



HAL
open science

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

N Ono, I van Der Heijden, G L Scheffer, K van de Wetering, E van Deemter, M de Haas, A Boerke, B M Gadella, D G de Rooij, J J Neefjes, et al.

► To cite this version:

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-00478766>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

3
4 Running title: Intracellular localization of MRP9.
5

6 Nobuhito ONO*[†], Ingrid VAN DER HEIJDEN[†], George L. SCHEFFER[‡], Koen VAN DE
7 WETERING[†], Elizabeth VAN DEEMTER[†], Marcel DE HAAS[†], Arjan BOERKE[§], Bart M.
8 GADELLA[§], Dirk G. DE ROOIJ^{||}, Jacques J. NEEFJES[¶], Tom A.M. GROOTHUIS[¶], Laurant
9 OOMEN[¶], Lenny BROCKS[¶], Toshihisa ISHIKAWA¹, Piet BORST^{†,2}

10

11 * Present address: The 2nd Department of Internal Medicine, Faculty of Medicine, Kagoshima University, 8-35-1
12 Sakuragoaka, Kagoshima 890-8520, Japan.

13 [†] Division of Molecular Biology and Center of Biomedical Genetics, The Netherlands Cancer Institute,
14 Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

15 [‡] Department of Pathology, Free University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The
16 Netherlands.

17 [§] Department of Biochemistry and Cell Biology, Department of Farm Animal Health, Faculty of Veterinary
18 Medicine, Utrecht University, Yalelaan 2, 3508 TD Utrecht, The Netherlands.

19 ^{||} Departments of Endocrinology, Faculty of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, and of
20 Cell Biology, UMCU, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands.

21 [¶] Division of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The
22 Netherlands.

23 ¹ Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of
24 Technology, 4259 Nagatsuta, Midozuku, Yokohama 226-8501, Japan.

25 ² To whom correspondence should be addressed (tel. +31-20-512 2880; fax: +31-20-669 1383; email
26 p.borst@nki.nl).

27 SYNOPSIS

28

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.

41

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

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57

58

59

60

61

62 **INTRODUCTION**

63

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

77 The most recent addition to the MRP family is MRP9 (ABCC12), independently
 78 described in 2001 by Yabuuchi et al. [21], Tammur et al. [22] and Bera et al. [23]. MRP9 is
 79 most closely related to MRP5 and MRP8. Human MRP9 is located next to MRP8 on
 80 chromosome 16q12.1 [21], presumably the result of a recent duplication [22]. Whereas no
 81 mouse homolog of MRP8 (ABCC11) has been found [24], the cDNA of murine Mrp9
 82 (Abcc12) was cloned and characterized by Shimizu et al. [24]. Both the human MRP9 and the
 83 mouse Mrp9 genes yield a plethora of alternatively spliced RNAs and this has led to
 84 uncertainty about the true nature of the protein encoded by MRP9. The gene structure looks
 85 unambiguous. It predicts 29 exons both in human and mouse, encoding a classical “short”
 86 MRP, lacking the N-terminal domain present in MRP1, 2, 3, 6, and 7, but absent in MRP4, 5,
 87 and 8. The predicted amino acid sequences of murine Mrp9 and human MRP9 are 84.5%
 88 identical and their hydropathy profiles are nearly identical [24]. With MRP5 the human
 89 MRP9 shares 42% identity and all conserved elements in MRPs are also present in MRP9
 90 [22].

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

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

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

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

114
115

116 MATERIALS AND METHODS

117

118 Chemicals and reagents

119 DMM (1-deoxymannojirimycin) was made by Broxterman et al. [26] and was supplied by
120 J.N. A polyclonal antibody against the human mitochondrial outer membrane protein Sam50
121 [27] was generated by T.G. and J.N. Sam50 fused to glutathione-S-transferase was made in
122 *Escherichia coli* (*E. coli*) BL 21, purified over glutathione beads, and eluted with thrombin.
123 The thrombin was removed with a heparin column (Amersham) and the purified Sam50 was
124 injected into rabbits according to standard protocols. The rabbit antisera recognized a
125 mitochondrial protein in confocal microscopy and stained a 50 kDa band on Western blots of
126 human, mouse and pig tissues.

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

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

136

137 **Protein immunoblot (Western) analysis**

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

145

146 **Transfected cell lines**

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

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

170 The presence of MRP9 in these vesicles was checked with our new anti-MRP9 Mab M₉I-27,
171 1 µl of vesicle protein producing a clear 150-kDa band on a Western blot.

172

173 **Cloning of rat Mrp9 (Abcc12) cDNA**

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

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

199

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

201 The expression of human MRP9, mouse *Mrp9* and rat *Mrp9* in different organs was examined
202 by quantitative PCR with human, mouse and rat Multiple Tissue cDNA (MTC) panel
203 (Clontech, Palo Alto, CA), respectively. The PCR was performed with TaKaRa Ex TaqTM R-
204 PCR version in a TaKaRa Smart CyclerTM System (TaKaRa, Japan), where amplification of
205 DNA was monitored with SYBR Green I. The reaction mixture contained 2 µl of cDNA
206 (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

207 primers, 0.3 μ l of 250 mM Mg²⁺ solution, 0.25 μ l of 1/300-diluted SYBR Green I (Bio-
 208 Whittaker Molecular Applications, Rockland, ME, USA), and 0.25 μ l of Ex Taq™
 209 polymerase in a total volume of 25 μ l. For this analysis, the following specific primers were
 210 used; for human (the forward primer 5'- GTAAGGTACAACCTTGGATCCCT -3' and the
 211 backward primer: 5'- TGCTGCTAGTAACATCGCAA -3'), for mouse (the forward primer
 212 5'- TATGGCCCGGG CACTTCTCCGTAA -3', the backward primer 5'-
 213 GACCTTTACAGTCCAACCTCTG CAGCTAGT -3') and for rat Mrp9 (the forward primer:
 214 5'- GTAGGAGAACGCCAGCTGCTTTGTATGG -3' and the backward primer: 5'-
 215 GACCTTTACAGTCCAACCTTCTGCAGCTAGT -3');. The PCR cycle consisted of four
 216 steps; i.e., denaturation (95°C, 15 sec), annealing (60°C, 15 sec), amplification (72°C, 15 sec)
 217 and quantification (87°C, 10 sec).

