A) Oct1, Oct2, Oct3 and (B) Mate1, Mate2 mRNA expression in the rat term placenta, HRP-1 rat trophoblast cells, and maternal kidney (positive control). The mRNA data normalized to those of GAPDH and Ywhaz are expressed as a target mRNA/housekeeping mRNA. Data represent means ± SD ($n = 4$, in each group). ***$p < 0.001$; statistically different from maternal kidney and ††$p < 0.01$, †††$p < 0.001$ statistically different from placental Oct3 (A) or Mate1 (B) (one-way ANOVA followed by Bonferroni test).
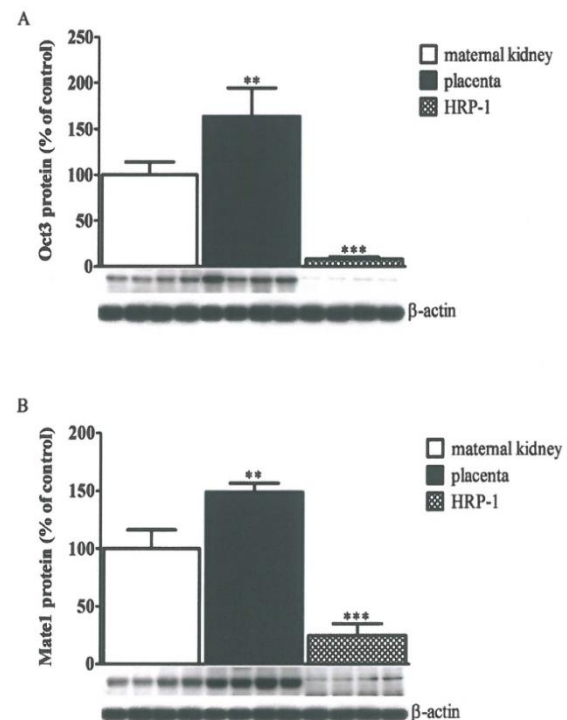

FIG. 2. Protein expression of (A) Oct3 and (B) Mate1 in the rat term placenta, maternal kidney, and HRP-1 rat trophoblast cells. Protein data after densitometric analysis are related to maternal kidney (positive control). Data are expressed as means ± SD; ($n = 4$), in each group. Equal loading of protein was confirmed by β-actin. **$p < 0.01$, ***$p < 0.001$; statistically significant difference from maternal kidney (one-way ANOVA followed by Bonferroni test).

Immunohistochemical Localization of Oct3 and Mate1 in the Rat Term Placenta

The antigen retrieval immunohistochemistry at the light microscopy level was carried out for localization of Oct3 and Mate1 in the rat term placenta using polyclonal primary antibody Rabbit anti-rat OCT3 and primary antibody Rabbit anti-rat MATE1, respectively. The positivity of the Oct3 in the rat term placenta is almost exclusively located in the syncytiotrophoblast of the labyrinth area, namely in layers II and III of the trophoblast cells (Fig. 3A). No positivity was detected in fetal capillaries, labyrinth zone, or spongiotrophoblast. These data support the hypothesis that Oct3 is present in the basolateral membrane of the syncytiotrophoblast layer of the rat term placenta. On the other hand, Mate1 positivity was located in syncytiotrophoblast of labyrinth area (predominantly on maternal side); no staining positivity of Mate1 was detected in fetal capillaries, in labyrinth zone, or in spongiotrophoblast (Fig. 3B). However, more precise visualization techniques will be required to specify the exact localization of the transporters in the placental tissue.

Open-Circuit Perfusion Experiments: Effect of Substrate Inflow Concentrations on Transplacental Clearance in Maternal-to-Fetal and Fetal-to-Maternal Direction

The maternal or fetal side of the placenta was infused with various concentration of MPP$^+$ (0.001, 0.01, 0.1, 1, 10, 100, or 1000μM) with trace amount of [^{3}H]MPP$^+$. In both maternal-to-fetal and fetal-to-maternal transport studies, increase in substrate concentration resulted in significant changes in transplacental clearance, confirming involvement of a capacity-
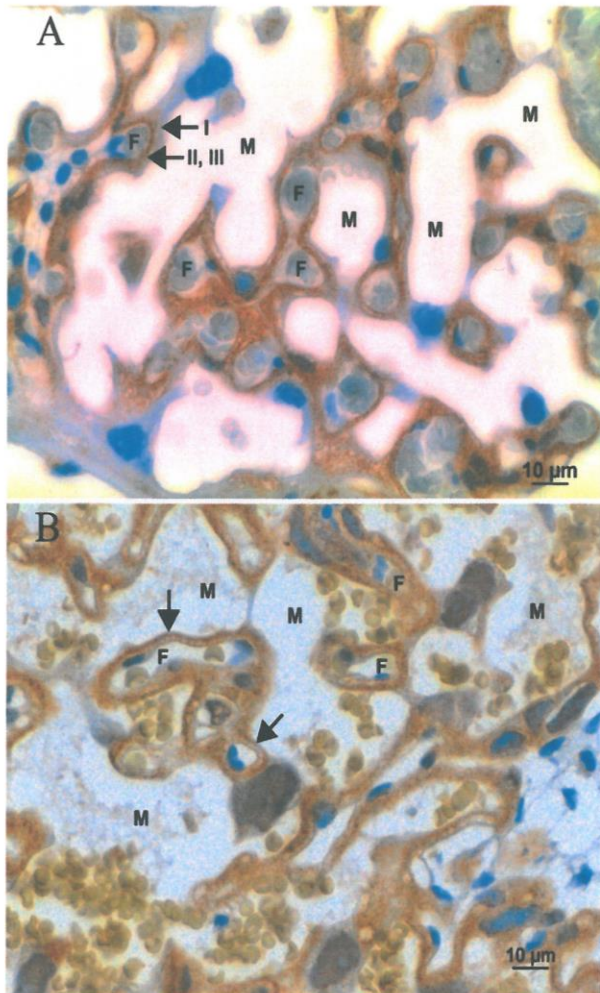

FIG. 3. Immunohistochemical detection of (A) Oct3 and (B) Mate1 in the rat term placenta. The Oct3 expression was detected only in layers II and III of syncytiotrophoblast in the labyrinth zone (see arrows). No staining was visible either in layer I of syncytiotrophoblast or in fetal capillaries. Mate1 expression was detected predominantly on the maternal side of syncytiotrophoblast in the labyrinth zone (see arrows). No staining was visible in fetal capillaries. M, maternal blood space; F, fetal blood space. The slides were counterstained with hematoxylin. Scale bar 10 μm.
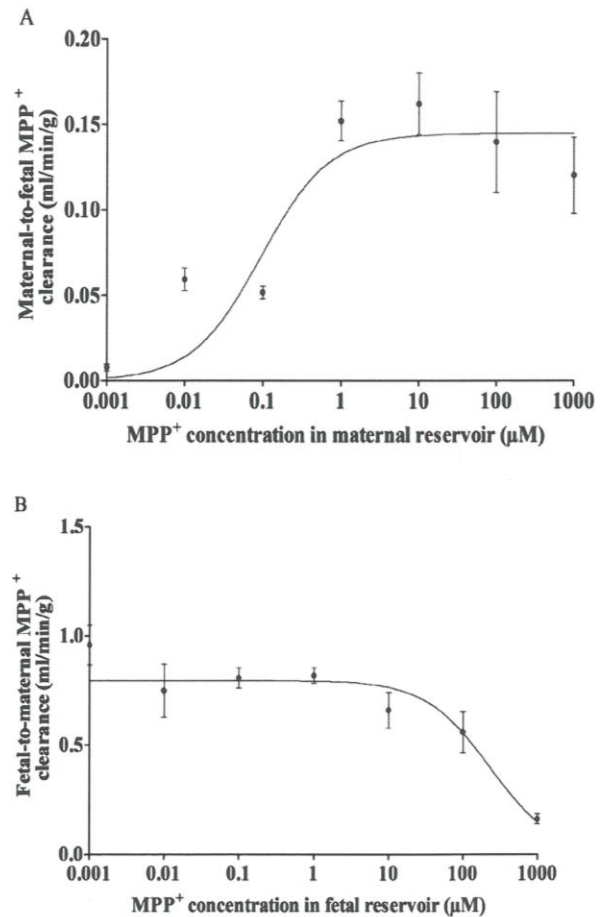

FIG. 4. Transport of MPP$^+$ across the dually perfused rat term placenta in the (A) maternal-to-fetal and (B) fetal-to-maternal directions. MPP$^+$ with [^{3}H]MPP$^+$ tracer was added to the maternal (A) or fetal (B) reservoir, and its radioactivity was measured in the fetal venous outflow. Changes of clearance with increasing MPP$^+$ concentration indicate the nonlinearity of the processes and involvement of a saturable mechanism. Total transplacental clearance was calculated by Equation 1 for maternal-to-fetal and by Equation 2 for fetal-to-maternal direction (see Materials and Methods section). Experimental values are presented as means ± SD of at least three experiments.

limited transport mechanism (Figs. 4A and B). Less than 1% of the total MPP$^+$ dose was detected in placentas after perfusion experiments, suggesting limited tissue binding and negligible effect on clearance calculations.

Comparing fetal-to-maternal and maternal-to-fetal clearances, we observed significant asymmetry in favor of fetal-to-maternal direction (Figs. 5A and B), suggesting an involvement of effective antiport system mediating transplacental permeation of cationic substrates from fetal to maternal circulation. This asymmetry was most pronounced at low MPP$^+$ concentration (0.001μM), where fetal-to-maternal clearance was 123 times higher than that in the opposite direction (Fig. 5A). On the other hand, at high MPP$^+$ concentration (1000μM), fetal-to-maternal and maternal-to-fetal clearances reached almost identical values, and the asymmetry was almost annulled
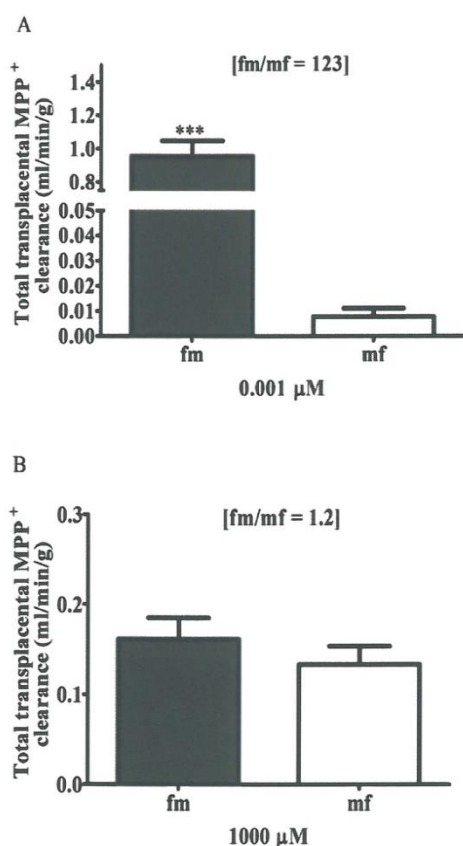

FIG. 5. Ratio of clearances between fetal-to-maternal (fm) and maternal-to-fetal (mf) directions at (A) low, nonsaturating and (B) high, saturating MPP$^+$ concentrations. MPP$^+$ with [^{3}H]MPP$^+$ tracer was added to the maternal or fetal compartment, and its radioactivity was measured in fetal venous outflow. Total transplacental clearances were calculated by Equations 1 and 2 (see Materials and Methods section). At low substrate concentration (0.001μM), significant asymmetry in transplacental clearance of MPP$^+$ was observed in favor of fetal-to-maternal direction. At high MPP$^+$ concentration (1000μM), this asymmetry was almost annulled, and no differences between fetal-to-maternal and maternal-to-fetal clearances were detected, suggesting saturation of the transport mechanisms. Numbers in brackets show the ratio between fm and mf clearances; data are presented as means ± SD of at least three experiments. Student's t-test was used to assess statistical significance; ***$p < 0.001$.

(Fig. 5B), confirming saturation of the transport proteins and limited role of their transport activities.

Closed-Circuit Perfusion Experiments: Effect of Inhibitors on Transplacental Passage of MPP$^+$

To investigate the potential of Oct3/Mate1 to remove their substrates from fetal circulation, MPP$^+$ was added to both maternal and fetal reservoirs at nonsaturating concentration of 0.001μM in closed-circuit experiment setup. A steady decrease in the MPP$^+$ concentration in the fetal reservoir was observed followed by concentration equilibration after approximately 40 min of perfusion (Fig. 6, inset), confirming the ability of the compound to cross the placenta in fetal-to-maternal direction even against the concentration gradient. This decline was fully blocked by coinfusion of MPP$^+$ (1000μM) or partially blocked by corticosterone (100μM), metformin (100μM), or cimetidine (100μM). No changes were observed when GF120918 (2μM), a P-glycoprotein inhibitor, was coinfused (Fig. 6).

Closed-Circuit Perfusion Experiments: Effect of pH on Transplacental Passage of MPP$^+$

To investigate the effect of pH on fetal-to-maternal transport of cations, MPP$^+$ was added to both maternal and fetal reservoirs at nonsaturating concentration of 0.001μM in closed-circuit experiment setup. pH in the maternal reservoir was set to 6.5, 7.3, or 8.5, whereas pH 7.3 was used in fetal reservoir. At pH 7.3 in both maternal and fetal reservoirs, a steady decrease in the MPP$^+$ concentration in the fetal reservoir was observed followed by concentration equilibration after approximately 40 min of perfusion. This decline was notably blocked by adjusting maternal pH to 8.5 resulting in significantly higher ratio between fetal and maternal MPP$^+$ concentrations, thus confirming proton-dependent transport of cations across the placenta (Fig. 7).

DISCUSSION

In this study, we describe the expression and localization of Oct3 and Mate1 transport proteins in the rat term placenta and investigate their role in transplacental pharmaco/toxicokinetics.

Available knowledge on the placental expression of OCT3 suggests that it is expressed in the fetal-facing basolateral membrane of the placenta (Kekuda *et al.*, 1998; Sata *et al.*, 2005), where it can function as an influx transporter of cationic drugs. However, contradictory views on the role of OCT3 in the transplacental pharmacokinetics can be found in the current literature (Ganapathy and Prasad, 2005; Kekuda *et al.*, 1998; Lee *et al.*, 2009). Considering that organic cations cannot cross biological membranes by passive diffusion, it is obvious that after OCT3-mediated influx of a cationic compound into the trophoblast cells, another membrane transporter is responsible for efflux of the molecule from the placenta. As the
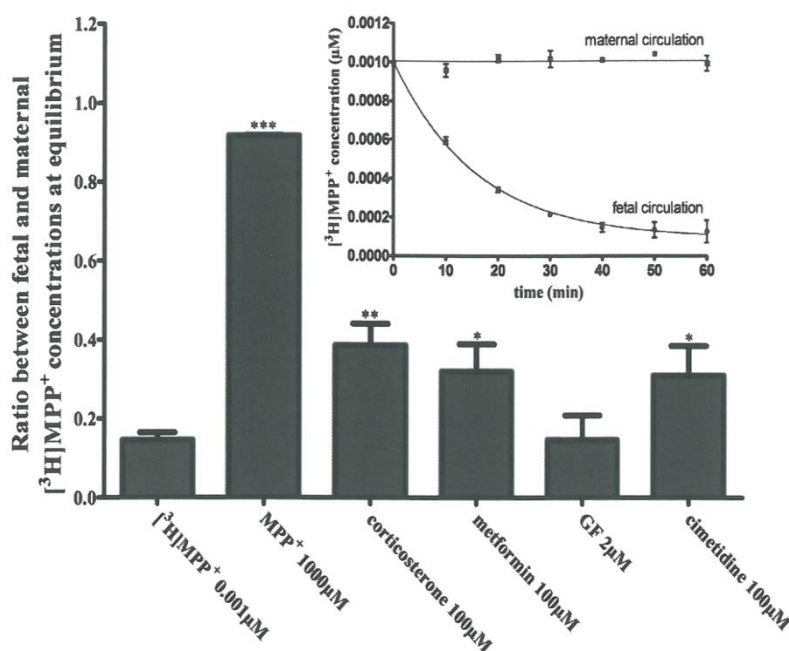
FIG. 6. Effect of inhibitors on elimination of MPP[+] from the fetal circulation. In the closed-circuit perfusion setup, [³H]MPP[+] was simultaneously infused to both the maternal and the fetal sides of the placenta at equal concentrations of $0.001 \mu M$, and fetal perfusate was recirculated for 60 min; at the end of the perfusion, fetal and maternal MPP[+] concentrations were compared. Fetal [³H]MPP[+] concentrations decreased from $0.001 \mu M$ down to $0.00015 \mu M$ and stabilized after 40 min of perfusion (see inset). This decrease was significantly inhibited by cold MPP[+] $(1000 \mu M)$, corticosterone $(100 \mu M)$, metformin $(100 \mu M)$, and cimetidine $(100 \mu M)$. GF120918 $(2 \mu M)$, on the other hand, had no effect on MPP[+] elimination from the fetal reservoir. Data are presented as means $\pm$ SD of at least three experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; statistically significant difference from MPP[+] $0.001 \mu M$ (ANOVA followed by Bonferroni test).
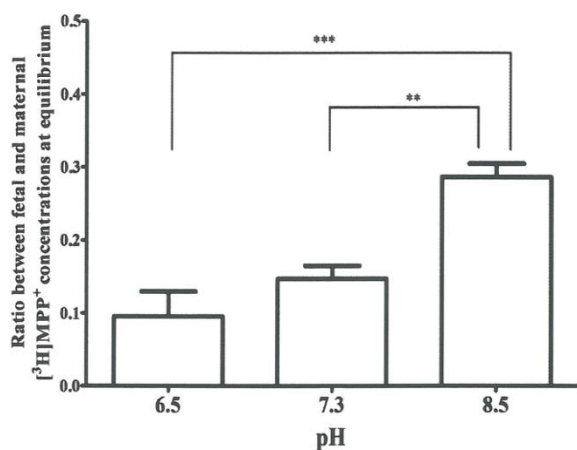

FIG. 7. Effect of maternal pH on elimination of MPP[+] from the fetal circulation. In the closed-circuit perfusion setup, [³H]MPP[+] was simultaneously infused to both the maternal and the fetal sides of the placenta at equal concentrations of $0.001 \mu M$, and the fetal perfusate was recirculated for 60 min. Fetal pH was set to 7.3, and the pH values in the maternal reservoir were set to 6.5, 7.3, or 8.5. At the end of the experiment, fetal and maternal MPP[+] concentrations were compared. It is evident that higher concentration of protons in the maternal circulation results in higher fetal-to-maternal transport of MPP[+], indicating the role of a proton-cation antiporter system on the apical side of the placenta. Data represent means $\pm$ SD of at least three experiments. $**p < 0.01$, $***p < 0.001$ (ANOVA followed by Bonferroni test).

transport of organic cations from the trophoblast has not been systematically investigated to date, we searched for a clue in other excretory organs such as the kidney and liver where efflux of organic cations across the apical membrane is thought to occur by a separate transporter-mediated process, either by an ABC drug efflux transporter (e.g., P-glycoprotein) (Bleasby *et al.*, 2000; Martel *et al.*, 1996) or by a cation-H^{+} exchange mechanism (e.g., OCTNs or recently discovered MATEs) (Giacomini *et al.*, 2010; Otsuka *et al.*, 2005; Yonezawa and Inui, 2011). We hypothesized that a similar scenario might occur in the placenta and, therefore, investigated Oct3-mediated influx and P-glycoprotein– and/or Mate1-mediated efflux of MPP[+] at the maternofetal interface.

First, we explored the expression and localization of Oct3 and Mate1 in the rat term placenta. Mate1 is a very recently identified transporter (Otsuka *et al.*, 2005), and only limited information regarding its placental expression is available in the current literature. No Mate1 mRNA was detected in the murine (Aleksunes *et al.*, 2008) and human placenta (Otsuka *et al.*, 2005). On the other hand, Terada *et al.* (2006) found abundant Mate1 mRNA expression in the rat placenta. Here, we observed prominent placental expression of Mate1 at both mRNA and protein levels, significantly exceeding those of maternal kidney. Similar results were obtained for Oct3 mRNA and protein expression. We also planned to characterize the rat trophoblast HRP-1 cells as an alternative model for *in vitro* studies of

placental transport of organic cations; however, negligible expression of Oct3 and Mate1 at both mRNA and protein levels were detected, and this model was, therefore, excluded from the following functional studies. Immunohistochemical visualization revealed preferential localization of Oct3 on the fetal side of the rat placenta, which is a finding consistent with the current literature (Sata et al., 2005). On the other hand, Mate1 appears to be localized preferentially toward the maternal circulation, which is in agreement with its apical localization in the kidney and liver (Otsuka et al., 2005), supporting our hypothesis of its role in trophoblast-to-mother efflux of substrates.

