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N Ono, I van Der Heijden, G L Scheffer, K van de Wetering, E van Deemter, et al.. Multidrug Resistance-associated Protein 9 (ABCC12) is present in mouse and boar sperm. *Biochemical Journal*, 2007, 406 (1), pp.31-40. <10.1042/BJ20070292>. <hal-00478766>

HAL Id: hal-00478766

<https://hal.science/hal-00478766v1>

Submitted on 30 Apr 2010

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Multidrug Resistance-associated Protein 9 (ABCC12) is present in mouse and boar sperm.

Running title: Intracellular localization of MRP9.

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27 SYNOPSIS

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29 The human and murine genes for Multidrug Resistance Protein 9 (ABCC12) yield many
30 alternatively spliced RNAs. Using a panel of monoclonal antibodies we detected full-length
31 Mrp9 only in testicular germ cells and mouse sperm; we obtained no evidence for the
32 existence of the truncated 100 kDa MRP9 protein previously reported. In contrast to other
33 MRPs, neither murine Mrp9, nor the human MRP9 produced in MRP9-transfected Human
34 Embryonic Kidney 293 (HEK 293) cells appear to contain N-linked carbohydrates. In mouse
35 and boar sperm Mrp9 localizes to the mid-piece, a structure containing all sperm
36 mitochondria. However, immuno-localization microscopy and cell fractionation studies on
37 transfected HEK cells and mouse testis show that MRP9/Mrp9 does not route to
38 mitochondria. In HEK cells it is predominantly localized in the endoplasmic reticulum. We
39 have been unable to demonstrate transport by MRP9 of substrates transported by other MRPs,
40 such as drug conjugates and other organic anions.

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42 Keywords: ABC-transporters, drug transport, MRP, testis, mammary cancer, sperm.

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INTRODUCTION

The largest family of mammalian primary active drug transporters is the Multidrug Resistance-associated Protein (MRP) family, belonging to the ABCC sub-group of ATP Binding Cassette (ABC) proteins. MRP1 (ABCC1) was discovered by Cole and Deeley and their co-workers [1] in 1992 and eight other MRPs have since followed one by one [2-4], as reviewed by us [5-12] and others [13-19]. The 8 human MRPs that have been characterized are all able to transport organic anions, such as drugs conjugated to glutathione, sulphate or glucuronate. In addition, selected MRPs may transport a variety of endogenous compounds, such as leukotriene C4 (MRP1), bilirubin glucuronides (MRP2, MRP3), prostaglandins E1 and E2 (MRP4), cGMP (MRP4, MRP5, MRP8), and several glucuronosyl-, or sulfatidyl steroids. The absence of two of these transporters is associated with human disease. Defective MRP2 causes the Dubin-Johnson syndrome; defective MRP6 pseudoxanthoma elasticum. A defect in MRP8 was recently associated with the inability to produce wet, brownish, sticky ear wax [20].

The most recent addition to the MRP family is MRP9 (ABCC12), independently described in 2001 by Yabuuchi et al. [21], Tammur et al. [22] and Bera et al. [23]. MRP9 is most closely related to MRP5 and MRP8. Human MRP9 is located next to MRP8 on chromosome 16q12.1 [21], presumably the result of a recent duplication [22]. Whereas no mouse homolog of MRP8 (ABCC11) has been found [24], the cDNA of murine Mrp9 (Abcc12) was cloned and characterized by Shimizu et al. [24]. Both the human MRP9 and the mouse Mrp9 genes yield a plethora of alternatively spliced RNAs and this has led to uncertainty about the true nature of the protein encoded by MRP9. The gene structure looks unambiguous. It predicts 29 exons both in human and mouse, encoding a classical "short" MRP, lacking the N-terminal domain present in MRP1, 2, 3, 6, and 7, but absent in MRP4, 5, and 8. The predicted amino acid sequences of murine Mrp9 and human MRP9 are 84.5% identical and their hydropathy profiles are nearly identical [24]. With MRP5 the human MRP9 shares 42% identity and all conserved elements in MRPs are also present in MRP9 [22].

A transcript encoding full-length human MRP9 has been hard to find, however. Tammur et al. [22] (2001) identified transcripts of about 5000 bp on blots of human testis, ovary and prostate RNA, but were unable to clone a full length cDNA containing all MRP9 exons. Yabuuchi et al. (2001) showed that the predominant MRP9 splice variants present in human tissues do not encode a canonical MRP, but they detected a small amount of RNA containing all putative exons in testis RNA [21]. The relevance of this RNA is supported by the results obtained with RNA from mouse testis [24]. The predominant transcript in this

tissue is a 5.4 kb RNA, which encodes the complete Mrp9 protein sequence. Two minor variants were cloned as well, both encoding truncated proteins [24].

In contrast, Bera et al. (2002) concentrated on the main splice products of the human *MRP9* gene [25]. They cloned two major RNAs: a 4.5 kb RNA lacking exons 5, 16, and 26, found only in testis and encoding a truncated protein of 930 amino acids; and a 1.3 kb RNA present at high levels in brain and encoding a putative protein of 233 amino acids. Interestingly, substantial amounts of the 4.5 kb RNA were also found in some breast cancer cell lines. Moreover, tumor cells in some samples of breast cancer showed strong RNA *in situ* hybridization with the *MRP9* probe. A band of about 100 kDa, presumably the 930 amino acid translation product of the 4.5 kb RNA, was detected in testis extracts and in a breast cancer cell line extract by an IgG fraction purified from rabbit antisera raised against MRP9 synthetic peptides [25].

We have focussed on the full-length canonical versions of murine Mrp9 as well as human MRP9. We have tried to determine whether these proteins are actually made *in vivo* and what their transport function could be. Here we report that murine Mrp9 is present in murine sperm and sperm cell precursors.

MATERIALS AND METHODS

Chemicals and reagents

DMM (1-deoxymannojirimycin) was made by Broxterman et al. [26] and was supplied by J.N. A polyclonal antibody against the human mitochondrial outer membrane protein Sam50 [27] was generated by T.G. and J.N. Sam50 fused to glutathione-S-transferase was made in *Escherichia coli* (*E. coli*) BL 21, purified over glutathione beads, and eluted with thrombin. The thrombin was removed with a heparin column (Amersham) and the purified Sam50 was injected into rabbits according to standard protocols. The rabbit antisera recognized a mitochondrial protein in confocal microscopy and stained a 50 kDa band on Western blots of human, mouse and pig tissues.