218

219 **Rat monoclonal antibodies**

220 For the generation of antibodies against MRP9, segments of MRP9 cDNA (Fig. 1) were
 221 subcloned into the pMal-c vector for production of a fusion protein of *E. coli* maltose binding
 222 protein with MRP9 segments. The fusion protein was produced in *E. coli* strain JM101 and
 223 purified by amylose affinity chromatography. The use of the fusion protein for the production
 224 of rat monoclonal antibodies was as described [31]. Briefly, the Mabs were selected by an
 225 ELISA test, and positive clones were cross-checked on cytospins of HEK293/MRP9 and
 226 HEK293 cells. The antibodies obtained were finally screened on Western blots of cellular
 227 lysates prepared from HEK293 cells transfected with MRP9 cDNA. Three mAbs of the M₀I
 228 series against the N-terminal segment, and 4 of the M₀II series against the linker segment
 229 were used for this study.

230

231 **Immunohistochemistry of cells and tissues**

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

238

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

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

241

242 **Isolation and immunostaining of sperm**

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

254 Boar semen was collected in the Cooperative Centre for Artificial Insemination in
255 Pigs "Utrecht en de Hollanden" (Bunnik, the Netherlands). Semen was filtered through gauze
256 to remove gelatinous material and diluted to 150 million cells/ml in Beltsville Thawing
257 Solution (BTS: 0.2 M glucose, 20 mM sodium citrate, 15 mM NaHCO₃, 3.36 mM Na₂EDTA,
258 10 mM KCl, 20 mM Hepes, pH 7.4) and kept at 17^o C for transport or storage in air-tight 80
259 ml insemination tubes. Prior to further experimentation the sperm samples were first washed
260 through BTS by centrifugation for 5 min at 400 x g and the resuspended sperm (1 billion
261 sperm/ml) was then spun through a discontinuous (35%/70%) Percoll® (Pharmacia, Uppsala,
262 Sweden) density gradient to remove epithelial cell contamination and immature sperm [32].
263 After washing the pellet with PBS, sperm smears were stained as for the mouse sperm, but
264 pre-blocking before adding anti-MRP Mabs was done with 4% BSA in PBS without the
265 donkey serum.

266

267 **Confocal microscopy of immunofluorescent cells**

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

279 cells were pre-blocked with 1% BSA in PBS for 30 min, incubated with primary antibody for
280 1 hr, washed with 1% BSA/PBS, incubated with the secondary antibody for 1 hr, washed with
281 1% BSA/PBS and PBS, and mounted in vectashield.

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

285

286 **Analysis of protein-linked oligosaccharides**

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

293

294 **Cell fractionation studies**

295 Mouse testis was dissected into small pieces in ice-cold mitobuffer (0.2 mM sodium-EDTA,
296 0.25 M sucrose, 10 mM Tris-chloride, pH 7.8) and homogenized by 15 strokes with a
297 Wheaton Dounce Tissue Grinder with tight pestle clearance (0.05 mm). The homogenate was
298 centrifuged for 10 min at 1000 g at 4⁰ C. The supernatant (S1) was centrifuged for 15 min at
299 8000 g at 4⁰ C (Sorvall, SS34 rotor), the pellet (P2) was washed twice with mitobuffer
300 containing protease inhibitors (Roche Complete tablets) and stored frozen. (P2) was thawed,
301 centrifuged and resuspended in a minimal volume of 0.8 M sucrose, containing 0.2 mM
302 EDTA / Tris pH 7.8 and layered on a 5-ml 0.9 – 2.0 M sucrose gradient (in 0.3 mM sodium
303 EDTA, 10 mM Tris, pH 7.8). After centrifugation to equilibrium (2 hrs, 4⁰ C, 80,000 g), about
304 15-20 fractions were collected through a hole punched in the bottom. The membranous
305 material in each fraction was collected by centrifugation for 10 min at 20,000 g. The pellets
306 were resuspended in mitobuffer.

307

308

309 **RESULTS**

310

311 **Generation of antibodies against MRP9**

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

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

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

331

332 **Detection of full-length Mrp9 in mouse tissues**

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

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

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

365 During mouse testis development, Mrp9 was first detected in mice three weeks of age
366 (fig. 5A). Only a few cells were positive in 3-week testis (Fig. 5B), the pachytene
367 spermatocytes. As the mice aged, more Mrp9-positive cells appeared (Fig. 5B). In the adult
368 seminiferous tubules, specific associations of subsequent generations of spermatogonia,
369 spermatocytes and spermatids at particular development steps, occur in a cyclic manner
370 referred to as stages of the cycle of the seminiferous epithelium [34]. To study which types of
371 cells during spermatogenesis were positive for Mrp9, we investigated sections of
372 seminiferous tubules in the different epithelial stages, of which there are 12 in the mouse.
373 During germ cell differentiation in the adult mouse testis, pachytene spermatocytes in stage
374 VI of the epithelial cycle were the first germ cells to show Mrp9 expression (Figure 6B).
375 Subsequently, Mrp9 stayed present during further development of the spermatocytes, round
376 spermatids and elongating spermatids (Figure 6A). During the maturation of the spermatids,
377 Mrp9 accumulated in the tail of elongating spermatids (Figure 6B). There was no significant
378 staining of spermatogonia or pachytene spermatocytes in stage I-IV (Figure 6A), of type B
379 spermatogonia in stage VI (Figure 6B), in leptotene spermatocytes in stage VIII-IX (Figure
380 6C) or in leptotene/zygotene spermatocytes in stage X (Figure 6D).