To investigate the function of Oct3 and Mate1 in the rat placenta and to quantify their role in transplacental pharmacokinetics, the in situ method of dually perfused rat term placenta was used in our study as a well-established model to investigate placental physiology (Kertschanska et al., 2000; Stulc et al., 1995), pathophysiology (Jakoubek et al., 2008), and pharmacology (Cygalova et al., 2009; Pavek et al., 2003; Staud et al., 2006). MPP^+ was chosen as a model toxin and prototypical organic cation substrate. MPP^+ is the active metabolite of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine that has been shown to cause symptoms that mimic Parkinson disease (Wang et al. 2011). It is an ideal candidate model for our studies as it is a well-established substrate of both OCT3 (Sata et al., 2005; Wu et al., 2000) and MATE1 (Terada and Inui, 2008). In addition, it is not subjected to metabolic degradation (Sayre, 1989), and little is known regarding its transplacental passage.

In the initial studies, a range of MPP^+ concentrations from 0.001 to 1000μM was tested in open-circuit perfusion to evaluate the effect of influx concentration on transplacental clearance. In the case of linear pharmacokinetics, clearance is independent of concentration; however, in both fetal-to-maternal and maternal-to-fetal directions, we observed strong dependence of transplacental clearance on MPP^+ concentration, which indicates nonlinear pharmacokinetics and involvement of a saturable transport system. When comparing fetal-to-maternal and maternal-to-fetal clearances at low, nonsaturating concentrations, we can quantify a measure of fetal protection by a transport system in the placenta. In the case of MPP^+, fetal-to-maternal clearance was 123 times faster than maternal-to-fetal one; we believe this huge asymmetry in transplacental clearances is caused by a concerted action of Oct3 and Mate1. In Figure 8, we compare asymmetries in transplacental clearances among several compounds that are substrates of other placental efflux transporters that we investigated previously under identical condition (Cygalova et al., 2009; Staud et al., 2006). This comparison shows that Oct3/Mate1 combo offers surprisingly high level of fetal protection; nevertheless, the role of other cationic transporters (such as Octn1, Octn2, and NET [Ganapathy and Prasad, 2005; Koepsell et al., 2007]) and lipid solubility of the compounds (Cygalova et al., 2009) may affect this clearance ratio comparison.

To investigate the ability of Oct3/Mate1 to remove MPP^+ already present in the fetal compartment, closed-circuit perfusion was used in which both maternal and fetal sides of the placenta were perfused with nonsaturating concentration of MPP^+ (0.001μM) and the fetal perfusate was recirculated. In the case of linear pharmacokinetics, both concentrations remain unchanged for the duration of the experiment as
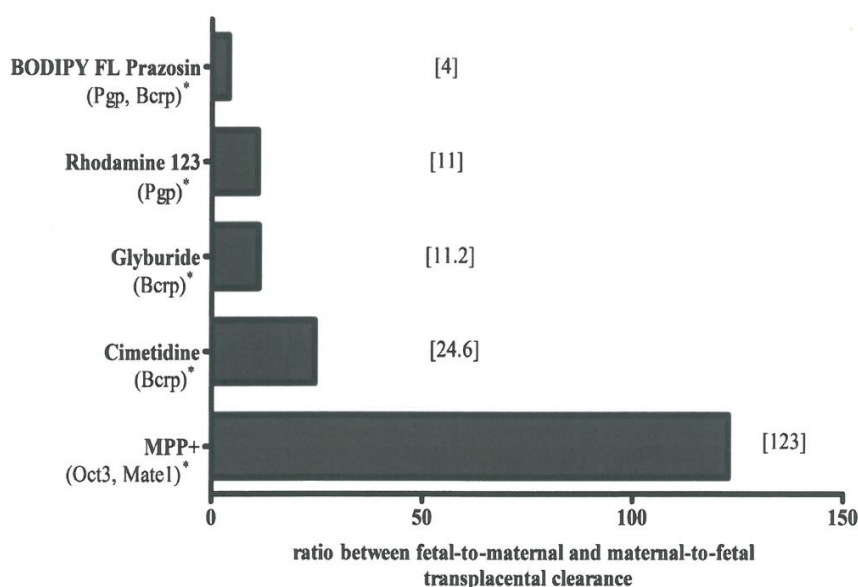

FIG. 8. Measure of fetal protection by placental transporters. The figure shows the ratios between the clearances in fetal-to-maternal and maternal-to-fetal directions of several xenobiotics. The values for BODIPY FL prazosin, glyburide, rhodamine 123, and cimetidine are taken from our previous studies using the same experimental method (Cygalova et al., 2009; Staud et al., 2006). The numbers in the brackets show the exact value of the clearance ratios. *Transporter(s) involved in placental passage of the compound.

demonstrated with antipyrine (Cygalova *et al.*, 2009). Here we observed considerable decrease in fetal MPP$^+$ concentrations, demonstrating the ability of MPP$^+$ to cross the placenta from fetus to mother even against its concentration gradient, thus confirming the involvement of an active transport mechanism. Once inside the trophoblast cells, MPP$^+$ can be eliminated into the maternal circulation across the apical membrane either by P-glycoprotein (Bleasby *et al.*, 2000; Martel *et al.*, 1996) or by organic cation-H$^+$ antiporter system (Ganapathy *et al.*, 1988). In closed-circuit perfusion setup, we first tested possible effect of P-glycoprotein on the transplacental passage of MPP$^+$ by employing GF120918 as an inhibitor. Although in our previous studies GF120918 effectively inhibited P-glycoprotein in the rat placenta (Cygalova *et al.*, 2009; Pavek *et al.*, 2003), here we did not record any change in transplacental passage of MPP$^+$, suggesting P-glycoprotein does not have a substantial role in elimination of the organic cation from the fetus (Fig. 6). We, therefore, further focused on the activity of placental Mate1 and evaluated the effect of proton concentration in the maternal circulation on MPP$^+$ placental transport. Changing pH values from 6.0 to 8.5, Terada *et al.* (2006) reported Mate1 transport activity to be pH-dependent *in vitro* in HEK293 cells transiently expressing MATE1. In our *in situ* study, we employed a similar range of pH on the maternal side of the placenta, showing that the oppositely directed H$^+$-gradient can drive the secretion of organic cations from the placenta to mother. These data indicate that Mate1 on the apical membrane is the collaborating partner of Oct3 in fetal-to-maternal excretion of cations (Fig. 9). We speculate that the H$^+$-gradient in the syncytiotrophoblast is regulated by an ATP-driven H$^+$ pump (Simon *et al.*, 1992) and/or by Na$^+$/H$^+$ exchangers (Sibley *et al.*, 2002) that have been localized in the placenta of several species as important mechanisms for syncytiotrophoblast homeostasis.

Apart from MPP$^+$, a model toxin used in this study, many other molecules have been recognized as substrates/inhibitors of OCT3 and/or MATE1 transporters, including endogenous compounds (e.g., corticosterone, estradiol, progesterone, dopamine, epinephrine, histamine, and serotonin), clinically used drugs (e.g., acyclovir, metformin, procainamide, imipramine, diltiazem, quinine, amantadine, cimetidine, topotecan, and tenofovir), and other toxins and environmental pollutants (e.g., cocaine, paraquat, ethidium, and nicotine) (Nies *et al.*, 2011). It is, therefore, very likely that drug-drug interactions or disruption of the Oct3/Mate1-mediated eliminatory pathway may affect transplacental pharmacokinetics of these substrates and limit the detoxication capacity of the placenta. We addressed this issue in our study by employing various inhibitors of Oct3 and/or Mate1 to investigate their role in the fetal-to-maternal elimination of MPP$^+$. In particular, we used metformin, a frequently prescribed oral antidiabetic and an Oct3 and Mate1 substrate/inhibitor (Tsuda *et al.*, 2009), cimetidine, an H2 antagonist and an inhibitor of Oct3 (Sata *et al.*, 2005) and Mate1 (Ito *et al.*, 2011), and corticosterone as an endogenous compound and inhibitor of Oct3 (Wu *et al.*, 1998). All of these compounds were capable of, at least partially, decreasing the fetal-to-maternal transplacental elimination of MPP$^+$. We, therefore, suggest that the Oct3/Mate1 excretory pathway of the placenta may be compromised by endogenous and exogenous compounds, which may eventually result in unpredictable outcome of medication during pregnancy or even toxicity to the sensitive fetoplacental unit.

In conclusion, we describe the expression and localization of Oct3 on the basolateral, fetus-facing side and of Mate1 on the apical, mother-facing side of the rat placenta. Furthermore, using *in situ* method of dually perfused rat term placenta, we provide evidence that these two transporters, in a
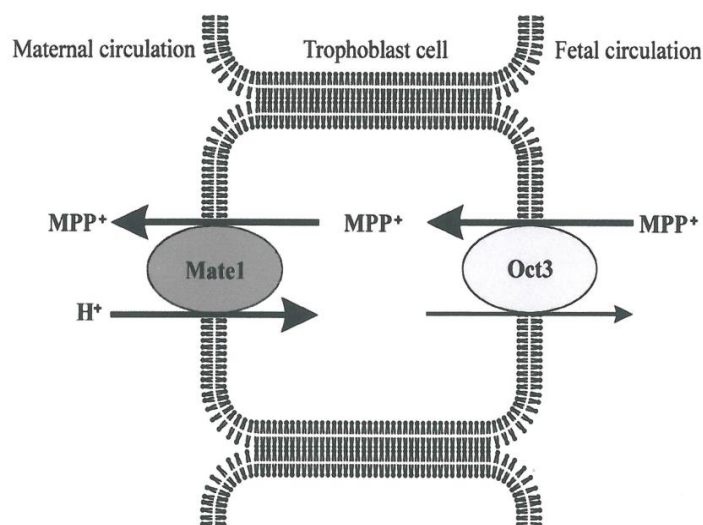
FIG. 9. Schematic depiction of synchronized activity of Oct3 and Mate1 in MPP$^+$ transport across the rat placenta. The localization and orientation of uptake (Oct3) and efflux (Mate1) transporters in the rat placenta are shown.

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concerted action, remove their substrates from the fetal circulation and pump them to the maternal one, even against concentration gradient. Based on these results, we propose that Oct3 and Mate1 form an efficient transplacental eliminatory pathway and play an important role in fetal protection and detoxication.

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II. Organic Cation Transporter 3 (OCT3/SLC22A3) and Multidrug and Toxin Extrusion 1 (MATE1/SLC47A1) Transporter in the Placenta and Fetal Tissues: Expression Profile and Fetus Protective Role at Different Stages of Gestation

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Organic Cation Transporter 3 (OCT3/SLC22A3) and Multidrug and Toxin Extrusion 1 (MATE1/SLC47A1) Transporter in the Placenta and Fetal Tissues: Expression Profile and Fetus Protective Role at Different Stages of Gestation[1]

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ABSTRACT

In our previous study, we described synchronized activity of organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion 1 (MATE1/SLC47A1) transporter in the passage of organic cations across the rat placenta and the role of these transporters in fetal defense; in this study, we hypothesized that changes in placental levels of OCT3 and MATE1 throughout gestation might affect the fetal protection and detoxification. Using quantitative RT-PCR, Western blot analysis, and immunohistochemistry, we were able to detect Oct3/OCT3 and Mate1/MATE1 expression in the rat placenta as early as on gestation day (gd) 12 with increasing tendency toward the end of pregnancy. Comparing first versus third trimester human placenta, we observed stable expression of OCT1 and decreasing expression of OCT2 and OCT3 isoforms. Contrary to the current literature, we were able to detect also MATE1/MATE2 isoforms in the human placenta, however, with considerable inter- and intraindividual variability. Using infusion of 1-methyl-4-phenyl-pyridinium (MPP$^+$), a substrate of OCT and MATE transporters, into pregnant dams, we investigated the protective function of the placenta against organic cations at different gds. The highest amount of MPP$^+$ reached the fetus on gd 12 while from gd 15 onward, maternal-to-fetal transport of MPP$^+$ decreased significantly. We conclude that increased expression of placental OCT3 and MATE1 along with general maturation of the placental tissues results in significantly lower transport of MPP$^+$ from mother to fetus. In contrast, decreasing expression of OCT3 and MATE1 in human placenta indicates these transporters may play a role in fetal protection preferentially at earlier stages of gestation.

INTRODUCTION

The placenta, among its many other functions, mediates the transfer of endogenous compounds as well as xenobiotics between the mother and her developing fetus; it is also an important organ of fetal protection and detoxification. To fulfill these roles, the placenta is equipped with many transport systems localized to both the maternal- and fetal-facing membranes [1, 2]. Understanding the exact mechanism(s) of placental transporters and their role in transplacental pharmacokinetics will aid in optimization of pharmacotherapy of pregnant women and prediction of toxicity risks for the fetus. While the placental expression and function of the drug transporters of the ATP-binding cassette (ABC) family, such as P-glycoprotein and breast cancer resistance protein, have been studied extensively, significantly less information has been gathered on the solute carrier (SLC) transporters [1].

The SLC family is the largest of all the transporter families, consisting of over 300 members. Some SLC transporters are substrate-specific, preferentially mediating transport of endogenous compounds such as amino acids, nucleosides, and sugars. Others, on the other hand, show wide substrate specificity, recognizing a broad spectrum of molecular structures and dimensions. These are called polyspecific transporters and have been shown to play a major role in drug disposition [3]. In particular, SLC transporters have been described as important components of eliminatory pathways for cations and anions in excretory organs. For example, vectorial transport of organic cations mediated by organic cation transporters (OCTs) and multidrug and toxin extrusion proteins (MATEs) was described in the proximal tubules of the kidney and hepatocytes [4, 5], suggesting that OCTs and MATEs work in a concert manner; OCTs being responsible for uptake and MATEs for efflux of organic cations [5–7].

In the placenta, SLC transporters mainly facilitate uptake of hydrophilic or charged molecules by the trophoblast cells; once in the trophoblast, the substrates are either utilized for the placenta's own metabolic needs (e.g., de novo synthesis of placental hormones) or pumped out of the trophoblast cell by another SLC or ABC transporter such as MATE or P-glycoprotein (for a review, see [1]). In our previous study [8], we described the expression and localization of organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion 1 (MATE1/SLC47A1) transporter in the rat term placenta: using immunohistochemical visualization. We re-

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vealed preferential localization of OCT3 on the basolateral, that is, fetus-facing side of the placenta, whereas MATE1 was located in the labyrinth area predominantly on the apical (i.e., maternal) side of the placenta. Furthermore, using the technique of dually perfused rat term placenta, we demonstrated that OCT3, in a concentration-dependent manner, takes up organic cations from the fetal circulation into the placenta and MATE1 is responsible for their efflux to the maternal circulation, even against a concentration gradient. We, therefore, proposed that OCT3 and MATE1 form an efficient transplacental eliminatory pathway for organic cations and play an important role in fetal protection and detoxification [8].

Pregnancy is a dynamic process where the physiological systems of both the mother and her developing fetus change continuously. Importantly, most data on placental drug transporters are obtained from term placentas. However, variations in expression of placental drug transporters and activity throughout pregnancy have been observed in several mammal species. Regarding SLC transporters, several papers reported on intraindividual variability of *Oct3* expression in the murine placenta, concluding that the levels of expression decline toward the end of gestation [9, 10]; however, no information on intraindividual changes in OCTs and/or MATEs expression in the human and rat placenta is available to date. Furthermore, little is known about expression and function of these transporters in fetal tissues.

In this study we hypothesized that changes in placental levels of OCT3 and/or MATE1 transporters throughout gestation might affect their transport capacity and, subsequently, provide variable fetal protection throughout fetal development. Therefore, we investigated the expression of OCT1, OCT2, OCT3, MATE1, and MATE2 transporters in the rat and human placenta and rat fetal tissues at different stages of pregnancy. In addition, using the infusion of 1-methyl-4-phenylpyridinium (MPP$^+$), a model toxin and well-established substrate of OCT3 and MATE1, into the pregnant rat, we also studied the protective effect of the placenta against organic cations at different stages of gestation.

MATERIALS AND METHODS

Reagents and Chemicals

MPP$^+$ and the loading control for Western blot analysis, rabbit polyclonal anti-β-actin antibody (42–45 kDa), were purchased from Sigma-Aldrich. The radiolabeled [^{3}H] MPP$^+$ was obtained from Perkin Elmer Life and Analytical Sciences. Rabbit polyclonal antibodies anti-Oct3, directed to the OCT3 (BS3359) (70 kDa) and anti-Mate1 directed to MATE1 (sc-138983) (65 kDa) were obtained from Bioworld Technology, Inc. and Santa Cruz Biotechnology, Inc., respectively. Horseradish peroxidase-linked donkey anti-rabbit immunoglobulin G (IgG), F (ab')$_2$ fragment, was purchased from GE Healthcare. All the other chemicals were of analytical grade.

Animals

Female Wistar rats were used for all the experiments. Pregnant rats were purchased from Biotest and maintained at 12L:12D standard conditions with water and pellets ad libitum. All the experiments were approved by the ethical committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strassbourg, 1986).

Gestation day (gd) 0 was established upon detection of a copulatory plug of sperm after overnight mating. Experiments were performed on gds 12, 15, 18, and 21 using three to four dams in each group. Fasted rats were anesthetized with pentobarbital (nembutal; Abbott Laboratories) in a dose of 40 mg/kg administered into the tail vein.

RNA Isolation and Real-Time RT-PCR Analysis in the Rat Placenta and Fetal Tissues

Four rat placentas were randomly dissected from four dams on each gd (12, 15, 18, and 21). The kidney, liver, brain, and intestine were sampled from three to four randomly selected fetuses of each dam on gds 18 and 21. The maternal kidney was selected as a positive control. The organs were frozen in liquid nitrogen immediately after dissection and stored at −70°C until analysis. Expression of *Oct1, Oct2, Oct3, Mate1,* and *Mate2* mRNA was analyzed using quantitative RT-PCR (qRT-PCR) on 7500HT Fast Real-Time PCR System (Applied Biosystems) as described previously [8]. Total RNA was isolated from collected tissue samples using the Qiagen RNeasy Mini Kit (Bio-Consult Laboratories); the purity of the isolated RNA was checked by the absorbance ratio at 260 and 280 nm (A260/A280) and RNA integrity was confirmed by electrophoresis on a 1% agarose gel. RNA was converted into cDNA via high-capacity cDNA reverse transcription kit (Life Technologies); the reaction mixture contained 30 ng of analyzed cDNA. The amplification of each sample was performed in triplicate using TaqMan Fast Universal PCR Master Mix and predesigned TaqMan Gene Expression Assay for *Oct1* (*Slc22a1*, Rn00562250_m1), *Oct2* (*Slc22a2*, Rn00580893_m1), *Oct3* (*Slc22a3*, Rn00570264_m1), *Mate1* (*Slc47a1*, Rn01460731_m1), and *Mate2* (*Slc47a2*, Rn02601013_m1) provided by Life Technologies. The time-temperature profile used in the fast mode was 95°C for 3 min followed by 40 cycles at 95°C for 7 sec and 60°C for 25 sec. For greater precision of the mRNA quantification results, two housekeeping genes were selected by the geNorm algorithm [11]: *Ywhaz* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) (Ywhaz_Q1, NM_013011, exon5/exon6; GENERI BIOTECH Ltd.) was used because of its stable expression in the placenta [12] and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) (4352338E; Life Technologies) was used because of its stability in other organs [13]. Expression values of each sample were obtained as described previously [11, 14]. The expression data are normalized by the geometric mean of Gapdh and Ywhaz expressions and presented as a ratio between target mRNA and housekeeping mRNA.