Commercial antibodies came from the following sources: Polyclonal rabbit anti-human calreticulin (Upstate); polyclonal rabbit anti-human EEA1 (Upstate); polyclonal rabbit anti-bovine Catalase (Abcam); monoclonal rat anti-mouse CD107a (LAMP-1) (BD Pharmingen); monoclonal mouse anti pigeon Cytochrome *c* (B D Pharmingen); polyclonal rabbit anti-human Calnexin (Santa Cruz); polyclonal goat anti-human GRASP65 (Santa Cruz); rabbit anti-PDI rec 442 (a kind gift of Dr. Hidde Ploegh, Boston); goat anti-rabbit-HRP (Santa Cruz); goat anti-rat-HRP (Santa Cruz); rabbit anti-mouse-HRP (DAKO); rabbit anti-

goat-HRP (DAKO); rabbit anti-rat (Vector Labs); goat anti-rat-Alexa 488 (Molecular Probes);
goat anti-rabbit-Texas Red (Molecular Probes).

Protein immunoblot (Western) analysis

Western blot analysis was done essentially as described before [28;29]. Protein was quantified using the Bio-Rad protein assay (Bio-Rad), fractionated on an 8% polyacrylamide slab gel and transferred to a nitrocellulose membrane by electroblotting. After blocking for 1 hour in PBS containing 1% nonfat milk powder, 1% bovine serum albumin, and 0.05% Tween 20, the blots were incubated with the MRP9 rat monoclonal antibodies, with subsequent use of a horseradish peroxidase-labeled goat anti-rat antibody (Santa Cruz Technology) and enhanced chemiluminescence for detection.

Transfected cell lines

HEK293 cells were grown in DMEM (GIBCO/BRFL) containing 10% FCS and penicillin/streptomycin. The cloning of the full-length human MRP9 cDNA has been described [21]. To make MRP9-expressing cell lines, the MRP9 cDNA was inserted into the *EcoRI* and *Hind* III sites of the pcDNA3.1(-)vector (Invitrogen). By using calcium phosphate precipitation, HEK293 cells were transfected with the pcDNA3.1(-)-MRP9 vector. After selection with G418 (1 mg/ml), positive colonies were picked and separately expanded in DMEM containing 10% FCS and penicillin/streptomycin for further studies.

Although details are not described in this manuscript, several other transfected cell lines were also generated to characterize human MRP9. Briefly, to generate pEGFP-N2-*MRP9*, the 5' part of an *EcoRI* and *PstI* *MRP9* cDNA fragment of PcDNA3.1 containing full-length *MRP9* cDNA was inserted into the corresponding restriction sites of the pEGFP-N2 vector. To replace the stop codon, 1327bp was amplified by PCR from an *MRP9* cDNA template. The PCR fragment was digested with *PstI* and *SmaI* and inserted into pEGFP-N2 containing the 5' part of *MRP9*. The orientation and fidelity of the fragment was verified by sequence analysis. By analogous procedures, we constructed a MscV-MRP9-IRES-EGFP construct and expressed this in HEK293 cells. Finally, we expressed *MRP9* in insect Sf9 cells using a baculovirus construct: The *EcoRI* *MRP9* cDNA fragment from plasmid pcDNA3.1 (-) was inserted into the corresponding restriction sites of the pFastBac-1 vector. After the orientation of the *MRP9* cDNA insert in the vector was verified, DH10Bac cells were transformed with the pFastBac-1-MRP9 construct to generate the recombinant bacmid DNA. The sequence of the resulting recombinant bacmid DNA was verified by PCR analysis. Sf9 cells were transfected with the bacmid DNA to produce recombinant baculovirus. The MRP9-producing Sf9 cells were used to prepare inside-out membrane vesicles for transport studies.

The presence of MRP9 in these vesicles was checked with our new anti-MRP9 Mab M₉I-27, 1 µl of vesicle protein producing a clear 150-kDa band on a Western blot.

Cloning of rat Mrp9 (*Abcc12*) cDNA

The rat *Mrp9* gene that is homologous to mouse *Mrp9* or human *MRP9* genes was identified in the NCBI mouse database as well as the EMBL/UCSC database. By using the GENSCAN program [30] (<http://genes.mit.edu/GENSCAN.html>), we have predicted plausible exons in the rat *Mrp9* gene. Based on the predicted exons, rat expressed sequence tag (EST) clones were extracted from the EST database. We have screened the multiple tissue cDNA panels (MTCTM, Clontech, Palo Alto, CA, USA) by means of PCR using the following primers: the forward primer 5'- GACTATCGGATGAGATACAGAGACAACAC -3' the backward primer 5'- CAAAGCAGCTGGCGTTCTCCTACTGAGAAG -3'. Among the tissues screened, the highest expression of rat *Mrp9* was detected in the testis.

To clone the rat *Mrp9* cDNA, we have designed the following four sets of PCR primers: c12-1 (the forward primer: 5'-GTCCACAGAGGAGGAAGCTAGAGTGAAC -3' and the backward primer: 5'-GACTCACCTGCCCTAGAAGAGCAGAAATG-3'), c12-2 (the forward primer: 5'- GATACGTCCAAAGTGGGAACTCAGCCCTG-3' and the backward primer: 5'- CTGCATGGAGGAGGTGATGTGAGAGAAC-3'), c12-3 (the forward primer: 5'- CACATGTACCAGTTGGTTTACATAGCAAG-3' and the backward primer: 5'- CAAAGCAGCTGGCGTTCTCCTACTGAGAAG-3'), and c12-4 (the forward primer: 5'- GACTATCGGATGAGATACAGAGACAACAC-3' and the backward primer: 5'- GCGAGGAGCGCTGTTACCTCCAGCCTTG-3'). The PCR reaction was performed with rat testis cDNA (Clontech, Palo Alto, CA) and Ex TaqTM polymerase (TaKaRa, Japan). The PCR conditions consisted of 30 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 90 sec. After agarose gel electrophoresis, the PCR products were extracted from the gels and subsequently inserted into TA cloning vectors (Invitrogen USA) according to manufacturer's protocol. The sequences of the resulting inserts were analyzed with an automated DNA sequencer (TOYOBO Gene Analysis, Japan). Finally, the whole cDNA of rat *Mrp9* was obtained by assembling those partial sequences.

