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RESEARCH ARTICLE

ABC transporters in gills of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Fish gills are a structurally and functionally complex organ at the interface between the organism and the aquatic environment. Gill functions include the transfer of organic molecules, both natural ones and xenobiotic compounds. Whether the branchial exchange of organic molecules involves active transporters is currently not known. Here, we investigated the presence, diversity and functional activity of ATP-binding cassette (ABC) transporters in gills of juvenile rainbow trout. By means of RT-qPCR, gene transcripts of members from the *abcb*, *abcc* and *abcg* subfamilies were identified. Comparisons with mRNA profiles from trout liver and kidney revealed that ABC transporters known to have an apical localization in polarized epithelia, especially *abcc2* and *abcb1*, were under-represented in the gills. In contrast, ABC transporters with mainly basolateral localization showed comparable gene transcript levels in the three organs. The most prominent ABC transporter in gills was an *abcb* subfamily member, which was annotated as *abcb5* based on the synteny and phylogeny. Functional *in vivo* assays pointed to a role of branchial ABC transporters in branchial solute exchange. We further assessed the utility of primary gill cell cultures to characterize transporter-mediated branchial exchange of organic molecules, by examining ABC transporter gene transcript patterns and functional activity in primary cultures. The gill cultures displayed functional transport activity, but the ABC mRNA expression patterns were different to those of the intact gills. Overall, the findings of this study provide evidence for the presence of functional ABC transporter activity in gills of fish.

KEY WORDS: ABC transporter, *abcb5*, Detoxification, Fish, Gills

INTRODUCTION

Tissues fulfilling transport, barrier and/or excretory functions such as the liver, kidney and blood–brain barrier possess a variety of membrane transporters, which function in active pumping of physiologically important solutes. A prominent family of membrane transporters are the ATP-binding cassette (ABC) transporters. The substrates for these transporters are mainly endogenous molecules but some of them are also active in the transport of xenobiotics and their metabolites. In mammals, three ABC transporter families are known to be involved in xenobiotic transport, namely the ABCB, ABCC and ABCG families (Leslie et al., 2005). For instance, the ABCB1 transporter has a broad

substrate range and is known to transport xenobiotics, similarly to ABCG2, which also plays a role in the first line of defense against xenobiotics at the blood–brain barrier and in the gut (Garg et al., 2015; Müller et al., 2017). The ABCC subfamily members predominantly transport anionic substrates including conjugated products of phase II biotransformation. It is assumed that the role of the three ABC families, ABCB, ABCC and ABCG, in xenobiotic membrane traffic is evolutionarily conserved between the vertebrate class of teleost fish and mammals (Luckenbach et al., 2014).

Fish gills serve as an important interface between the organism and the surrounding water (Evans et al., 2005). Gills have a complex organ architecture and they have a variety of physiological functions, such as respiration, ionoregulation and osmoregulation. The large epithelial surface of the gills, with its short diffusion distances, as well as the continuous flow of water and blood, provides excellent conditions for gas and ion exchange. Fish gills are also a major site for the transfer of environmental chemicals from water into fish and vice versa. While the uptake of metal ions at the gills involves specific transport proteins (Grosell and Wood, 2002), the branchial uptake and excretion of organic chemicals is usually considered to represent a passive diffusion process, which is driven by parameters such as chemical hydrophobicity, water ventilation and blood perfusion (McKim et al., 1985; Erickson and McKim, 1990; Bradbury et al., 1986). However, as in other barrier epithelia, ABC transporters – in particular, transporters of the abcb, abcc and abcg families – may also be involved in the branchial uptake, transfer and/or elimination of organic contaminants. In fact, in a study with primary gill cell cultures, Stott et al. (2015) found evidence for carrier-mediated transport of pharmaceuticals, and suggested the involvement of specific members of solute carrier and ABC transporter families. In this context, it is of interest that fish gills have the capability for biotransformation of xenobiotics (Olson, 2002; Carlsson and Pärt, 2001; Olson, 2002; Carlsson and Pärt, 2001). The resulting need to transport xenobiotic metabolites and conjugates also argues for the presence of branchial ABC transporters.

Knowledge on the presence of ABC transporters in the gills of fish is sparse. To date, research on ABC transporters in the organs of fish focused mainly on liver, kidney and brain (Miller et al., 2002, 1998; Sturm et al., 2001; Zaja et al., 2008a). Lončar et al. (2010) found mRNAs encoding several ABC transporters in rainbow trout gills, including *abcc2*, *abccc3*, *abcg2* and *abcc5*. Immunohistochemical and western blot analyses of fish gills using the antibody C219, which targets mammalian ABCB, provided no conclusive information on whether ABC transporter proteins are expressed in the branchial tissue (Bard et al., 2002; Hemmer et al., 1995). Virtually no studies are available on the functional activity of ABC transporters in fish gills. Stott et al. (2015) observed that the transfer of pharmaceuticals across cultured gill epithelia is sensitive to known inhibitors of drug transport proteins, but the presence of ABC transporters was not shown. Furthermore, data on the localization of ABC transporters in fish gills are lacking and it is therefore not known whether they have a cell-specific expression.

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The aim of the present study is to examine gene transcript profiles of a series of ABC transporters in the gills of rainbow trout, *Oncorhynchus mykiss*, and to compare them to the corresponding profiles in kidney and liver. *In situ* hybridization and immunofluorescence methods were applied for the cellular localization of a selected ABC transporter in the gills. In addition, we assessed *in vivo* the functional activity of ABC transporters in trout gills. Finally, the expression and activity of ABC transporters in primary gill cell models as a potential tool for the characterization of branchial ABC transporters was investigated. Together with the analysis of the ABC transporters, biotransformation enzymes were determined, both *in vivo* and *in vitro*, in order to estimate the branchial capability to produce xenobiotic metabolites as potential ABC transporter substrates.

MATERIALS AND METHODS
Chemicals
Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland). The sterile and pyrogenic free Aqua B. Braun water was used for buffer and reconstituted water preparations (B. Braun Medical AG, Sempach, Switzerland). Compounds for ABC transporter assays were dissolved in DMSO, except MK-571 which was dissolved in H_2O, and were stored at −20°C until use. The cell culture media were purchased from Life Technologies, whereas culture plastic material was from TPP® (Techno Plastic Products, Trasadingen, Switzerland). Translucent Falcon® permeable inserts were utilized in 12- and 24-well culture plates.

Maintenance of rainbow trout
All-female rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792); 10–150 g, 4–15 months] were hatched and kept at the fish facility of the Centre for Fish and Wildlife Health, Bern, Switzerland in a partially recirculating water system equipped with a UV unit and biofilter. Oxygen, temperature and pH values were permanently monitored by a sensor system. Periodically, oxygen values were additionally determined manually using Oxy Guard, and water quality parameters (ammonium, nitrite, nitrate) were assessed using commercial test kits (Aquamerck #1.11102.0001). The fish were kept in 2000 l tanks at 10–19°C at natural photoperiodic conditions and fed daily with 1.5% body weight of commercial dry pellets (Hokovit, Bützberg, Switzerland). A day prior to the experiments, animals were fasted. Experiments were carried out according to the Swiss Animal Welfare regulations with the animal permission number BE13/16.

RNA extraction, reverse transcription, primer design and quantitative real-time PCR
Tissue and cell samples were stored in RNAlater (−80°C) and cells were lysed and stored in RTL buffer (−80°C). RNA was extracted using RNeasy kit (Qiagen) following the manufacturer's instructions (Qiagen, Basel, Switzerland). For RNA isolation from blood cells, a pre-isolation was performed using TRI Reagent (Sigma, Buchs, Switzerland). The amount and purity of isolated RNA was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The quality of some random selected samples was tested by the Agilent RNA 6000 Nano Kit, in combination with the Agilent 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany). Potential traces of genomic DNA contamination were removed using RQ1 RNase-Free DNase (Promega AG, Dübendorf, Switzerland). 1000 ng DNA-free RNA was used for cDNA synthesis with the iScript™ cDNA synthesis kit (Bio-Rad, Reinach, Switzerland).

Primers were used as previously described (Kropf et al., 2016). The oligonucleotides were all purchased from Microsynth (Basel, Switzerland) and dissolved in nuclease free water at a concentration of 100 µmol l^{-1} and stored at −20°C.

Real-time PCR quantification was performed on an Applied Biosystems 7300 analyzer (Applied Biosystems, Foster City, CA, USA). The total reaction volume was 12.5 µl, containing 6.25 µl FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics Schweiz AG, Rotkreuz, Switzerland), 1 µl of 3.75 µmol l^{-1} primer stock, and 5.25 µl Nuclease-Free water (Qiagen, Basel, Switzerland) containing 0.4 µl of the cDNA synthesis mix. qPCR was performed using the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of amplification. Each cycle consisted of 8 s of denaturation at 95°C, annealing and elongation at 60°C for 40 s. PCR was always terminated with a melting curve analysis starting with a denaturation step of 95°C followed by the start ramping temperature of 60°C for 30 s.

The data were analyzed with the 7300 system sequence detection software version 1.3.1 of the Applied Biosystems qPCR machine. The calculated Ct values were exported to Microsoft Excel for further analysis. *Ef1α* was the most stable among the tested reference genes in different tissues of *O. mykiss* (Kropf et al., 2016) and was thus taken as reference to which all genes were related. Data were presented in relative quantification method of Q-Gene (Muller et al., 2002; Simon, 2003) multiplied by the factor of 10^3 for better presentation of the values.

Cloning of rainbow trout *abcb5* transporter cDNA
Based on results of this study, the gene sequence initially named as *abcb1b* was found to be rather a family b5 member, more specifically *abcb5*. Therefore, here we use for this gene sequence the terminology *abcb5* rather than *abcb1b*. Rainbow trout initial *abcb5* sequences were obtained from total RNA extracts of the rainbow trout cell line, RTgill-W1, using reverse transcription polymerase chain reaction (RT-PCR). The primer pairs were designed based on expressed sequence tags (ESTs) for trout *abcb1b* that were identified with NCBI. For the RT-PCR, Advantage 2 (Clontech, California, USA) and Phusion (Finnzymes, Espo, Finland) polymerases were used. The PCR products were purified by gel electrophoresis, cloned and sequenced. Sequences were edited and assembled using Sequencher 4.10 (Genecodes).

Based on partial amino acid sequences, *Oncorhynchus mykiss* abcb5 transporter protein domain structures were analyzed using Prosite (http://prosite.expasy.org) from the Expert Protein Analysis System (Expasy) Proteomics Server (http://expasy.org) and Polyphobius (http://phobius.sbc.su.se/poly.html). Phylogenetic trees based on multiple ABCB transporter amino acid sequence alignments using TCoffee (http://tcoffee.crg.cat; Notredame et al., 2000) were generated with MEGAX (http://www.megasoftware.net) using the neighbor-joining, maximum likelihood and minimum evolution methods.

Construction of *abcb5 in situ* hybridization probe
The following primer pair was used to produce a 589 base pair long fragment of *abcb5*: 5′–3′: forward primer, GAA CGT GAG GGT TCT CCA GG and reverse primer CGC TAT CTG GTC TGC GTT CT. For PCR, the HotStar Taq Master Mix (Qiagen) was used and cDNA of gills served as a template. An aliquot of the reaction product was loaded on a 1.5% agarose gel while another aliquot was used for the purification using the High Pure PCR Product Purification Kit (Qiagen). One part of the purified product was

used for sequencing to prove specificity, and 1 µl of purified PCR product (25 ng µl^{-1}) was ligated in the vector pGEM-T Easy vector system (Promega) (50 ng). TOP10 chemically competent *E. coli* (Fisher Scientific) were transfected with the plasmid and selected on ampicillin plate and S-gal plates. White colonies were picked and cultured for plasmid isolation following the manufacturer's instructions (PureYield™ Plasmid Miniprep System, Promega). The plasmid was sequenced using the T7 primer pair to confirm identity and determine orientation of the cloned *abcb5* sequence. The antisense DIG-labeled RNA was synthesized using T7 RNA polymerase and the SP6 RNA polymerase was used to label the sense DIG-labeled RNA probe, both using linearization plasmid as template following the manufacturer's instructions (Roche DIG RNA Labeling Kit SP6/T7, #1175025).