RNA Isolation and Real-Time RT-PCR Analysis in the Human Placenta

All the human placentas were obtained from uncomplicated pregnancies in accordance with ethical standards at University Hospital, Hradec Kralove. All the participants provided written informed consent, and their details are summarized in Table 1. Ten term placentas were collected following elective cesarean section at term (38–41 wk of gestation) and seven first trimester placentas were acquired from interruption of the pregnancy between 9 and 13 wk of gestation. The placental samples were frozen in liquid nitrogen immediately after surgery and stored at −70°C until analysis. Total RNA was isolated from preweighed placenta using Tri Reagent solution (Molecular Research Centre) according to the manufacturer's instructions. The purity of the isolated RNA was checked by A260/A280, and RNA integrity was confirmed by electrophoresis on a 1% agarose gel. The concentration of RNA was calculated by A260 measurement. RNA was converted into cDNA using the Tetro cDNA Synthesis Kit (Bioline). Real-time PCR analysis of *OCT1, OCT2, OCT3, MATE1,* and *MATE2* mRNA expression was performed on iCycler machine (Bio-Rad). Complementary DNA (40 ng) was amplified using 2× Probe Master Mix (GENERI BIOTECH Ltd.) and predesigned TaqMan PCR assays for *OCT1* (hSLC22A1_Q3), *OCT2* (hSLC22A2_Q1), *OCT3* (hSLC22A1_Q2), *MATE1* (hSLC47A1_Q1), and *MATE2* (hSLC47A2_Q1). For greater precision of the mRNA quantification, four housekeeping genes were selected: *B2M* (beta-2-microglobulin) because of its high and relatively stable expression in the term placenta [12], *HPRT* (hypoxanthine phosphoribosyl-transferase 1) and *TBP* (TATA box binding protein) for their stable expression in the term placenta [12], and *18S rRNA* (hRN18S1_Q1) because of its high and consistent expression throughout gestation and low variability between individuals [15–17]. All the assays were purchased from GENERI BIOTECH Ltd. The amplification of each sample was performed in triplicate. The time-temperature profile used was 95°C for 3 min followed by 50 cycles at 95°C for 15 sec and 60°C for 45 sec. Expression values of each sample were obtained as described previously [11, 14].

Membrane Preparation

A modified method of Chen et al. [18] was used for the membrane preparation. Briefly, the maternal kidney, placenta, fetal brain, and intestine were minced in ice-cold RIPA buffer (Sigma-Aldrich), containing 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 2 μg/ml aprotinin, and 50 μg/ml benzamidine, and then were homogenized using a Magna Lyser Instrument (Roche

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TABLE 1. Characteristics of the pregnant women and their offspring.

	3rd trimester (cesarean delivery)[a]	1st trimester (surgical abortion)[a]
Maternal age (yr)*	31.5 (25–38)	35.0 (21–41)
Maternal BMI (kg/m^2)*[b]	23.8 (19.3–40.8)	24.8 (18.93–26.57)
Ethnicity (no.)	Caucasian (10)	Caucasian (7)
Gestational age (wk)[c]	38.9 (37.9–39.9)	10.9 (8.5–11.6)
Neonate weight (g)	3265 (2200–4120)	N.A.
Neonate height (cm)	49 (46–52)	N.A.

* No statistical differences were found for the maternal age or body mass index (BMI) between the two groups of pregnant women ($P > 0.05$, Mann-Whitney test).
[a] Data are given as median (range) with the exception of ethnicity (number).
[b] BMI was calculated from maternal parameters (body height and weight) at the beginning of pregnancy.
[c] Gestational age at the time of delivery/abortion was based on early ultrasonography examination.
[d] N.A., not available.

Diagnostics) at 6500 rpm for 2 × 30 seconds. The supernatants were obtained after a 3000 × g centrifugation at 4°C for 10 min. The protein concentration was determined with the BCA assay (Pierce), and samples were stored at −80°C.

Western Blot Analysis

Crude membrane-containing homogenates of maternal kidney, placenta, fetal brain, and intestine (20 µg) were incubated with sample buffer at room temperature for 30 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) on 7.5% and 10% polyacrylamide gel for OCT3 and MATE1, respectively. After the proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad), the membrane was blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). The membranes were then incubated with primary antibodies for 1 h at concentrations of 1:1000 and 1:100 for OCT3 and MATE1, respectively. Then the membranes were washed four times with TBST and incubated for 1 h with a horseradish peroxidase-linked donkey anti-rabbit IgG antibody at concentrations 1:2000 and 1:200 for OCT3 and MATE1, respectively. After washing with TBST buffer, the membranes were developed using enhanced chemiluminescent reagent (GE Healthcare) and subjected to autoluminography for 1–5 min. The immunoreactive bands on the exposed films were quantified as described previously [19]. Equal loading of proteins onto the gel was confirmed by immunodetection of β-actin.

Immunohistochemistry

Preparations of the placental tissues on gds 12, 15, 18, and 21 were performed as described previously [8]. Specimens of the placenta were fixed in 4% paraformaldehyde and then paraffin-embedded for immunohistochemistry. Sections of the placenta (thickness, 7 µm) were rehydrated through a series from xylene to ethanol solutions. The antigen OCT3 and MATE1 were unmasked by heating the specimens in sodium citrate buffer (pH 6.0) in a microwave oven at 750 W. Blocking of nonspecific background staining was performed with 10% normal goat serum (Sigma-Aldrich) in phosphate-buffered saline (pH 7.4) for 30 min. Slides were incubated for 1 h at room temperature with polyclonal primary antibody rabbit anti-rat Oct3 (Alpha Diagnostic) diluted 1:50 in bovine serum albumin (BSA). Likewise, the slides were incubated for 1 h at room temperature with primary antibody rabbit anti-rat Mate1 (Santa Cruz Biotechnology) diluted 1:50 in BSA. Endogenous peroxidase activity was blocked with 3% H$_2$O$_2$ in PBS solution for 15 min. Subsequently, the slides were developed with a secondary antibody: goat anti-rabbit IgG conjugated to peroxidase-labeled polymer (DAKO EnVision$^+$) in the presence of rat IgG (Sigma-Aldrich) for 30 min. The reaction was visualized using diaminobenzidine (DAB substrate-chromogen solution; DAKO), and the sections were counterstained by hematoxylin. The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins. The MATE1 immunohistochemistry was performed in the same manner.

Photo documentation and image digitizing were performed using Olympus AX 70 with a digital firewire camera Pixelink PL-A642 (Vitana Corp.) and image analysis software NIS (Laboratory Imaging).

Fetal Exposure to MPP$^+$ During Pregnancy

The jugular vein (for the injection of radioisotope) and the carotid artery (for blood sampling) of pregnant rats on gds 12, 15, 18, and 21 were cannulated after anesthetizing the rats [20]. The rats were infused for 60 min with a solution containing 1.48 µg of MPP$^+$ in 1 ml of physiological solution including trace amount of radiolabeled [^{3}H] MPP$^+$; 1 ml of this solution was injected as a loading dose at the beginning of the infusion. The steady state, as determined by maternal blood sampling, was reached 40 min after the beginning of the experiment. The concentration of MPP$^+$ in the maternal blood at the steady state ranged from 50 to 80 nM, which is well below the saturating concentration [8].

At the end of the infusion (60 min), the animals were sacrificed, and one to three randomly selected fetuses of each dam were sampled. On gds 18 and 21, the fetal brain, kidney, liver, and intestine from three fetuses were excised. Fetal tissue and blood samples were treated with tissue solubilizer Solvable (PerkinElmer Life and Analytical Sciences), and after mixing with scintillation liquid (PerkinElmer Life and Analytical Sciences), their radioactivity was measured (Tri-Carb 2900TR; Perkin Elmer). The amount of MPP$^+$ that reached the fetus was expressed as the feto-maternal ratio of the radioactivity of [^{3}H] MPP$^+$ in 1 g of fetal tissue over the radioactivity of [^{3}H] MPP$^+$ in maternal plasma at the steady state.

Statistical Analysis

The animal experiments are based on four rats (n = 4) for each stage of gestation. The number of the human placenta samples was n = 7 (first trimester) and n = 10 (third trimester). All the results are presented as means ± SD. Assuming nonnormal data distribution, nonparametric permutation test was used to assess statistical significance [21, 22]. $P < 0.05$ was considered statistically significant.

RESULTS

Oct1, Oct2, Oct3, Mate1, and Mate2 mRNA Expression in the Rat Placenta and Fetal Tissues at Different Stages of Gestation

The mRNA levels of Oct and Mate isoforms in the placentas and fetal organs at different stages of gestation (gds 12, 15, 18, and 21) as evaluated by qRT-PCR analysis are shown in Figures 1–3. All the Oct isoforms were detected in the rat placenta on all the gds (Fig. 1A). While the levels of Oct1 and Oct2 remained constant throughout gestation, Oct3 mRNA levels increased significantly toward the end of gestation, reaching values almost nine times higher in the term placenta compared with those on gd 12 (Fig. 1A). Similarly, both Mate1 and Mate2 mRNA were expressed on all the gds but only Mate1 levels increased during gestation, reaching values almost 17 times higher in the term placenta compared with those on gd 12 (Fig. 1B). Mate2 levels, on the other hand, remained unchanged throughout gestation (Fig. 1B).

In the fetal organs, the highest expression of Oct1 was detected in the liver and kidney with significant increase between gd 18 and 21 (Fig. 2A). Oct2 mRNA was highly expressed in the fetal brain and kidney with increase in the expression from gd 18 to 21 (Fig. 2B). Oct3 was found to be
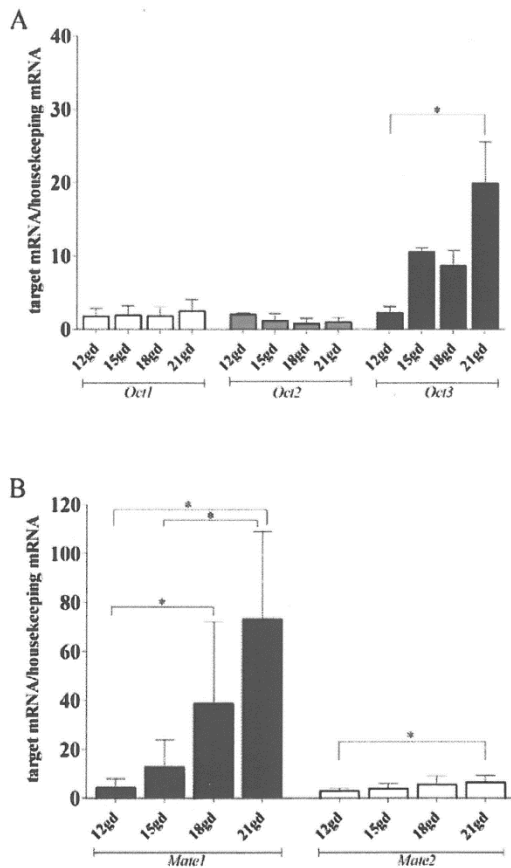
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FIG. 1. Quantitative RT-PCR analyses of *Oct1, Oct2,* and *Oct3* (**A**), and *Mate1* and *Mate2* (**B**) mRNA expression in the rat placenta on different gestational days (gds). The mRNA data normalized to those of *Gapdh* and *Ywhaz* are expressed as a target mRNA/housekeeping mRNA. Data represent means ± SD (n = 4 in each group); *P < 0.05.

highly expressed in the fetal brain while its expression in other organs was relatively low. Furthermore, its levels on gd 21 were almost five times higher than those on gd 18 (Fig. 2C). The highest expression of *Mate1* was observed in the fetal kidney; again, its levels increased from gd 18 to 21 approaching those of the adult maternal kidney (Fig. 3A). *Mate2* was found to be expressed predominantly in the fetal brain compared with other fetal organs with increasing trend from gd 18 to 21 (Fig. 3B).

OCT1, OCT2, OCT3, MATE1, and MATE2 mRNA Expression in the First Trimester and Term Human Placenta

The mRNA expression of *OCT* and *MATE* isoforms in the human placenta as evaluated by qRT-PCR is shown in Figure 4. In the first trimester placenta, expression of all the genes of interest was detected, however, with a relatively high level of variability. In the term placenta, we were not able to detect *MATE1* mRNA expression while *MATE2* mRNA expression was observed. Comparing expression of *OCT* and *MATE* isoforms between first trimester and term placentas, statistically significant decreases were observed for *OCT2, OCT3,* and *MATE1* (Fig. 4).
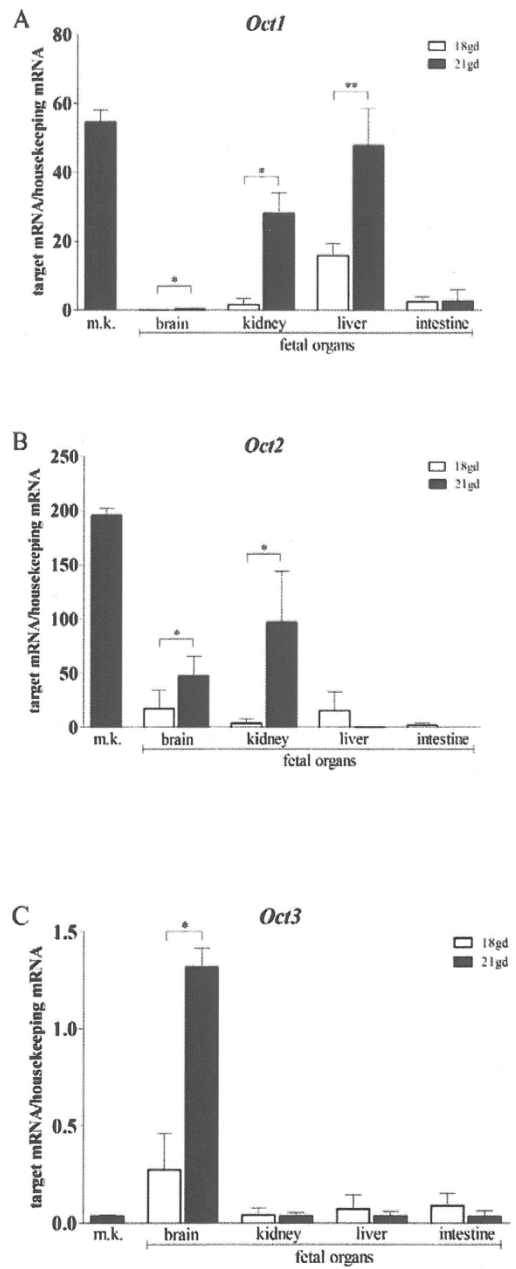

FIG. 2. Quantitative RT-PCR analyses of *Oct1* (**A**), *Oct2* (**B**), and *Oct3* (**C**) mRNA expression in the fetal organs on gestation days (gds) 18 and 21 and in maternal kidney (m.k.). The mRNA data normalized to those of *Gapdh* and *Ywhaz* are expressed as a target mRNA/housekeeping mRNA. Data represent means ± SD (n = 4 in each group); *P < 0.05, **P < 0.01.

Western Blot Analysis of OCT3 and MATE1 Transporters in the Rat Placenta and Fetal Tissues at Different Stages of Gestation

Protein quantification was performed in homogenates of the maternal kidney, placenta, fetal brain, and intestine (Figs. 5 and 6). The relative amount of OCT3 and MATE1 protein was expressed as a percentage of the maternal kidney values. The OCT3 and MATE1 protein expression in the placenta showed

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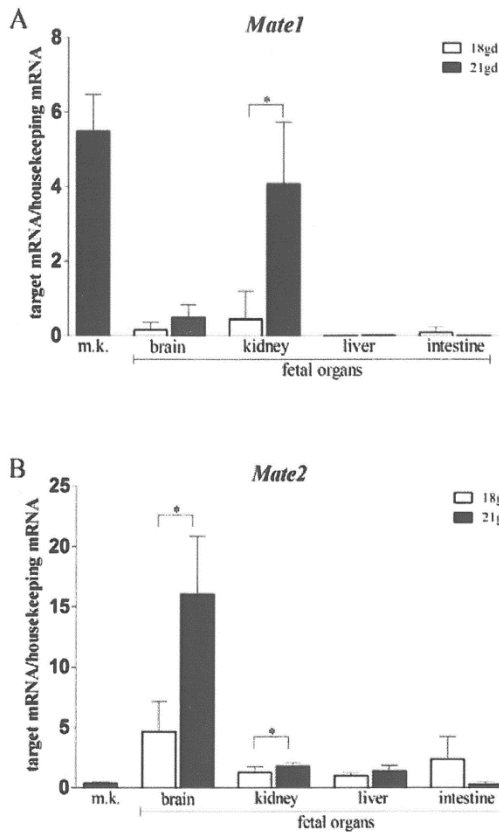FIG. 3. Quantitative RT-PCR analyses of Mate1 (A) and Mate2 (B) mRNA expression in the fetal organs on gestation days (gds) 18 and 21 and in maternal kidney (m.k.). The mRNA data normalized to those of Gapdh and Ywhaz are expressed as a target mRNA/housekeeping mRNA. Data represent means ± SD (n = 4 in each group); *P < 0.05.

FIG. 4. Quantitative RT-PCR analysis of OCT1, OCT2, OCT3, MATE1, and MATE2 mRNA expression in the first trimester and term human placenta. The mRNA expression data are normalized to those of B2M, HPRT, TBP, and 18S rRNA and are presented as a target mRNA/housekeeping mRNA. Data represent mean ± SD (n = 7 for first trimester placentas and n = 10 for term placentas). *P < 0.05, **P < 0.01; statistical difference between mRNA expression in the first trimester and term placenta. ND, not detected.

an increasing trend toward the end of gestation, reaching 1.88- and 1.63-fold higher levels on gd 21 as compared to gd 12, respectively (Fig. 5, A and B). An almost 1.4-fold increase in OCT3 protein expression in the fetal brain was observed from gd 18 to 21 (Fig. 6A).

Immunohistochemical Localization of OCT3 and MATE1 in the Rat Placenta at Different Stages of Gestation

The expression patterns of OCT3 and MATE1 in the rat placenta as investigated by immunohistochemistry at the light microscopy level are shown in Figures 7 and 8. On gd 12, MATE1 and OCT3 staining patterns were similar; the staining was detected in the cytoplasm of giant cells and in trophoblast cells in the area of the forming labyrinth area. A similar pattern was also detected on gd 15; OCT3 and MATE1 positive responses were shown in the cytoplasm of giant cells and in trophoblast cells in the area of the forming labyrinth zone but not in fetal capillaries (Figs. 7 and 8). On gd 18, OCT3 and MATE1 staining was very weak in giant cells because giant cells disappear from the placenta during gds 17–19. A strong positive OCT3 response was detected in the labyrinth area (Fig. 7). Staining was predominantly visible in layers II and III of syncytiotrophoblast but was not visible in layer I (Fig. 7). MATE1 staining was also visible in syncytiotrophoblast but it was not possible to differentiate the exact layer in which it was expressed (Fig. 8). No OCT3 and MATE1 positive responses
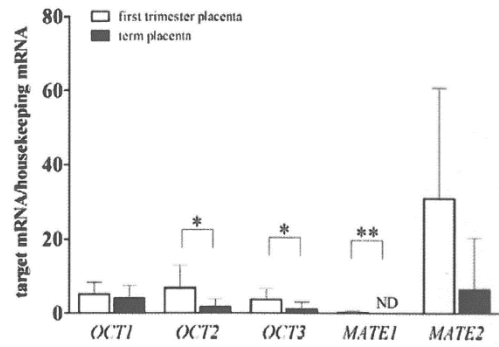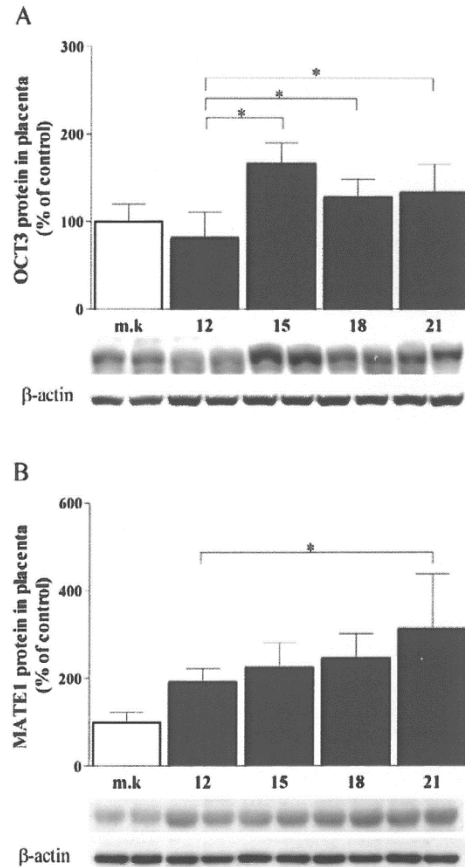

FIG. 5. Protein expression of OCT3 (A) and MATE1 (B) in the rat placenta at different stages of gestation. Protein was isolated from the placenta on gestation days (gds) 12, 15, 18, and 21. Protein data after densitometric analysis are related to maternal kidney (m.k.) (control). Data are expressed as means ± SD (n = 4 in each group). Equal loading of protein was confirmed by β-actin; *P < 0.05. In the Western blot images, the top line shows densitometric analysis (maternal kidney = 100%), and the bottom line shows a representative blot of the reference protein.