Quantitative PCR analysis of human MRP9, mouse Mrp9 and rat Mrp9

The expression of human MRP9, mouse *Mrp9* and rat *Mrp9* in different organs was examined by quantitative PCR with human, mouse and rat Multiple Tissue cDNA (MTC) panel (Clontech, Palo Alto, CA), respectively. The PCR was performed with TaKaRa Ex TaqTM R-PCR version in a TaKaRa Smart CyclerTM System (TaKaRa, Japan), where amplification of DNA was monitored with SYBR Green I. The reaction mixture contained 2 µl of cDNA (MTC panel), 2.5 µl of 10 x PCR buffer, 0.75 µl of 10 mM dNTP mix, 0.75 µl of 10 µM PCR

primers, 0.3 µl of 250 mM Mg²⁺ solution, 0.25 µl of 1/300-diluted SYBR Green I (Bio-Whittaker Molecular Applications, Rockland, ME, USA), and 0.25 µl of Ex TaqTM polymerase in a total volume of 25 µl. For this analysis, the following specific primers were used; for human (the forward primer 5'- GTAAGGTACAACCTTGGATCCCT -3' and the backward primer: 5'- TGCTGCTAGTAACATCGCAA -3'), for mouse (the forward primer 5'- TATGGCCCGGG CACTTCTCCGTAA -3', the backward primer 5'- GACCTTTACAGTCCAACCTCTG CAGCTAGT -3') and for rat Mrp9 (the forward primer: 5'- GTAGGAGAACGCCAGCTGCTTTGTATGG -3' and the backward primer: 5'- GACCTTTACAGTCCAACCTTCTGCAGCTAGT -3');. The PCR cycle consisted of four steps; i.e., denaturation (95°C, 15 sec), annealing (60°C, 15 sec), amplification (72°C, 15 sec) and quantification (87°C, 10 sec).

Rat monoclonal antibodies

For the generation of antibodies against MRP9, segments of MRP9 cDNA (Fig. 1) were subcloned into the pMal-c vector for production of a fusion protein of *E. coli* maltose binding protein with MRP9 segments. The fusion protein was produced in *E. coli* strain JM101 and purified by amylose affinity chromatography. The use of the fusion protein for the production of rat monoclonal antibodies was as described [31]. Briefly, the Mabs were selected by an ELISA test, and positive clones were cross-checked on cytospins of HEK293/MRP9 and HEK293 cells. The antibodies obtained were finally screened on Western blots of cellular lysates prepared from HEK293 cells transfected with MRP9 cDNA. Three mAbs of the M₉I series against the N-terminal segment, and 4 of the M₉II series against the linker segment were used for this study.

Immunohistochemistry of cells and tissues

For immunolocalization of the MRP9 protein in cells, HEK293 cells were grown for 2 days on glass slides. After fixation with acetone, we used the DAKO EnVision Plus HRP system (K4008; DAKO), which uses AEC as substrate, with minor modifications for staining. Several rat monoclonal anti-MRP9 antibodies were used for MRP9 immunostaining. A rabbit anti-rat-IgG (Vector lab AI-4001, pre-absorbed with mouse IgG, 1:200 dilution) was used as the secondary antibody. Before mounting, slides were counterstained with hematoxylin.

Adult FVB mouse were used for the isolation of testis and epididymis.

Immunohistochemistry of testis cyto-sections fixed with acetone was performed as described for HEK293 cells.

Isolation and immunostaining of sperm

Mouse sperm were washed out of the caudal epididymis and centrifuged at 400 x g. The pellet was washed in PBS and a drop was placed on a glass slide, smeared out like a blood smear, and air dried. After fixation in acetone for 10 min. at room temperature, the slide was rinsed with PBS/0.05% Tween 20 for 5 min. and the smear was then stained using the DAKO EnVision Plus HRP system (DAKO). Preblocking was done with a mixture of PBS, 0.05% Tween and 5% normal donkey serum (Jackson ImmunoResearch). Rat anti-MRP9 Mabs were used in undiluted form (culture supernatant) for 2 hours at room temperature; after rinsing with PBS/0.05% Tween, the slide was pre-incubated with rabbit anti-rat IgG (Vector lab, pre-absorbed with mouse IgG in 1:200 dilution), followed by the polymer-HRP incubation with AEC substrate, as specified by DAKO. The slides were counterstained for 5 sec with hematoxylin solution.

Boar semen was collected in the Cooperative Centre for Artificial Insemination in Pigs "Utrecht en de Hollanden" (Bunnik, the Netherlands). Semen was filtered through gauze to remove gelatinous material and diluted to 150 million cells/ml in Beltsville Thawing Solution (BTS: 0.2 M glucose, 20 mM sodium citrate, 15 mM NaHCO₃, 3.36 mM Na₂EDTA, 10 mM KCl, 20 mM Hepes, pH 7.4) and kept at 17° C for transport or storage in air-tight 80 ml insemination tubes. Prior to further experimentation the sperm samples were first washed through BTS by centrifugation for 5 min at 400 x g and the resuspended sperm (1 billion sperm/ml) was then spun through a discontinuous (35%/70%) Percoll® (Pharmacia, Uppsala, Sweden) density gradient to remove epithelial cell contamination and immature sperm [32]. After washing the pellet with PBS, sperm smears were stained as for the mouse sperm, but pre-blocking before adding anti-MRP Mabs was done with 4% BSA in PBS without the donkey serum.

Confocal microscopy of immunofluorescent cells

Confocal fluorescence images were obtained on a Leica TCS SP2-AOBS (Leica Microsystems, Heidelberg, Germany) confocal system, using the 488 nm line from an Argon laser and the 594 nm line from a HeNe laser as excitation source. Images were taken using a HCX PL APO CS 63x NA 1.4 oil objective. Possible crosstalk between the different fluorochromes, which could give rise to false-positive colocalisation of the signals, was totally avoided by careful selection of the imaging conditions. For the analysis of the intracellular location of MRP9 in transfected cells, the MRP9-HEK293 cells were grown overnight on poly-L-lysine-coated coverslips. After rinsing with PBS, the cells were fixed with either methanol (5 min icecold methanol, rehydration in PBS for 5 min) or formalin (20 min formalin at room temp., 3 PBS washes, 30 min PBS incubation, permeabilization for 5 min in a mixture of 1% BSA and 0.2% Tween X-100 in PBS). For antibody incubations, the

cells were pre-blocked with 1% BSA in PBS for 30 min, incubated with primary antibody for 1 hr, washed with 1% BSA/PBS, incubated with the secondary antibody for 1 hr, washed with 1% BSA/PBS and PBS, and mounted in vectashield.

The rat anti-MRP9 antibodies M₉I-38 and M₉II-19 were used undiluted on formalin-fixed cells and 1:2 diluted on methanol-fixed cells. As secondary antibodies we used goat anti-rat-Alexa488 or anti-rabbit-Texas Red.

Analysis of protein-linked oligosaccharides

Total cellular lysates were incubated with an N-glycosidase cocktail (2.67mU/μl), a mixture of endoglycosidase F and peptide-N-glycosidase (Boehringer Mannheim, Mannheim, Germany), at 30°C overnight, to remove N-linked oligosaccharides. High-mannose oligosaccharides were removed by incubation with endoglycosidase H (2 units/ml, Boehringer Mannheim, Mannheim, Germany) in 50mM sodium citrate (pH 5.5) and 0.2% SDS, at 37°C overnight.