381

382 **Analysis of Mrp9 in mouse sperm**

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

389 shows specific staining in the lumen of the epididymal ducts and originates from the sperm
 390 that are migrating towards the ductus deferens, but not in the epithelium. That the sperm cells
 391 are responsible for the luminal staining is confirmed in the lower half of Fig. 7B and in Fig.
 392 7C. The Mrp9-specific Mabs stain the midpiece of sperm; the control Mab M₉II-26 gives no
 393 staining at all.

394

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

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

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

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

416

417 **Localization of Mrp9 in murine and boar sperm**

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

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

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

433
 434

435 **DISCUSSION**

436

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

446 We have been unable to detect any form of MRP9 in any human tissue or cell line.
 447 Given the fact that MRP9 RNA was readily detected in many tissues and in cell lines from
 448 various tumors, this is puzzling. Each of the 7 Mabs that we raised against human MRP9 (Fig.
 449 1A) should have sensitively detected both full-length MRP9 and all long splice variants
 450 described. This includes the 100-kDa band detected by Bera et al. [25] in testis and in a breast
 451 cancer cell line. It is possible (but unlikely) that the single polyclonal antibody used by Bera
 452 et al. [25] was a more sensitive tool than the 7 Mabs we generated. It is also possible that the
 453 Bera antiserum reacted with a 100-kDa cross-reacting band and that there is very little
 454 translation of the MRP9 RNA detected with PCR. It may be significant that there are hardly
 455 any published RNA blots containing MRP9 transcripts; most MRP9 transcripts were thusfar
 456 detected by PCR, indicating low expression of MRP9.

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

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

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

472

473 ACKNOWLEDGEMENTS

474

475 We are grateful to Elly Mesman and Dr. P. Peters (NKI-AVL) and to Dr. L. Looijinga and Dr.
476 J.W. Oosterhuis (Erasmus Medical Centre) for advice and help with microscopy; to M.
477 Dekker and Dr. H. te Riele (NKI-AVL) for help in the generation of mice with a truncated
478 form of Mrp9; and to Dr. J. Wijnholds (Netherlands Institute for Neuroscience) for advice and
479 assistance. Dr. N. Ono was supported by a postdoctoral fellowship from the Japan Research
480 Foundation for Clinical Pharmacology. This work was also supported by grants NKI 2001-
481 2473 and -2474 of the Dutch Cancer Society to P. Borst and J. Wijnholds.

482

483 REFERENCES

484

485

- 486 1 Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K.
487 C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V. and Deeley, R. G. (1992)
488 Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell
489 line. *Science* **258**, 1650-1654
- 490 2 Büchler, M., König, J., Brom, M., Kartenbeck, J., Spring, H., Horie, T. and Keppler, D.
491 (1996) cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance
492 protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic
493 mutant rats. *J. Biol. Chem.* **271**, 15091-15098
- 494 3 Paulusma, C. C., Bosma, P. J., Zaman, G. J. R., Bakker, C. T. M., Otter, M., Scheffer,
495 G. L., Scheper, R. J., Borst, P. and Oude Elferink, R. P. J. (1996) Congenital jaundice in
496 rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271**,
497 1126-1128
- 498 4 Kool, M., De Haas, M., Scheffer, G. L., Scheper, R. J., Van Eijk, M. J. T., Juijn, J. A.,
499 Baas, F. and Borst, P. (1997) Analysis of expression of *cMOAT* (*MRP2*), *MRP3*, *MRP4*,
500 and *MRP5*, homologs of the multidrug resistance-associated protein gene (*MRP1*), in
501 human cancer cell lines. *Cancer Res.* **57**, 3537-3547