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FIG. 6. Protein expression of OCT3 (**A**) and MATE1 (**B**) in the fetal brain and intestine on gestation days (gds) 18 and 21. Protein was isolated from the rat fetal brain and intestine. Protein data after densitometric analysis are related to maternal kidney (m.k.) (control). Data are expressed as means ± SD (n = 4 in each group). Equal loading of protein was confirmed by β-actin. In the Western blot images, the top line shows densitometric analysis (maternal kidney = 100%), and the bottom line shows a representative blot of the reference protein.
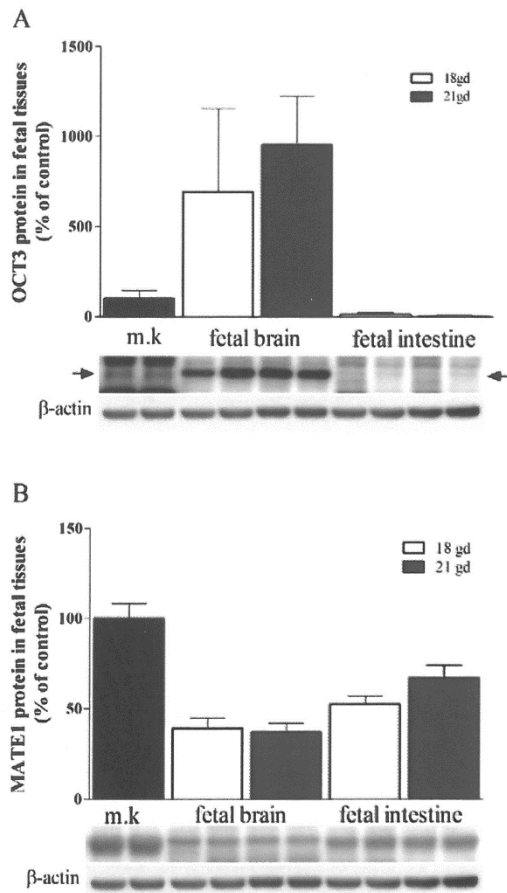
were detected in fetal capillaries (Figs. 7 and 8). On gd 21, the OCT3 response was almost exclusively detected in syncytio-trophoblast of the labyrinth area, namely in layer II and III of trophoblast cells (Fig. 7). MATE1 staining was also visible in syncytiotrophoblast, however, their precise localization with respect to the layers was not possible (Fig. 8). No MATE1 and OCT3 staining was detected in fetal capillaries in the labyrinth zone and spongiotrophoblast (Figs. 7 and 8).

Transport of MPP$^+$ Across the Rat Placenta and Its Distribution in Fetal Tissues at Different Stages of Gestation

MPP$^+$ was infused intravenously into pregnant rats at gd 12, 15, 18, or 21; its concentrations at steady state, as determined by maternal blood sampling, were measured in fetal tissues and compared to those in the maternal plasma. The highest amount of MPP$^+$ crossed the placenta and reached the fetus on gd 12; after that, a significant drop in fetal exposure to maternal MPP$^+$ was recorded. The amount of MPP$^+$ reaching the fetus on gds 15, 18, and 21 was approximately 10-fold lower than on gd 12 (Fig. 9), suggesting greater protection of the fetus against

maternal MPP$^+$ beyond gd 12. In the fetal tissues, MPP$^+$ was detected in all the organs tested, that is, the kidney, liver, intestine, and brain.

DISCUSSION

Transporter proteins in the placenta are involved in the regulation of fetus exposure to xenobiotics by selectively transporting toxic substrates between maternal and fetal circulations. Many ABC and SLC transporters have been found to be functionally expressed in the placenta [1]; it is thus obvious that detailed understanding of the role of these transporters in transplacental pharmacokinetics is crucial for the assessment of the risk of fetal drug exposure and toxicity. To date, SLC transporters have mainly been investigated in the excretory organs, such as kidney and liver [5, 23], but relatively little attention has been paid to their expression and function in the placenta. In our previous report, we described the synchronized activity of two SLC transporters, OCT3 and MATE1, in the passage of MPP$^+$ across the rat term placenta [8]. Since changes in transporter expression during pregnancy are of great importance, the objectives of this follow-up study were to quantitatively determine the expression, localization, and function of OCT and MATE isoforms in the placenta and fetal tissues at different stages of gestation.

OCTs are facilitative diffusion systems that transport organic cations in a bidirectional manner across the plasma membrane [3]. Of all the isoforms, OCT3 (also known as the extraneuronal monoamine transporter and encoded by the *SLC22A3* gene) shows very high expression in the placenta of all the species tested [8, 9, 24–27], suggesting that OCT3 is a placenta-specific OCT. It was found to be localized to the basolateral, fetus-facing, membrane of the trophoblasts in the human [26] and rat [8] placenta. Based on RT-PCR quantification, we confirm in this study the abundant expression of *OCT3/Oct3* in both the human and rat placenta. However, when investigating changes in placental expression of *OCT3/Oct3* mRNA at different stages of gestation, opposite trends were observed in the human and rat. In the human placenta, *OCT3* expression declined from first trimester to term; on the other hand, in the rat placenta, *Oct3* expression showed an increasing trend toward the end of gestation. Similar interspecies differences in the profiles of transporter expression throughout pregnancy have previously been observed for other placental transporters, for example, P-glycoprotein [28] or BCRP [29]. This issue is still controversial, however, as many research groups reach different conclusions. It is obvious from our data that SLC transporter expression follows different regulation schemes in rat and human placentas, suggesting different fetal protective mechanisms between species. The regulation steps are not known to date; what is clear, however, is that these differences should be borne in mind when extrapolating animal data to human conditions.

MATE transporters have been discovered recently and have been described as H$^+$/organic cation antiporters [7]. Despite belonging to the SLC family of transporters, they function as efflux proteins and often couple with OCTs in excretory organs, such as kidney and liver. To date, placental expression of *Mate1* has been revealed only in the rat [8, 30]; negligible or zero expression of *Mate1/MATE1* mRNA was detected in the murine [31, 32] or human term placenta [7], raising the question as to the mechanism of transplacental transport of organic cations in these species (see, e.g., [30, 33]). We believe a common consensus on MATE1 expression and function in the human placenta is yet to be reached. While our data are consistent with Otsuka and colleagues' [7] findings with
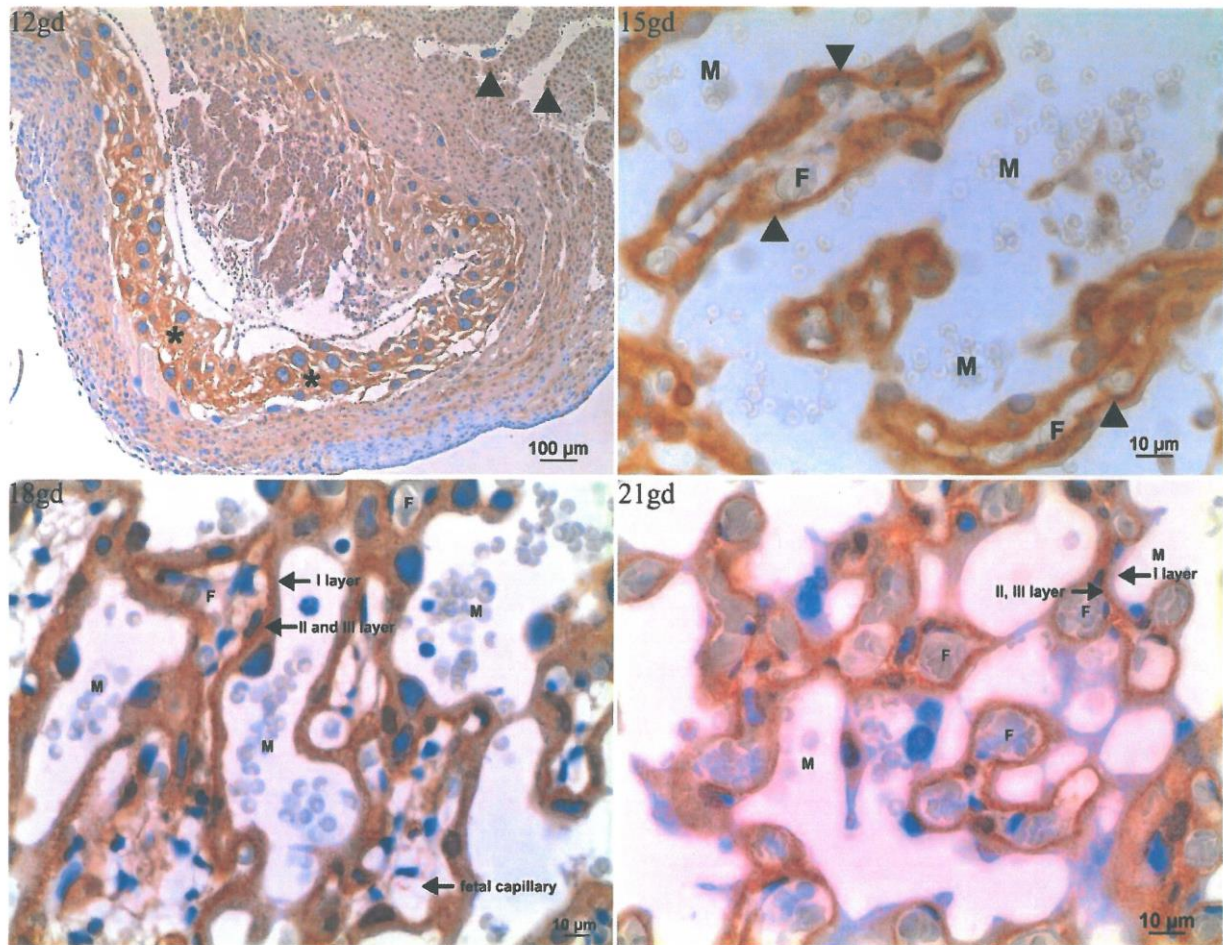
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FIG. 7. Representative microphotographs of OCT3 staining during placental development. The expression of OCT3 was detected in giant cells (asterisks) and trophoblast cells (arrowheads) on gestation day (gd) 12. On gd 15, OCT3 staining was detected in trophoblast cells (arrowheads) but not in fetal capillaries in the forming labyrinth zone. Similar staining patterns were visible on the preterm gd 18 and term gd 21. OCT3 expression was detected only in layer II and III of syncytiotrophoblast in the labyrinth zone (arrows). No staining was visible either in layer I of syncytiotrophoblast or fetal capillaries. M, maternal blood space; F, fetal blood space. The slides were counterstained with hematoxylin. Bars = 100 μm (12 gd) and 10 μm (15, 18, and 21 gds).

respect to negligible expression of *MATE1* in the term placentas, we analyzed also immature placentas, observing *MATE1* expression in the first trimester placentas and *MATE2* expression in the first and third trimester placentas. We, therefore, speculate that vectorial transport of organic cations across the placenta recently described in rat [8] may be mediated by a OCT3-MATE1/2 pathway also in human. However, further studies are required to elucidate this issue in detail.

Protein analyses by Western blots and immunohistochemical visualization were in a reasonable agreement with the gene expression studies. OCT3 and MATE1 protein expression in the rat placenta on different gds were similar to those of *Oct3* and *Mate1* mRNA, but the differences were still statistically significant, confirming an increase in OCT3 and MATE1 expression toward the end of gestation. Consistent with our previous study [8], immunohistochemical visualization on gd 21 showed OCT3 expression preferentially localized to the fetal side and MATE1 to the maternal side of the trophoblast cells but not in fetal capillaries. During earlier stages of pregnancy (gds 12 and 15), we detected OCT3 and MATE1

expression in the giant cells and trophoblast cells in the area of the forming placental labyrinth. As pregnancy proceeds, giant cells disappear from the placenta, and the staining on gds 18 and 21 was detected only in the syncytiotrophoblast in the labyrinth zone. In human placenta, the techniques for proteomic analyses were not available and protein expression/function data need to be elucidated in further studies; nevertheless, because mRNA expression is often an excellent proxy for the presence of a protein [34], it is reasonable to assume the expression and function of OCT and MATE transporters on the materno-fetal interface of the human placenta.

MPP^+ is a model toxin and a well-established substrate of OCT3 [26, 27] and MATE1 [35]. Furthermore, it is not subject to metabolic degradation [36], which makes it a suitable model compound for functional analysis of OCT and MATE transporters. In our previous study, using dually perfused rat term placenta, we described OCT3-MATE1-mediated transport of MPP^+ in the fetal-to-maternal direction even against a concentration gradient [8]. We concluded that placental OCT3-MATE1 form an efficient excretory pathway for cations,
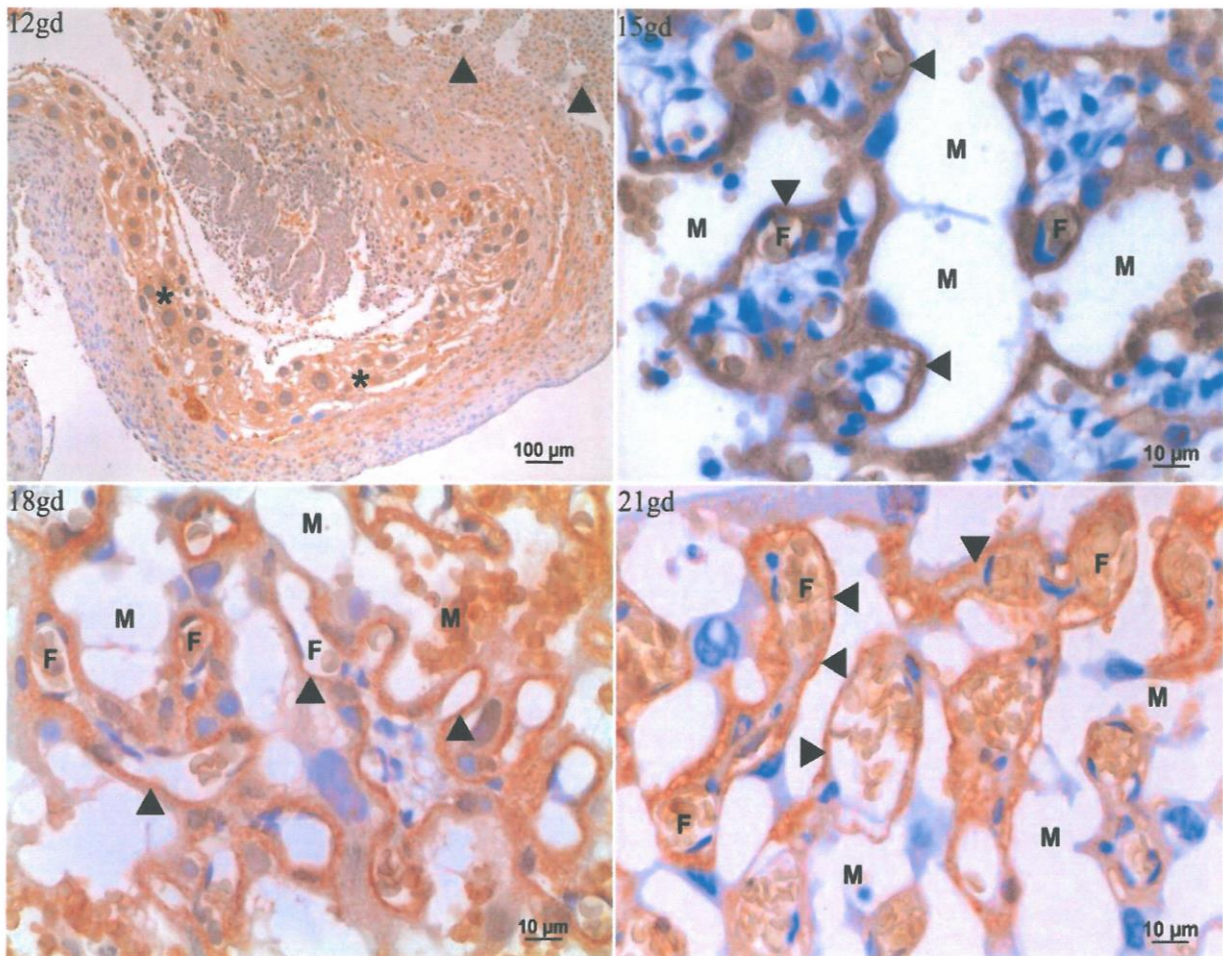
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FIG. 8. Representative microphotographs of MATE1 staining during placental development. MATE1 positivity was detected in giant cells (asterisks) and trophoblast cells (arrowheads) on gestation day (gd) 12. On gd 15, MATE1 staining was detected in trophoblast cells (arrowheads) but not in fetal capillaries in the forming labyrinth zone. Similar staining patterns for MATE1 were visible on gds 18 and 21. MATE1 expression was visible in the syncytiotrophoblast in the labyrinth zone (arrowheads); however, precise localization with respect to layers was not possible. No staining was visible in fetal capillaries. M, maternal blood space; F, fetal blood space. The slides were counterstained with hematoxylin. Bars = 100 μm (12 gd) and 10 μm (15, 18, and 21 gds).

similar to the kidney or liver, and play an important role in fetal protection and detoxification. However, the drawback of experiments using perfused placenta is that they can be performed only on the last day of gestation. To extend our knowledge beyond the final phase of pregnancy and to investigate the protective role of placental OCT3 and MATE1 also at earlier gestational stages, in this paper, we infused MPP$^+$ intravenously into mothers at different stages of gestation and measured at the steady state the MPP$^+$ concentration in the fetal tissues. We observed significant leakage of MPP$^+$ into fetal tissues only in the midgestational stage, that is, on gd 12. From 15 gd onward, relatively low levels of MPP$^+$ were detected in the fetuses (Fig. 9). These findings indicate that the fetus is more vulnerable to cationic compounds in maternal circulation in the first half of pregnancy. As pregnancy proceeds, the protective role of the placenta against organic cations increases; we believe this is due to maturation of both active and passive components of the placenta, that is, higher expression of placental OCT3/MATE1 transporters as observed in this study, as well as increasing compactness of the placental tissues.

Once in the fetal compartment, the fate of MPP$^+$ or other toxic cations may be partly controlled by the expression of SLC transporters in the fetal tissues. Therefore, in this study, we investigated the expression of OCT and MATE transporters in the developing fetus. Near term, significant levels of Oct and Mate isoforms were detected in the fetal excretory organs with similar patterns to those described in adults, that is, Oct1 was detected mainly in the kidney and liver while Oct2 and Mate1 were detected predominantly in the kidney. These observations suggest some capacity of the fetus to eliminate organic cations through its kidney and liver. However, in addition to excretory organs, Oct2, Oct3, and Mate2 showed significant expression in the fetal brain. These findings, on the other hand, indicate the possibility of MPP$^+$ crossing the blood-brain barrier, which was confirmed in our study by accumulation of maternally administered MPP$^+$ in the fetal brain. In addition, central nervous system toxicity (Parkinson's disease) after administration of MPP$^+$ or its precursor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine to pregnant dams has previously been reported in rats [37] and mice [38, 39].
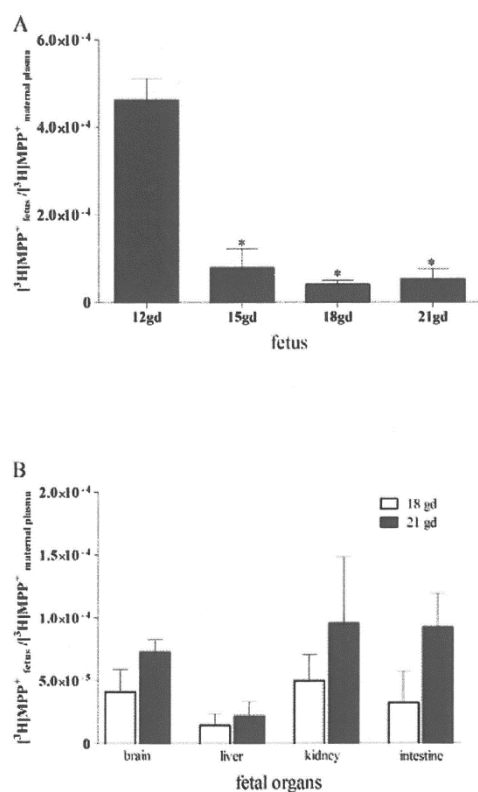
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FIG. 9. Exposure of fetus and fetal organs to MPP$^+$ during pregnancy. MPP$^+$ with [^{3}H] MPP$^+$ tracer were infused intravenously to pregnant rats on gestation days (gds) 12, 15, 18, and 21. One hour after the beginning of the infusion, radioactivity in fetus and fetal organs and maternal plasma was measured. Bars indicate means with their SDs (n = 4). *$P < 0.05$, statistically different from gd 12.

