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Complete Knockout of Endogenous Mdr1 (*Abcb1*) in MDCK Cells by CRISPR-Cas9

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ABSTRACT

Madin-Darby canine kidney II cells transfected with one or several transport proteins are commonly used models to study drug transport. In these cells, however, endogenous transporters such as canine Mdr1/P-glycoprotein (*Abcb1*) complicate the interpretation of transport studies. The aim of this investigation was to establish a Madin-Darby canine kidney II cell line using CRISPR-Cas9 gene-editing technology to knock out endogenous canine Mdr1 (cMdr1) expression. CRISPR-Cas9-mediated *Abcb1* homozygous disruption occurred at frequencies of around 20% and resulted in several genotypes. We selected 1 clonal cell line, cMdr1 KO Cl2, for further examination. Consistent with an on-target effect of CRISPR-Cas9 in specific regions of the endogenous canine *Abcb1* gene, we obtained a cell clone with *Abcb1* gene alterations and without any cMdr1 expression, as confirmed by genome sequencing and quantitative protein analysis. Functional studies of these cells, using digoxin and other prototypic MDR1 substrates, showed close to identical transport in the apical-to-basolateral and basolateral-to-apical directions, resulting in efflux ratios indistinguishable from unity.

Introduction

Transporter-mediated efflux of exogenous compounds and metabolites is an important cellular defense mechanism and plays a central role in the barrier and excretory functions of tissues such as the intestinal mucosa, the blood-brain and blood-testis barriers, the renal proximal tubules, and the liver. Several members of the ATP-Binding Cassette (ABC) transporter superfamily—including multidrug resistance protein 1 (MDR1/P-glycoprotein; *ABCB1*), breast cancer resistance protein (BCRP; *ABCG2*), and several members of the multidrug resistance-associated protein family (MRP; *ABCC*)—are highly expressed in such barrier tissues, and their importance for drug disposition is undisputed.[1]

Monolayer-forming epithelial cell lines are often used as model systems to study the impact of ABC efflux transporters on drug disposition. One commonly used cell line is Madine-Darby Canine Kidney (MDCK II), which forms tight, confluent monolayers when cultured on filter supports, thereby allowing studies of bidirectional transepithelial transport. Its protein sorting machinery provides the opportunity for stable heterologous introduction of the human efflux transporter of interest—either in isolation or in combination with specific uptake transporters from the Solute Carrier superfamily.[2-6]

A major drawback of MDCK II is its endogenous background of canine transporters, mainly cMdr1, which may influence the fluxes of studied compounds. Further complicating the interpretation of results, this background expression can also differ between clones, between transfected and untransfected cells, between laboratories, and as a result of culturing conditions.[7,8] The contribution from canine Mdr1 is usually subtracted by including MDCK II wild-type (wt) cells as a negative control in the transport experiment to obtain a net flux ratio (NFR), that is, the efflux ratio (ER) in MDCK II-hMDR1 cells is divided by the ER of the wt. As the ER in MDCK II-hMDR1 and MDCK II wt cells can vary due to the aforementioned reasons, the NFR will also vary tremendously. For instance, NFRs

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ranging from 1.8 to 12 have been reported for the MDR1-substrate digoxin.[8-11] Hence, for accurate estimations of the contribution of human transporters, a complete knockout of canine Mdr1 would be advantageous.

Over the years, attempts have been made to knock out endogenous Mdr1 in MDCK II and other cell lines using, for example, inhibitory RNA,[12] clonal selection based on intracellular retention of fluorescent MDR1 substrates,[13,14] and most recently, zinc-finger nucleases (ZFNs).[15] Depending on the technology, higher or lower degrees of expression of cMdr1 were obtained, but even with the recent application of ZFN technology, background correction using wt cells is necessary.

Here, we present the use of CRISPR-Cas9 gene-editing technology[16-19] to knock out endogenous canine Mdr1 expression at the genome level, resulting in the establishment of an MDCK II cell line without any cMdr1 background.

Methods

Cells

Madin-Darby canine kidney cells (MDCK II wt) obtained from American Type Culture Collection (ATCC CRL-2936, Rockville, MD) were cultured in Dulbecco's Modified Eagle Medium with GlutaMax™ supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin solutions (100 units/mL penicillin and 100 μg/mL streptomycin) at 37°C and 5% CO_2. All cell culture media and supplements were from Invitrogen (Carlsbad, CA). For transport and proteomic experiments, 5×10^5 MDCK II wt (passages 42-54) or cMdr1 KO Cl2 cells (passages 8-16) were seeded onto 12 mm, 0.4-μm Transwell filter inserts (Corning, Amsterdam, Netherlands), and cells were allowed to differentiate for 4 days before the experiment.