- 502 5 Raggars, R. J., van Helvoort, A., Evers, R. and van Meer, G. (1999) The human
503 multidrug resistance protein MRP1 translocates sphingolipid analogs across the plasma
504 membrane. *J. Cell Sci.* **112**, 415-422
- 505 6 Borst, P. and Oude Elferink, R. (2002) Mammalian ABC transporters in health and
506 disease. In *Ann. Rev. Biochem.* (Richardson, C. C., Kornberg, R., Raetz, C. H. R. and
507 Thorstensen, K., eds.), pp. 537-592, Science, California
- 508 7 Borst, P., Reid, G., Saeki, T., Wielinga, P. and Zelcer, N. (2003) The multidrug
509 resistance proteins 3-7. In *ABC proteins: from bacteria to man* (Holland, I. B., Kuchler,
510 K., Higgins, C. F. and Cole, S. P. C., eds.), pp. 445-458, Elsevier Science Ltd, London
- 511 8 Borst, P., Balzarini, J., Ono, N., Reid, G., De Vries, H., Wielinga, P., Wijnholds, J. and
512 Zelcer, N. (2004) The potential impact of drug transporters on nucleoside-analog-based
513 antiviral chemotherapy. *Antiviral Res.* **62**, 1-7
- 514 9 Borst, P., Zelcer, N., Van de Wetering, K. and Poolman, B. (2006) On the putative co-
515 transport of drugs by multidrug resistance proteins. *FEBS Letters* **580**, 1085-1093
- 516 10 Borst, P., De Wolf, C. and Van de Wetering, K. (2007) Multidrug Resistance associated
517 Proteins 3, 4 and 5. *Pflugers Arch.* **453**, 661-673
- 518 11 Ishikawa, T., Li, Z.-S., Lu, Y.-P. and Rea, P. A. (1997) The GS-X pump in plant, yeast,
519 and animal cells: structure, function, and gene expression. *Bioscience Rep.* **17**, 189-207
- 520 12 Ishikawa, T., Tien Kuo, M., Furuta, K. and Suzuki, M. (2000) The human multidrug
521 resistance-associated protein (MRP) gene family: from biological function to drug
522 molecular design. *Clin. Chem. Lab. Med.* **38**, 893-897
- 523 13 Adachi, M., Reid, G. and Schuetz, J. D. (2002) Therapeutic and biological importance
524 of getting nucleotides out of cells: a case for the ABC transporters, MRP4 and 5. *Adv.*
525 *Drug Deliv. Rev.* **54**, 1333-1342
- 526 14 Bodo, A., Bakos, E., Szeri, F., Varadi, A. and Sarkadi, B. (2003) The role of multidrug
527 transporters in drug availability, metabolism and toxicity. *Toxicol. Lett.* **140-141**, 133-
528 143
- 529 15 Haimeur, A., Conseil, G., Deeley, R. G. and Cole, S. P. (2004) The MRP-related and
530 BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and
531 regulation. *Curr. Drug. Metab.* **5**, 21-53
- 532 16 König, J., Nies, A. T., Cui, Y. and Keppler, D. (2003) MRP2, the apical export pump
533 for anionic conjugates. In *ABC Proteins: From Bacteria to Man* (Holland, I. B., Cole, S.
534 P. C., Kuchler, K. and Higgins, C. F., eds.), pp. 423-443, Elsevier Science Ltd., London
- 535 17 Kruh, G. D. and Belinsky, M. G. (2003) The MRP family of drug efflux pumps.
536 *Oncogene* **22**, 7537-7552
- 537 18 Renes, J., De Vries, E. G. E., Jansen, P. L. M. and Müller, M. (2000) The
538 (patho)physiological functions of the MRP family. *Drug Resist. Updates* **3**, 289-302
- 539 19 Deeley, R. G., Westlake, C. and Cole, S. P. (2006) Transmembrane Transport of Endo-
540 and Xenobiotics by Mammalian ATP-Binding Cassette Multidrug Resistance Proteins.
541 *Physiol. Rev.* **86**, 849-899

- 542 20 Yoshiura, K. I., Kinoshita, A., Ishida, T., Ninokata, A., Ishikawa, T., Kaname, T.,
543 Bannai, M., Tokunaga, K., Sonoda, S., Komaki, R., Ihara, M., Saenko, V. A., Alipov,
544 G. K., Sekine, I., Komatsu, K., Takahashi, H., Nakashima, M., Sosonkina, N.,
545 Mapendano, C. K., Ghadami, M., Nomura, M., Liang, D. S., Miwa, N., Kim, D. K.,
546 Garidkhuu, A., Natsume, N., Ohta, T., Tomita, H., Kaneko, A., Kikuchi, M.,
547 Russomando, G., Hirayama, K., Ishibashi, M., Takahashi, A., Saitou, N., Murray, J. C.,
548 Saito, S., Nakamura, Y. and Niikawa, N. (2006) A SNP in the *ABCC11* gene is the
549 determinant of human earwax type. *Nat. Genet.* **38**, 324-330
- 550 21 Yabuuchi, H., Shimizu, H., Takayanagi, S. and Ishikawa, T. (2001) Multiple splicing
551 variants of two new human ATP-binding cassette transporters, *ABCC11* and *ABCC12*.
552 *Biochem. Biophys. Res. Comm.* **288**, 933-939
- 553 22 Tammur, J., Prades, C., Arnould, I., Rzhetsky, A., Hutchinson, A., Adachi, M., Schuetz,
554 J. D., Swoboda, K. J., Ptacek, L. J., Rosier, M., Dean, M. and Allikmets, R. (2001) Two
555 new genes from the human ATP-binding cassette transporter superfamily, *ABCC11* and
556 *ABCC12*, tandemly duplicated on chromosome 16q12. *Gene* **273**, 89-96
- 557 23 Bera, T. K., Lee, S., Salvatore, G., Lee, B. and Pastan, I. (2001) *MRP8*, a new member
558 of ABC transporter superfamily, identified by EST database mining and gene prediction
559 program, is highly expressed in breast cancer. *Mol. Medicine* **7**, 509-516
- 560 24 Shimizu, H., Taniguchi, K., Hippo, Y., Hayashizaki, Y., Aburatani, H. and Ishikawa, T.
561 (2003) Characterization of the mouse *ABcc12* gene and its transcript encoding an ATP-
562 binding transporter, an orthologue of human *ABCC12*. *Gene* **310**, 17-28
- 563 25 Bera, T. K., Iavarone, C., Kumar, V., Lee, S., Lee, B. and Pastan, I. (2002) *MRP9*, an
564 unusual truncated member of the ABC transporter superfamily, is highly expressed in
565 breast cancer. *Proc. Natl. Acad. Sci. USA* **99**, 6997-7002
- 566 26 Broxterman, H. J. G., Neefjes, J. J., van der Marel, G. A., Ploegh, H. L. and Van Boom,
567 J. H. (1988) Synthesis of the antibiotic 1,5-dideoxy-1,5-imino-D-mannitol. *J.*
568 *Carbohydrate Chemistry* **7**, 593-603
- 569 27 Kozjak, V., Wiedemann, N., Milenkovic, D., Lohaus, C., Meyer, H. E., Guiard, B.,
570 Meisinger, C. and Pfanner, N. (2003) An essential role of Sam50 in the protein sorting
571 and assembly machinery of the mitochondrial outer membrane. *J. Biol. Chem.* **278**,
572 48520-48523
- 573 28 Wielinga, P. R., Reid, G., Challa, E. E., Van der Heijden, I., Van Deemter, L., De Haas,
574 M., Mol, C., Kuil, A. J., Groeneveld, E., Schuetz, J. D., Brouwer, C., De Abreu R.A.,
575 Wijnholds, J., Beijnen, J. H. and Borst, P. (2002) Thiopurine metabolism and
576 identification of the thiopurine metabolites transported by *MRP4* and *MRP5*
577 overexpressed in human embryonic kidney cells. *Mol. Pharmacol.* **62**, 1321-1332
- 578 29 Wielinga, P. R., Van der Heijden, I., Reid, G., Beijnen, J. H., Wijnholds, J. and Borst,
579 P. (2003) Characterization of the *MRP4*- and *MRP5*-mediated transport of cyclic
580 nucleotides from intact cells. *J. Biol. Chem.* **278**, 17664-17671
- 581 30 Burge, C. and Karlin, S. (1997) Prediction of complete gene structures in human
582 genomic DNA. *J. Mol. Biol.* **268**, 78-94
- 583 31 Scheffer, G. L., Kool, M., Heijn, M., De Haas, M., Pijnenborg, A. C. L. M., Wijnholds,
584 J., van Helvoort, A., de Jong, M. C., Hooijberg, J. H., Mol, C. A. A. M., Van der
585 Linden, M., De Vree, J. M. L., Van der Valk, P., Oude Elferink, R. P. J., Borst, P. and