Tissue preparation for *in situ* hybridization and immunofluorescence staining for Na⁺/K⁺-ATPase and C219

Fish were euthanized with MS222 (150 mg l^{-1}) in water followed by bleeding and removal of the gill arches. The gills were dissected and rinsed quickly in water to remove blood and excess mucus. Gill filaments were cut and placed in precooled 4% buffered formaldehyde containing 18% sucrose for at least 2 h on ice before cryo-slides for *in situ* hybridization were cut. For immunostaining, tissues were placed in 4% buffered formaldehyde for at least 4 h and were then transferred to PBS containing 18% sucrose for overnight incubation. All gill filaments were carefully embedded in O.C.T. compound (Tissue Tek, Skura Finetek USA, Inc. Torrance, CA 90501, USA) and incubated for at least 15 min at −20°C in cryostat (Leica CM1950) before sectioning. The tissue sections were placed on SuperFrost® plus positively charged glass slides (Thermo Scientific) and dried at room temperature (RT) before storage at −80°C.

In situ hybridization (ISH)

Cryosections were washed in 2× SSC (saline-sodium citrate buffer) for 5 min followed by a treatment with 4× SSC for another 5 min. The slides were placed in an incubator for pre-hybridization in 5× SSC, 50% deionized formamide, 1% Denhardt's solution, 1.1 mg ml^{-1} tRNA and 10% dextran sulfate at 49°C for 1 h in a humid chamber. The probe (50 ng) was incubated for 2 min at 80°C mixed in 50% deionized formamide in DEPC water and added to the slide for the hybridization, which took place at 49°C for 16 h. The slides were placed at 54°C and washed with 2× SSC for 30 min followed by another wash with 0.1× SSC for 45 min. Finally, slides were rinsed with Tris-buffered saline containing 0.1% Tween 20 (TBST). For immunohistochemical staining, the slides were blocked with 1× Roti-Block (Carl Roth GmbH, Karlsruhe, Germany) in TBST containing 1% donkey serum for 60 min at room temperature. Sheep anti-DIG (Roche, #11214667001) was used at 1 µg ml^{-1} and was incubated for 60 min followed by washing three times for 10 min in TBST. The secondary antibody was donkey anti-sheep conjugated to DyLight 488 at 5 µg ml^{-1} (Jackson ImmunoResearch, #713-486-157) and was incubated for 60 min. Slides were washed three times with TBST for 10 min and covered with fluorescence mounting medium (Dako Fluorescent Mounting Medium, #S3023). The coverslipped slides were stored at 4°C.

Na⁺/K⁺-ATPase and C219 immunostaining on cryosections

For antibody staining, cryosections were thawed and dried at RT followed by incubation for 1 h with 1× RotiBlock (Carl Roth, Karlsruhe, Germany). Staining with the primary antibody

mouse monoclonal antibody (1:50) against the α-subunit of chicken Na⁺/K⁺-ATPase antibody (University of Iowa Hybridoma Bank, AB2166869) for 1 h at room temperature was followed by the secondary incubation for 1 h with goat anti-mouse IgG conjugated to Texas Red (Jackson ImmunoResearch, Texas Red® AffiniPure). For detection of *O. mykiss abcb* family members, the crossreactivity of the C219 antibody (Thermo Fisher, Mouse IgG2a, MA1-26528) was used instead. For negative controls, cryosections were incubated in absence of primary antibody. Finally, sections were mounted in DAKO mounting medium. Fluorescence images were taken using Nikon Eclipse 80i microscope equipped with a Retiga 2000R CCD camera (Qimaging) using 60× and 100× Plan Apo objectives (Nikon) and Openlab 5 software (Improvision, Coventry, UK).

In vivo fish exposure to Rhodamine B and MK-571

To assess the effect of an ABC transporter inhibitor in water on the uptake and distribution of a classical ABC transporter substrate, fish were exposed to Rhodamine B in absence or presence of the ABCC transporter inhibitor MK-571. Glass tanks containing 1.9 l water were prepared, aerated and temperature adapted to 13°C by the use of a Biocenter 2001 incubator (SalvisLab, Rotkreuz, Switzerland) connected to a cooling system. Fish (10 cm, 10–15 g) were transferred to experimental tanks to adapt for 30 min followed by the addition of 50 ml water with or without the ABC transporter inhibitor MK-571 to reach a final nominal concentration of 10 µmol l^{-1}. After 15 min exposure, Rhodamine B dissolved in 50 ml water was well distributed in the tank to reach a final concentration of 1 µmol l^{-1}. At the beginning and after 10, 30, 60 and 90 min, fish were randomly sampled from the exposure tank and anesthetized with an MS222 overdose for 2 min. Blood was taken from the caudal vein and gall bladder content was sampled. Liver and gills were placed in 800 µl PBS, 0.2% Triton X-100. All samples were stored on ice and weights were determined. Homogenization took place in TissueLyser (Qiagen) in presence of 5 mm steel beads at 30 Hz for 2 min followed by a centrifugation step at 15,000 *g* for 10 min at 4°C. From each sample, 100 µl of supernatant were measured for Rhodamine B fluorescence in duplicates in black 96-well plates using wavelengths of 555 nm for excitation and 574 nm for emission in the EnSpire multimode plate reader (PerkinElmer, Waltham, MA, USA). All values were referred to mg tissue (wet weight).

Primary gill cell culture

Upon excision of the gill arches, sterile working conditions were used throughout. Buffers, mesh filters and equipment were autoclaved before use. The primary gill cell isolation protocol was modified from the methods described by Kelly et al. (2000). In brief, gill filaments were washed in Hanks' balanced salt solution buffer and antibiotic treated for 10 min prior to trypsin treatment. Enzymatic digestion using trypsin at the concentration of 0.05% was performed for 3×7 min on an orbital shaker. To remove undigested tissue parts, the cell suspension was flushed through 31 µm nylon filters (Sefar AG, Heiden, Switzerland) into L-15 medium containing FBS. After centrifugation at 110 *g* and 4°C the cell pellet was resuspended in L-15 medium and centrifuged for a second time. Finally, the cells were resuspended in L-15 medium (containing FBS and antibiotics) and counted in a Neubauer chamber using trypan blue solution for viability assessment. All primary gill cell culture experiments have been performed with single-seeded cultures.

The primary gill cells were seeded on solid or permeable support at a density of 1.5×10^6 cells cm^{-2} and were cultured at 17°C. Tissue culture

test plates (12 or 24 wells) from TPP® served as the solid support. The permeable supports were polyethylene terephthalate (PET) membrane inserts (Falcon®) with 0.4 μm pores and a culture area of 0.9 cm² or 0.3 cm². Development of a tight and intact *in vitro* epithelium on membrane inserts was assessed by monitoring the transepithelial electrical resistance (TEER) using a voltmeter (EVOMX; World Precision Instruments, Berlin, Germany) equipped with a chopstick electrode (STX-2). The measurements were blank corrected using cell culturing inserts without cells.

Vimentin immunohistochemistry

To identify fibroblast cells in primary gill cell culture, immunostaining of vimentin was used, which is an established mesenchymal cell marker in mammals (Chang et al., 2002). Gill tissue and the established cell line RTG-2 were used to assess specificity of the marker. Gill filaments were cut and placed in precooled 4% buffered formalin for at least 5 h. The fixed tissues were then transferred to PBS containing 18% sucrose and were incubated overnight. For cryosectioning, gill filaments were embedded in O.C.T. compound (VWR) in a cryostat (Leica CM3000) at −20°C. 4.5 μm tissue cryosections were cut, placed on SuperFrost® plus positively charged glass slides and dried for 1 h at room temperature before storing at −20°C until use. Primary gill cells cultured on permeable insert were harvested using trypsin digestion, fixed by buffered 4% formalin and cytospun on Superfrost Plus slides. Cells grown on permeable support were washed and fixed directly onto the insert.

The samples were permeabilized using 0.1% Triton X-100 for 5 min followed by blocking for 1 h using 1× RotiBlock (Carl Roth, Karlsruhe, Germany). The primary antibody goat anti-vimentin (Sigma) was diluted 1:20 in PBS (containing 10% of RotiBlock) and incubated for 1 h at room temperature. Following three washing steps for 5 min each, incubation was carried out with secondary antibody anti-goat conjugated to FITC diluted 1:100 with PBS for 1 h at room temperature. Following washing, samples were mounted in DAKO mounting media (California, USA) and coverslipped. For negative controls, samples were incubated in the absence of the primary antibody.

Fluorescence images were taken using Nikon Eclipse 80i microscope equipped with a Retiga 2000R CCD camera (Qimaging) using 60× and 100× Plan Apo objectives (Nikon) and Openlab 5 software (Improvision).

ABC transporter substrate accumulation assays in primary gill cells

To assess the functional activity of ABC transporters in primary gill cells, the fluorescent transporter substrates Rhodamine-123 (Rh123) and calcein acetoxymethylester (CaAM) were used in combination with pharmacological ABC transporter inhibitors (Daoud et al., 2000; Essodaïgui et al., 1998; Holló et al., 1996). ABC transporter activity is indicated by an increase in the accumulation of the ABC substrates in the cells in the presence of an inhibitor. Broad range ABC transporter inhibitors used in this setup were cyclosporin A (CyA) (inhibitor of ABCB, ABCC, and ABCG subfamily members) (Germann et al., 1997; Rautio et al., 2006; Robey et al., 2004), vinblastine (Vinbl) (Miller, 1995; Zaja et al., 2008a) and doxorubicin (Doxo) (Sturm et al., 2001; Zaja et al., 2008b). More specific inhibitors applied were reversin 205 (Rev205; an ABCB1-specific inhibitor) (Sharom et al., 1999) and MK-571 (an ABCC subfamily-specific inhibitor) (Chen et al., 1999; Gekeler et al., 1995; Reid et al., 2003).

Primary gill cells cultured on solid support in 96-well plates were washed with fresh L-15 medium followed by the addition of 100 μl medium containing the respective inhibitor. After a 5 min pre-incubation period, one of the fluorescent ABC transporter substrates were added in 100 μl reaching a final concentration of 1 μmol l⁻¹ Rhodamine-123 and 0.5 μmol l⁻¹ for CaAM. The final solvent concentration never exceeded 0.1%, which was found to impact neither the cell viability nor the transport activity. Compared with Rh123 where the substrate must be removed to assess accumulation, the increase in fluorescence of hydrolyzed CaAM can be continuously screened without the need of additional handling. The optimal incubation period for Rh123 accumulation was found to be 1 h (data not shown), therefore the assays were terminated after 60 min of incubation by a short centrifugation step followed by at least two washing steps using L-15 medium. Cells were lysed by 0.1% Triton X-100 in PBS. Compared with the end point measurement for Rhodamine-123, CaM measurements were performed as kinetic measurements of fluorescence increase in the treated cells. Accumulation of the substrates was assessed by measuring the fluorescence signals at 485 nm excitation and 530 nm emission wavelengths. Fluorescence analysis took place in the EnSpire multimode plate reader (PerkinElmer, Waltham, MA, USA). The dye accumulation data were expressed as percentage fluorescence compared with control cells incubated in the absence of inhibitors.

Statistical analyses

The statistical analyses were performed using SigmaPlot 12.0 (Systat Software, San Jose, CA, USA) and for graphical presentations using GraphPad Prism software (version 4; GraphPad Software Inc., San Diego, CA, USA). The differences in Rhodamine B exposure assay between control and MK-571-treated group were compared and tested for significant differences using a *t*-test. For the other experimental data, one-way ANOVA analysis was applied followed by a Tukey's test for multiple comparisons. Data sets failing normality tests and displaying heterogeneity of variance, the non-parametric Kruskal–Wallis ANOVA on ranks was applied in combination with Dunn's *post hoc* test for non-parametric multiple comparisons in case of significant data. Data were considered to be significant at *P*<0.05.