Cell fractionation studies

Mouse testis was dissected into small pieces in ice-cold mitobuffer (0.2 mM sodium-EDTA, 0.25 M sucrose, 10 mM Tris-chloride, pH 7.8) and homogenized by 15 strokes with a Wheaton Dounce Tissue Grinder with tight pestle clearance (0.05 mm). The homogenate was centrifuged for 10 min at 1000 g at 4°C. The supernatant (S1) was centrifuged for 15 min at 8000 g at 4°C (Sorvall, SS34 rotor), the pellet (P2) was washed twice with mitobuffer containing protease inhibitors (Roche Complete tablets) and stored frozen. (P2) was thawed, centrifuged and resuspended in a minimal volume of 0.8 M sucrose, containing 0.2 mM EDTA / Tris pH 7.8 and layered on a 5-ml 0.9 – 2.0 M sucrose gradient (in 0.3 mM sodium EDTA, 10 mM Tris, pH 7.8). After centrifugation to equilibrium (2 hrs, 4°C, 80,000 g), about 15-20 fractions were collected through a hole punched in the bottom. The membranous material in each fraction was collected by centrifugation for 10 min at 20,000 g. The pellets were resuspended in mitobuffer.

RESULTS

Generation of antibodies against MRP9

Whereas transcripts of *MRP9* have been detected in various human, rat and mouse tissues including brain, the expression levels are relatively low with the exception of rat and mouse testes (Fig. 2). Most of the human testis RNA consists of alternative splice products, but our previous analysis has also shown the presence of a minor fraction of an RNA able to encode

full-length MRP9 [21]. To test whether this protein is actually made, we generated a series of monoclonal antibodies (MAbs) and two polyclonal rabbit antibodies against human MRP9 segments with little sequence identity with other MRPs (Fig. 1). The MRP segments were fused to the *E. coli* maltose binding protein, and purified fusion proteins were used as immunogen (see Methods). The Mabs were raised in rats in the hope of getting some antibodies that would not only react with human MRP9, but also with murine Mrp9.

To characterize the antibodies, we used Human Embryonic Kidney 293 (HEK293) cells transfected with a MRP9 cDNA construct. Fig. 3A shows the results with 4 of the 7 different Mabs. Each of the Mabs recognizes a single 150 kDa band in the transfected cells, but not in the parental cells and the same result was obtained with the 3 Mabs not shown in Fig. 3A. Fig. 3B presents immunohistochemical results with 3 of the Mabs. In the HEK293 cells MRP9 does not localize to the plasma membrane, as other MRPs do, but remains intracellular. The two rabbit antisera raised against MRP9 segments also recognized a 150 kDa band, but as these sera gave more background than the MAbs, they were not used in most further experiments.

Detection of full-length Mrp9 in mouse tissues

With the antibodies generated we were unable to detect any form of MRP9 in human tissues or cell lines, or on Western blots of extracts from tissues or cell lines. These included testis and mammary tumor cell lines in which a short 100-kDa form of MRP9 has been detected by others. We therefore turned to murine tissues. Fig. 4A shows a Western blot of mouse tissue extracts, incubated with Mab M₉II-19. Only testis extracts contained a 150-kDa band co-migrating with the 150-kDa band in HEK293/MRP9 cell extract. More rapidly migrating bands were observed in mouse liver extracts, but these were non-specific as they were also present in the control with second antibody only (results not shown). The 150 kDa band in mouse testis reacted with 6 of our 7 anti-MRP9 Mabs; only M₉II-26 was negative (Fig. 4B), although it reacted as intensely with human MRP9 as the other Mabs (Fig. 3A). Mab₉II-26 therefore provided a convenient control for further mouse studies. Whereas the Mabs directed against the N-terminus of MRP9 (M₉I series) only stained a 150-kDa band, the Mabs directed against the linker region (M₉II series; see Fig. 1) also stained a band of about 105 kDa (Fig. 4 A and B, indicated by arrow). This is unlikely to be a protein corresponding to one of the known alternatively spliced RNAs of mouse Mrp9 [24], as both RNAs encode the N-terminal sequence of Mrp9 and the corresponding proteins should therefore also react with the Mabs of the M₉I series (Fig. 1A) It is therefore either a degradation product of Mrp9 or the result of an internal translation start. The variable amount of this band and its absence in other tissues than testis makes the degradation product alternative the more likely one.

In a (failed) attempt to generate a mouse Mrp9 KO by oligonucleotide targeting [33] we obtained mice producing MRP9 without the N-terminal segment used to get the M₉I series of antibodies. Western blots of testis extracts of these mice contained a 150-kDa band that reacted with the M₉II series of Mabs, but not the M₉I series, confirming the specificity of the M₉I series for MRP9 (see supplementary Fig. 1). We estimate that the missing segment in truncated Mrp9 is only 31 aa (see supplementary material), explaining why the truncated Mrp9 co-migrates with wild-type Mrp9.

We studied the distribution of Mrp9 in mouse testis by immunocytochemistry using 3 Mabs. Fig 4C shows strong staining of the testicular tubules with the Mrp9-specific Mabs M₉I-38 and M₉II-3, but not with M₉II-26, which does not react with murine Mrp9 (Fig 4B). No significant staining was observed in Leydig cells, or other interstitial elements. Within the seminiferous tubules we found no staining of the Sertoli cells and Mrp9 staining appeared to be specific for testicular germ cells.

During mouse testis development, Mrp9 was first detected in mice three weeks of age (fig. 5A). Only a few cells were positive in 3-week testis (Fig. 5B), the pachytene spermatocytes. As the mice aged, more Mrp9-positive cells appeared (Fig. 5B). In the adult seminiferous tubules, specific associations of subsequent generations of spermatogonia, spermatocytes and spermatids at particular development steps, occur in a cyclic manner referred to as stages of the cycle of the seminiferous epithelium [34]. To study which types of cells during spermatogenesis were positive for Mrp9, we investigated sections of seminiferous tubules in the different epithelial stages, of which there are 12 in the mouse. During germ cell differentiation in the adult mouse testis, pachytene spermatocytes in stage VI of the epithelial cycle were the first germ cells to show Mrp9 expression (Figure 6B). Subsequently, Mrp9 stayed present during further development of the spermatocytes, round spermatids and elongating spermatids (Figure 6A). During the maturation of the spermatids, Mrp9 accumulated in the tail of elongating spermatids (Figure 6B). There was no significant staining of spermatogonia or pachytene spermatocytes in stage I-IV (Figure 6A), of type B spermatogonia in stage VI (Figure 6B), in leptotene spermatocytes in stage VIII-IX (Figure 6C) or in leptotene/zygotene spermatocytes in stage X (Figure 6D).