- 586 Scheper, R. J. (2000) Specific detection of multidrug resistance proteins MRP1, MRP2,
587 MRP3, MRP5 and MDR3 P-glycoprotein with a panel of monoclonal antibodies.
588 *Cancer Res.* **60**, 5269-5277
- 589 32 Gadella, B. M. and Harrison, R. A. (2002) Capacitation induces cyclic adenosine 3',5'-
590 monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at
591 the apical head plasma membrane of boar sperm cells. *Biol. Reprod.* **67**, 340-350
- 592 33 Dekker, M., Brouwers, C., Aarts, M., van der Torre, J., de Vries, S., van de Vrugt, H.
593 and Te Riele, H. (2006) Effective oligonucleotide-mediated gene disruption in ES cells
594 lacking the mismatch repair protein MSH3. *Gene Ther.* **13**, 686-694
- 595 34 Russel, L. D., Ettlin, R. A., Sinha Hikim, A. P. and Clegg, E. D. (1990) Histological
596 and histopathological evaluation of the testis. pp. 119-161, Cache River Press,
597 Clearwater
- 598 35 Borst, P., Evers, R., Kool, M. and Wijnholds, J. (1999) The multidrug resistance protein
599 family. *Biochim. Biophys. Acta* **1461**, 347-357
- 600 36 Yabuuchi, H., Takayanagi, S., Yoshinaga, K., Taniguchi, N., Aburatani, H. and
601 Ishikawa, T. (2002) ABCC13, an unusual truncated ABC transporter, is highly
602 expressed in fetal human liver. *Biochem. Biophys. Res. Commun.* **299**, 410-417
- 603 37 Annilo, T. and Dean, M. (2004) Degeneration of an ATP-binding cassette transporter
604 gene, ABCC13, in different mammalian lineages. *Genomics.* **84**, 34-46
- 605 38 Olson, G. E. and Winfrey, V. P. (1990) Mitochondria-cytoskeleton interactions in the
606 sperm midpiece. *J Struct. Biol* **103**, 13-22
607

608 **Legends to Figures**

609

610 **Figure 1 Segments of MRP9 used for raising antibodies**

611 The figure shows the putative topology of MRP9 in the membrane (23). Arrows above the
612 membrane indicate the protein segments used to raise rabbit polyclonal antibodies against a
613 fusion protein containing amino-acids 1-33 (9 FP-A) or amino acids 1317-1349 (9 FP-D).
614 Arrows below the membrane indicate the MRP9 segments used to generate rat monoclonal
615 antibodies. Series I was raised against a mixture of fusion proteins containing amino acids 1-
616 33 and 1-42; series II against amino acids 690-734. All series I antibodies react with the
617 fusion protein containing amino acids 1-33. See Methods for details.

618

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

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

622

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

625 (A) Western blot of cell lysates from transfected and parental HEK 293 cells stained with
626 4 different rat anti-MRP9 MAbs.

627 (B) Immunocytochemical staining of transfected and parental HEK 293 cells stained with 3
628 different rat MAbs. See Methods for details.

629

630 **Figure 4 Tissue distribution of MRP9 in mice**

631 (A) Western blot analysis of MRP9 in protein extracts of various mouse tissues, using rat
632 Mab M₉II-19. The asterisk indicates a non-specific band reacting with the second
633 antibody. The arrowhead indicates the 105 kDa band.

634 (B) Western blot analysis of MRP9 in mouse testis extracts, using three different rat anti-
635 MRP9 MAbs, as indicated. The arrowhead indicates the 105 kDa band.

636 (C) Sections of adult mouse testis, stained with two rat MAbs that detect MRP9 and a control
637 Mab M₉II-26 that does not react with mouse MRP9, as shown in panel B. See Methods
638 for details.

639

640 **Figure 5 Appearance of Mrp9 during mouse development**

641 (A) Western blots of testis extracts from mice 2 (two different mice) or 3 weeks after birth
642 with adult testis extract for comparison.

643 (B) Immunocytochemistry of testis sections of mice of the age indicated. AEC was used
644 instead of DAB to visualize the antibody signal. Mab₉I-38 was used in both panels.

645

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

648 (A) stage I-IV;

649 (B) stage VI;

650 (C) stage VIII-IX;

651 (D) stage X.