RESULTS

ABC transporter mRNA in gills, liver and kidney

The mRNA levels of the selected ABC transporters were assessed in gills and compared with the corresponding mRNA levels in two reference organs, kidney and liver, which display prominent ABC transporter expression. In addition, we measured ABC transporter mRNA levels in the blood, as blood cells constitute a major proportion of the gill tissue cellularity and thus may influence the qPCR results from the gill tissue extracts. The abundance of *abcb1a* gene transcripts did not differ between the gills, liver and kidney (Fig. 1A), but the gills showed the highest mRNA levels of *abcb5* relative to the other organs (Fig. 1B). Likewise, the gene transcript levels of *abcg2* were similar in different organs (Fig. 1H). High levels of *abcc1* (Fig. 1C), *abcc3* (Fig. 1E), *abcc4* (Fig. 1F) and *abcc5* (Fig. 1G) mRNA were detected in the gills and kidney compared with the liver. The *abcc2* gene transcripts (Fig. 1D), tended to have lower levels in gills than in the kidney and liver.

The mRNA levels of the *abcb* subfamily members were substantially lower in blood cells compared to gills, liver and kidney, which was also seen for the *abcg2* gene transcripts. Other genes showed comparable levels in blood cells and organs, except
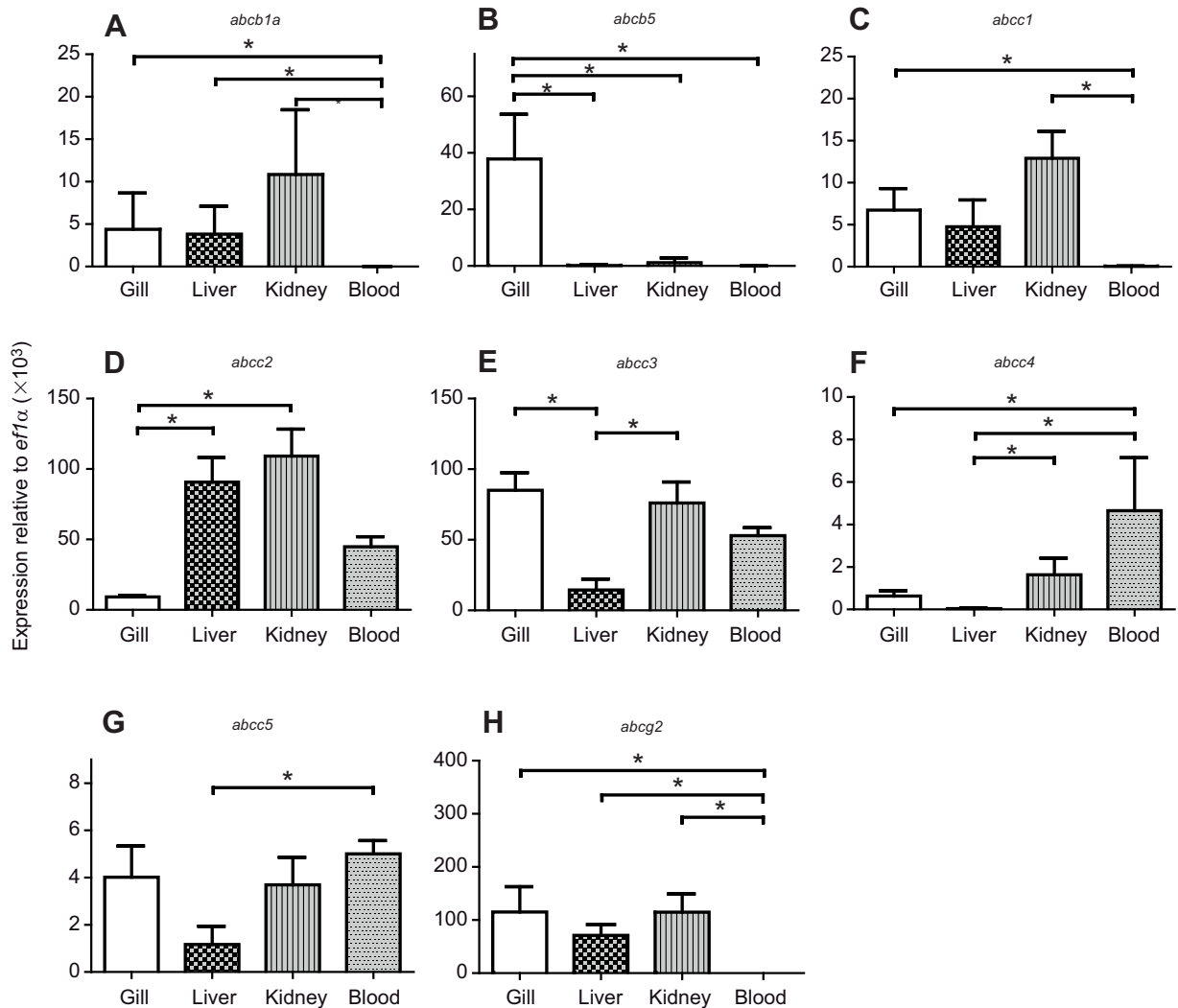
Fig. 1. ABC transporter mRNA expression profiles in gill, liver, kidney and blood of rainbow trout (*Oncorhynchus mykiss*). (A–H) The relative quantification of ABC transporter gene transcripts in whole tissue RNA extracts of gill, liver and kidney as well as blood cells from juvenile *O. mykiss* (*n*=7). The mRNA levels are given as mean normalized expression (MNE)±s.d. using *ef1α* as reference gene; asterisks indicate significant difference (*P*<0.05).

the *abcc4* gene transcripts, which were detected at significantly higher levels in blood cells compared with liver, kidney and gills.

Sequencing and localization of *abcb5* mRNA in gills

Based on published sequences for *O. mykiss abcb1b*, we designed primers for RT-PCR. Sequencing the resulting product, however, gave indications that this gene putatively may be *abcb5* rather than *abcb1b*. Therefore, further analyses were performed in order to obtain more genomic information to confirm or reject the *abcb5* annotation. To obtain a partial sequence of *abcb5*, primers were designed based on expressed sequence tags from the *O. mykiss* database (Salem et al., 2010) and were assembled to a contig of 2880 base pairs. To this end, the PCR product of a reverse transcription PCR was cloned and sequenced (Dataset 1). Analyses of the rainbow trout abcb5 partial amino acid sequences with Prosite and Polyphobius showed a partial transmembrane domain 1 (TMD) and full TMD2 and nucleotide-binding domains (NBD1 and 2) (Fig. 2A). The cytoplasmic NBD1 and NBD2 of rainbow trout abcb5 consists of distinctive and highly conserved motifs of ABC transporter proteins, Walker A, Walker B, ABC signatures and A loops. The presence of classical ABC transporter domains and

membrane integration (Fig. 2B) is in accordance with the topology of mammalian ABCB 'full' transporters. Furthermore, at the level of chromosomal gene localization, *abcb5* (former *abcb1b*) of *O. mykiss* (Gene ID: 100653442) is flanked by the genes *macc1* and *sp8*, whose orthologues are also found in this location around ABCB5 in *H. sapiens* (Gene ID: 340273) and other vertebrates.

Initial NCBI BLAST analyses of nucleotide as well as amino acid sequences of the previously abcb1b annotated transporter showed close matches with abcb5 transporter orthologues from various vertebrates. Phylogenetic comparison of abcb1, abcb4, abcb5 and abcb11 amino acid sequences from different vertebrates based on amino acid sequence alignments resulted in three clusters according to each Abcb transporter sub-type (Fig. 3, neighbor-joining method; Fig. S2, maximum likelihood method; and Fig. S3, minimum evolution method). The rainbow trout protein previously annotated as abcb1b transporter showed a close evolutionary relationship and a significant grouping to corresponding Abcb5 orthologues of *Danio rerio*, *Astyanax mexicanus* and *Lepisosteus oculatus*, which is an evolutionarily old fish species.

To assess whether the high mRNA levels of *abcb5* are associated with a particular branchial cell type, ISH was used. ISH staining of
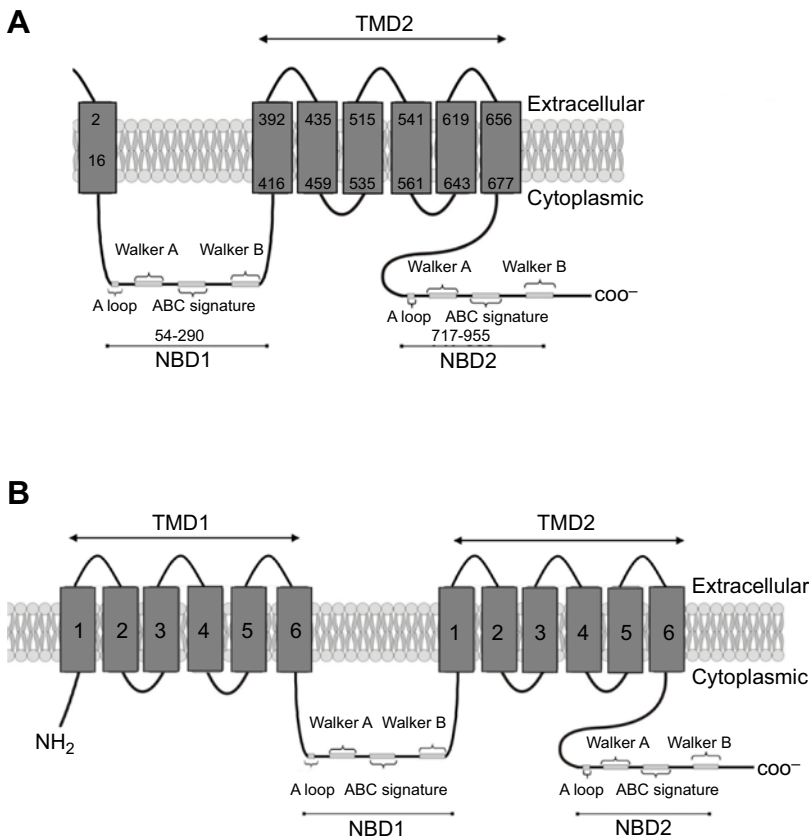
Fig. 2. Predicted ABC transporter structures of *O. mykiss* abcb5. (A) Topology of rainbow trout abcb5 partial protein with partial transmembrane domain 1 (TMD) and full TMD2 as predicted by the Polyphobius algorithm and nucleotide-binding domains (NBD1 and NBD2) that are indicated by A loop, Walker A and B, and the ABC signature motifs (predicted by Prosite). (B) Predicted ABCB 'full' transporters have two TMDs and two NBDs, with the NBDs located on the cytoplasmic side of the membrane.

gill cryosections using the antisense *abcb5* probe gave a strong and specific signal (Fig. 4A), whereas no signal was detectable in samples incubated with the sense probe (data not shown). The *abcb5* mRNA positive cells were located in the interlamellar space of the gill filaments (Fig. 4B). These positive cells do not have direct contact with the apical or basal surface of gills. No staining was found neither in topmost apical nor in basolateral cell layers. Immunohistochemical staining of gill cryosections using an antibody against trout Na^+/K^+-ATPase was used to evaluate, whether the *abcb5* mRNA positive cells are identical or different to mitochondria-rich cells (Fig. 4C,D). When comparing the staining results, it appears that the Na^+/K^+-ATPase is located in a different cell type and layer than the *abcb5* signal.

Phase I and II biotransformation enzyme mRNA in gills
The mRNA levels of the phase I enzyme, cytochrome P450 1A (*cyp1a*) showed significant differences between the investigated organs (Fig. 5A). For *cyp3a*, however, the gills showed markedly lower gene transcript levels than the liver whereas the difference from the kidney was not significant (Fig. 5B). The phase II enzyme *gstp* displayed the highest mRNA levels in the kidney, while expression levels were low in the other organs, including the gills (Fig. 5C).