Analysis of Mrp9 in mouse sperm

To study whether mature mouse sperm contain Mrp9, we analysed sperm from the epididymis. Fig. 7A shows a Western blot of mature sperm lysates analysed with 6 different antibodies. All three antibodies of the M₉I series detect the characteristic 150 kDa Mrp9 band, M₉II-3 and -19 detect this band and the additional 105 kDa band, whereas the control Mab M₉II-26 yields an “empty” lane. The background with control Mab M₉II-26 is somewhat higher in epididymis (Fig. 7B) than in testis (Fig. 4C). The upper part of panel B of Fig. 7

shows specific staining in the lumen of the epididymal ducts and originates from the sperm that are migrating towards the ductus deferens, but not in the epithelium. That the sperm cells are responsible for the luminal staining is confirmed in the lower half of Fig. 7B and in Fig. 7C. The Mrp9-specific Mabs stain the midpiece of sperm; the control Mab M₉II-26 gives no staining at all.

Post-translational modification and intra-cellular location of MRP9 in transfected HEK cells

In polyacrylamide gels human MRP9 and murine Mrp9 run faster than MRP5, although these proteins are of similar size on the basis of their amino acid sequences. The MRP9 band is also much sharper on Western blots than of the other MRPs, which are all heavily glycosylated.

To test whether any MRP9 glycosylation was detectable, we incubated cell lysates of HEK293/MRP9 and of mouse testis with endoglycosidases, prior to Western blot analysis. As shown in supplementary Fig. 2, no indication for glycosylation of MRP9/Mrp9 was obtained.

The lack of detectable N-linked glycosylation of MRP9 suggests that it is not routed through the Golgi cisternae to the plasma membrane or to a post-Golgi endosomal compartment. Indeed, Fig. 3B does not show MRP9 in the plasma membrane or in lysosomes, the usual final destinations of MRPs in transfected cells [35]. To analyse the distribution of MRP9 in the transfected HEK cells in more detail, we did co-localization studies with markers for intra-cellular membranous compartments. As shown in Fig. 8, MRP9 is clearly not located in mitochondria (represented by the mitochondrial outer membrane marker Sam50) and also not in lysosomes (analysed with the lysosomal marker LAMP-1; results not shown). Co-localization was most extensive with the ER markers protein disulfide isomerase (PDI) and calnexin, as also shown by the pixel analysis in supplementary Fig. 3. Lack of colocalization of MRP9 with mitochondrial markers was also observed in HEK293 cells transfected with other MRP9 constructs and with an EGFP-MRP9 fusion construct (see Methods).

Localization of Mrp9 in murine and boar sperm

Although the staining for Mrp9 of sperm midpiece shown in Fig. 7B is clear, we were unable to resolve the intracellular localization. The staining was too weak for reliable immunofluorescence staining; none of our 6 MAbs were suitable for (cryo) electron microscopy and neither were the two anti-MRP9 sera made. We therefore turned to cell fractionation. Sucrose gradients of sub-cellular fractions of mouse testis showed that Mrp9 clearly separated from the mitochondrial markers Sam50 and cytochrome *c*, confirming that Mrp9 is not in mitochondria (supplementary Fig. 4). In contrast, MRP9 did not separate

unambiguously from the lysosomal marker LAMP1 or the ER markers calnexin (Supplementary Fig. 4) or calreticulin (not shown).

Attempts to fractionate mouse sperm cells failed for lack of material and we therefore turned to boar sperm. Of our 7 MRP9-specific Mabs, 2 reacted well with the boar sperm midpiece, as shown in Fig. 9, and these antibodies also detected a band of approximately 150 kDa on Western (protein) blots (results not shown). Unfortunately, our attempts to separate a boar sperm homogenate into sub-cellular fractions failed, as the membranous fractions aggregated so strongly that we were unable to separate them in a sucrose gradient.

DISCUSSION

MRP9 was the last member of the MRP family cloned and no more will follow. The genome sequence is complete and the only other candidate MRP left, - MRP10 (ABCC13) – is clearly a pseudo-gene [36;37]. MRP9 is an unusual MRP in many respects; it contains no detectable N-linked glycosylation; the full-length protein is only made in detectable amounts in testicular germ cells and sperm; it appears to be localized mainly in the ER in transfected HEK293 cells and in a still poorly defined (non-mitochondrial) membrane fraction in sperm. We have tried to find a substrate for MRP9 by testing the ability of MRP9 to induce cellular resistance to cytotoxic compounds and to transport known MRP substrates in vesicular transport, without any positive result thusfar (see supplementary materials).

We have been unable to detect any form of MRP9 in any human tissue or cell line. Given the fact that MRP9 RNA was readily detected in many tissues and in cell lines from various tumors, this is puzzling. Each of the 7 Mabs that we raised against human MRP9 (Fig. 1A) should have sensitively detected both full-length MRP9 and all long splice variants described. This includes the 100-kDa band detected by Bera et al. [25] in testis and in a breast cancer cell line. It is possible (but unlikely) that the single polyclonal antibody used by Bera et al. [25] was a more sensitive tool than the 7 Mabs we generated. It is also possible that the Bera antiserum reacted with a 100-kDa cross-reacting band and that there is very little translation of the MRP9 RNA detected with PCR. It may be significant that there are hardly any published RNA blots containing MRP9 transcripts; most MRP9 transcripts were thusfar detected by PCR, indicating low expression of MRP9.

MRPs evolve rapidly and substantial differences have been observed between human MRPs and their murine counterparts. The most striking thus far is the absence of MRP8 in mice [20]. Nevertheless, it seems unlikely that MRP9 is a pseudo-gene in humans, as the basic structure of MRPs is fully conserved in MRP9 and as it is 84.5 % identical in amino

acid sequence to murine Mrp9, a high level of identity conserved over all the 29 putative exons of MRP9.

Our results suggest a role for MRP9 during the latter part of the male meiotic prophase, the development of spermatids and/or possibly in sperm function. MRP9 is clearly and exclusively localized in the sperm mid-piece, the anterior part of the flagellum characteristically containing all sperm's mitochondria. Nevertheless we are confident that MRP9 is not in mitochondria. Conventional wisdom has it that sperm does not contain ER, the sub-cellular fraction in which we find most of the MRP9 made in transfected HEK293 cells. However, Olson and Winfrey have described a mid-piece specific sub-mitochondrial reticulum in mammalian spermatozoa [38], and this structure remains a possible location for MRP9. The generation of a Mrp9 KO mouse may shed more light on MRP9 function.