652

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

654 (A) Western blot of murine sperm extracts probed with 6 different rat anti-MRP MAbs. For
655 comparison an extract of HEK 293 / MRP9 cells probed with Mab M₉I-38 is shown. The
656 arrowhead indicates the 105 kDa band.

657 (B) The upper panel shows sections of mouse epididymis stained with the rat MAbs
658 indicated. The control was only treated with the second antibody. The lower panel shows
659 mouse sperm stained with the same set of antibodies. See Methods for details.

660 (C) A single mouse sperm stained with Mab M₉II-19 at higher magnification.

661

662 **Figure 8 Immunolocalization of MRP9 in HEK 293 / MRP9 cells**

663 In the upper 2 panels, cells were fixed for 20 minutes with formalin at room temperature.
664 After washing and permeabilization they were incubated with Mab M₉II-19 (undiluted) or
665 rabbit anti-PDI (protein disulfide isomerase, 1:200) (upper panel) or rabbit anti-human-
666 calnexin (middle panel) for 2 hours at room temperature. After washing the MRP9 Mab was
667 detected with goat anti-rat Ig coupled to Alexa 488 (green); the PDI antibody with goat anti-
668 rabbit Ig coupled to Texas Red (upper panel; 1:200); and the calnexin antibody with the Texas
669 Red Ig (middle panel; 1:400) by incubation for 1 hour at room temperature. In the lower panel
670 cells were fixed for 5 minutes with icecold methanol. MRP9 was detected with Mab M₉I-38
671 (1:2 diluted); Sam50 was detected with a rabbit polyclonal Ig raised against human Sam50
672 (1:200). The secondary antibodies were the same as in the upper panels. See Methods for
673 further details.

674

675 **Figure 9 Immunolocalization of MRP9 in boar sperm, using rat MAbs directed**
676 **against human MRP9**