In vivo exposure to Rhodamine B and MK-571
For functional assessment of ABC-mediated transport activity in trout gills, we performed an *in vivo* exposure to the classical ABC substrate, Rhodamine B, in the presence or absence of the ABCC inhibitor, MK-571 (Fig. 6). Juvenile *O. mykiss* were exposed in glass tanks to control conditions or the inhibitor MK-571, followed by exposure of fish from both treatments to Rhodamine B and determination of its accumulation in the gills. Since the gills are not

a storage organ, but a transfer organ, we additionally analysed Rhodamine B accumuluation in blood, liver and gall bladder.

The results did not show enhanced Rhodamine B accumulation in the gills of MK-571-pretreated trout, and there was pronounced inter-individual variation (Fig. 6A). The Rhodamine B levels in blood plasma were significantly higher in MK-571 pretreated fish compared with control fish (Fig. 6B). An increase in Rhodamine B accumulation was also observed in the liver after 10 min Rhodamine B exposure and it persisted up to 90 min (Fig. 6C). Rhodamine B accumulation in the gall bladder was delayed compared with its localization to the other organs (Fig. 6D), but no significant accumulation differences were detected between treatments.

ABC transporter mRNA profiles in primary gill cell cultures
The aim of this part of the study was to assess the mRNA expression patterns of ABC transporters during primary culture of isolated gill cells. The most prominent ABC transporter in terms of mRNA levels in the intact gill tissue was *abcb5*. After 2 days of primary culture of the isolated gill cells, the mRNA levels were significantly decreased (Fig. 7). To test for the possible influence of a different behavior of reference genes on *in vitro* and *in vivo* results, two reference genes were measured, namely *ef1α* and *β-actin*, which resulted in the same trend of significant *abcb5* decline in the cell culture system (data not shown).

To assess whether the cell culture conditions influenced the conservation of ABC transporters, we analysed gene transcript levels of several ABC transporters in gill cells that were cultured on either permeable inserts (TEER data, Fig. S5) or solid supports. When comparing intact gill tissue with freshly, isolated gill cells, comparable mRNA levels of *abcg2* and *abcc3* were found (Fig. 8). However, during cell culture, mRNA levels of these genes strongly
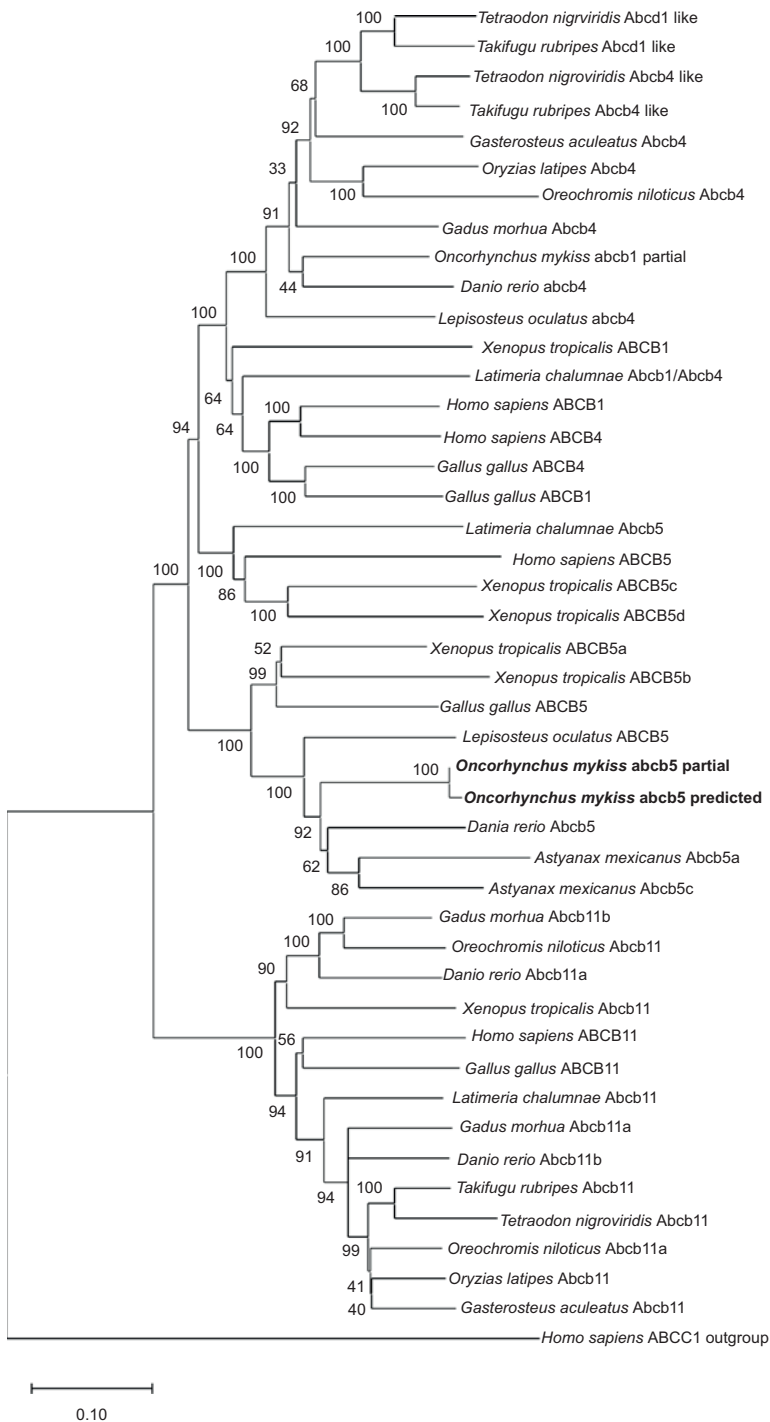
Fig. 3. Phylogenetic analysis of abcb isoforms. Phylogenetic tree based on multiple alignments (TCoffee) of Abcb1, Abcb4, Abcb5 and Abcb11 ABC transporter amino acid sequences from vertebrates. The tree was generated using neighbor-joining method and the percentage concordance based on 1000 bootstrap iterations is shown at the nodes. Trees generated with the maximum likelihood and minimum evolution methods showed very similar topologies (Figs S2 and S3), indicating that the results are robust.

declined irrespective of the cell culture system used. Interestingly, the gene transcript levels of biotransformation enzymes, especially *cyp1a* and *cyp3a*, showed only weak changes over culture duration (Fig. S6).

In order to assess whether the decline of ABC gene transcript levels during primary culture might be related to a change of cell type composition, in particular to a possible proliferation of fibroblasts versus a loss of branchial cells, we examined by means of immunostaining the intensity of vimentin expression in the cell cultures (Fig. S4). Vimentin is used as a mesenchymal marker in mammalian studies, and we obtained positive staining with vimentin in the *O. mykiss* fibroblast cell line RTG-2. Furthermore,

while the anti-vimentin antibody stained a small number of cells in cryosections of gill tissue without staining of branchial epithelial cells, it stained all cells in the primary culture, both on solid and permeable supports.

ABC transporter activity in primary gill cells

The activity of the ABC transporters was determined based on the accumulation of two ABC transporter substrates in the presence or absence of ABC transporter inhibitors at different concentrations (Fig. 9). The substrates CaAM and Rh123 are two ABC transporter substrates widely used for ABCB1- and ABCC-like transport activity measurements (Essodaïgui et al., 1998; Holló et al., 1996).
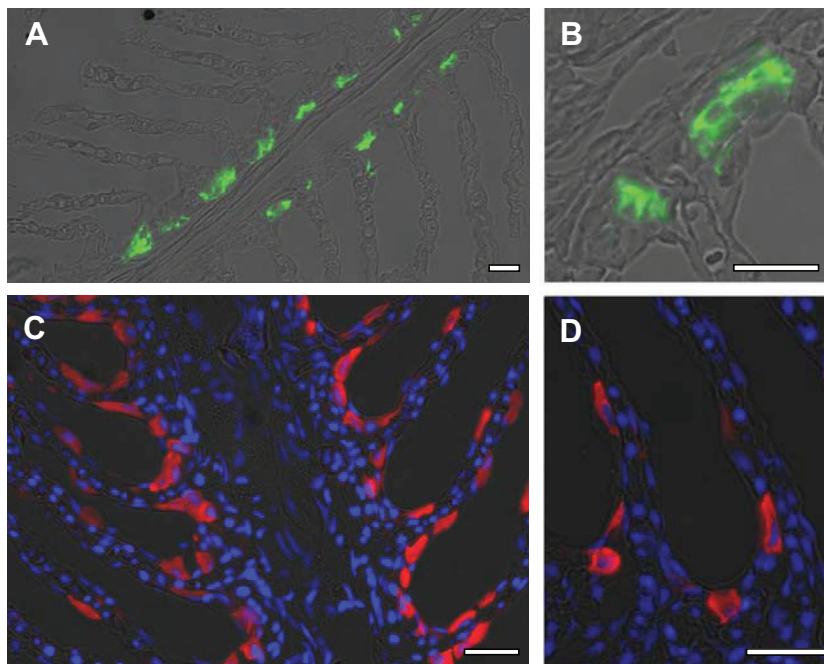
Fig. 4. Cellular localization of *abcb5* mRNA and Na⁺/K⁺-ATPase. (A,B) Detection of *abcb5* by *in situ* hybridization in gills of *O. mykiss*. The ISH probe for *abcb5 mRNA* was detected by sheep anti-DIG antibody in combination with an anti-sheep antibody conjugated to DyLight 488 nm (green). Positive cells are found along the primary gill filament (A). Higher magnification reveals the ISH signals from an intermediate cell layer in the interlamellar space of gills (B). (C) Identification of mitochondria-rich cell in gill cryosections of juvenile *O. mykiss* (red). (D) The majority of Na⁺/K⁺-ATPase is found near the base of the lamellae, whereas a few positive cells are distal on the lamellae. Scale bars: 25 μm.

The primary gill cells were pretreated with a set of known ABC transporter inhibitors, namely cyclosporine A, MK-571, reversin 205, vinblastine and doxorubicin, before the exposure to the model substrates.

The alkaloid vinblastine and the anthracycline doxorubicin are broad-scale ABC transporter inhibitors as well as substrates. They did not significantly impact the accumulation of ABC transporter substrates in the cultured gill cells. The ABCB1-specific inhibitor reversin 205 also had minor effect on ABC transporter-dependent Rh123 and CaAM accumulation. Cyclosporin A with a broad-scale inhibitory action resulted in a concentration-dependent accumulation of the ABC transporter substrate CaAM, with a significant inhibition at 15 μmol l⁻¹. The most pronounced effects were detected for the inhibitor MK-571 (Fig. 9B,D), which in mammalian systems acts as inhibitor of ABCC transporters.

DISCUSSION

Gills are a key interface between fish and their environment. The large branchial surface, the counter-current physiology and the ventilation are excellent conditions for efficient gas and solute exchange. Many functions performed in mammalian kidneys are performed by the gills in fish (Evans et al., 2005; Rombough, 2007).

Unlike other gill functions such as ion exchange and pH regulation in the branchial tissue, the presence and function of ABC transporters in the gills of fish is virtually unstudied to date. Membrane transporters have an important homeostatic function, as their activities at physiological barriers regulate the internal distribution of solutes and protect from potential harmful effects of endogenous and exogenous molecules. Accordingly, in liver and kidney, the membrane transfer of physiological compounds as well as numerous foreign compounds and their metabolites is mediated to a large extent by ABC transporters. The results of the present study provide evidence that the gills of rainbow trout similarly harbor a diverse range of ABC transporters, and that they are functionally active.

