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Localization of Multidrug Resistance-Associated Proteins along the Blood-Testis Barrier in Rat, Macaque, and Human Testis

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ABSTRACT

The blood-testis barrier (BTB) prevents the entry of many drugs into seminiferous tubules, which can be beneficial for therapy not intended for the testis but may decrease drug efficacy for medications requiring entry to the testis. Previous data have shown that some of the transporters in the multidrug resistance-associated protein (MRP) family (ABCC) are expressed in the testis. By determining the subcellular localization of these transporters, their physiologic function and effect on drug disposition may be better predicted. Using immunohistochemistry (IHC), we determined the site of expression of the MRP transporters expressed in the testis, namely, MRP1, MRP4, MRP5, and MRP8, from immature and mature rats, rhesus macaques, and adult humans. We determined that in all species MRP1 was restricted to the basolateral membrane of Sertoli cells, MRP5 is located in Leydig cells, and MRP8 is located in round spermatids, whereas MRP4 showed species-specific localization. MRP4 is expressed on the basolateral membrane of Sertoli cells in human and nonhuman primates, but on the apical membrane of Sertoli cells in immature and mature rats, representing a potential caution when using rat models as a means for studying drug disposition across the BTB. These data suggest that MRP1 may limit drug disposition into seminiferous tubules, as may MRP4 in human and nonhuman primates but not in rats. These data also suggest that MRP5 and MRP8 may not have a major impact on the penetration of drugs across the BTB.

Introduction

The epithelial cells that form most of the static cellular mass in seminiferous tubules are called Sertoli cells. Sertoli cells possess a basolateral membrane that faces the outside of the tubule that is exposed to nutrients from the blood, and an apical membrane that is in contact with germ cells (Mruk and Cheng, 2011; Caballero et al., 2012; Franca et al., 2012; Mruk et al., 2013). It is the primary job of Sertoli cells to nurture and protect the developing germ cells (Kato et al., 2009). Germ cell development is a dynamic process that produces several distinct morphologic types, starting from the spermatogonia developing into the haploid round spermatids, and ending with release of immature spermatozoan into the lumen of the tubule (Gerton and Millette, 1986; Olivia, 2006; Su et al., 2011). During this development, the germ cells are sensitive to toxic agents that may be able damage the sperm or may have genotoxic effects on the offspring. To help protect the germ cells from potential toxicants, the Sertoli cells form a blood-testis barrier (BTB) (Su et al., 2012; Chihara et al., 2013; Wang et al., 2013).

The anatomic portion of the BTB is composed of tight junctions between the Sertoli cells (Mital, et al., 2011; Pelletier, 2011; Li et al., 2012). These tight junctions are located near the outside edge of the seminiferous tubule, just apical of the spermatogonia. This barrier prevents many exogenous agents from gaining entry into the lumen of the seminiferous tubules and contacting germ cells (Mann and Lutwak-Mann, 1982; Mruk and Cheng 2010). Although this barrier is beneficial for sperm cell development, it can be an obstacle for drugs that are required to bypass the BTB to achieve full therapeutic effect (Klein et al., 2013). Examples of such drugs include many antiretroviral medications used to treat infection of human immunodeficiency virus (HIV). By limiting the entry of many antiretrovirals into the seminiferous tubules, the BTB may be contributing to the testes' serving as a sanctuary site for HIV (Byrn and Kiessling, 1998; Anderson et al., 2000; Olson, 2002; Dahl et al., 2010; Avery et al., 2011). Because the tight junctions of the BTB prevent or reduce paracellular diffusion of hydrophilic drugs, transcellular transport through the Sertoli cells is required for antiretrovirals to bypass the BTB.