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

681

Figure 1

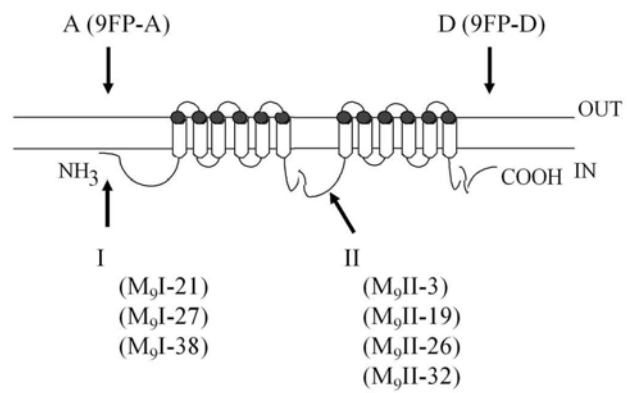


Figure 2

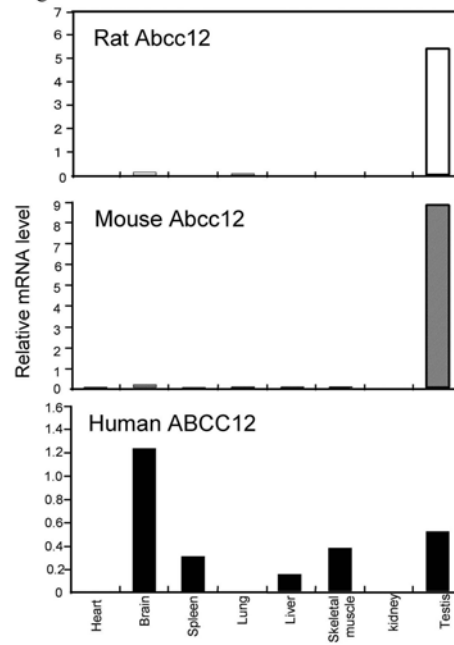


Figure 3

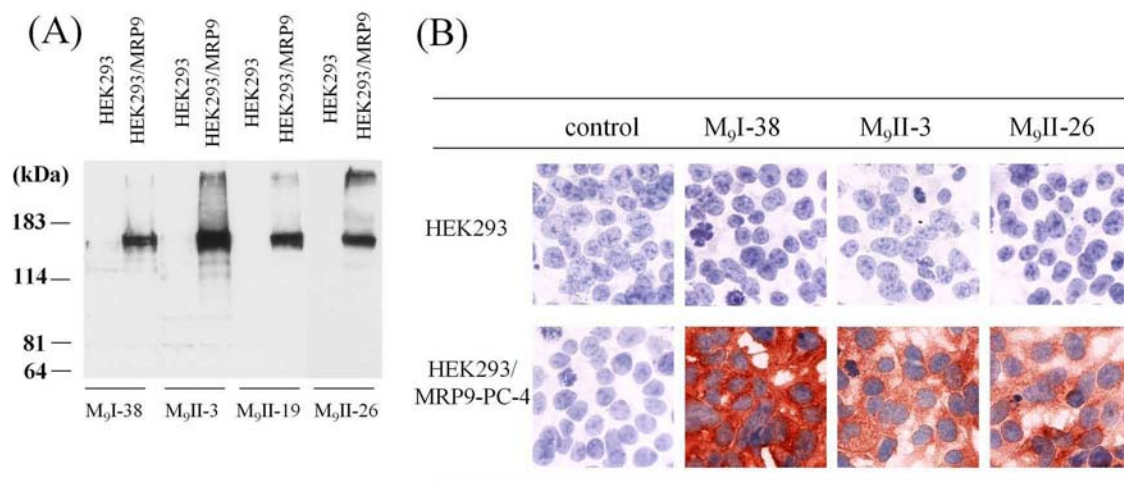


Figure 4

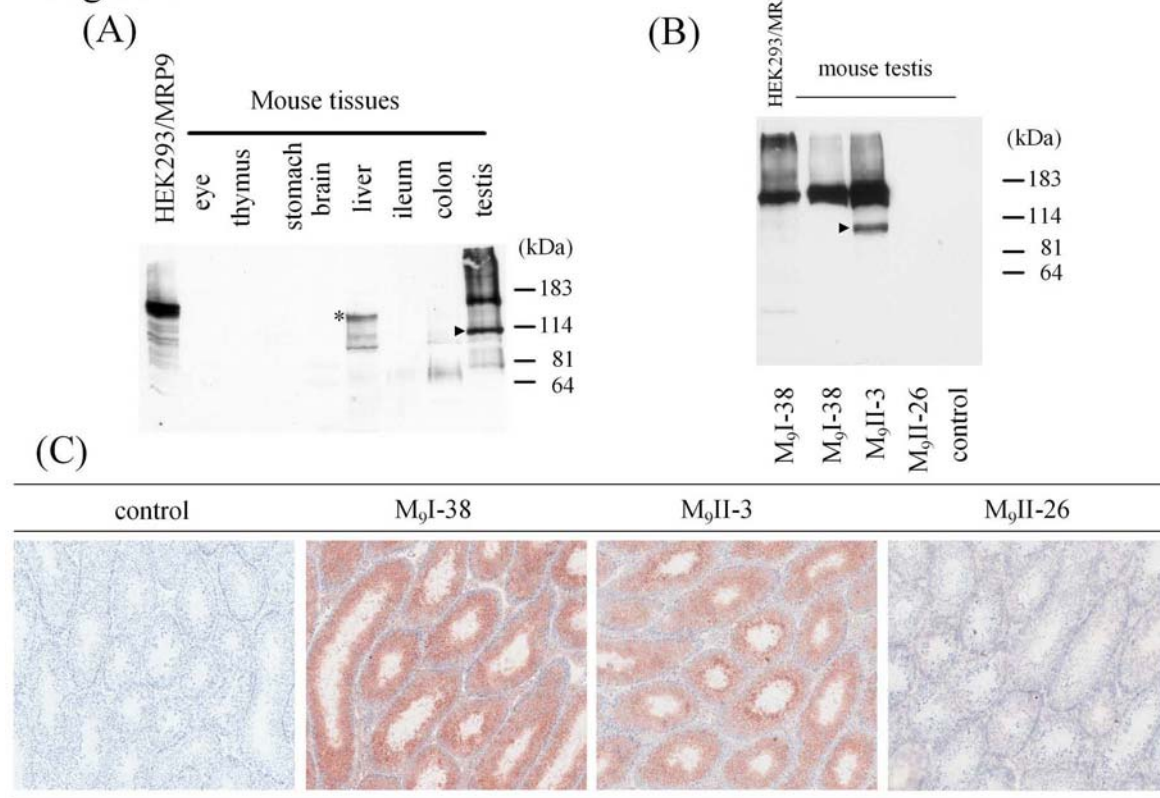
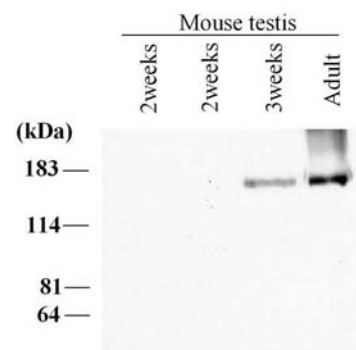


Figure 5

(A)



(B)