The mRNA profiles of ABC transporters and biotransformation enzymes in branchial tissue in comparison to liver and kidney

We compared ABC transporter mRNA profiles in the gills with those of liver and kidney, which have key functions in solute transport and for which information on the occurrence of ABC transporters is available for teleost fish. For all ABC transporters investigated in this study, mRNAs were found in the gills.
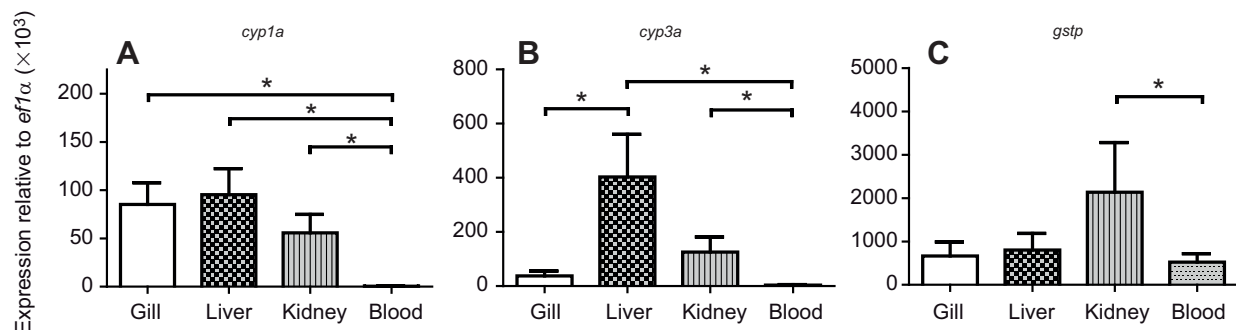

Fig. 5. mRNA profiles of biotransformation enzymes in gill, liver, kidney and blood. Relative quantification of gene transcripts of *cyp1a* (A), *cyp3a* (B) and *gstp* (C) in whole tissue RNA extracts of gill, liver and kidney as well as blood cells from juvenile *O. mykiss* (*n*=7). The mRNA levels are given as mean normalized expression (MNE)±s.d. to the reference gene *ef1α*; asterisks indicate significant difference (*P*<0.05).
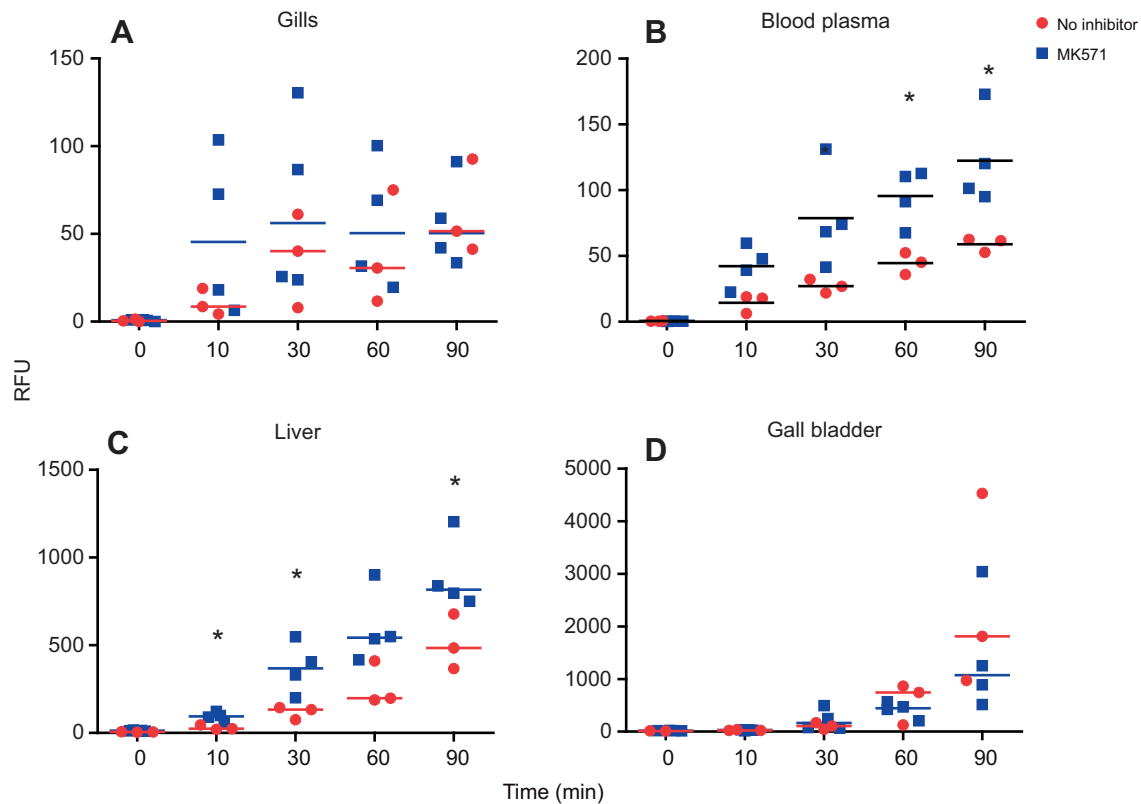
Fig. 6. Accumulation of Rhodamine B in *O. mykiss* in the presence and absence of the ABC transporter inhibitor MK-571. The ABC transporter substrate accumulation was tested in bath exposed juvenile *O. mykiss* with 1 µmol l^{-1} Rhodamine B in the absence and presence of 10 µmol l^{-1} MK-571 (nominal concentrations). The accumulation of Rhodamine B was assessed in gills (A), blood plasma (B), liver (C), and gall bladder (D) after 0, 10, 30, 60, and 90 min of exposure. Data are presented in relative fluorescence units (RFU) per mg of tissue extract or per µl bile or blood plasma ±s.d. (*n*=4 for MK-571 and *n*=3 for control); asterisks indicate significant difference from no inhibitor control (*P*<0.05).

Compared to liver and kidney, the gills displayed the highest gene transcript levels of *abcb5* whereas they had the lowest levels of *abcc2*. The abundance of branchial *abcc3* and *abcc5* mRNAs was similar to levels in the kidney but higher than in the liver, while *abcb1a* and *abcg2* showed similar mRNA levels in all three organs.

The high level of branchial expression of *abcb5* attracted our attention to this gene. To date, only 229 nucleotide base pairs

Fig. 7. Relative mRNA abundance of *abcb5* in gills compared with *in vitro* culture. The mRNA levels of *abcb5* were measured in whole gills RNA extracts and in primary culture with solid support 2, 7 and 12 days post isolation (dpi). The mRNA levels are given as mean normalized expression (MNE)±s.d. from independent experiments (*n*=4) using *ef1α* as reference gene; asterisks indicate significant difference compared with gills (*P*<0.05).

(HQ400613.1) of the *abcb5*, former *abcb1b* sequence, were known (Fischer et al., 2011). We extended the sequence, checked for gene annotation and ABC transporter domains. Membrane integration predictions based on the translated mRNA sequence revealed the presence of partial TMD1, TMD2 and two NBDs at the C-terminus of the ABC transporter domains, whereas the predicted integration from the contig revealed the full length of the transporter with two TMD and two NBD domains. The correct sequential arrangement suggests that *abcb5* encodes a functional ABC transporter.

The *O. mykiss* amino acid sequence formerly annotated as abcb1b clustered with abcb5 of other aquatic species. This clade is clearly distinct from other abcb subfamilies, which suggested reannotation of this *O. mykiss* abcb isoform to abcb5. The close evolutionary relationship and a significant grouping to corresponding Abcb5 orthologues from other species suggested a common ancestor of Abcb5 transporters in vertebrates. Furthermore, chromosomal gene localization of the predicted *O. mykiss* abcb5 showed close proximity to the genes *macc1* and *sp8*, which is in line with their localization in humans near the homologue *ABCB5*. In contrast to *ABCB5*, human and other vertebrate *ABCB1* genes, as well as the predicted *O. mykiss abcb1* (Gene ID: 110507796) are surrounded by *ABCB4* and *RUNDc3B*. According to these results, the current annotation on the Genbank database (https://www.ncbi.nlm.nih.gov/genbank) needs to be adapted, the former *abcb1b* is designated as *abcb5*.

Mammalian ABCB5 is a negative regulator of cell fusion maintaining hyperpolarization and could have some chemoresistance activity (Frank et al., 2005, 2003). Hypothesizing the same function in fish, one would expect this basic physiological cell renewal process to
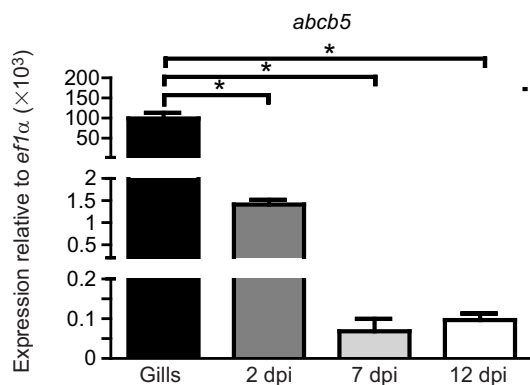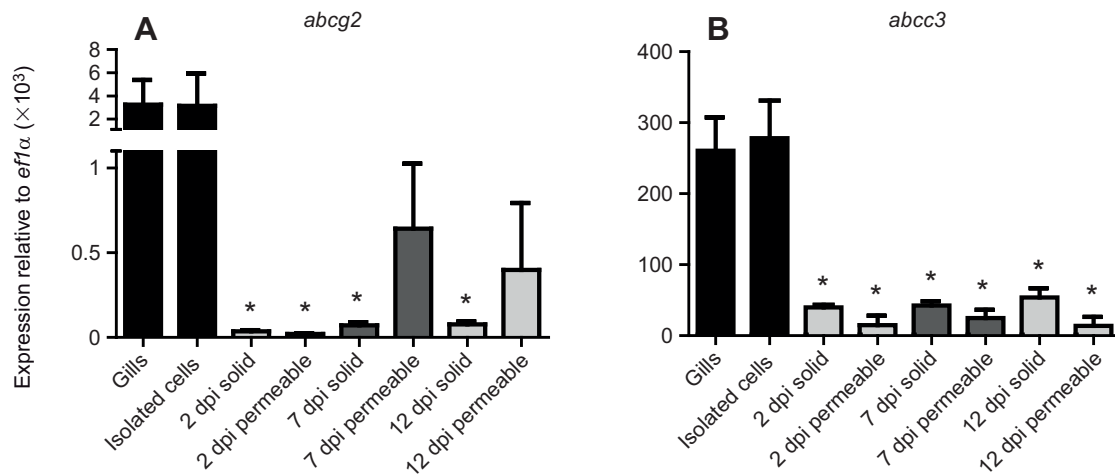
Fig. 8. Relative mRNA abundance of selected ABC transporter genes in gills and during primary gill cell culture. The mRNA levels of selected genes in gills, isolated gill cells and from cultured cells on solid and permeable supports at 2, 7 and 12 days post isolation. Isolated gill cell populations were treated with red blood cell lysis buffer to prevent an impact of erythrocyte cell RNA on mRNA profiles of isolated cells. The experiments were performed in 12-well format for solid and permeable supports (0.9 cm^2 inserts). The mRNA levels of *abcg2* (A) and *abcc3* (B) are given as mean normalized expression (MNE) to the reference gene *ef1α* ±s.d. from independent experiments ($n=5$, except gills $n=3$, isolated gill cells $n=3$); asterisks indicate significant difference compared with gills ($P<0.05$).

be present among all fish species. But many species, such as medaka and minnow, do not have *abcb5* gene sequences in their genome (Luckenbach et al., 2014). The abcb5 clade of teleost is different from that of *Homo sapiens* and *Latimeria*, which could indicate an evolutionary change of physiological function. The skin is a very important exchange organ for cave fish (*Astyanax mexicanus*) and *Xenopus*, and both possess several abcb5 isoforms in their genome which might indicate diversified functions of the *abcb5* gene family members in some aquatic species. Furthermore, in the head region of early life stages of rainbow trout, *abcb5* mRNA was found to rise drastically over the first 20 days post-hatch, and this increase occurred simultaneously to the development of the gills (Kropf et al., 2016).