ACKNOWLEDGEMENTS

We are grateful to Elly Mesman and Dr. P. Peters (NKI-AVL) and to Dr. L. Looijinga and Dr. J.W. Oosterhuis (Erasmus Medical Centre) for advice and help with microscopy; to M. Dekker and Dr. H. te Riele (NKI-AVL) for help in the generation of mice with a truncated form of Mrp9; and to Dr. J. Wijnholds (Netherlands Institute for Neuroscience) for advice and assistance. Dr. N. Ono was supported by a postdoctoral fellowship from the Japan Research Foundation for Clinical Pharmacology. This work was also supported by grants NKI 2001-2473 and -2474 of the Dutch Cancer Society to P. Borst and J. Wijnholds.

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607

Legends to Figures

Figure 1 Segments of MRP9 used for raising antibodies

The figure shows the putative topology of MRP9 in the membrane (23). Arrows above the membrane indicate the protein segments used to raise rabbit polyclonal antibodies against a fusion protein containing amino-acids 1-33 (9 FP-A) or amino acids 1317-1349 (9 FP-D). Arrows below the membrane indicate the MRP9 segments used to generate rat monoclonal antibodies. Series I was raised against a mixture of fusion proteins containing amino acids 1-33 and 1-42; series II against amino acids 690-734. All series I antibodies react with the fusion protein containing amino acids 1-33. See Methods for details.

Figure 2 MRP RNA levels in rat, mouse and human tissues, determined by semi-quantitative RT-PCR.

Note differences in scale for human versus rodent tissues. See Methods for details.

Figure 3 Detection of MRP9 in HEK 293 cells transfected with a MRP9 cDNA construct

(A) Western blot of cell lysates from transfected and parental HEK 293 cells stained with 4 different rat anti-MRP9 MABs.
(B) Immunocytochemical staining of transfected and parental HEK 293 cells stained with 3 different rat MABs. See Methods for details.

Figure 4 Tissue distribution of MRP9 in mice

(A) Western blot analysis of MRP9 in protein extracts of various mouse tissues, using rat Mab M₉II-19. The asterisk indicates a non-specific band reacting with the second antibody. The arrowhead indicates the 105 kDa band.
(B) Western blot analysis of MRP9 in mouse testis extracts, using three different rat anti-MRP9 MABs, as indicated. The arrowhead indicates the 105 kDa band.
(C) Sections of adult mouse testis, stained with two rat MABs that detect MRP9 and a control Mab M₉II-26 that does not react with mouse MRP9, as shown in panel B. See Methods for details.

Figure 5 Appearance of Mrp9 during mouse development

(A) Western blots of testis extracts from mice 2 (two different mice) or 3 weeks after birth with adult testis extract for comparison.
(B) Immunocytochemistry of testis sections of mice of the age indicated. AEC was used instead of DAB to visualize the antibody signal. Mab₉I-38 was used in both panels.

Figure 6 Immunohistochemical staining of Mrp9 in different stages of the seminiferous tubule cycle, using anti-MRP Mab M₉II-3

- (A) stage I-IV;
- (B) stage VI;
- (C) stage VIII-IX;
- (D) stage X.

Figure 7 MRP9 in murine epididymis and in aspirated cauda epididymal sperm

- (A) Western blot of murine sperm extracts probed with 6 different rat anti-MRP MABs. For comparison an extract of HEK 293 / MRP9 cells probed with Mab M₉I-38 is shown. The arrowhead indicates the 105 kDa band.
- (B) The upper panel shows sections of mouse epididymis stained with the rat MABs indicated. The control was only treated with the second antibody. The lower panel shows mouse sperm stained with the same set of antibodies. See Methods for details.
- (C) A single mouse sperm stained with Mab M₉II-19 at higher magnification.

Figure 8 Immunolocalization of MRP9 in HEK 293 / MRP9 cells

In the upper 2 panels, cells were fixed for 20 minutes with formalin at room temperature. After washing and permeabilization they were incubated with Mab M₉II-19 (undiluted) or rabbit anti-PDI (protein disulfide isomerase, 1:200) (upper panel) or rabbit anti-human-calnexin (middle panel) for 2 hours at room temperature. After washing the MRP9 Mab was detected with goat anti-rat Ig coupled to Alexa 488 (green); the PDI antibody with goat anti-rabbit Ig coupled to Texas Red (upper panel; 1:200); and the calnexin antibody with the Texas Red Ig (middle panel; 1:400) by incubation for 1 hour at room temperature. In the lower panel cells were fixed for 5 minutes with icecold methanol. MRP9 was detected with Mab M₉I-38 (1:2 diluted); Sam50 was detected with a rabbit polyclonal Ig raised against human Sam50 (1:200). The secondary antibodies were the same as in the upper panels. See Methods for further details.

Figure 9 Immunolocalization of MRP9 in boar sperm, using rat MABs directed against human MRP9

The panel shows from top to bottom: boar sperm stained with Mab M₉II-3, M₉II-19, or M₉II-26 (which does not react with pig MRP9). The presence of the anti-MRP9 Mab was detected by peroxidase staining as described in Methods, resulting in coarse granular precipitates of the peroxidase reaction product.