Knockout of Canine Mdr1 in MDCK II Cells

Custom-designed CRISPR-Cas9 vectors, targeting 3 specific regions of canine *Abcb1*, were obtained from Sigma-Aldrich (St. Louis, MO); see Supplementary Material for details. Guide RNAs for the respective CRISPR were under the regulation of a U6 promoter, whereas a cytomegalovirus promoter drove coexpression of the Cas9 enzyme and green fluorescent protein (GFP). The MDCK II wt cells (passage 43) were grown to 70%-90% confluence and transfected with all 3 CRISPR plasmids. Cells were sorted based on high coexpression of GFP and then seeded as single cells. After clonal expansion of the cells, genomic DNA was extracted, and the target regions were amplified by PCR using primer pairs for the expected mutation sites. The PCR product was sequenced by Sanger sequencing at Uppsala Genome Center, Uppsala University.

Protein Quantification of ABC Drug Transporters

For proteomic analysis, 8 Transwell membranes of MDCK II wt or cMdr1 KO Cl2 cells were collected 4 days after seeding. For details, see the Supplementary Material. Briefly, membranes with attached cell monolayers were rinsed, immersed in lysis buffer (4 filters per lysis reaction), and immediately boiled for 10 min. The lysates were sonicated and then clarified by centrifugation. The total protein concentration was determined using the tryptophan fluorescence assay (WF)[20] assuming 1.17% total tryptophan content. The filter-aided sample preparation protocol[21] was used for trypsin digestion. Briefly, 50-μg total protein lysates were depleted from detergent by successive washes using 30-kDa MWCO filtration units, and proteins were digested with trypsin. Peptides were eluted and quantified using WF[20] and resuspended in the LC-MS/MS mobile phase solvent before analysis. The digestion yields were all greater than 78%.

Transport Assays

For transport experiments, MDCK II wt or cMdr1 KO Cl2 cells were seeded onto Transwell membrane inserts and allowed to differentiate for 4 days before the experiment. Transport experiments were performed with digoxin (PerkinElmer, Wiltham, MA), digoxin in the presence of the MDR1 inhibitor elacridar, or prazosin, labetalol, loperamide, quinidine, vinblastine, ritonavir, and saquinavir (all from Sigma-Aldrich) in Hanks' balanced salt solution, pH 7.4. Experiments were essentially performed as described in Hubatsch[22]; for details, see the Supplementary Material. Transepithelial electrical resistance was measured before and after the transport experiments to verify the integrity of the cell monolayers. The Student t-test was used for statistical comparisons between each pair of independent mean values.

Results

Knockout of Canine Mdr1 in MDCK II Cells

Three canine Mdr1-specific CRISPR-Cas9 gRNAs were designed, targeting the fourth and thirteenth exon of the canine *Abcb1* gene (Gene ID: 403879). MDCK II wt cells were transiently transfected with a combination of the 3 CRISPR-Cas9 vectors. Surviving cells were sorted by FACS on the basis of high GFP expression, then seeded as single cells into 96-well plates for clonal expansion and cryopreservation. These cell clones were further analyzed by PCR amplification and Sanger sequencing of the targeted genomic regions in exons 4 and 13. Of the 25 clones analyzed by genomic sequencing, gene editing with clear homozygous indels of variable size occurred in 5 clones (data not shown). The remaining clones exhibited an unaltered genotype or were heterozygous at exon 4 and/or 13. We chose to continue our functional assays with cMdr1 KO Cl2 as it had a 4-nucleotide deletion in exon 4 (Fig. 1), leading to a shift of the reading frame and 3 consecutive stop codons downstream in exon 4, thus resulting in a truncated protein.

Protein Quantification of ABC Drug Transporters

To verify the absence of canine Mdr1, a quantitative mass-spectrometry–based protein analysis method was set up for Mdr1 and 3 additional transporters of the ABC family (Mrp2, Mrp3, and Bcrp). All selected proteotypic peptides and corresponding stable isotope-labeled standards were representative of both the human and canine sequence.[23] Whole cell digests of filter-grown MDCK II wt and cMdr1 KO Cl2 clones were analyzed. The limits of quantification were 0.1 fmol/μmol total protein for Mdr1, Mrp2, and Bcrp and 0.01 for Mrp2, that is, comparable to state-of-the-art targeted proteomics.[23-25] LC-MS/MS data showed that cMdr1 KO Cl2 lacked detectable levels of endogenous cMdr1 expression (Table 1), whereas wt cells had high cMdr1 protein concentrations. cMrp2 was expressed at a similar level as cMdr1 in wt cells, but the levels were lower in the cMdr1 KO Cl2 cells. cMrp3 expression was similar in both cell lines, and cBcrp was below the limit of quantification.

MDR1-Mediated Transport

Transport experiments with the MDR1-substrate digoxin showed that the MDCK II wt cells gave a higher P_{app} in the