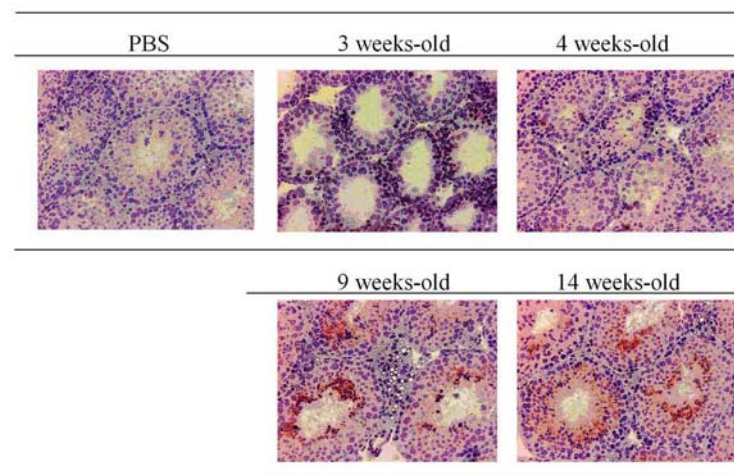


Figure 6

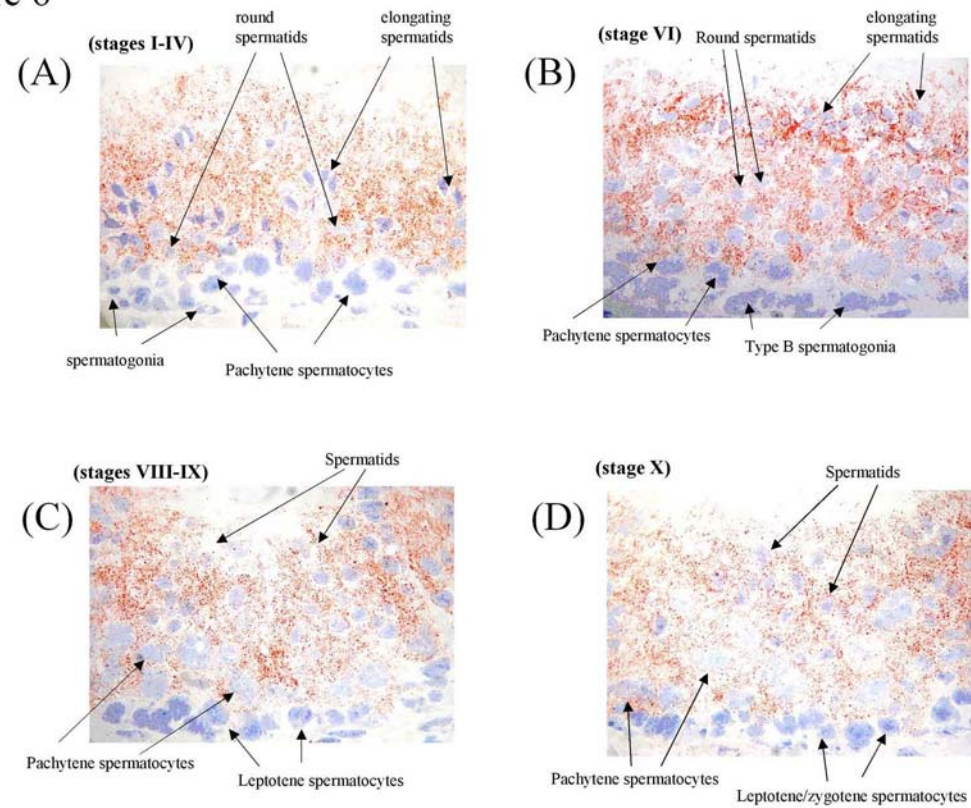


Figure 7

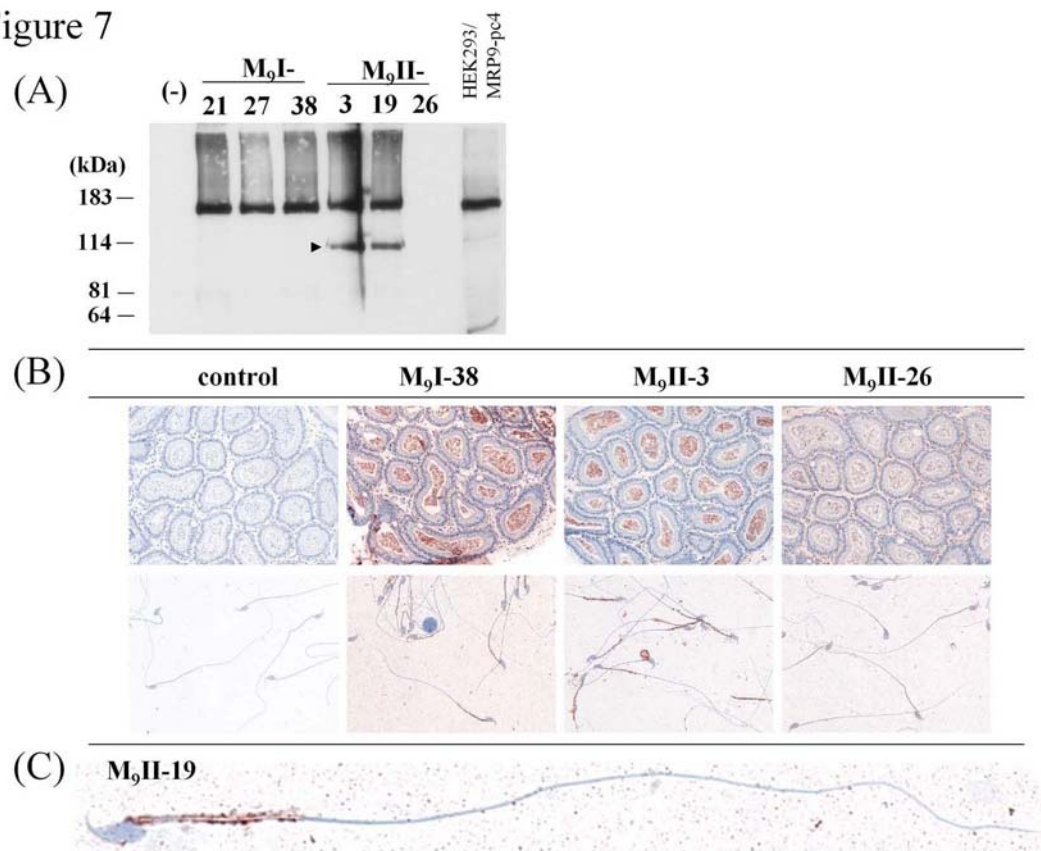
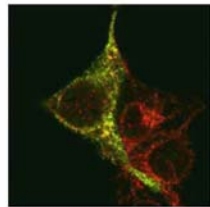
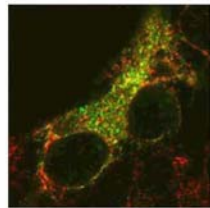


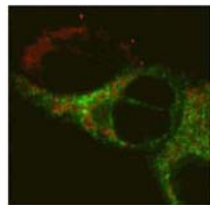
Figure 8



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



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

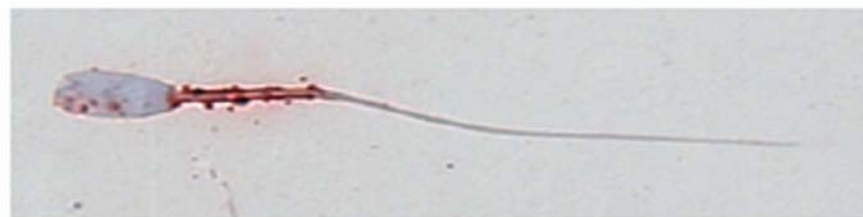


HEK293-MRP9 cells
MRP9 (green) / Sam50 (red)

Figure 9



M₉II-3



M₉II-19



M₉II-26