In conclusion, it is apparent that throughout gestation both the placenta and fetus express OCT and MATE transporters in a dynamic manner. In the rat placenta and fetal tissues, expression of OCT3 and MATE1 tends to increase toward the end of gestation. This fact, along with the general maturation of the placental tissue results in significantly lower transport of MPP$^+$ across the placenta from mother to fetus after gd 12. In contrast, human placenta shows decreasing expression of *OCT2, OCT3,* and *MATE1* mRNA, suggesting these transporters may play a role in fetal protection preferentially at earlier stages of gestation.

ACKNOWLEDGMENT

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III. Transfer of Metformin across the Rat Placenta is Mediated by Organic Cation Transporter 3 (OCT3/SLC22A3) and Multidrug and Toxin Extrusion 1 (MATE1/SLC47A1) Protein

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Transfer of Metformin across the Rat Placenta is Mediated by Organic Cation Transporter 3 (OCT3/SLC22A3) and Multidrug and Toxin Extrusion 1 (MATE1/SLC47A1) Protein

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Keywords: Metformin; Organic cation transporter 3; Multidrug and toxin extrusion 1; Placenta; Pregnancy; Gestational diabetes mellitus; Pharmacokinetics

Abbreviations

GDM, Gestational diabetes mellitus; MATE, multidrug and toxin extrusion proteins; MPP^+, 1-methyl-4-phenylpyridinium iodide; OCT, organic cation transporter; SLC, Solute carrier.

Abstract

In our previous studies we described functional expression of organic cation transporter 3 (OCT3) and multidrug and toxin extrusion 1 (MATE1) protein in the rat placenta. Since metformin is a substrate of both OCT3 and MATE1, in this study we used the model of dually perfused rat placenta to investigate the role of these transporters in metformin passage across the placenta. We observed concentration-dependent transplacental clearance of metformin in both maternal-to-fetal and fetal-to-maternal directions; in addition metformin crossed the placenta from the fetal to maternal compartment even against its concentration gradient. This transport was completely inhibited by MPP^+, a common OCT3 and MATE1 inhibitor. Furthermore, we observed that the oppositely directed H^+-gradient can drive the secretion of metformin from placenta to maternal circulation, confirming apical efflux of metformin from trophoblast by MATE1. In conclusion, we suggest an important role of OCT3 and MATE1 in the transplacental transfer of metformin.

1. Introduction

Gestational diabetes mellitus (GDM) is a complication that develops in about 5% of pregnant women [1] and has been associated with a risk of long-term diabetes in both the mother and offspring [2]. If diet and exercise fail to maintain normoglycemia, insulin is the first choice to treat GDM; however, the use of this peptide hormone presents several limitations, such as immune response, hypoglycemia or weight gain. In addition, pain and discomfort associated with insulin administration may result in non-compliance [3]. Low-molecular oral hypoglycemic agents are, therefore, searched for as a "more comfortable" alternative and, indeed, recent evidence based on trials and meta-analyses show that GDM can be safely and effectively treated with oral hypoglycemic agents such as glyburide or metformin [4-7]. For example, in a recent randomized controlled open trial of 751 women with gestational diabetes, Rowan et al. [5] evaluated the efficiency and safety of insulin versus metformin treatment; the authors reported no significant differences between the metformin and insulin groups. In addition, the women preferred metformin treatment to insulin. Apart from GDM, metformin is frequently used in pregnant women with polycystic ovary syndrome [8-10] and new indications are still emerging [11]. Metformin has for long been considered to be non-teratogenic [12, 13]; however, the recommendation for its use in pregnancy was introduced without proper knowledge of its transplacental passage.

Recently, several research groups have investigated the transport of metformin across the human placenta [14-17]; the studies were performed within five years and used identical

model of dually perfused ex vivo placental cotyledon. Yet, the results are inconclusive and often contradictory: while some authors suggested metformin easily crosses the placenta from mother to fetus by simple passive diffusion [16], others speculated, but did not confirm, involvement of an organic cation transporter (OCT) such as OCT2 or OCT3 [14, 15, 17]. However, these papers often lack dose-dependent studies to reveal non-linear pharmacokinetics in both feto-maternal and materno-fetal directions. In addition, none of these reports considered a second transporter for vectorial passage of metformin across the placenta which is a necessity for the transport of cations across trophoblast [18]. While OCTs can influx organic cations into the trophoblast cell across the basolateral membrane from fetal circulation, another transporter, typically multidrug and toxin extrusion 1 (MATE1) protein is responsible for cation efflux across the apical membrane into the maternal circulation [18]. The exact mechanism(s) of the transplacental transfer of metformin thus remains to be elucidated.

We can search for a hint in the excretory organs, such as the kidney, in which vectorial transport of organic cations, including metformin, is mediated by the cooperating action of OCT [19, 20] and MATE [21-23] transporters. While OCTs facilitate the first step of cation excretion, i.e. uptake of organic cation by proximal tubules, MATEs are responsible for their secretion across the brush-border membrane [24]. Analogously, we have recently reported on the expression and localization of OCT3 (SLC22A3) and MATE1 (SLC47A1) transporters in the rat placenta concluding that OCT3 is preferentially located on the basolateral (fetus-facing) side of the placenta, whereas MATE1 is mainly found on the apical (mother-facing) side of the placenta [25]. We further demonstrated functional activity of these placental transporters confirming that OCT3 takes up organic cations from the fetal circulation into the placenta and MATE1 pumps them out from trophoblast to the maternal circulation, even against a concentration gradient. We, therefore, proposed that OCT3 and MATE1 form an efficient transplacental eliminatory pathway for organic cations and play an important role in fetal protection and detoxication [25].

Since metformin is a recognized substrate of both OCT3 and MATE1 [26, 27], in this study we hypothesized these transporters may affect placental passage of metformin. Using the model of dually perfused rat term placenta we investigated concentration-dependent transport of metformin in both materno-fetal and feto-maternal directions. We also determined the effect of OCT/MATE inhibitor as well as proton concentration on transplacental pharmacokinetics of metformin.

2. Materials and Methods

2.1. Chemicals

1-methyl-4-phenylpyridinium iodide (MPP^+) and metformin hydrochloride were obtained from Sigma-Aldrich (St Louis, MO). The radiolabeled [14C]metformin hydrochloride was purchased from Moravek Biochemicals (Brea, CA). All other chemicals were of analytical grade.

2.2. Animals

All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were carried out in agreement with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986). This study was performed on pregnant Wistar rats obtained from Biotest Ltd (Konárovice, Czech Republic), and kept under standard laboratory condition (in 12-/12-h day/night and with water and pellets ad libitum). Gestation day (gd) 0 was established upon detection of copulatory plug of sperm after overnight mating. Experiments were carried out on the 21st gestation day. Fasted rats were anesthetized with 40 mg/kg of pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL) administered into the tail vein.

2.3. Dual perfusion of the rat placenta

In this study, the dually perfused rat term placenta method was employed as described previously [28]. In brief, one uterine horn was excised and submerged in heated Ringer's saline. The uterine artery proximal to the blood vessel supplying a selected placenta was catheterized and connected with the peristaltic pump. Krebs perfusion liquid containing 1% dextran was brought from the maternal reservoir at a rate of 1 ml/min. The uterine vein, including the anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighboring fetuses by ligatures. Using 24-gauge catheter the umbilical artery was catheterized and connected to the fetal reservoir and perfused at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner, and the selected fetus was removed. After successful umbilical catheterization and before the start of each experiment, the fetal vein effluent was collected into preweighed glass vial to check for a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was terminated. Maternal and fetal perfusion pressures were maintained at levels close to physiological values and monitored continuously throughout the perfusion experiments; pH values in the perfusion

reservoirs were maintained by controlled oxygenation with a mixture of 5% CO2/95% O2 as described previously [29]. At the end of experiment, the placenta was perfused with radioactivity-free buffer for 10 minutes, excised from the uterine tissue, dissolved in tissue solubilizer (Solvable; Perkin Elmer Life and analytical Sciences), and its radioactivity was measured to detect tissue-bound metformin (Tri-Carb 2900TR; Perkin Elmer).

In this study, two types of perfusion system were used [30]:

1. The open-circuit perfusion system was employed for pharmacokinetic analysis of concentration-dependent transplacental passage of metformin at both fetal and maternal sides of the placenta. This method was used to study maternal-to-fetal and fetal-to-maternal clearances of metformin at various concentrations. In this experimental setup, metformin with a trace amount of [14C]metformin was added to either maternal (maternal-to-fetal studies) or fetal (fetal-to-maternal studies) reservoir immediately after successful surgery. The sample collection was initiated after a 5 minute stabilization period. Fetal effluent was sampled into preweighed vials in 5 minute intervals; radioactivity was measured, and transplacental clearance was calculated.

2. The closed-circuit (recirculation) perfusion system was used to explore the potential of placenta to remove metformin from the fetal circulation. In recirculation perfusion system, after successful catheterization and 5 minute stabilization, both the maternal and fetal sides of the placenta were infused with equal concentrations of metformin and the fetal perfusate (10 ml) was recirculated for 60 min. Samples (250 μl) were collected every 10 min from both the maternal and fetal reservoirs, and metformin concentration was measured. This experimental setup ensures steady metformin concentration on the maternal side of the placenta and enables investigations of maternal/fetal concentration ratio at equilibrium; any net transfer of the substrate implies transport against a concentration gradient and is evidence for active transport.

2.4. Effect of metformin concentration on transplacental clearance

The open-circuit perfusion system was employed to investigate the effect of various metformin concentrations on maternal-to-fetal and fetal-to-maternal clearances. Metformin with a trace amount of [14C]metformin was added to the maternal or fetal reservoir in the following concentrations: 0.1μM, 1mM, or 10mM. The inflowing metformin concentration was maintained constant for the duration of the experiment; transplacental clearances of metformin were calculated as described below.

In closed-circuit system, both maternal and fetal sides of the placenta were perfused with metformin in the same concentration (0.1 μM or 10 mM) and changes in metformin concentrations in the fetal reservoir were monitored.

2.5. Effect of 1-methyl-4-phenylpyridinium iodide (MPP⁺) on transplacental passage of metformin

In closed-circuit system, both maternal and fetal sides of the placenta were perfused with metformin in the same concentration of 0.1 μM. After a 5 min stabilization period, MPP⁺ (1000μM) was added to both maternal and fetal reservoirs and changes in metformin concentrations in the fetal reservoir were monitored.

2.6. Effect of pH on transplacental passage of metformin

To study the effect of proton concentration, and thus the effect of MATE, on fetomaternal transport of metformin, both maternal and fetal sides of the placenta were perfused with low, nonsaturating concentration (0.1μM) of metformin in the closed-circuit perfusion system. The pH in the maternal reservoir was adjusted to 6.5, 7.4, or 8.5; in the fetal reservoir, pH 7.4 was used in all experiments as described previously [25].

2.7. Pharmacokinetic analysis of transport activity in the placenta

In the open-circuit perfusion system, the organ clearance concept was applied to mathematically describe maternal-to-fetal and fetal-to-maternal transport of metformin [30]. Average data from the intervals of 20 to 35 min of placenta perfusion were used for the following calculations. Maternal-to-fetal transplacental clearance (Cl_{mf}) was calculated according to Equation 1.

$$Cl_{mf} = \frac{C_{fv} \cdot Q_f}{C_{ma} \cdot W_p} \qquad (1),$$

where C_{fv} is drug concentration in the umbilical vein effluent, Q_f is the umbilical flow rate, Cma is drug concentration in the maternal reservoir, and W_p is the wet weight of the placenta. Fetal-to-maternal transplacental clearance (Cl_{fm}) was calculated according to Equation 2.

$$Cl_{fm} = \frac{(C_{fa} - C_{fv}) \cdot Q_f}{C_{fa} \cdot W_p} \qquad (2),$$

where C_{fa} is drug concentration in the fetal reservoir entering the perfused placenta via the umbilical artery.

2.8. Statistical analysis

For each group of placental perfusion experiments, the number of animals was $n \geq 3$. Statistical significance was examined by unpaired Student's t-test or one-way ANOVA followed by Bonferroni's test using Graphpad Prism 5.0 software (Graphpad Software, Inc., San Diego, CA). A difference of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of metformin concentrations on transplacental transport

The maternal or fetal side of the placenta was infused with various concentration of metformin (0.1μM, 1mM, or 10mM) with a trace amount of [^{14}C]metformin. In both maternal-to-fetal and fetal-to-maternal transport studies, an increase in substrate concentration resulted in significant changes in transplacental clearance, confirming the involvement of a capacity-limited transport mechanism (Figs. 1A and B). When comparing the fetal-to-maternal and maternal-to-fetal clearances, we observed significant asymmetry in favor of the fetal-to-maternal direction (Figs. 2A and B). This asymmetry was most pronounced at low metformin concentration (0.1μM), where fetal-to-maternal clearance was almost 7.3 times higher than that in the opposite direction (Fig. 2A). On the other hand, at high metformin concentration (10mM), fetal-to-maternal and maternal-to-fetal clearances reached almost identical values, and the asymmetry was annulled (Fig. 2B), confirming saturation of the transport proteins and limited role of their transport activities.
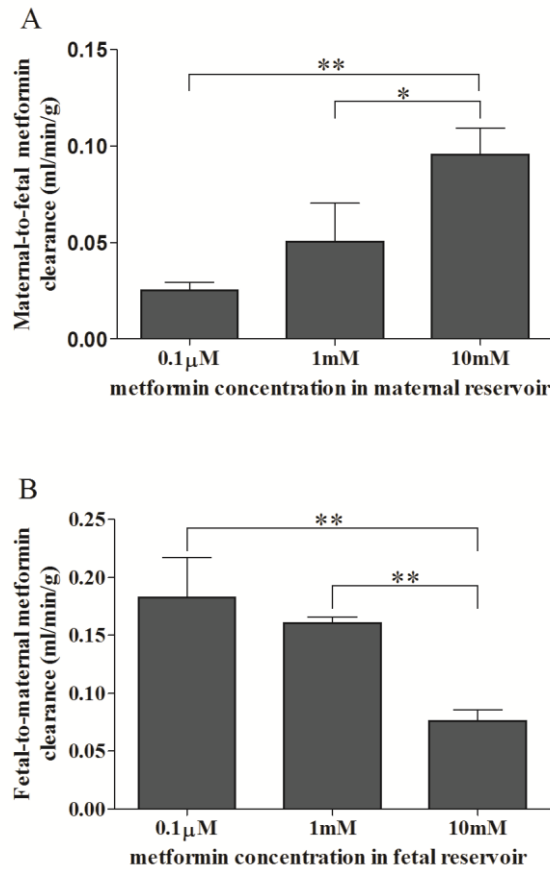
Fig. 1. Concentration-dependent transport of metformin across the dually perfused rat term placenta in the (A) maternal-to-fetal and (B) fetal-to-maternal directions. Metformin with [^{14}C]metformin tracer was added to the maternal (A) or fetal (B) reservoir and its radioactivity was measured in the fetal venous outflow. Changes of clearance with increasing metformin concentration indicate the nonlinearity of the processes and involvement of a saturable mechanism. Total transplacental clearance was calculated by Equation 1 for maternal-to-fetal and by Equation 2 for fetal-to-maternal direction (see Materials and Methods section). Experimental values are presented as means ± SD, n ≥ 3; *p < 0.05, **p < 0.01.
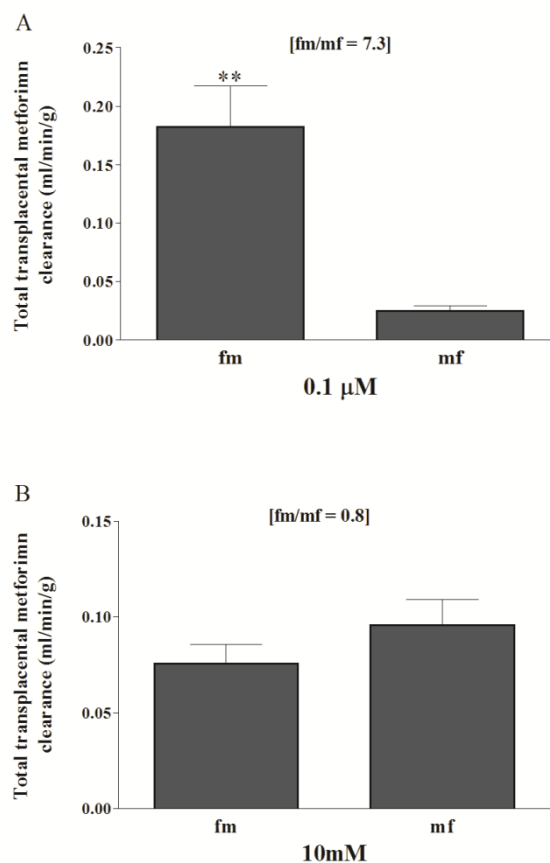
Fig. 2. Ratio of clearances between fetal-to-maternal (fm) and maternal-to-fetal (mf) directions at (A) low, nonsaturating and (B) high, saturating metformin concentrations. Metformin with [^{14}C]metformin tracer was added to the maternal or fetal compartment, and its radioactivity was measured in fetal venous outflow. Total transplacental clearances were calculated by Equations 1 and 2 (see Materials and Methods section). At low substrate concentration (0.1μM), significant asymmetry in transplacental clearance of metformin was observed in favor of fetal-to-maternal direction. At high drug concentration (10mM), this asymmetry was almost annulled, and no differences between fetal-to-maternal and maternal-to-fetal clearances were detected, suggesting saturation of the transport mechanisms. Numbers in brackets show the ratio between fm and mf clearances; data are presented as means ± SD, $n \geq 3$; **$p < 0.01$.

3.2. Inhibitory effect of MPP$^+$ on transplacental passage of metformin

To investigate the potential of OCT3/MATE1 in removing metformin from fetal circulation, metformin was added to both maternal and fetal reservoirs at nonsaturating concentration of 0.1μM in closed-circuit experiment setup. A steady decrease in the metformin concentration in the fetal reservoir was observed followed by concentration equilibration after approximately 40 min of perfusion (Fig. 3A), confirming the ability of the compound to cross the placenta in fetal-to-maternal direction even against the concentration gradient. When MPP$^+$ (1000μM) was added to both maternal and fetal reservoirs, the transport of metformin from fetal to maternal compartment was completely blocked by (Fig. 3B).
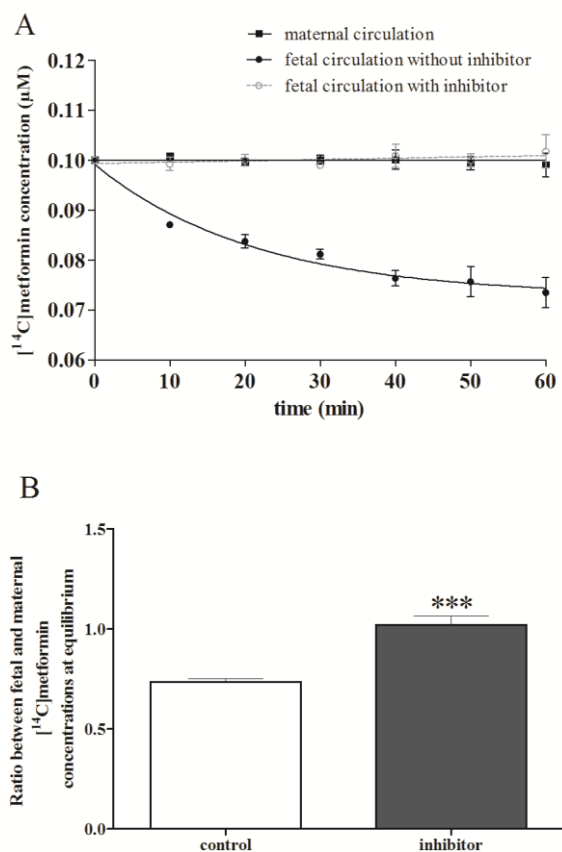
Fig. 3. Effect of OCT3 and MATE1 inhibitor on elimination of metformin from the fetal circulation. In the closed-circuit perfusion setup, [14C]metformin was simultaneously infused to both the maternal and the fetal sides of the placenta at equal concentrations of 0.1μM, and fetal perfusate was recirculated for 60 min; at the end of the perfusion, fetal and maternal metformin concentrations were compared. Without addition of MPP+ as an OCT3/MATE1 inhibitor, fetal [14C] metformin concentrations steadily decreased from 0.1μM to 0.073μM (A). This decrease was fully inhibited by addition of MPP+ (1000μM). (B) Effect of MPP+ on feto-maternal concentration ratio of metformin at the end of experiment (60 min). Data are presented as means ± SD, n ≥ 3; ***$p < 0.001$.