In addition to the tight junctions between Sertoli cells, it has also been reported that there is a transport portion of the BTB to counteract passive diffusion (Bart et al., 2004). Many of the transporters that line the BTB belong to the ATP-binding cassette (ABC) family, which uses energy from ATP hydrolysis to actively efflux a wide variety of substrates (Beringer and Slaughter, 2005; Klaassen and Aleksunes, 2010; Michaud et al., 2012). This family includes transporters such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and members of the multidrug resistance-associated protein (MRP) subfamily (Bart et al., 2004). Within this family, P-gp (*ABCB1*), MRP1 (*ABCC1*), MRP4 (*ABCC4*), MRP5 (*ABCC5*), and MRP8 (*ABCC11*) mRNA expression has been found in rat Sertoli cells, and MRP2 and MRP3 were found to be expressed at low amounts (Bart et al., 2004; Augustine et al., 2005). Additionally, BCRP and P-gp have been localized in human testis to the peritubular myoid cells (Bart et al., 2004; Qian et al., 2013). The

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ABBREVIATIONS: ABC, ATP-binding cassette; BTB, blood-testis barrier; HIV, human immunodeficiency virus; IHC, immunohistochemistry; MGT, male genital tract; MRP, multidrug resistance protein; NRTI, nucleoside reverse transcriptase inhibitor; P-gp, P-glycoprotein.

physiologic functions of MRP1, MRP4, and MRP5 are cytoprotective in many tissues and are known to efflux a wide variety of compounds (Klaassen and Aleksunes, 2010). MRP8 is known to play a role in ear wax synthesis, auxiliary body odor, and breast cancer due to the transport of many pro-growth hormones and amino acids (Guo et al., 2003; Bortfeld et al., 2006; Kruh et al., 2007). In the testis, it has been speculated that these transporters contribute to keeping xenobiotic compounds out of the BTB, thereby protecting dividing germ cells from potential toxicants (Kato et al., 2005).

Many drugs used to treat HIV have been shown to be transported by MRP transporters (Yao et al., 2001, Reid et al., 2003, Kohler et al., 2011; Michaud et al., 2012). This would imply that the MRPs expressed by Sertoli cells could influence the ability of HIV drugs to bypass the BTB. However, it is difficult to determine the effect MRP transport has on disposition across the BTB until the localization of each member is known. Our study used immunohistochemical analysis (IHC) of rat, macaque, and human tissue to determine the subcellular location in the testis of MRP transporters that may interact with HIV drugs.

Materials and Methods

The MACH4 IHC staining kit was acquired from Biocare Medical (St. Louis, MO). MRP1 (*ABCC1*), MRP5 (*ABCC5*), and MRP8 (*ABCC11*) antibodies were purchased from Abcam (Cambridge, MA), and MRP4 (*ABCC4)* antibodies were purchased from Lifespan Biosciences (Seattle, WA). Testis from MRP4$^{-/-}$ mice was a generous gift from Dr. J. Schuetz (St. Jude Children's Research Hospital). All other reagents were purchased from a standard scientific supplier at the highest available purity.

Sample Collection. Rat samples were collected from euthanized male Sprague Dawley rats either at 3 weeks (immature) or at least 12 weeks (mature) old. The samples were fixed in 10% neutral buffered formalin overnight. A small incision was made in the tunica the next day, and the samples remained in 10% neutral buffered formalin for another night. The next day, formalin was replaced with 70% ethanol until the samples were embedded in paraffin. Paraffin-embedded rhesus macaque testis from an 8-year-old Macaque was purchased from Oregon National Primate Research Center (ONPRC) tissue distribution program. Paraffin-embedded human samples were purchased from the National Disease Research Interchange (NDRI) or were provided from the University of Arizona Medical Center pathology department. Sectioning of all paraffin-embedded tissue was accomplished using a microtome with sections sliced 5-microns thick with one section per slide. Protocols for obtaining samples were approved by the University of Arizona institutional review board or the institutional animal care and use committee (IACUC).