The high abundance of branchial *abcb5* mRNA raised the question of the site of expression within the gills. The *abcb5* ISH probe revealed predominance of the mRNA in the interlamellar space of gills known to contain progenitor cells of pavement cells and mitochondria-rich cells but seems to be absent in differentiated mitochondria-rich cells located in the apical cell layer. In addition to *in situ* hybridization, we performed immunohistochemical staining using the C219 antibody, which is directed against an epitope shared by ABCB1 and ABCB11. Positive immunoreactivity was obtained in liver and kidney, but not in the gills (Fig. S1). This is a further indication that the gills do not express *abcb1* (Kropf et al., 2016). Overall, the reclassification of the *O. mykiss* isoform to abcb5 and the localization to intermediate gill layers, does not point to a major involvement of this transporter in apical branchial excretion of organic molecules.

Important for the physiological function of the ABC transporters is their asymmetrical distribution in polarized epithelia. With respect to xenobiotics, apically located ABC transporters in gills could prevent chemicals from entering the organism through direct efflux at the surface. Alternatively, branchial ABC transporters may function in the excretion of xenobiotics and their metabolites. In mammals, the ABC transporters ABCB1, ABCC2 and ABCG2 are known to prevent chemicals from entering tissues. Thus, they are part of a first line of toxicological defense. ABCG2 prevents cellular uptake of molecules as well as transporting products from phase II

biotransformation (Suzuki et al., 2003), steroid hormones (Dankers et al., 2012), heme (Desuzinges-Mandon et al., 2010) as well as uric acid (Woodward et al., 2009). In mammals, ABCG2 is present in sinusoids and capillaries of the blood–brain barrier and in endocrine organs (Dankers et al., 2012), while it shows apical expression in liver and kidney. In trout gills, *abcg2* displayed mRNA levels comparable to those in the liver and kidney. A similar pattern of substrate specificity of trout abcg2 and human ABCG2 were recently confirmed (Zaja et al., 2016). Since gills are involved in hormone metabolism (Olson, 1998), branchial abcg2 transporter may function in the transfer of steroids, and as such might be located on the apical and/or basolateral side of gills.

In contrast to the apical pumping out of ABC substrates entering the gills from water, basolaterally located ABC transporters may alternatively function in the transport of compounds from the gill cells into the blood. ABC transporters often colocalize with biotransformation enzymes for a subsequent efflux of metabolites and conjugates out of the cell as found for example in mammalian enterocytes. Gill cells appear to be active in xenobiotic biotransformation, therefore basolateral ABC transporters may function in transferring the metabolite conjugates from the gill epithelium into the blood. An important transporter for conjugates in the kidney and liver is the ABCC2 transporter, which often co-localizes with ABCB1. Our mRNA data from juvenile *O. mykiss* indicate that gene transcription of *abcc2* occurs in gills, although at low levels, which is in accordance with findings in zebrafish (Long et al., 2011). The ABCC1 and ABCC3 transporters are known for their basolateral expression in liver, kidney, digestive tract of mammals, and for their involvement in xenobiotic transport. ABCC3 extrudes bile acids, a variety of glucuronide conjugates and steroid conjugates (Chu et al., 2004; van de Wetering et al., 2009) to the sinusoidal side of a tissue. Notably, the ABC transporters ABCC3 and ABCC2 have substantial substrate overlap but are present on opposite membranes. A decrease in hepatic ABCC2 function or choleostatis leads to an upregulation of ABCC3 (Scheffer et al., 2002; Soroka et al., 2001; van der Schoor et al., 2015). The low abundance of *abcc2* mRNA in gills therefore could support the importance of basolateral ABC transporters such as abcc3 in performing active blood elimination of conjugates that build up in gill cells.
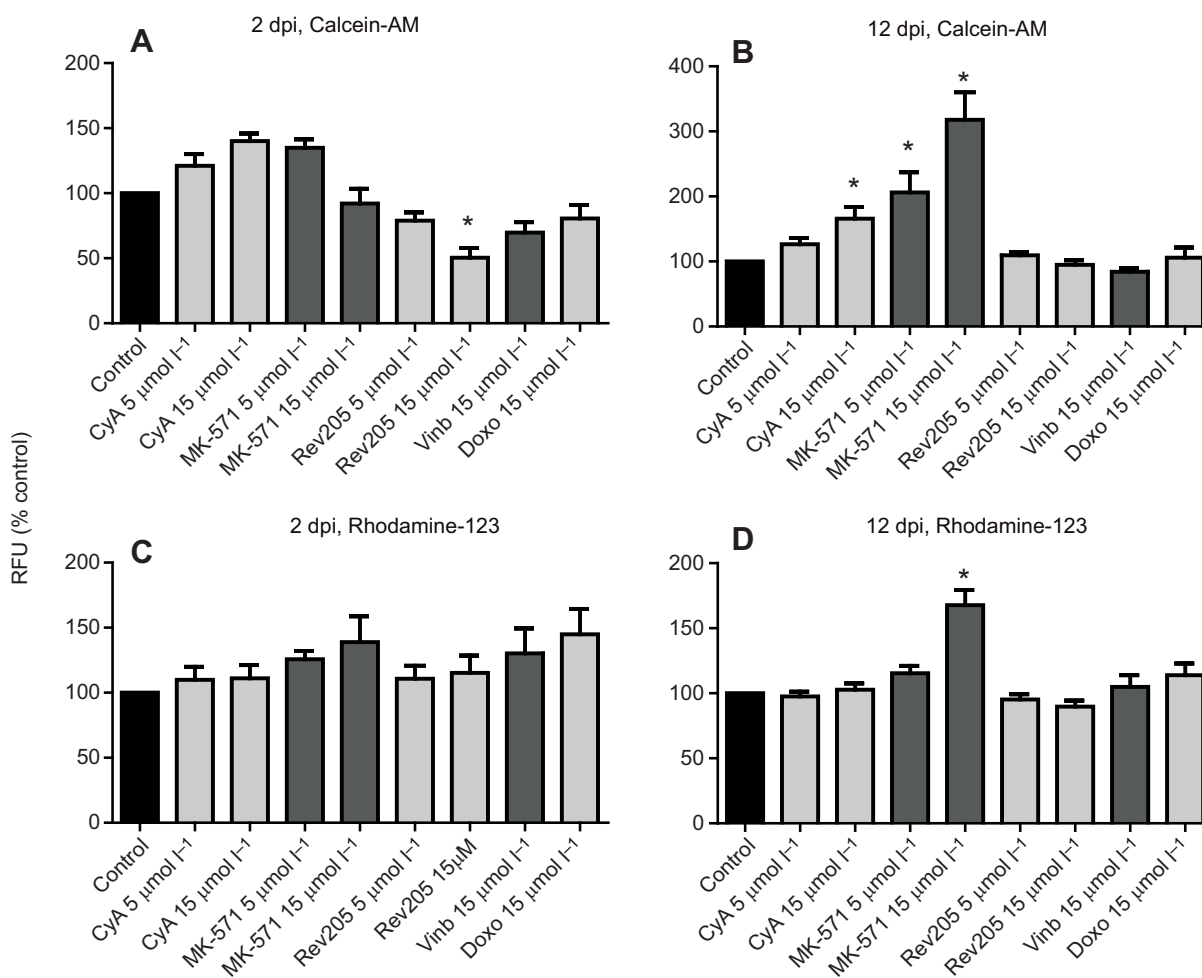
Fig. 9. The effect of ABC transporter inhibitors on the accumulation of ABC transporter substrates in primary gill cells. Primary gill cells cultured on solid supports were exposed to inhibitors and substrates of ABC transporters after 2 and 12 days of culture. The cells were pretreated with either cyclosporin A, MK-571, reversin 205, vinblastine or doxorubicin followed by the model ABC transporter substrate incubation. The accumulation of either calcein-AM at 2 dpi (A) and 12 dpi (B) or Rhodamine-123 at 2 dpi (C) and 12 dpi (D) was determined using fluorescence intensity measurement. The data are presented as relative fluorescence units (RFU) as a percentage of control accumulation. The values are mean±s.d. from independent experiments (n=5). Statistically significant differences to solvent control group are indicated by asterisks (P<0.05).

Several studies showed the presence of cyp1a in pavement and pillar cells, especially after exposure to certain chemicals leading to cyp induction (Husoy et al., 1994; Miller et al., 1989). Our mRNA data from juvenile rainbow trout for *cyp1a* and *gstp* support the expression and presence of phase I and II biotransformation in gills. This pinpoints a first-pass biotransformation activity in fish gills, which probably reduces the amount of certain xenobiotics from reaching other organs (Barron et al., 1989; Bartram et al., 2012). Biotransformation can act directly on the concentration gradient, influencing the uptake dynamic of molecules. Comparing the biotransformation per mg protein, the fish liver has higher activity (Kennedy and Walsh, 1994), but this value does not take in to account the high perfusion and ventilation rate present in gills.

In vivo indications of functional branchial ABC transporter activity

To assess functional ABC transporter activity in trout gills, the uptake of a general ABC transporter substrate – Rhodamine B – was assessed in combination with the known ABC transporter inhibitor MK-571. In fish, ABC transporter activity measurements are always based on the assumption that substrates and inhibitors used have the

same or at least similar specificities as in mammals to provide evidence for the presence or absence ABC membrane protein. A caveat that has to be kept in mind with respect to *in vivo* studies on branchial ABC transport is the potential influence of stress, which may lead to an increased ventilation rate. Rhodamine B enters the cells via passive diffusion and is actively extruded from cells by ABC transporters. MK-571 is a known inhibitor of ABCC1, ABCC2, ABCC4 and ABCC5 (Chen et al., 1999; Gekeler et al., 1995; Reid et al., 2003) but at elevated concentrations, it can impact other ABC transporters, as seen for ABCC3 (Bodó et al., 2003).

Exposure of juvenile *O. mykiss* to Rhodamine B via water resulted in its rapid accumulation in gills, blood, liver and, with a delay, in the gall bladder. The accumulation was sensitive to the ABC transporter inhibitor in a tissue-specific way and therefore affected Rhodamine B distribution in the body. MK-571 increased the concentration of Rhodamine B in blood plasma and liver but not in gills. There might be three explanations for this. Firstly, MK-571 may have inhibited apical branchial ABC transporters pumping out Rhodamine B from the apical gill cells into the water leading to elevated rhodamine B uptake into the fish. Secondly, there could be no effect at the gill level but a rapid transfer of the absorbed substrate from the gill cells into the

blood. Thirdly, MK-571 might reduce the ABC transporter-mediated hepatobiliary or renal efflux of Rhodamine B, resulting in its increased accumulation in the blood plasma and in the liver. This final hypothesis is supported by the findings on the gall bladder, which showed a trend of higher Rhodamine B levels in the absence compared with in the presence of MK-571.

ABC transporters in primary gill cell model

To obtain deeper insight into the ABC transport activities at the branchial epithelium and the characteristics of the involved transporters, an *in vitro* gill cell culture system would be advantageous. However, there is a lack of literature concerning ABC transporters in primary gill cell cultures of fish. The main finding from our *in vitro* gill cell culture experiments is that we saw a pronounced shift in the ABC transporter expression profile between the intact *in vivo* gill tissue and the cultured cells. When gill cells were cultured until they formed a tight epithelium *in vitro* (as evidenced from the TEER), a lowering of gene transcript levels of ABC transporters can be detected upon culturing, especially for *abcb5*. This change in expression profile was independent of the culture method, either solid support or permeable support. Based on our mRNA data, a shift in the mRNA profile during the cell isolation process can be excluded. An alternative hypothesis for the loss of the ABC expression in cell culture, might be that gill tissue is dominated by the blood cells, as blood cells are a major constituent of the gills *in vivo*, but are absent in the *in vitro* culture. However, the measurements of ABC gene transcripts in isolated blood cells (Fig. 1B) argues against a significant contribution of the blood cells towards the ABC transporter profile of the intact gills. Therefore, the absence of blood cells in the *in vitro* system does not explain the reduced mRNA levels of ABC transporters in cultured cells.