Figure 1

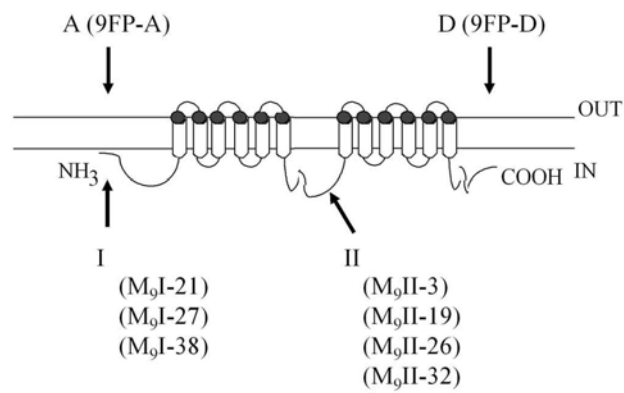


Figure 2

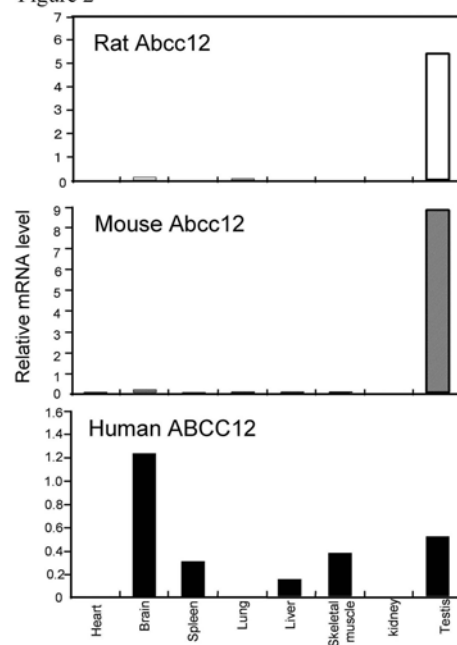


Figure 3

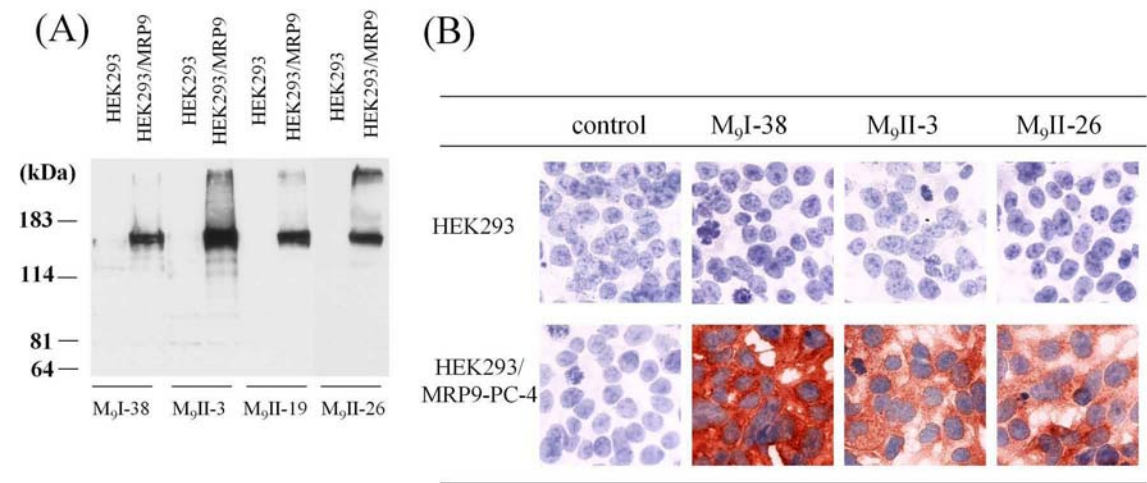


Figure 4

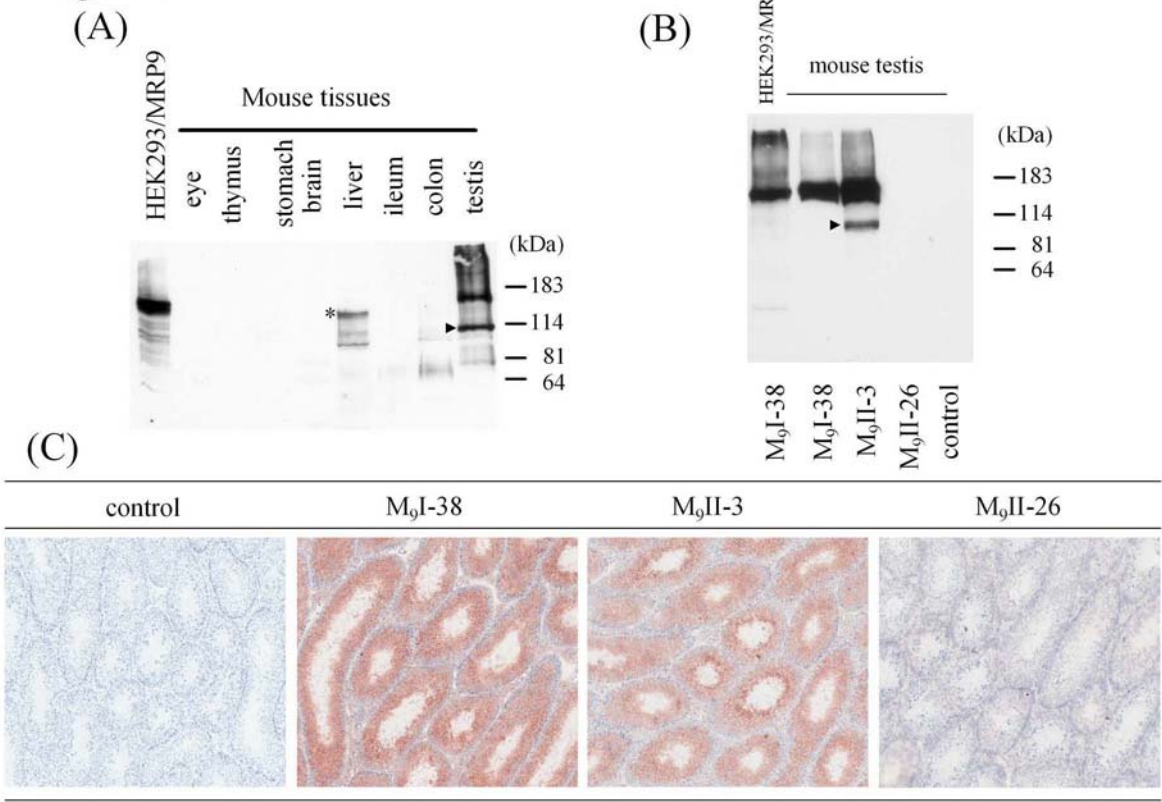
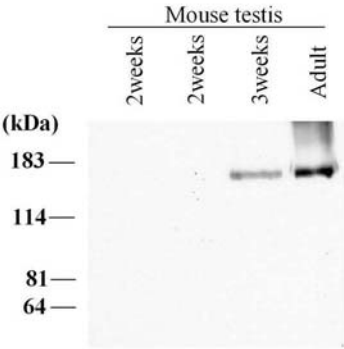


Figure 5

(A)



(B)