Immunohistochemistry. IHC staining was performed on formalin-fixed, paraffin-embedded samples. Slides were deparaffinized with xylene and rehydrated with ethanol. The samples were then heated in an antigen retrieval buffer: citrate (pH 6.0) for MRP1 and MRP5, or tris-EGTA (pH 9.0) for MRP4 and MRP8. Endogenous peroxide activity was blocked by a 0.3% hydrogen peroxide/methanol solution. Staining for all antibodies was performed with the MACH4 kit according to the manufacturer's instructions (Biocare Medical). All slides were imaged with a Leica DM4000B microscope and a DFC450 camera (Leica Microsystems Inc., Buffalo Grove, IL). Each experiment also contained a negative control slide that was not exposed to any primary antibodies but otherwise was treated the same as every other slide. The negative slides contained very little to no positive (brown) staining.

Results

Immunohistochemical Staining of Rat, Macaques, and Human Testes for MRP1. IHC staining for MRP1 was performed on testes tissue to determine the subcellular distribution of this transporter. MRP1 was localized in Sertoli cells using IHC staining on immature (Fig. 1A) and mature (Fig. 1B) rat, mature rhesus macaque primate (Fig. 1C), and mature human (1D) testis. In all cases, MRP1 was located on the basolateral membrane of Sertoli cells (Fig. 1). Positive staining can also be observed in Leydig cells located in the interstitial region.

Immunohistochemical Staining of Rat, Macaques, and Human Testes for MRP4. IHC staining for MRP4 was also performed on testicular tissue acquired from immature (Fig. 2A) and mature (Fig. 2B) rats as well as adult primates (Fig. 2C) and humans (Fig. 2D). Interestingly, the data demonstrated a species difference in MRP4 localization. Positive staining was observed on the apical membrane of

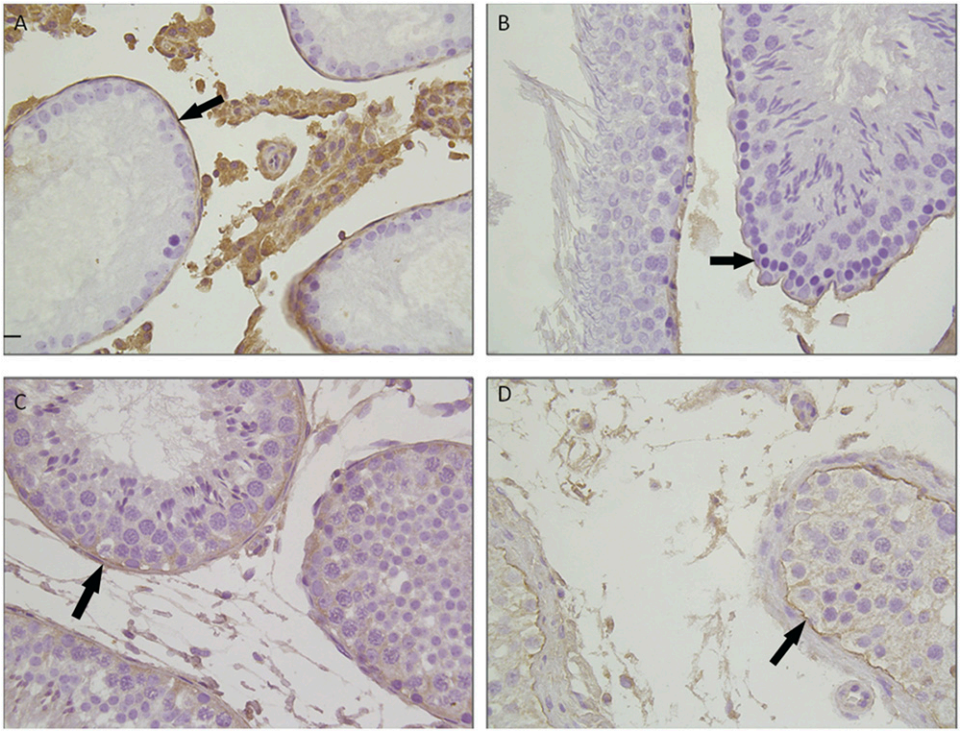

Fig. 1. MRP1 localization in the testis. Immunohistochemical staining for MRP1 in formalin-fixed paraffin-embedded immature (A) or mature (B) rat testes, mature rhesus macaque (C), or mature human (D) is shown at magnification 40×. Black bar in panel A indicates length of 10 microns. Arrows indicate positive (brown) staining for proteins.