3.3. Effect of pH on transplacental passage of metformin

To investigate the effect of maternal pH on fetal-to-maternal transport of drug, metformin was added to both maternal and fetal reservoirs at nonsaturating concentration of 0.1μM in the closed-circuit experiment setup. The pH in the maternal reservoir was set to 6.5, 7.4, or 8.5, whereas pH 7.4 was used in the fetal reservoir. At pH 7.4 in both maternal and fetal reservoirs, a steady decrease in the metformin concentration in the fetal reservoir was observed followed by concentration equilibration after approximately 40 min of perfusion (Fig. 3A). This decline was considerably affected by adjusting pH on the maternal side of the placenta: lower pH (i.e. higher proton concentration) induced faster metformin transport from

fetus to mother and resulted in significantly lower ratio between fetal and maternal metformin concentrations (Fig. 4). In contrast, at higher pH (i.e. lower proton concentration) on the maternal side, fetal-to-maternal passage of metformin was reduced resulting in significantly higher feto-maternal concentration ratio, thus confirming proton-dependent transport of metformin across the placenta (Fig. 4).
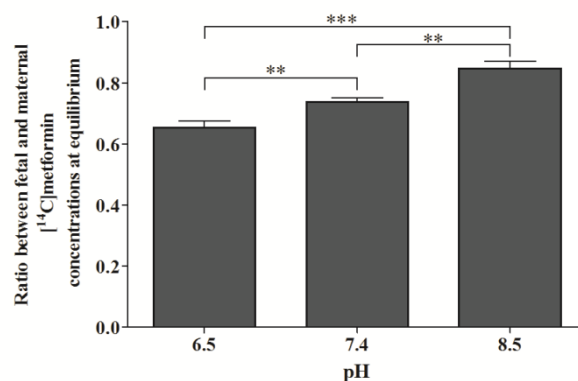

Fig. 4. Effect of maternal pH on elimination of metformin from the fetal circulation. In the closed-circuit perfusion setup, [^{14}C]metformin was simultaneously infused to both the maternal and fetal sides of the placenta at equal concentrations of 0.1μM, and the fetal perfusate was recirculated for 60 min. Fetal pH was set to 7.4, and the pH values in the maternal reservoir were set to 6.5, 7.4, or 8.5. At the end of the experiment (60 min), fetal and maternal metformin concentrations were compared. Higher concentration of protons in the maternal circulation results in higher fetal-to-maternal transport of metformin, indicating the role of a proton-cation antiporter system on the apical side of the placenta. Data represent means ± SD, n ≥ 3; **$p < 0.01$, ***$p < 0.001$.

4. Discussion

Metformin is a polar compound that is positively charged at physiological pH. It exists mainly as a water soluble cation with less than 0.01% unionized in blood. Furthermore, the lipid solubility of the unionized portion is low due to its octanol/water partition coefficient (-1.43) [21]. Therefore, passive diffusion of metformin through biological membranes, including placenta, is rather unlikely as recently demonstrated by Kovo and colleagues using the parallel artificial membrane permeation assay (PAMPA) [15]. To be able to cross biological barriers, metformin must utilize membrane transporter(s); in excretory organs, e.g. kidney, metformin is transported by coordinated activity of influx (OCT2) and efflux (MATE) transporters [23, 24]. In this study, we hypothesized that OCT3 and MATE1 transporters mediate transport of metformin also across the placenta.

In our previous studies, we have described abundant expression and activity of OCT3 and MATE1 transporters in the rat placenta and their role in transplacental passage of organic cations [25]; we have also reported on gestation stage-dependent expression of OCT and MATE isoforms in human and rat placenta [31]. In the present study, we investigated the role

of OCT and MATE transporters in transplacental passage of metformin using the technique of dually perfused rat term placenta, which is a well-established model to investigate placental physiology [32-34], pathophysiology [35] and pharmacology [29, 30, 36]. In open-circuit perfusion setup we observed concentration-dependent clearance of metformin in both maternal-to-fetal and fetal-to-maternal directions; furthermore, at low, non-saturating metformin concentration, transplacental clearance in fetal-to-maternal direction was 7.3 times higher than that in maternal-to-fetal direction. This is comparable with a study by Kovo et al [15] who revealed similar asymmetry in metformin transport using the model of perfused human placenta cotyledon. These data indicate nonlinear pharmacokinetics and contribution of a saturable transport system oriented in fetal-to-maternal direction.

We further confirmed these results using closed-circuit perfusion setup in which both fetal and maternal sides of the placenta were perfused with a non-saturating concentration of metformin ($0.1\mu M$) and the fetal perfusate was recirculated. In the case of compounds that cross the placenta by passive diffusion, such as antipyrine, the concentrations in both fetal and maternal compartments remain unchanged for the duration of the experiment [28]. However, here we observed a steady decrease in fetal metformin concentrations indicating the ability of metformin to cross the rat placenta from fetal to maternal compartment even against its concentration gradient. At high metformin concentration (10mM), the transport system was saturated and this asymmetry was annulled; the saturating concentration is very close to that observed in human placenta by Kovo et al [15] who indicated that metformin transport across the human placenta is nearly saturated at 6mM concentration. Assuming the asymmetry is caused by placental SLC transporters, we further used MPP^+, a molecule which can, at a concentration of $1000\mu M$, fully inhibit placental OCT3/MATE1 pathway [25]. Adding this compound into both maternal and fetal circulations, we observed its significant inhibitory effect on the transplacental passage of metformin indicating the involvement of OCT3/MATE1 vectorial pathway in the passage of metformin from the fetal to maternal circulation.

Recently, three different research groups have investigated the passage of metformin across the human placenta using the model of dually perfused ex vivo placental cotyledon [14-17]; however, the results are unexpectedly variable. Nanovskaya et al [16] concluded that metformin crosses the human placenta from mother to fetus freely by passive diffusion, which is a surprising outcome considering physical-chemical properties of the molecule. Kovo et al [14] and Tertti et al [17] presumed the involvement of OCT3 and/or OCT2 in transplacental passage of metformin but either failed to confirm [14] or refuted the hypothesis [17]. Only in

the second paper by Kovo et al [15] the authors used various metformin concentrations in maternal-to-fetal transport to observe dose-dependent pharmacokinetics; they also detected higher transport from fetus to mother compared with that in the opposite direction and suggested OCT3 and to some extent OCT2 mediate metformin transport from fetus to mother.

Surprisingly, none of the above mentioned reports considered a second transporter for transcellular movement of metformin across the human placenta. It must be stressed out that OCT or any other cationic uptake transporter alone cannot explain the transport of metformin, or any other organic cation, across biological membranes including placenta. In excretory organs such as the liver and kidney, OCTs on the basolateral side have been found to be linked with MATE transporters on the apical side [37]. MATE proteins, members of the SLC family, function as efflux transporters by utilizing an oppositely directed H^+-gradient as a driving force for transport of organic cations across cell membrane [24, 38]. The expression of MATE transporters in the placenta of various species is still somewhat controversial: while no Mate1/MATE1 mRNA was detected in murine placenta [39, 40] and human term placenta [41], we have recently detected Mate1/MATE1 and Mate2/MATE2 expression in the rat placenta [25] as well as human first trimester placenta [31].

Since metformin is a recognized substrate of MATE transporters [22, 26, 27], in this study we aimed to confirm the involvement of MATE1 in elimination of metformin from trophoblast cells to the maternal compartment. As MATE1 is a cation-proton exchanger, we investigated the effect of proton concentrations in the maternal circulation on metformin transport. In our previous study [25], we employed pH values from 6.5 to 8.5 on the maternal side of the placenta to demonstrate that the oppositely directed H^+-gradient can drive the transport of organic cations from placenta to maternal circulation. Similar effect was observed also in the placental transport of metformin in this study; with increasing pH on the maternal side of the placenta, the transport of metformin from fetus to mother decreased significantly, confirming metformin elimination from trophoblast cells by MATE1.

Many endogenous and exogenous compounds have been described as substrates or inhibitors of OCT and MATE transporters [27] and recent studies have suggested drug-drug interactions on both OCT3 and MATE1 [27, 42]. In our experiments, adding another OCT3/MATE1 substrate, MPP^+, resulted in significantly reduced transport of metformin from fetus to mother. Similarly, metformin blocks fetal-to-maternal excretion of other organic cations [25]. We, therefore, presume that concomitant administration of metformin with another drug which is a substrate or inhibitor of OCT3 and/or MATE1 may significantly affect the transplacental disposition of metformin, resulting in unpredictable outcome of

medication during pregnancy or even toxicity to the fetus. In addition, the NCBI-SNP database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/SNP) currently lists over 2000 and over 800 SNPs for OCT3 and MATE1 genes, respectively and several studies have indicated effect of these polymorphisms on metformin pharmacology [43, 44]. It is obvious that genetic variants in these transporters may also account for interindividual variability in transplacental pharmacokinetics of metformin and compromise the placental protective function.

Comparing expression of OCT and MATE transporters between rat and human placenta, we have recently described considerable dissimilarities that must be reflected when extrapolating rodent data to human conditions [31]. In the rat placenta, OCT3 and MATE1 seem to be the predominant isoforms and their expressions increase significantly towards the end of gestation. On the contrary, human placenta expresses full spectrum of organic cation transporters, including OCT1, OCT 2, OCT 3, MATE1, and MATE 2 that may, to a certain extent affect transplacental pharmacokinetics of organic cations such as metformin. In addition, decreasing expression of OCT2, OCT3 and MATE1 mRNA from first to third trimester was observed, suggesting these transporters may play a role in fetal protection preferentially at earlier stages of gestation [31].

In conclusion, based on our findings we suggest that OCT3 takes up metformin from the fetal circulation into the trophoblast and MATE1 is responsible for its efflux into the maternal circulation (schematically depicted in Fig. 5). OCT3/MATE1 in the rat placenta thus provide an excretory pathway for metformin transport from fetus to mother, representing a protective mechanism for the fetus. Translation of our results to human environment must, however, be performed carefully due to different expression patterns of OCT and MATE transporters between rat and human placenta [31]. It can be presumed that the OCT3/MATE1-based protective role of the placenta may be compromised by concurrent administration of other substrates/inhibitors of these transporters. In addition, genetic polymorphisms in OCT and/or MATE transporters can significantly affect their placental expression and/or function leading to interindividual variability in placental disposition of metformin.
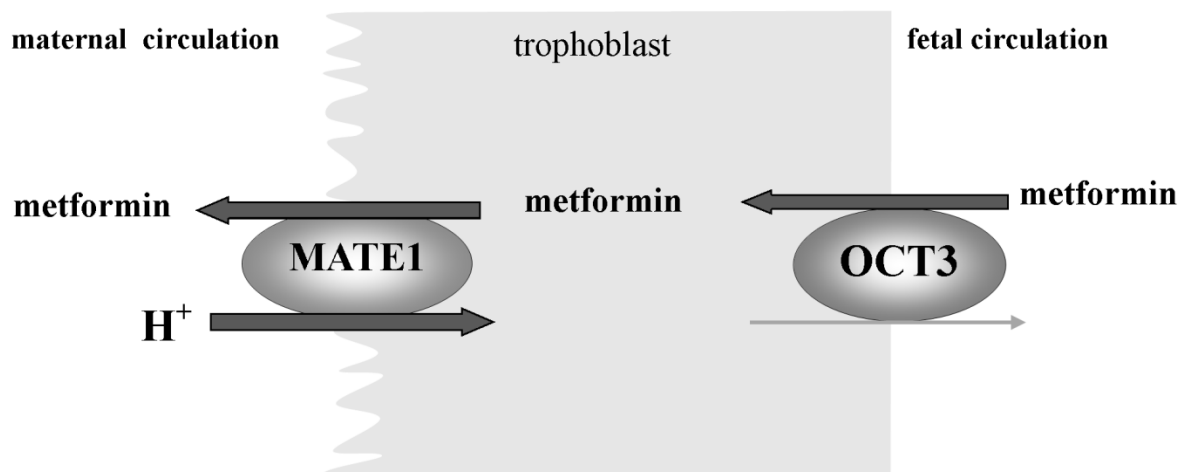
Fig. 5. Schematic depiction of metformin transport across the rat placenta by coordinated activity of OCT3 and MATE1. The uptake (OCT3) and efflux (MATE1) transporters localization and direction in the rat placenta are shown.

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IV. Multidrug and toxin extrusion protein (MATE/SLC47); role in pharmacokinetics

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Multidrug and toxin extrusion proteins (MATE/SLC47); role in pharmacokinetics

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Abstract

Mammal multidrug and toxin extrusion protein 1 (MATE 1) encoded by *SLC47A1* gene was described in 2005 as an efflux transporter that mediates proton-coupled organic cation secretion. Shortly after, other isoforms (MATE2 and MATE2-K, both encoded by *SLC47A2* gene) were identified. In the kidney and liver, MATEs work in concert with organic cation transporters (OCTs), together representing an eliminatory pathway for organic cations. Over 40 clinically used drugs and several endogenous compounds are known substrates or inhibitors of MATEs and the list is constantly growing. These transporters, therefore, play a significant role in pharmacokinetics, toxicokinetics, drug resistance and (patho)physiological processes. Drug-drug interactions on MATE transporters and polymorphisms in *SLC47A* genes have been shown to affect renal excretion of substrate drugs, such as metformin, resulting in inadequate pharmacotherapy or occurrence of toxic effects. Expression and function of MATEs in tissues other than kidney and liver remain to be elucidated.

List of key facts about MATEs:

- recently discovered secondary active efflux transporters
- important for renal and biliary excretion of organic cations
- determine pharmacokinetics and toxicokinetics of various compounds
- over 40 clinically used drugs are substrates/inhibitors of MATEs
- drug-drug interactions and polymorphisms may result in drug toxicity

Keywords: multidrug and toxin extrusion; efflux transporter; drug-drug interactions; pharmacokinetics

1. Introduction

Multidrug and toxin extrusion (MATE) proteins are the long anticipated transporters that are responsible for efflux of organic cations from cells. They were first described in bacteria in 1998 and named NorM and YdhE (Morita et al., 1998). In 2005, the first human orthologue, MATE1, was identified and described as a transporter that mediates the final excretion step for organic cations (Otsuka et al., 2005); shortly thereafter, MATE2, MATE2-K and MATE2-B were discovered. Although MATEs belong to the family of solute carriers (SLC), they function as efflux transporters pumping their substrates out of cells (Aleksunes et al., 2008). The driving force for MATE-mediated transport is provided by oppositely directed proton gradient (Otsuka et al., 2005); MATEs are, therefore, considered secondary active transporters.

The widespread distribution of MATE proteins in various living organisms, including prokaryotes, plants and mammals, and their capacity to transport wide variety of endo-/exogenous substrates indicates the importance of these transporters in physiological and/or pharmacological processes such as drug body disposition and excretion, resistance in bacteria or tumor tissues, and hormone secretion.

Although MATEs are among the "youngest" of drug transporters, their importance has recently been recognized by the International Transporter Consortium that suggested to incorporate MATEs into the existing decision trees for identifying clinically relevant drug-drug interactions (DDIs) (Zamek-Gliszczynski et al., 2012).

2. Structure

The human SLC47A1 and SLC47A2 genes are located in tandem on chromosome 17p11.2, both consisting of 17 exons (ranging from 43 to 1708 bp for SLC47A1 and 619 bp for SLC47A2) and 16 introns spanning 45.18 kb and 38.32 kb, respectively. SLC47A1 encodes MATE1 (NP_060712.2; 570 amino acids) (Figure 1A). SLC47A2 produces two functional isoforms: MATE2 (NP_690872.2, 602 amino acids) and MATE2-K (NP_001093116.1, 566 amino acids) a shorter variant with partial deletion of exon 7 (Figure 1B) (Komatsu et al., 2011). MATE2 and MATE2-K share 94% amino acid similarity and the sequence identity with MATE1 is 48% and 51%, respectively. Structurally, human MATEs are folded into 13 transmembrane helices (TMH). First twelve TMHs are required for proper transport function while the 13th TMH is likely needed for protein turnover (Zhang et al., 2012) (Figure 1A-B).
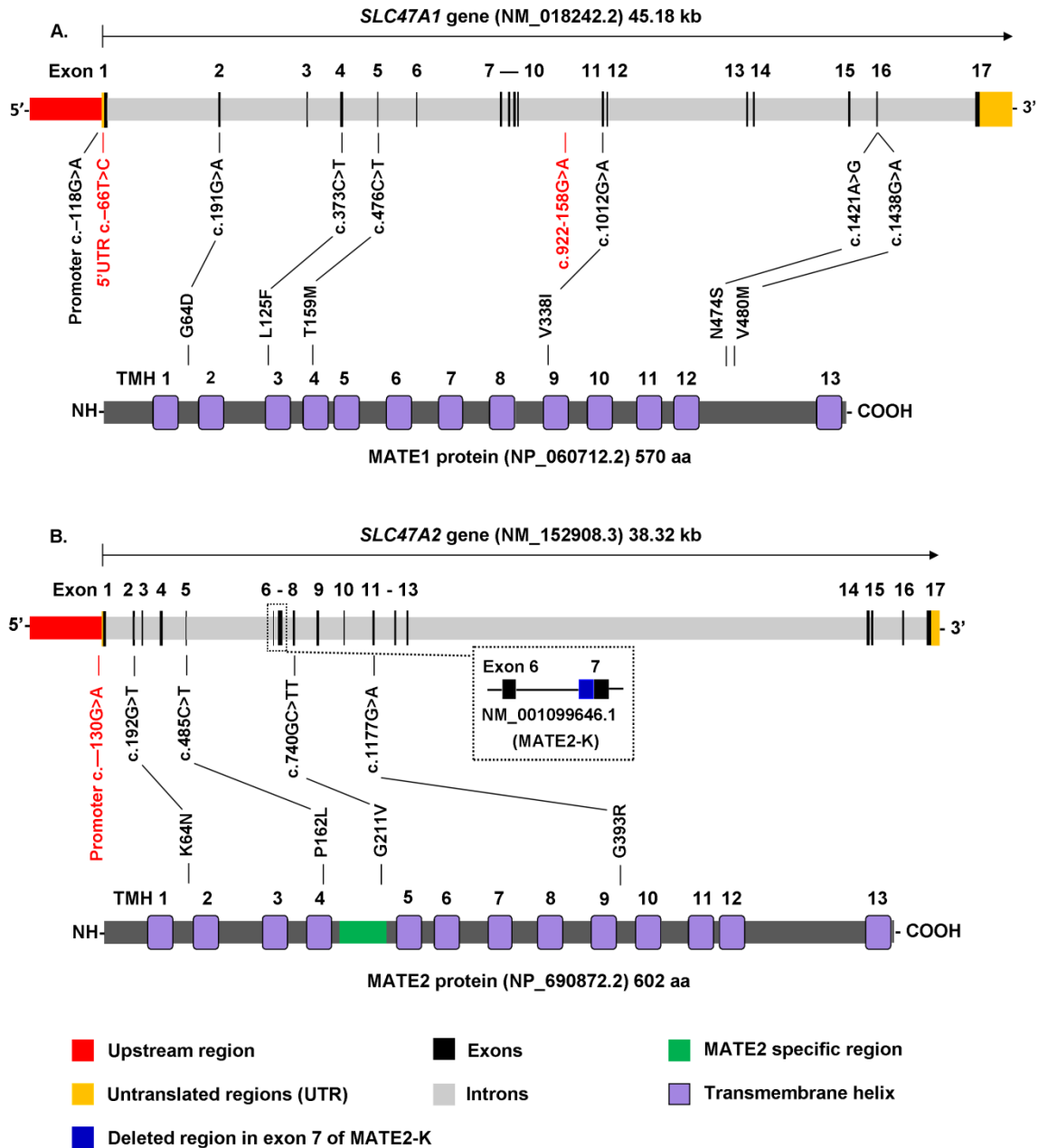
Figure 1. Schematic size-scaled diagram of gene organization and primary protein structure of human *SLC47A1* (MATE1) (A) and *SLC47A2* (MATE2) (B). The figure shows exons (numbered 1 - 17) and introns of both genes and arrangement of transmembrane helices (TMH) (numbered 1 - 13) of encoded proteins. The region of *SLC47A2* containing exons 6 and 7 is enlarged highlighting in blue the deletion in exon 7 of MATE2-K isoform; corresponding protein truncation of MATE2-K (36 amino acid residues) is highlighted in green. Examples of the non-synonymous single nucleotide polymorphisms (SNPs) that affect functioning of MATE transporters are depicted (Damme et al., 2011, Yonezawa and Inui, 2011, Choi et al., 2011). The clinically relevant SNPs discussed in the review (5'-UTR c.-66T>C and intronic c.922-158G>A for *SLC47A1* and c. -130G>A for *SLC47A2*) are indicated in red.