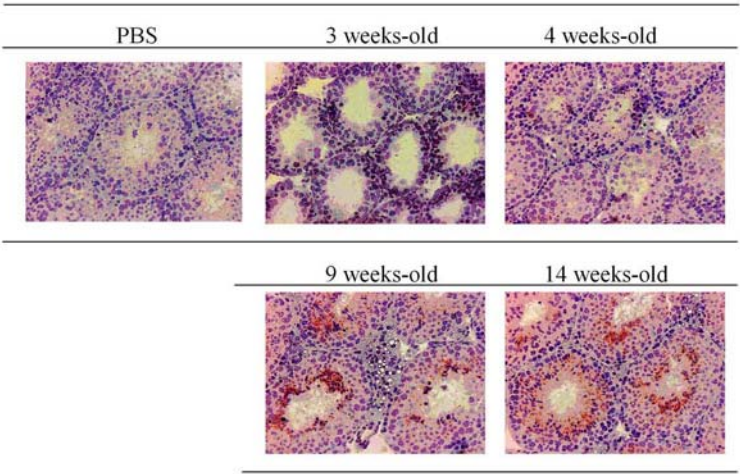


Figure 6

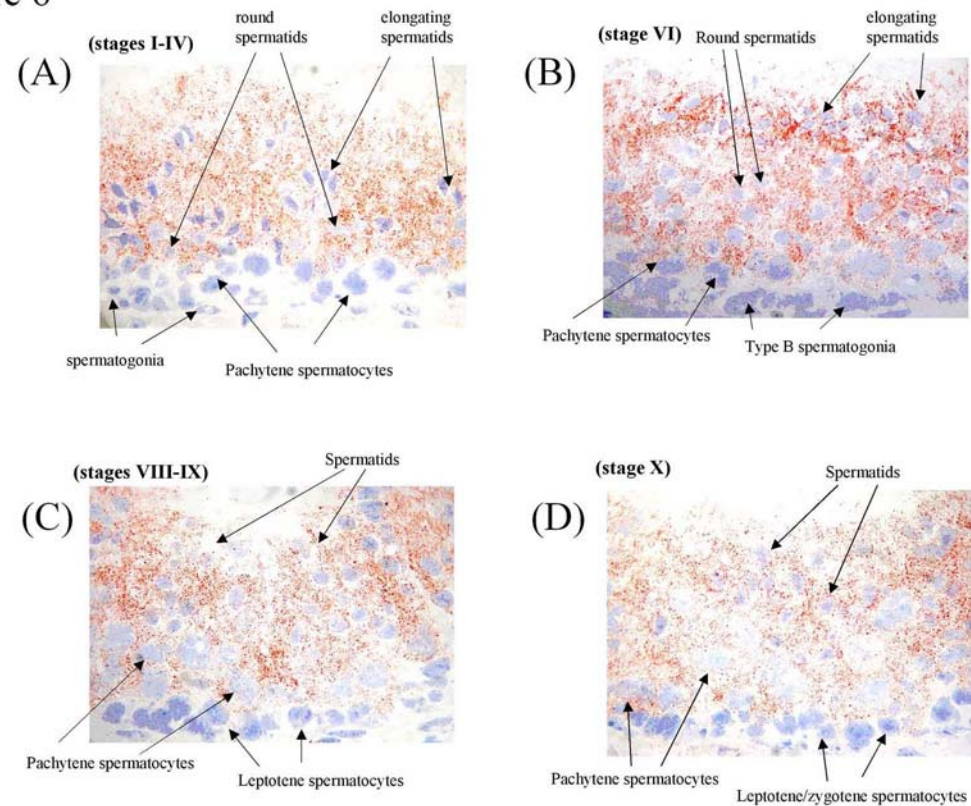


Figure 7

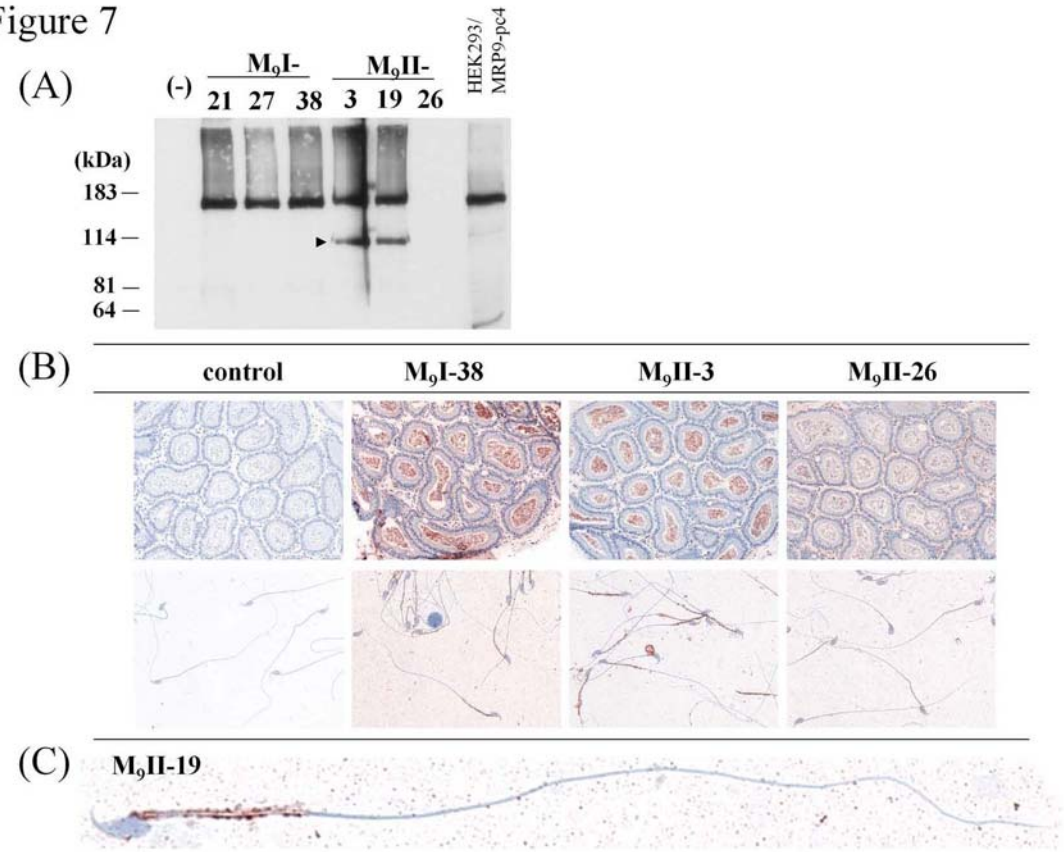
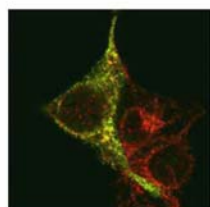
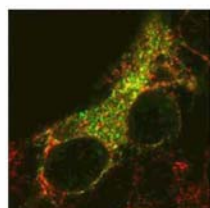


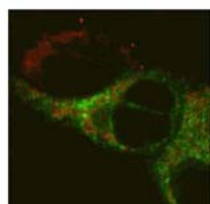
Figure 8



HEK293-MRP9 cells
MRP9 (green) / PDI (red)



HEK293-MRP9 cells
MRP9 (green) / Calnexin (red)

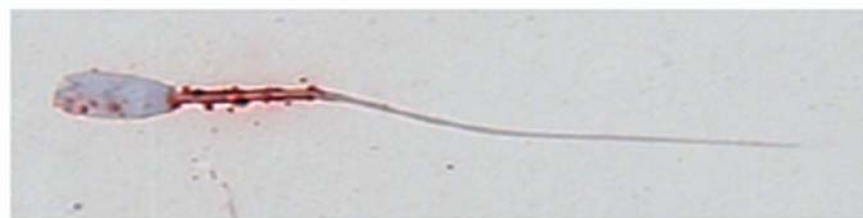


HEK293-MRP9 cells
MRP9 (green) / Sam50 (red)

Figure 9



M₉II-3



M₉II-19



M₉II-26