both immature and mature rats, but in macaques and human tissue the staining appeared basolateral. Due to the unexpected nature of these results, we verified specificity of the MRP4 antibody by performing IHC on normal mouse tissue (Fig. 2E) and MRP4$^{-/-}$ mouse tissue (Fig. 2F). No positive staining was observed in the MRP4$^{-/-}$ tissue, indicating that the MRP4 antibody used is specific for MRP4.

Immunohistochemical Staining of Rat, Macaques, and Human Testes for MRP5. Previous work performed in our laboratory indicated that MRP5 is expressed in testis (Augustine, et al., 2005). However, in all species no positive staining was observed in Sertoli cells (Fig. 3). There was positive staining in Leydig cells for mature and immature rats (arrows in Fig. 3, A and B), which accounts for the previous data indicating testicular expression of MRP5 (Augustine et al., 2005). Interestingly, there was no staining in the macaques tissue (Fig. 3C), and only minimal staining was observed in human tissue (Fig. 3D). As a positive control, rat kidney, which is known to express MRP5 on the apical membrane of proximal tubule cells, was stained and apical staining was observed, indicating that the MRP5 antibody was functional (data not shown).

Immunohistochemical Staining of Macaques and Human Testes for MRP8. Because rodents do not express an MRP8 ortholog, only human and rhesus macaque tissue was stained for MRP8 (Fig. 4). Interestingly, both species demonstrated distinct staining on round spermatids, which are germ cells that have undergone meiotic division but have not yet developed the characteristic sperm cell morphology. Only this stage of germ cell development seems to express MRP8, and MRP8 does not seem to be expressed by Sertoli cells.

Discussion

We present novel information concerning the localization of MRP transporters in the testis of three species: rats, rhesus macaques, and humans. We stained tissue isolated from immature (prepuberty) and mature (postpuberty) rats, but due to difficulty in obtaining immature macaque and human tissue and the lack of age-dependent differences in rats, we only stained mature macaque and human tissues. The MRP transport function can be difficult to study at the blood-testis because of the overlapping substrate specificity, the lack of specific inhibitors, the difficult in obtaining enough human tissue to culture primary Sertoli cells, and technical challenges in studying efflux transport. Although this information is novel for the testis, many of these transporters have been localized in other barrier tissues. In the blood-brain barrier, MRP1, MRP4, and MRP5 are expressed on the apical side of capillary endothelial cells. In choroid plexus epithelial cells,