Primary gill cells might experience changes in gene expression in response to the *in vitro* environment. This observation agrees with findings on primary culture of rainbow trout hepatocytes where a significant mRNA decline of *abcb1a*, *abcb11* and *abcc2* occurred after 24 h in culture (Zaja et al., 2008a). Factors that may be relevant for the maintenance of ABC gene expression in cultured cells include media composition and two- versus three-dimensional structure. Another factor for which our findings suggest that it has a minor influence is the culture system, i.e. solid versus insert culture. This is in accordance with Leguen et al. (2007) who found that ion transporters and hormone receptors displayed similar culture-related changes on gene expression levels regardless of whether the gill cells were cultured on solid or permeable inserts. Finally, the *in vitro* results may vary between single-seeded and double-seeded culture systems, since the latter contain a higher percentage of mitochondria-rich cells (Fletcher et al., 2000). In our unpublished preliminary experiments we found no clear difference in terms of the investigated ABC transporters, which is in line with our immunohistochemical findings that the mitochondria-rich cells are not a site of abcb5 expression. Nevertheless, it will be worthwhile to further investigate the seeding system in future studies.

A dominance of abcc transporters was found in permanent cell lines of *O. mykiss* (Fischer et al., 2011) when compared to profiles found in the tissues of origin (Lončar et al., 2010). A similar trend of abcc transporter predominance was also seen in our data from primary cultured gill cells. Some reasons for this preference for abcc in cultured cells might be the absence of physiological substrates in culture, the presence of new stimulators affecting gene regulation, as well as changes in energy metabolism (Donner and Keppler, 2001). Concerning the decline of *abcb5* mRNA in cell culture, a possible explanation might be the cellular differentiation processes. In

mammals, ABCB5 was found to maintain membrane hyperpolarization in skin progenitor cells (Frank et al., 2003). The *abcb5* mRNA expression found in the gill region containing progenitor cell layers and the loss of *abcb5* gene transcripts in primary gill culture could therefore be a result of progenitor cell decline through differentiation processes initiated by *in vitro* culturing.

Another factor indicating a change in gene expression during cell culture was the presence of vimentin in primary gill cells. Our initial intention was to use vimentin as mesenchymal marker, but this failed, as the primary gill cells changed their epithelial expression profile upon culturing. Vimentin expression can be induced by serum but is also an indirect sign of proliferation and dedifferentiation (Pieper et al., 1992). Also other studies found that eukaryotic cells of epithelial origin start to express vimentin upon *in vitro* culturing (Connell and Rheinwald, 1983; Franke et al., 1979). The phenomenon is not understood but it is hypothesized that intermediate-sized filaments in cultured cells develop owing to the loss of the three-dimensional structure.

The cultured gill cells were also assessed for functional ABC transporter activity, as this may give a different picture than the mRNA profiles, because mRNA expression patterns do not infer functionality. As mentioned above, our ABC transporter activity measurements are based on the assumption that substrates and inhibitors applied on fish have the same or at least similar specificities as in mammals. This assumption is only partially confirmed (Sturm et al., 2001; Zaja et al., 2007, 2008b). However, the intention of the functional assays was not to measure the activity of specific transporters, but to provide evidence for the presence or absence of ABC membrane protein-dependent transport in primary gill cell cultures. The results revealed inhibitor-mediated accumulation of ABC transporter substrates in the gill cells, providing evidence for functional transport. A concentration-dependent accumulation was seen for the inhibitor MK-571, which was found to be the most potent among the used inhibitors. MK-571 was shown in fish to be more specific for abcc and less potent in abcb1a inhibition (Zaja et al., 2008b), which is in line with higher prominence of abcc transporter mRNA found during culture of primary gill cells. Indication of functional ABC transporters was already seen in another study where ABC transporter inhibitor lead to change in propranolol uptake across the primary gill cell culture epithelium (Stott et al., 2015).

Conclusion

In conclusion, the results of this first comprehensive study on ABC transporters in fish gills provide evidence that the gills of rainbow trout possess diverse ABC transporter mRNAs as well as functional ABC-mediated transport activity. The mRNA expression profiles of ABC transporters that show basolateral localization in mammalian systems are similar between gills, liver and kidney. In contrast, gene transcript profiles of ABC transporters with apical localization differ between gills and the other organs. Branchial localization and phylogenetic analyses of an abcb transporter with the most prominent expression in the gills, namely *abcb1b*, argue for a reannotation as *abcb5*. Both the localization in the gill tissue as well as the physiological function of the mammalian homologue ABCB5 imply a minor role of this ABC form in transepithelial transport in gills. The *in vitro* environment in primary gill cell culture leads to change in the expression of most investigated ABC transporters. The pronounced decline of ABC transporter mRNA in single-seeded cultures limits the utility of this method for studying branchial transport processes; however, the model might be of use for studies targeting specific ABC transporters.

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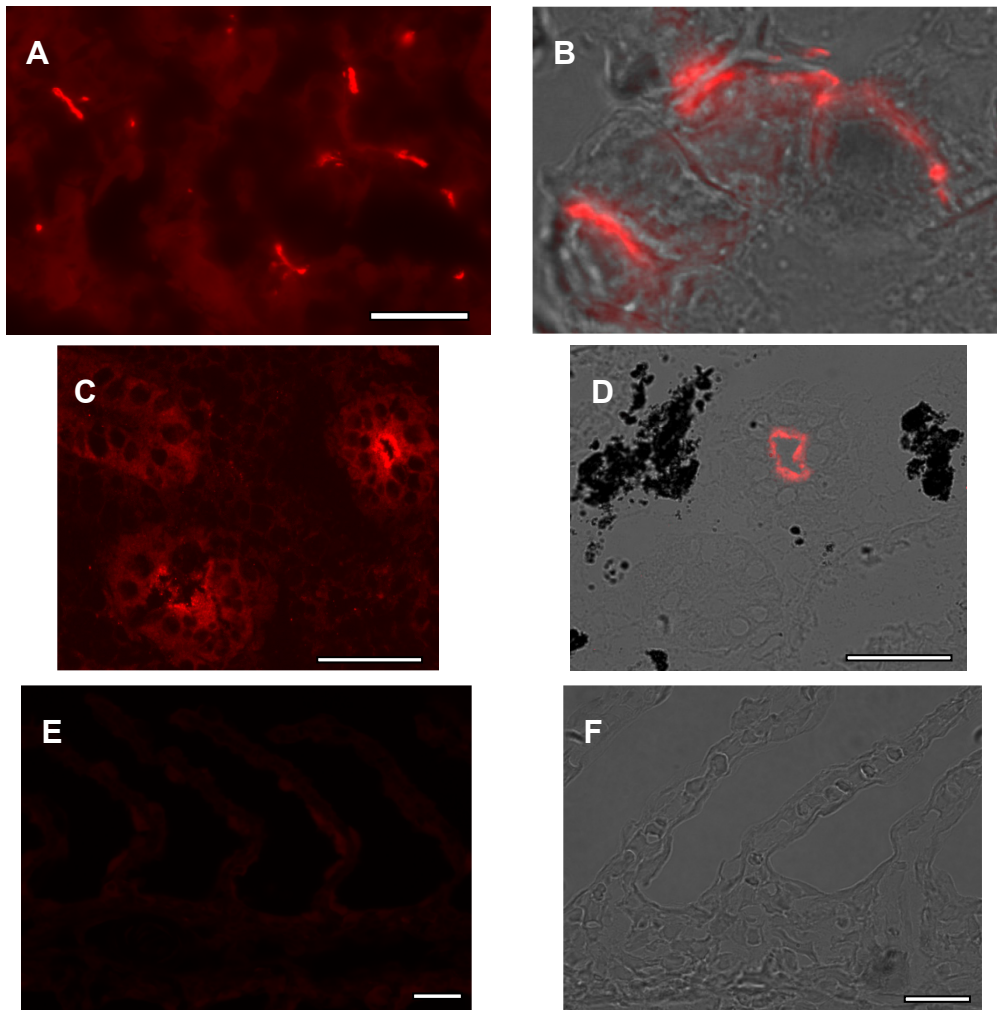
Figure S1

Figure S1: C219 antibody staining in liver, kidney and gills of *O. mykiss*. The cross reactivity of the anti-ABCB1 antibody C219 was used to localize abcb1 of *O. mykiss* on cryosections of liver, kidney and gills. **A/B**: C219 staining in the liver. Positive staining for abcb1 was detected on cell membranes of adjacent hepatocytes (red) forming the bile canaliculus. Scale bar in A, 20 µm and in B, 10 µm. **C/D**: C219 staining in the posterior kidney: The abcb1 positive staining was identified on apical membranes of kidney tubule cells (red). Scale bar in A and in B, 30 µm. **E/F**: C219 staining in the gills. No positive cells or cell membranes could be identified. Scale bar in A and in B, 20 µm.
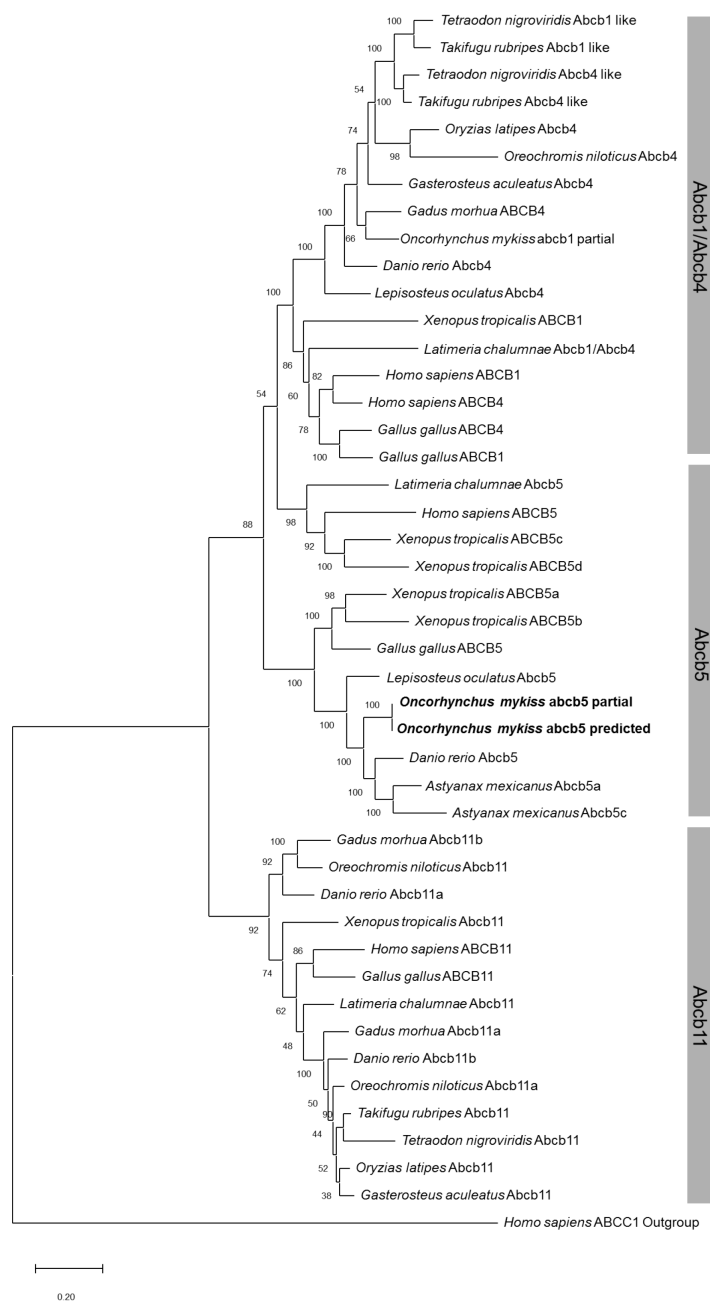
Figure S2