3. Expression and activation

Analysis of the gene promoter revealed that SLC47A1 lacks canonical TATA and CCAAT box. The role of these boxes is substituted by the presence of a region characterized by two GC-rich sites. Specific protein 1 (Sp1) binds the GC-rich sites and recruits TATA-binding proteins to fix the transcription start site (Kajiwara et al., 2007). Other factors, such as AP 1 and AP-2rep, have been shown to regulate transcription of human SLC47A1 (Ha Choi et al., 2009). Investigation of sequence variability of SLC47A2 promoter contributed to identification of myeloid zinc finger 1 (MZF-1) functioning as transcription repressor (Choi et al., 2011). Of nuclear receptors only hepatocyte nuclear factor 4 alpha (HNF4α) has been suggested to regulate Slc47a2 in HNF4α-null mice (Lu et al., 2010). Further studies are needed to investigate involvement of other factors in the transcriptional regulation of MATE transporters.

4. Polymorphism

The NCBI-SNP database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/SNP) currently lists over 980 and over 900 synonymous and non-synonymous single nucleotide polymorphisms (SNPs) for SLC47A1 and SLC47A2 genes, respectively. Of the non-synonymous SNPs identified, frequently occurring alleles c.-66T>C (rs2252281), intronic c.922-158G>A (rs2289669) for SLC47A1 and c.-130G>A (rs12943590) for SLC47A2 have been proposed to have clinical relevancy. SLC47A1 polymorphism c. 66T>C (allelic frequency 23.1-44.5%) and the intronic c.922-158G>A (allelic frequency 43%) are associated with greater glucose-lowering response to treatment with metformin, a prototypical MATE substrate. Role of c. 66T>C can be explained by lower expression of MATE1 variant in the liver leading to increased accumulation of metformin in target cells. Information about glucose-lowering mechanism of intronic c.922-158G>A is lacking. On the other hand, SLC47A2 polymorphism 130G>A (allelic frequency 22-28%) enhances MATE2 expression in the kidney and is related to reduced response to metformin treatment in diabetic patients (Becker et al., 2009, Stocker et al., 2013). Position of the non-synonymous SNPs discussed and other less frequent polymorphisms affecting transport activity are depicted in figure 1.

5. Body distribution; roles in biology, pathology and pharmacology

In their early work, Otsuka et al., (2005) and Hiasa et al., (2006) detected MATE proteins in many mammal tissues and predicted their role in diverse biological functions. In human, MATE1 is highly expressed in the kidney and liver; it is also expressed in other tissues such as adrenal gland, skeletal muscle, testis (Yonezawa and Inui, 2011) and first trimester placenta (Ahmadimoghaddam et al., 2013). MATE2 and MATE2-K are preferentially localized in the kidney (Komatsu et al., 2011); MATE2 has also been detected in the placenta (Ahmadimoghaddam et al., 2013). MATEs have been identified in several mammals, however, with notable interspecies differences. For details on tissue-specific expression of MATEs in various species the reader is referred to comprehensive reviews by Yonezawa and Inui (2011) and Damme et al., (2011). To date, MATE function has been best described in the kidney and liver; their role in other tissues remains to be elucidated.

In the excretory organs, MATEs appear to work in concert with other SLC transporters, typically organic cation transporters (OCT/SLC22A). Together, they represent an eliminatory pathway for an array of structurally diverse molecules including clinically used drugs, toxins, and endogenous metabolites and play a crucial role in pharmacokinetics and toxicokinetics (Figure 2). OCTs are localized to the basolateral membrane of proximal tubules of the kidney and canalicular membrane of hepatocytes where they influx their substrates into the cell; MATEs, on the other hand, are expressed in the apical membrane of the polarized cells pumping their substrates out of the cell thus finalizing the vectorial transport. This arrangement is, however, not unique to the kidney and liver. Recently, we have localized Mate1 in the apical (mother-facing) and Oct3 in basolateral (fetus-facing) membrane of the rat placenta (Ahmadimoghaddam et al., 2012) and functionally confirmed their cooperative activity in the fetus-to-mother transport of MPP+ (Ahmadimoghaddam et al., 2012) and metformin (Ahmadimoghaddam and Staud, 2013).

Importantly, in several tissues, MATEs are co-localized with drug efflux transporters from the ABC (ATP-binding cassette) family, such as P-glycoprotein (MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2) or MRP2 (ABCC2). Since several drugs are substrates of two or more of these transporters (Figure 2) it can be speculated that the transporters of ABC and SLC families join their forces in cellular detoxication and strengthen the excretory mechanisms in the kidney, liver or placenta. It must be borne in mind that transporter co-localization may contaminate pharmacokinetic studies in MATE knock-out mice as other transporters can, to some extent, substitute the function of MATEs.

Little is known about MATE role in pathological situations; nevertheless, Mate1 knockout mice are viable and fertile, without major genotype-related abnormalities (Tsuda et al., 2009). In animal models of human diseases, Mate1 expression was found to be decreased in renal failure (Nishihara et al., 2007) or increased in metabolic acidosis (Gaowa et al., 2011) suggesting that pathological conditions may affect MATE1 expression and subsequently pharmacokinetics of its substrates.
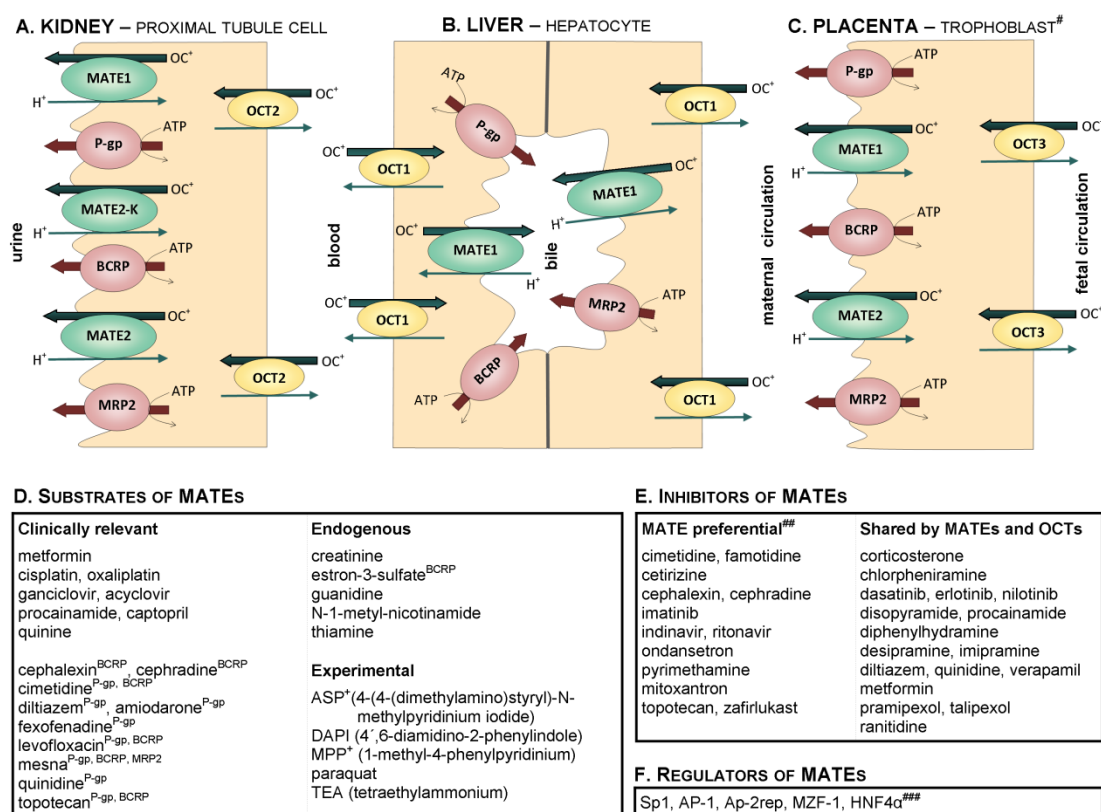

Figure 2. Schematic depiction of MATE expression and activity in the kidney (A), liver (B) and placenta (C). This figure shows the coordinated activity of MATEs and OCTs in transcellular passage of organic cations (OC$^+$) as well as collaboration between MATE and ABC transporters on the apical membranes (see text for details). Selected substrates of MATEs including clinically relevant drugs, endogenous molecules and experimental compounds (D), as well as clinically relevant MATE inhibitors (E) and currently known regulators (F) are shown. Superscripts in box D indicate the ABC efflux transporters that may participate in apical transport (P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP2, multidrug resistance-associated protein 2).
[#]Functional expression of placental MATEs and their collaboration with OCT3 transporter has been described in rat. In human placenta, MATE1, MATE2 and OCT3 expression has been shown; however, their role in vectorial transport of cations remains to be elucidated.
[##]A substance was considered as preferential MATE inhibitor in case its reported inhibitory IC_{50} values for MATE1 or MATE2-K-mediated efflux were at least 5 times lower than those for OCT1-3 transporters.
[###]described in mice
Based on: (Ahmadimoghaddam et al., 2012, Ahmadimoghaddam et al., 2013, Damme et al., 2011, Motohashi and Inui, 2013, Wittwer et al., 2013, Yonezawa and Inui, 2011)

6. Substrates and inhibitors

Although pharmacophore(s) characterizing MATE substrates/inhibitors has(ve) not been thoroughly characterized to date, combined in vitro/in silico and QSAR models suggest that MATE preferentially bind large, lipophilic and positively charged molecules (Astorga et al., 2012, Wittwer et al., 2013). These studies furthermore propose presence of more than one binding sites for ligands in the MATE molecule. Correspondingly, currently identified substrates of MATEs are of cationic character (TEA, MPP+), weak bases with positive charge (metformin, cimetidine, procainamide), zwitterions (cephalexin, cephradine) or even anions (estrone-3-sulphate) of molecular weight ranging from 50 to over 500. Individual MATE isoforms share many common substrates, although with different Km values (Damme et al., 2011); likewise, as MATEs and OCTs cooperate in transcellular transport, considerable overlap in substrate specificity exists between these transporters. To date, over 20 clinically used drugs are known to be transported by human MATE transporters and the list is sure to expand.

Wittwer et al., (2013) have recently identified several MATE1 selective inhibitors with clinical relevance. It should be stressed out that compounds that preferentially inhibit MATE transporters may disrupt the balance between OCT-mediated uptake and MATE-mediated efflux of their common substrates, which can eventually lead to intracellular accumulation of drugs (Figure 2). For example, cimetidine had for long been considered an inhibitor of renal OCT2; however, Ito et al., (2012) have recently suggested that the inhibition of MATEs, not OCT2, is the mechanism underlying the drug-drug interactions of cimetidine in renal elimination. Preferential MATE inhibitors thus may not only decrease renal clearance but also result in nephrotoxicity.

Interspecies differences were observed not only in tissue distribution but also in the interaction of drugs with mouse versus human orthologues of MATE1 (Minematsu and Giacomini, 2011) questioning the suitability of murine model for MATE-based DDIs studies.

7. Possible medical applications

Up to 40% of clinically used drugs carry a net positive charge at physiological pH and, therefore, must utilize carriers for their absorption, distribution and excretion. Logically, any change in the expression (e.g. genetic polymorphisms) and/or function (e.g. inhibition, DDIs) of these transporters might considerably affect pharmacokinetics/toxicokinetics of their substrates and result in unexpected pharmacological/toxic effect. To date the best described

compounds in terms of clinically relevant interactions with mammal MATE transporters are metformin and platinum-based chemotherapeutics.

Metformin, an oral hypoglycemic agent, is one of the most frequently prescribed drugs; as it is not metabolized, its elimination from the body solely depends on renal excretion mediated by MATE and OCT transporters. Changes in MATE expression/function may result in drug accumulation and manifestation of its serious adverse drug reaction, lactic acidosis, as suggested in Mate1(-/-) mice in which marked elevation in the metformin concentration in the liver was detected resulting in profound toxicity (Toyama et al., 2012). The considerable interindividual variability observed in response to metformin treatment may be partly attributed to the variants localized in SLC47A genes. Stocker et al., (2013) suggested that promoter variants in SLC47A1 and SLC47A2 genes are important determinants of metformin disposition and modulate response to treatment in healthy volunteers as well as diabetic patients. Likewise, Becker et al. (2009) demonstrated that genetic variation in MATE1 was associated with A1C-lowering effect of metformin. In addition to genetic polymorphisms, DDIs can also affect pharmacokinetics and/or dynamics of metformin; most recently, trimethoprim, pyrimethamine and cimetidine, recognized MATE inhibitors, reduced metformin elimination and increased metformin exposure in healthy volunteers (Grun et al., 2013).

Of all platinum analogs, cisplatin is the only agent that produces severe nephrotoxicity (Yonezawa et al., 2006). This interesting phenomenon can be explained by different affinity of platinum-based drugs to OCT and MATE transporters in the kidney cells. Cisplatin and oxaliplatin are both substrates of OCT2 and, therefore, transported into the renal tubular cells. Oxaliplatin is a good substrate of renal MATE2-K and is, therefore, effectively eliminated across the apical membrane into urine. Cisplatin, a poor substrate of MATE2-K, on the other hand, accumulates in the kidney cells resulting in pronounced nephrotoxicity (Motohashi and Inui, 2013). Recently, Grottker et al., (2011) have observed inhibition of MATE1 by several antineoplastic agents such as mitoxantrone and irinotecan suggesting that this transporter could play a role in chemosensitivity of tumor cells. OCT and MATE transporters may, therefore, become therapeutic targets to prevent resistance and/or avoid toxicity during chemotherapy.

In conclusion, the role of MATEs in renal and biliary handling of drugs is indisputable and the clinical importance thereof has been established for several drugs. Further investigation of MATE localization and function in other tissues is necessary to fully comprehend the importance of this transporter. However, the research is complicated by

various factors such as (i) the presence of isoforms, variants and SNPs in SLC47A genes, (ii) co-localization of MATEs with other efflux transporters that may substitute the role of MATE in case of disruption or inhibition as well as (iii) by large interspecies differences in tissue distribution and substrate specificity that complicate extrapolation of animal data to human conditions.

Acknowledgments

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7. Summary

The placenta is a crucial organ for proper fetus development, enabling communication between the mother and her fetus. Transporter proteins in the placenta fulfill many vital physiologic roles, essentially the exchange of nutrients and metabolites between fetal and maternal circulations. In addition, many transporters modulate the drug transport across the placenta and provide protective/detoxication function for the developing fetus. Good knowledge of placental transporters and their interactions with drugs is, therefore, important for optimizing pharmacotherapy during gestation and for predicting the risks thereof. Whereas the placental expression and function of the ABC drug transporters have been studied extensively, significantly less information has been gathered on SLC transporters. In the present project, we focused on investigating the expression, localization and function of OCT3 and MATE1 transporters in the rat placenta.

Organic cation transporters (OCTs) and multidrug and toxin extrusion proteins (MATEs) are polyspecific cationic transporters, belonging to the SLC family, encoded by *SLC22* and *SLC47* genes, respectively. OCTs are facilitative diffusion systems that transport organic cations in bidirectional manner across the plasma membrane. The driving forces are provided jointly by the concentration gradient of the transported substrate and by the membrane potential. Of all OCT isoforms, OCT3 is the one most abundantly expressed in the placenta; however, its exact function in the placental tissue is still not fully elucidated. Based on OCT function in the kidney, we hypothesized that OCT3 may be responsible for the first step in the transplacental passage, taking up cationic compounds from the fetal circulation into the trophoblast cell. After OCT3-mediated influx, another membrane transporter is responsible for the efflux of the molecule to the maternal circulation. We assumed that MATE1 might be the collaborating transporter as it often couples with OCTs in the major excretory organs, such as the kidney and liver.

In the initial study, we described the expression and localization of OCT3/*SLC22A3* and MATE1/*SLC47A1* transporters in the rat term placenta: using qRT-PCR and Western blot analysis, we observed prominent placental expression of *Mate1*/MATE1 at both mRNA and protein levels, significantly exceeding those of maternal kidney. Similar results were obtained for *Oct3*/OCT3 mRNA and protein expression. Using immunohistochemical visualization, we further revealed preferential localization of OCT3 on the basolateral, i.e.,

fetus-facing side of the placenta, whereas MATE1 positivity was located in the labyrinth area predominantly on the apical, i.e., maternal side of the rat term placenta.

To analyze the function of OCT3 and MATE1 transporters, we used the technique of dually perfused rat term placenta, in which the maternal or fetal side of the placenta was infused with various concentrations (0.001, 0.01, 0.1, 1, 10, 100 or 1000μM) of a well-established substrate of OCT3 and MATE1, 1-methyl-4-phenylpyridinum (MPP$^+$). We observed strong dependence of MPP$^+$ transplacental clearance on influx concentrations which indicates nonlinear pharmacokinetics and involvement of a saturable transport system. The fetal-to-maternal clearance of MPP$^+$ at low, non-saturating concentration (0.001μM), was 123 times faster than maternal-to-fetal one; we believe this huge asymmetry in transplacental clearances is caused by a concerted action of OCT3 and MATE1. At high MPP$^+$ concentration (1000μM), fetal-to-maternal and maternal-to-fetal clearances reached almost identical values, and the asymmetry was almost annulled, indicating saturation of the transport processes. In closed-circuit perfusion setup, in which both fetal and maternal sides of the placenta were perfused with a non-saturating concentration of MPP$^+$ (0.001μM) and the fetal perfusate was recirculated, we observed considerable decrease in fetal MPP$^+$ concentrations, demonstrating the ability of MPP$^+$ to cross the placenta from fetus to mother even against its concentration gradient, thus confirming the involvement of an active transport mechanism. Once inside the trophoblast cells, MPP$^+$ can be eliminated into the maternal circulation across the apical membrane either by P-gp or by organic cation-H$^+$ antiporter system. Using GF120918, a P-gp inhibitor, we did not observe any change in transplacental passage of MPP$^+$, suggesting P-gp does not have a substantial role in the elimination of the organic cation from the fetus. We, therefore, further focused on the activity of placental MATE1 and evaluated the effect of proton concentration in the maternal circulation on MPP$^+$ placental transport. We employed a range of pH values from 6.5 to 8.5 on the maternal side of the placenta, showing that the oppositely directed H$^+$-gradient can drive the secretion of MPP$^+$ from the placenta to mother. These data indicate that MATE1 on the apical membrane is the collaborating partner of OCT3 in fetal-to-maternal excretion of cations (fig. 8).