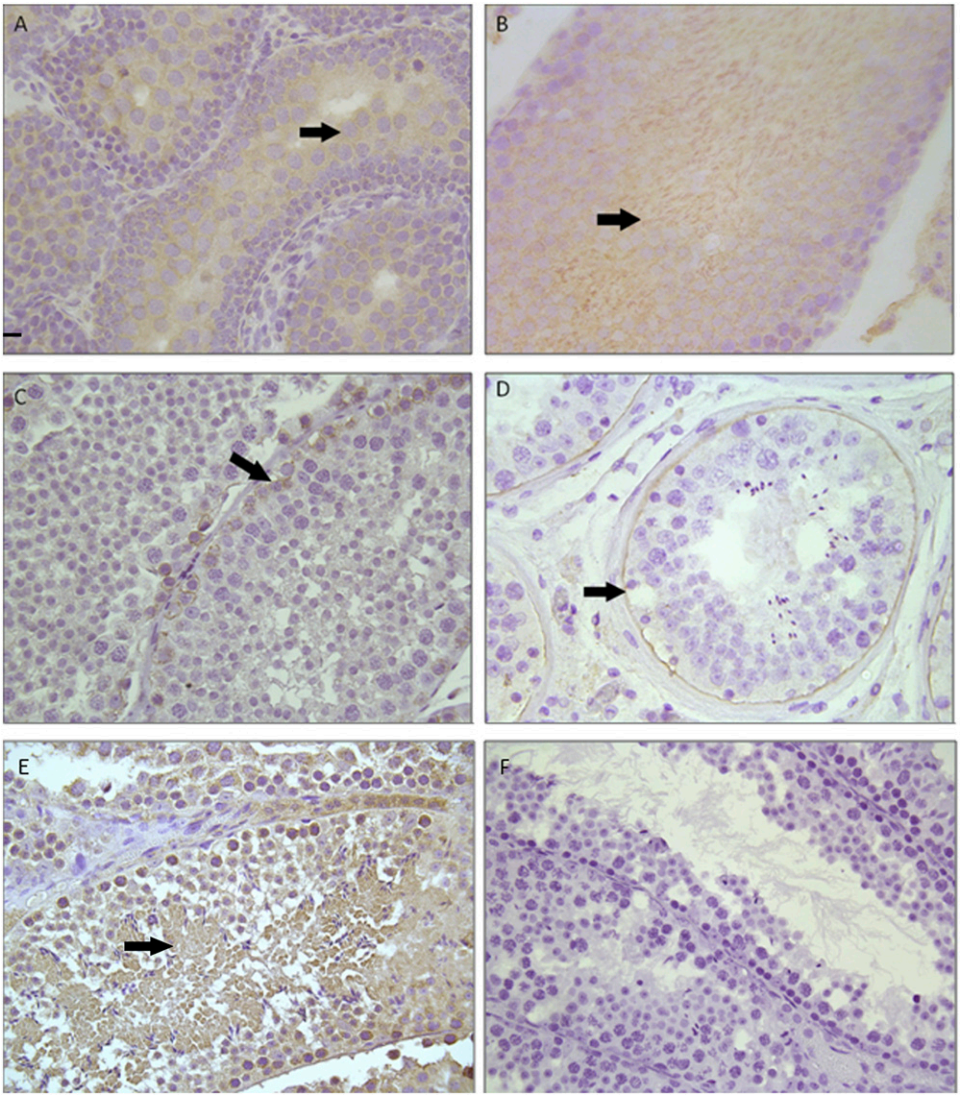

Fig. 2. MRP4 localization in the testis. Immunohistochemical staining for MRP4 in formalin-fixed paraffin-embedded immature (A) or mature (B) rat testes, mature rhesus macaque (C), mature human (D), mature mouse (E), or MRP4$^{-/-}$ mouse (F) is shown at magnification 40×. Black bar in panel A indicates length of 10 microns. Arrows indicate positive (brown) staining for proteins.