Figure S2: Phylogenetic analysis of abcb isoforms. Phylogenetic tree based on multiple alignments (TCoffee) of Abcb1, Abcb4, Abcb5 and Abcb11 ABC transporter amino acid sequences from vertebrates. The tree was generated using maximum likelihood method and the percentage concordance based on 500 bootstrap iterations is shown at the nodes.
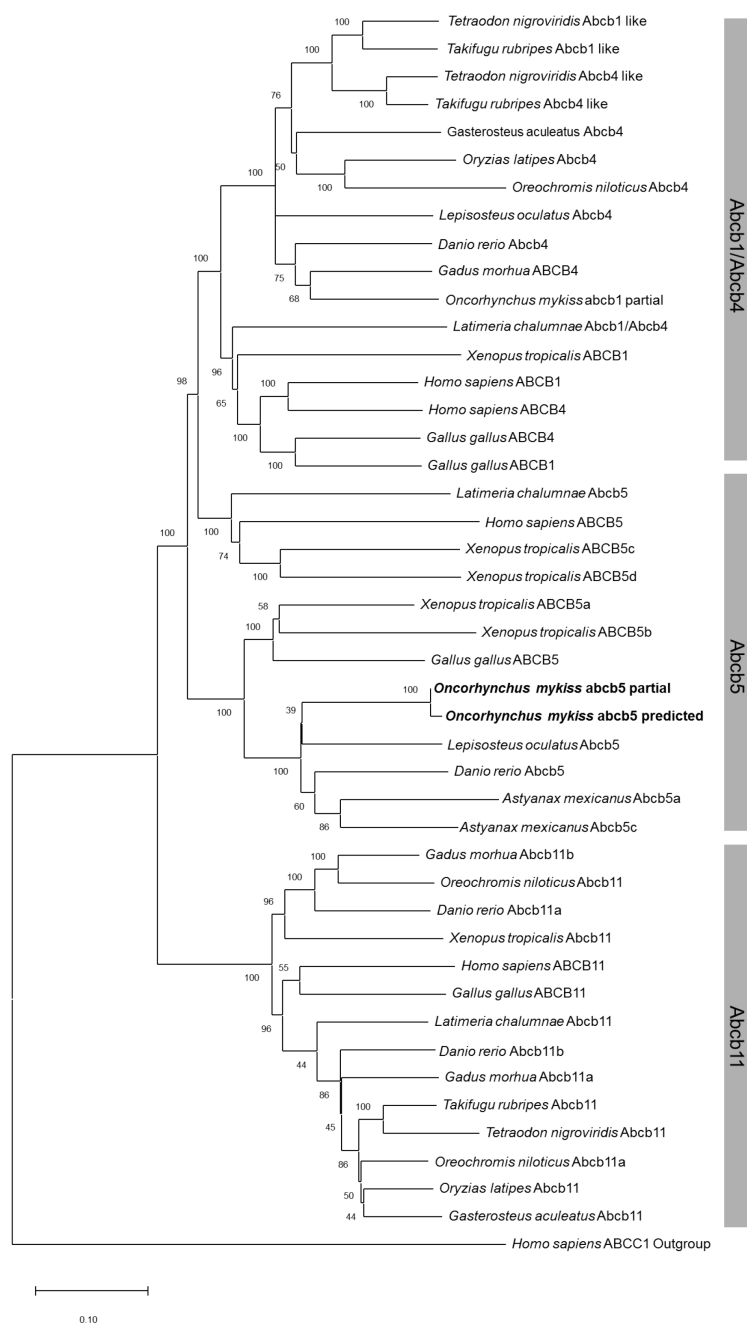
Figure S3

Figure S3: Phylogenetic analysis of abcb isoforms. Phylogenetic tree based on multiple alignments (TCoffee) of Abcb1, Abcb4, Abcb5 and Abcb11 ABC transporter amino acid sequences from vertebrates. The tree was generated using minimum evolution method and the percentage concordance based on 1000 bootstrap iterations is shown at the nodes
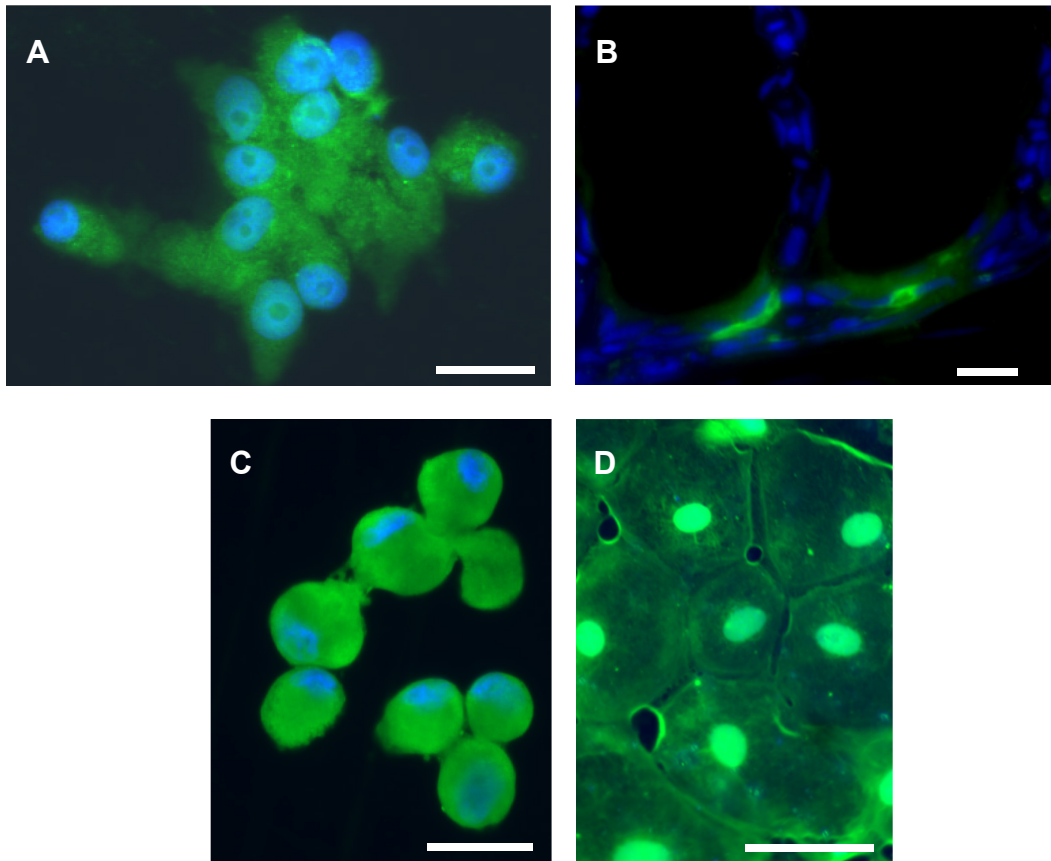
Figure S4

Figure S4: Vimentin as a marker for mesenchymal cells in gills and cultured primary gill cells. An anti-vimentin antibody was used as a marker to detected cells of mesenchymal origin, namely fibroblasts. The antibody staining was tested in the fibroblast cell line RTG2 (**A**) as well as on cryosections of gills (**B**) of juvenile O. mykiss. The presence of vimentin was assessed in primary gill cells at the third day post isolation cultured on solid support (**C**) and on permeable support (**D**). Scale bar 10 μm in A, C, D and 20 μm in B.
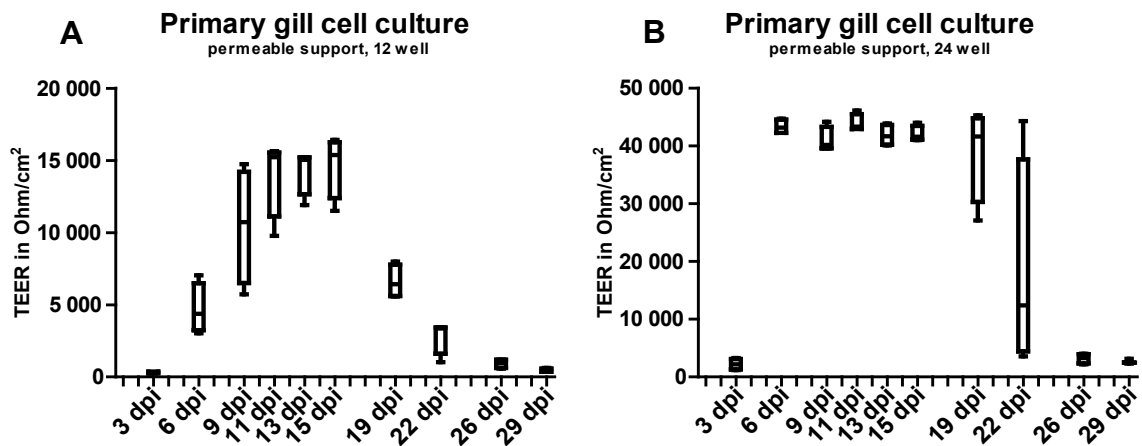
Figure S5

Figure S5: Development of a tight *in vitro* epithelium in primary gill culture on permeable support. The development of epithelium tightness on permeable culture was assessed measuring the transepithelial electrical resistance. Primary gill cells of juvenile *O. mykiss* were cultured on **A**: single seeded insert on 12 well inserts 0.9 cm^2 (n = 4) and **B**: 24 well insert 0.3 cm^2 (n = 4) and the TEER is given as Ohm/cm^2 of primary gill cell cultures. Whiskers show minimum and maximum TEER values.
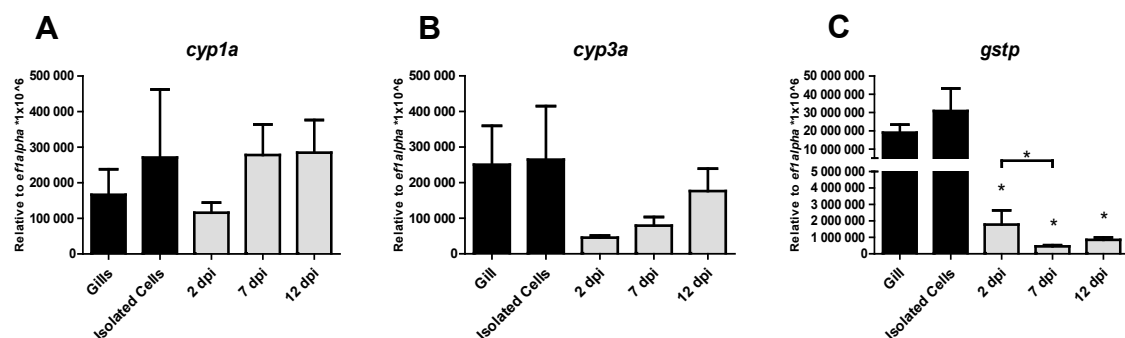
Figure S6

Figure S6: Relative mRNA abundance of biotransformation enzymes *cyp1a*, cyp3 and *gstp* in gills and during primary gill cell culturing. The mRNA levels of selected genes in gills, isolated cells of gills and from cultured cells on solid support at 2, 7, and 12 days post isolated. Isolated gill cell populations were red blood cell treated in order to prevent an impact of blood cells on mRNA profiles. The mRNA levels of *cyp1a* (**A**), *cyp3* (**B**) and *gstp* (**C**) are given as mean normalized expression (MNE) to the reference gene *ef1α* ± s.d. from independent experiments (n = 6, except gills n = 3, isolated gill cells n = 3); significant differences compared to gills is indicated by asterisks or asterisks linked with lines when compared among culture time points (p<0.05).

Dataset 1. Nucleotide sequences.

abcb5 O. mykiss partial CDS nucleotide sequence

ATGATTGGGGCGTTCTCTTTGGGCCAGGGTGCGCCTAACTTGGAAGCCATCGCCAAAG
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abcb5 O. mykiss partial CDS amino acid sequence

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abcb5 O. mykiss predicted nucleotide sequence

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abcb5 O. mykiss predicted amino acid sequence

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