In the following part of this study we hypothesized that changes in placental levels of OCT3 and MATE1 transporters throughout gestation might affect their transport capacity and, subsequently, provide variable fetal protection throughout the fetal development. Therefore, we investigated the expression of OCT and MATE isoforms in the rat and human placenta as well as rat fetal tissues at different stages of pregnancy. In addition, using infusion of MPP$^+$

into the pregnant rat we studied the protective effect of placenta against organic cations at different gestation days (gds). We observed an increase in *Oct3*/OCT3 and *Mate1*/MATE1 mRNA and protein expression towards the end of gestation in the rat placenta (fig. 5). We also confirm abundant expression of *OCT3* mRNA in the human placenta, however, *OCT3* mRNA expression declined from first trimester to term (fig. 6). In contrast to the current knowledge, we observed *MATE1* mRNA expression in the first trimester human placentas and *MATE2* mRNA expression in the first and third trimester human placentas (fig. 6). It is obvious from our data that SLC transporter expression follows different regulation schemes in the human and rat placentas suggesting different fetal protective mechanisms between species; therefore, these differences should be borne in mind when extrapolating animal data to human conditions. Consequently, we speculate that vectorial transport of organic cations across the human placenta may be mediated by OCT3-MATE1,2 pathway during early stages of pregnancy as well. However, further studies are required to elucidate this issue in detail.
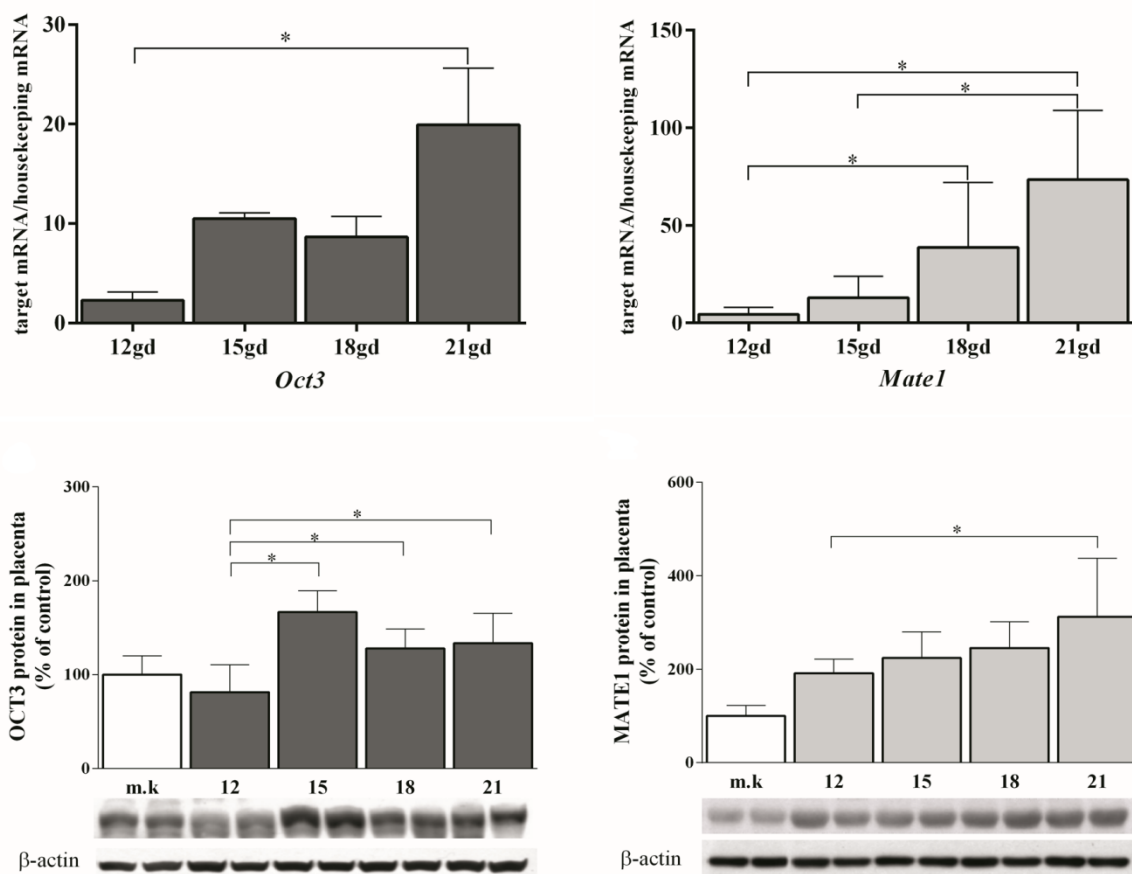

Fig. 5. qRT-PCR and western blot analyses of *Oct3*/OCT3 and *Mate1*/MATE1 mRNA and protein expression in the rat placenta on different gestational days (gds).
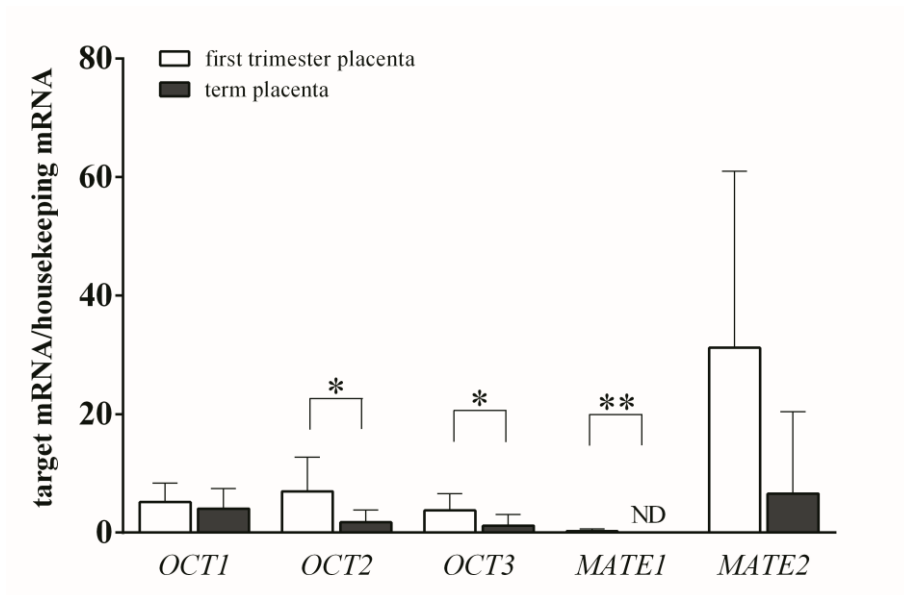
Fig. 6. qRT-PCR of *OCT3* and *MATE1* mRNA expression in the first trimester and term human placenta

We infused MPP$^+$ intravenously into the pregnant rats at different gds and measured at the steady state the MPP$^+$ concentration in the fetal tissues. We observed significant leak of MPP$^+$ into fetal tissues only in mid-gestation, i.e. on gd 12. From 15 gd onwards, relatively low levels of MPP$^+$ were detected in the fetuses (fig. 7). These findings indicate that the fetus is more vulnerable to cationic compounds in maternal circulation in the first half of pregnancy.
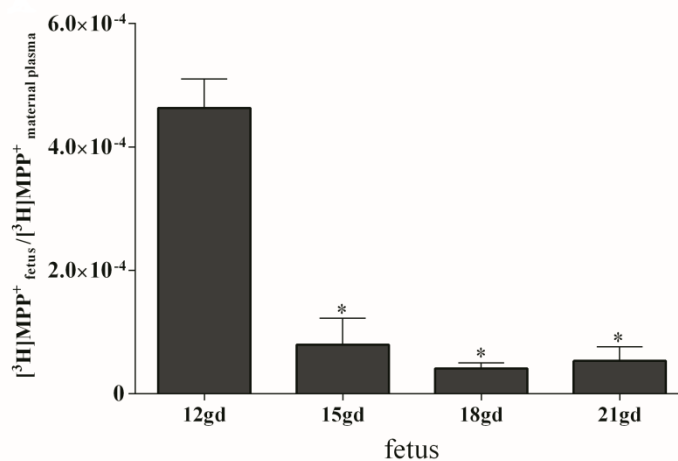

FIG. 7. Exposure of fetus to MPP$^+$ during pregnancy. MPP$^+$ with [^{3}H] MPP$^+$ tracer were infused intravenously to pregnant rats on gestation days (gds) 12, 15, 18, and 21. One hour after the beginning of the infusion, radioactivity in fetus and maternal plasma was measured.

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Having confirmed the functional expression of OCT3 and MATE1 in the rat placenta, in the final part of this study, we used the model of dually perfused rat placenta to investigate the role of these transporters in transplacental passage of a clinically relevant drug, metformin, that is often prescribed in pregnancy and is an established substrate of both OCT3 and MATE1. In open-circuit perfusion setup, in which maternal or fetal side of the placenta was infused with various concentrations of metformin (0.1μM, 1mM, or 10mM), we observed concentration-dependent clearance of metformin across the placenta in both maternal-to-fetal and fetal-to-maternal directions; furthermore, at low, non-saturating metformin concentration (0.1μM), transplacental clearance in fetal-to-maternal direction was 7.3 times higher than that in maternal-to-fetal direction. These data indicate nonlinear pharmacokinetics and contribution of a saturable transport system oriented in fetal-to-maternal direction. These indications were further confirmed using closed-circuit perfusion setup in which both fetal and maternal sides of the placenta were perfused with a non-saturating concentration of metformin (0.1μM) and the fetal perfusate was recirculated; we observed a steady decrease in fetal metformin concentrations indicating the ability of metformin to cross the placenta from fetal to maternal compartment even against its concentration gradient. At high metformin concentration, this asymmetry was annulled. Assuming this asymmetry was caused by placental SLC transporters, we further used MPP^+, a molecule which can, at a concentration of 1000μM, fully inhibit placental OCT3/MATE1 pathway. We observed significant inhibitory effect of MPP^+ on the transplacental passage of metformin indicating the involvement of OCT3/MATE1 vectorial pathway in passage of metformin from the fetal to maternal circulation. In this study, we employed pH values from 6.5 to 8.5 on the maternal side of the placenta to confirm the involvement of MATE1 in elimination of metformin from trophoblast cell to the maternal compartment. We observed that the oppositely directed H^+-gradient can drive the transport of metformin from placenta to maternal circulation, confirming metformin elimination from trophoblast cells by MATE1 (fig. 8).

In conclusion, we demonstrate that OCT3, in a concentration-dependent manner, takes up organic cations from the fetal circulation into the placenta and MATE1 is responsible for organic cation efflux to the maternal circulation, even against a concentration gradient (fig. 8). Furthermore, it is apparent that throughout gestation placenta expresses OCT and MATE transporters in a dynamic manner. In the rat placenta, expression of OCT3 and MATE1 tends to increase towards the end of gestation. This fact, along with the general maturation of the placental tissue results in significantly lower transport of MPP^+ across the placenta from

mother to fetus after gestation day 12. On the contrary, human placenta shows decreasing expression of *OCT2,3* and *MATE1* mRNA suggesting these transporters may play a vital role in fetal protection preferentially at earlier stages of gestation. Moreover, our findings suggest an important role of OCT3 and MATE1 in the transplacental transfer of metformin across the rat placenta. We, therefore, propose that OCT3 and MATE1 form an efficient transplacental eliminatory pathway for organic cations and play an important role in fetal protection and detoxication. We are the first research group that defined the synchronized activity of OCT3 and MATE1 in the placenta.
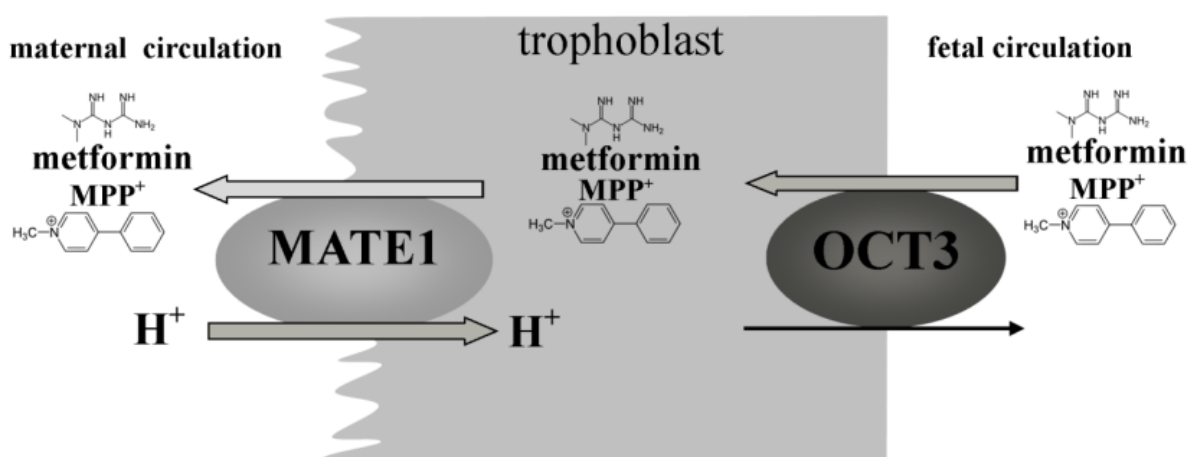

Fig. 8. Schematic depiction of synchronized activity of OCT3 and MATE1 in MPP^+ and metformin transport across the rat placenta. The localization and orientation of uptake (OCT3) and efflux (MATE1) transporters in the rat placenta are shown.

8. List of published works
8.1. Original articles related to the topic of dissertation

1) **Ahmadimoghaddam D.**, Hofman J., Zemankova L., Nachtigal P., Dolezelova E., Cerveny L., Ceckova M., Micuda S., Staud F.: *Synchronized activity of organic cation transporter 3 (Oct3/SLC22A3) and multidrug and toxin extrusion 1 (Mate 1/SLC47A1) transporter in transplacental passage of MPP+ in rat.* Toxicological Sciences 128(2), 471–481 (2012), $IF_{[2011]} = 4.652$.

2) **Ahmadimoghaddam D.**, Zemankova L., Nachtigal P., Dolezelova E., Neumanova Z., Cerveny L., Ceckova M., Kacerovský M., Micuda S., Staud F.: *Organic Cation Transporter 3 (OCT3/SLC22A3) and Multidrug and Toxin Extrusion 1 (MATE1/SLC47A1) Transporter in the Placenta and Fetal Tissues; Expression Profile and Fetus Protective Role at Different Stages of Gestation.* Biology of Reproduction, 7 January 2013; doi:10.1095/biolreprod.112.105064, $IF_{[2011]} = 4.009$.

3) **Ahmadimoghaddam D.**, Staud F.: *Transfer of Metformin across the Rat Placenta is Mediated by Organic Cation Transporter 3 (OCT3/SLC22A3) and Multidrug and Toxin Extrusion 1 (MATE1/SLC47A1) Protein.* Reproductive Toxicology (in press). 9 March 2013;DOI:10.1016/j.reprotox.2013.03.001, $IF_{[2011]} = 3.226$

4) Staud F., Cerveny L., **Ahmadimoghaddam D.**, Ceckova M.: *Multidrug and toxin extrusion protein (MATE/SLC47); role in pharmacokinetics.* The International Journal of Biochemistry & Cell Biology (Under review). This is an "invited review" by the Journal. $IF_{[2011]} = 4.634$.

8.2. Original article unrelated to the topic of dissertation

- Hofman J., **Ahmadimoghaddam D.**, Hahnova L., Pavek P., Ceckova M., Staud F.: *Olomoucine II and purvalanol A inhibit ABCG2 transporter in vitro and in situ and synergistically potentiate cytostatic effect of mitoxantrone.* Pharmacol Res 2012; 65 (3): $312 - 9$, $IF_{[2011]} = 4,436$.

8.3 Poster presentations and abstracts published in the national and international conferences

1) **Ahmadimoghaddam D.**, Hofman J., Brcakova E., Ceckova M., Micuda S., Staud F.: *Localization and functional analysis of organic cation transporter 3 (OCT3) in rat placenta.* 9th International ISSX Meeting, 4–8 September 2010, Istanbul, Turkey.

2) Hofman J., **Ahmadimoghaddam D.**, Hahnova L., Ceckova M., Staud F.: *Inhibition of breast cancer resistance protein (BCRP/ABCG2) by purine cyclin-dependent kinase inhibitors in vitro and in situ.* 9th International ISSX Meeting, 4–8 September 2010, Istanbul, Turkey.

3) **Ahmadimoghaddam D.**, Hofman J., Brcakova E., Ceckova M., Micuda S., Staud F.: *Expression and function of organic cation transporter 3 (OCT3) in rat placenta during pregnancy.* 60th Czech-Slovak pharmacological days, 15–17 September 2010, Hradec Králové, Czech Republic.

4) Hofman J., **Ahmadimoghaddam D.**, Hahnova L., Ceckova M., Staud F.: *On interactions between cyclin-dependent kinase inhibitors and breast cancer resistance protein BCRP (ABCG2).* 60th Czech-Slovak pharmacological days, 15–17 September 2010, Hradec Králové, Czech Republic.

5) **Ahmadimoghaddam D.**, Hofman J., Brcakova E., Ceckova M., Micuda S., Staud F.: *The role of organic cation transporter (OCT3) in placental transfer of MPP^+ at different stages of gestation.* Gordon Research Conference, Multi-Drug Efflux Systems, 12–17 June 2011, Les Diablerets, Switzerland.

6) Hofman J., Kucera R., **Ahmadimoghaddam D.**, Ceckova M., Klimes J., Staud F.: *Interactions of olomoucine II and purvalanol A with ABCG2 transporter in vitro and in situ.* Gordon Research Conference, Multi-Drug Efflux Systems, 12–17 June 2011, Les Diablerets, Switzerland.

7) **Ahmadimoghaddam D.**, Hofman J., Brcakova E., Ceckova M., Micuda S., Staud F.: *The role of organic cation transporter 3 (OCT3) in fetal protection throughout gestation in rat.* 61st Czech-Slovak pharmacological days, 14–16 September 2011, Brno, Czech Republic.

8) Hofman J., Kucera R., **Ahmadimoghaddam D.**, Ceckova M., Klimes J., Staud F.: *Olomoucine II and purvalanol A are inhibitors and substrates of ABCG2 transporter in vitro and in situ.* 61st Czech-Slovak pharmacological days, 14–16 September 2011, Brno Czech Republic.

9) **Ahmadimoghaddam D.**, Hofman J., Zemankova L., Nachtigal P., Dolezelova E., Cerveny L., Ceckova M., Micuda S., and Staud F.: *Synchronized Activity of Organic Cation Transporter 3 (Oct3/Slc22a3) and Multidrug and Toxin Extrusion Protein 1 (Mate1/Slc47a1) in transport of organic cations across the placenta.* 19th MDO Meeting and 12th European ISSX Meeting, 17-21 June 2012, Noordwijk aan Zee, Netherlands.

10) **Ahmadimoghaddam D.**, Neumanova Z., Cerveny L., Ceckova M., and Staud F.: *Transfer of Metformin across Rat Term Placenta.* 62nd Pharmacological day, 25-27 June, Košice, Slovakia.

11) **Ahmadimoghaddam D.**, Zemankova L., Neumanova Z., Nachtigal P., Dolezelova E., Cerveny L., Ceckova M., Micuda S., and Staud F.: *Expression Profile and Fetus Protective Role of Multidrug and Toxin Extrusion Transporter 1 (Mate1/SLC47A1) and Organic Cation Transporter 3 (Oct3/SLC22A3) at Different Stages of Gestation in Rat.* 62nd Pharmacological day, 25-27 June, Košice, Slovakia.

8.5. Oral presentation

1) **Ahmadimoghaddam D.**, Hofman J., Brcakova E., Ceckova M., Micuda S., Staud F.: *Transport of 1-methyl-4-phenylpyridinium (MPP$^+$) across rat term placenta.* 1st Postgraduate Scientific Conference of the Faculty of Pharmacy, Charles University, 1–2 February 2011, Hradec Králové, Czech Republic.

2) Hofman J., **Ahmadimoghaddam D.**, Hahnova L., Ceckova M., Staud F.: *Olomoucine II and purvalanol A inhibit ABCG2 and synergistically potentiate cytotoxic effect of mitoxantrone.* 1st Postgraduate Scientific Conference of the Faculty of Pharmacy, Charles University, 1–2 February 2011, Hradec Králové, Czech Republic.

3) **Ahmadimoghaddam D.**, Hofman J., Zemankova L., Nachtigal P., Dolezelova E., Cerveny L., Ceckova M., Micuda S., Staud F.: *Cooperation of organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion transporter 1*

(Mate1/SLC47A1) in transplacental passage of MPP$^+$. 2nd Postgraduate Scientific Conference of the Faculty of Pharmacy, Charles University, 31 January– 1 February 2011, Hradec Králové, Czech Republic.

4) **Ahmadimoghaddam D.**, Neumanova Z., Cerveny L., Ceckova M., and Staud F.: *Transfer of Metformin across Rat Term Placenta.* 62nd Pharmacological day, 25-27 June, Košice, Slovakia.

5) **Ahmadimoghaddam D.**, and Staud F.: *Metformin Transport across the Rat Placenta by cooperative activity of Organic Cation Transporter 3 (OCT3/SLC22A3) and Multidrug and Toxin Extrusion 1 (MATE1/SLC47A1).* 3rd Postgraduate Scientific Conference of the Faculty of Pharmacy, Charles University, 29-30 January 2013, Hradec Králové, Czech Republic.