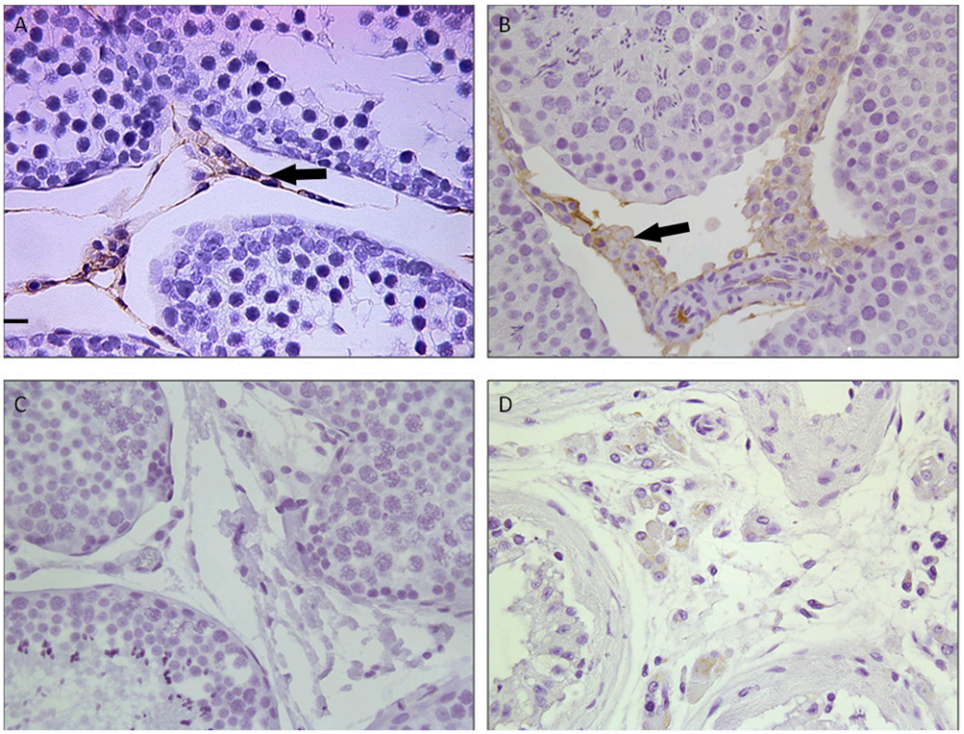

Fig. 3. MRP5 localization in the testis. Immunohistochemical staining for MRP5 in formalin-fixed paraffin-embedded immature (A) or mature (B) rat testes, mature rhesus macaque (C), or mature human (D) is shown at magnification 40×. Black bar in panel A indicates length of 10 microns. Arrows indicate positive (brown) staining for proteins.

MRP1 is expressed only on the basolateral membrane whereas MRP4 is on both apical and basolateral membranes. In the placenta, MRP1 is on the apical side of syncytiotrophoblasts and on the basolateral side of fetal membranes along with MRP4 and MRP5 (Klaassen and Aleksunes, 2010). Each transporter appeared to have a different staining pattern, indicating different physiologic functions and different effects on drug disposition past the BTB.

MRP1 staining likely represents the expected function of MRP transporters along the BTB. The basolateral localization indicates that MRP1 acts as part of the transporter portion of the BTB in effluxing xenobiotics out of the seminiferous tubule, thereby representing a spermatoprotective response. This would suggest that MRP1 would likely act as an obstacle for getting the antiviral drugs or other chemotherapeutics that are substrates for MRP1 into the testes.

In humans and nonhuman primates, MRP4 has the same localization and presumably function as MRP1—that is, acting as a spermatoprotective response to potentially toxic agents. Unexpectedly, we discovered a different localization for MRP4 in rats at both mature and immature ages. It is difficult to speculate on the potential function of MRP4 in rats. Because this transporter is known to transport a wide variety of substrates, including secondary signaling molecules such as cGMP, perhaps it is involved in paracrine signaling to the nearby germ cells (Kruh et al., 2007; Sager and Ravna, 2009). Nonetheless, it is clear that this represents a potential issue in using rats as a model for BTB disposition of drugs, as MRP4 could be aiding drug disposition into the seminiferous tubule in a manner not representative of the human condition. More studies are needed to assess the impact this species difference has on HIV drug transport.

In all species tested, MRP5 was not expressed along the BTB, but positive staining was observed in Leydig cells for rats. One of the primary functions of the Leydig cell is steroidogenesis (McGee and Narayan, 2013). Like MRP4, MRP5 is known to transport a wide variety of compounds and signaling molecules (Kruh et al., 2007; Sager and Ravna, 2009). It is likely that these transporters may play a role in aiding hormone signaling. Another possibility is that MRP5 is simply cytoprotective for the Leydig cells. Whatever its physiologic function may be, it is apparent that MRP5 would not be expected to have a significant impact on drug disposition across the BTB.

MRP8 also displayed an interesting and unexpected localization. Instead of being localized to the Sertoli cells, it was restricted to round

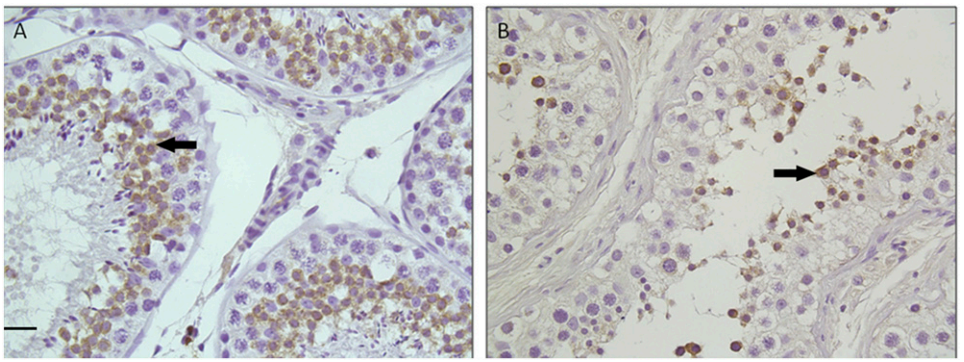

Fig. 4. MRP8 localization in the testis. Immunohistochemical staining for MRP8 in formalin-fixed paraffin-embedded mature rhesus macaque (A) or mature human (B) is shown at 40 × magnification. Black bar in panel A indicates length of 10 microns. Arrows indicate positive (brown) staining for proteins.

spermatids. These spermatids are haploid but, as the name suggests, still possess a spherical morphology. It is at this stage that the spermatids are down-regulating prodivision signals so that they may begin restructuring to a more elongated morphology (Pang et al., 2006). MRP8 is known to transport steroid sulfates and neurosteroids such as dehydroepiandrosterone sulfate (DHEAS) (Kruh et al., 2007). Because many MRP8 substrates are pro-growth hormones, it is possible that the spermatids are expressing MRP8 as a means of effluxing cell division signals that are no longer needed. If this is true, it may be expected that a defect in MRP8 would result in an increased incidence in germ cell tumors.

A single-nucleotide polymorphism (SNP) is known to exist in the human population for this gene, and although it has been linked to breast cancer, no information is available regarding its correlation to germ cell tumors (Toyoda and Ishikawa, 2010).

Although it could be speculated that this transporter is serving a cytoprotective function in round spermatids, this seems unlikely because MRP8 is not expressed throughout germ cell development and there are no apparent reasons why round spermatids would be any more sensitive to toxic agents than any other stage of germ cell development. What our study does conclude about MRP8 is that although this transporter may limit drug distribution to the round spermatids, it would not be expected to play a role in disposition of drugs past the BTB.

In conclusion, with the intent of furthering the field's understanding of drug transport at the BTB, this study provides novel data demonstrating the localization of MRP1, MRP4, MRP5, and MRP8 in four types of testicular tissue originating from three different species. Based on our data, we drew three major conclusions: 1) MRP1 may limit drug penetration into the seminiferous tubules; 2) MRP4 has a species-specific difference localization and may be expected to limit drug disposition in humans and nonhuman primates but facilitate disposition of (selected) drugs in the rat testis; 3) neither MRP5 nor MRP8 are likely to have a major effect on drug transport at the BTB. Based on our data, further research can also be performed to better understand the physiologic function(s) of these MRP transporters.

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Authorship Contributions

Participated in research design: Klein, Wright, Cherrington.

Conducted experiments: Klein.

Performed data analysis: Klein, Cherrington.

Wrote or contributed to the writing of the manuscript: Klein, Wright, Cherrington.

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