

Special Section – New Models in Drug Metabolism and Transport—Minireview

CRISPR-Cas9: A New Addition to the Drug Metabolism and Disposition Tool Box

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ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR associated protein 9 (Cas9), i.e., CRISPR-Cas9, has been extensively used as a gene-editing technology during recent years. Unlike earlier technologies for gene editing or gene knockdown, such as zinc finger nucleases and RNA interference, CRISPR-Cas9 is comparably easy to use, affordable, and versatile. Recently, CRISPR-Cas9 has been applied in studies of drug absorption, distribution, metabolism, and excretion (ADME) and for ADME model generation. To date, about 50 papers have been published describing *in vitro* or *in vivo* CRISPR-Cas9 gene editing of ADME and

ADME-related genes. Twenty of these papers describe gene editing of clinically relevant genes, such as ATP-binding cassette drug transporters and cytochrome P450 drug-metabolizing enzymes. With CRISPR-Cas9, the ADME tool box has been substantially expanded. This new technology allows us to develop better and more predictive *in vitro* and *in vivo* ADME models and map previously underexplored ADME genes and gene families. In this mini-review, we give an overview of the CRISPR-Cas9 technology and summarize recent applications of CRISPR-Cas9 within the ADME field. We also speculate about future applications of CRISPR-Cas9 in ADME research.

Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR associated protein 9 (Cas9) is an easily adapted gene-editing technology that can be designed to target most genomic sequences. The major difference between CRISPR-Cas9 and previously favored gene-editing systems—such as the zinc finger nucleases (ZFNs) and the transcription activator–like effector nucleases (TALENs)—is its simplicity. CRISPR-Cas9 DNA targeting only requires the design of a short guide RNA molecule compared with the more complex and expensive protein engineering needed for retargeting ZFNs and TALENs (cf. Gaj et al., 2013; Kim, 2016; Editorial, 2017). The availability and affordability of commercial kits and services means that many biomedical research fields are now using CRISPR-Cas9.

In applied genome editing, researchers have traditionally relied heavily on model organisms such as bacteria, yeast, fruit fly, and rodents. In part,

this was because these were the only species for which good tools for genetic manipulation were available. The need for faster gene editing led to the development of custom nucleases like ZFNs and TALENs that induce double strand breaks (DSBs) in a predefined region in the gene of interest (Gaj et al., 2013). Only the ZFN technology has been applied within the field of absorption, distribution, metabolism, and excretion (ADME). There, it has been used for selective knockout of drug transporters in C2BBE1 cells (a subclone of Caco-2, which is a widely used model for investigating transporter-mediated intestinal efflux of drugs) (Pratt et al., 2012; Sampson et al., 2015). Similarly, ZFN has been used to knock out endogenous canine transporters in Madin-Darby canine kidney (MDCK) II cells (Gartzke et al., 2015). It has also been used in HepaRG cells, an alternative to primary human hepatocytes, to knock out the nuclear pregnane X receptor (PXR), a receptor that regulates the expression of drug-metabolizing enzymes and drug transporters (Williamson et al., 2016). Despite these successful studies, neither ZFN nor TALEN have become widespread methods within the field of ADME. One reason may be the expensive and demanding protein engineering needed.

CRISPR-Cas9 has made it possible to edit genes in virtually all organisms for a fraction of the cost compared with alternative technologies. Moreover, CRISPR-Cas9 allows modification of the genes of interest at more physiologically relevant levels (i.e., regulated by an

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ABBREVIATIONS: ADME, absorption, distribution, metabolism, and excretion; Cas9, clustered regularly interspaced short palindromic repeats associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; dCas9, nuclease deficient clustered regularly interspaced short palindromic repeats associated protein 9; DSB, double strand break; HDR, homology directed repair; iPSC, induced pluripotent stem cell; KO, knockout; MDCK, Madin-Darby canine kidney; NHEJ, nonhomologous end joining; P450, cytochrome P450; PAM, protospacer adjacent motif; PXR, pregnane X receptor; sgRNA, single guide RNA; TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nuclease.

indigenous promoter) rather than by overexpression of the gene of interest. In addition to manipulation of individual genes, CRISPR-Cas9 can even be used to eliminate an entire chromosome (Zuo et al., 2017). This has been demonstrated in human pluripotent stem cells with chromosome 21 trisomy and in cancer cells; highlighting potential therapeutic applications for the CRISPR-Cas9 technology. Furthermore, it is possible to study multiple genes simultaneously, thus creating an intricate network for studying or even curing diseases. One example of such simultaneous gene editing is the knockout of 62 porcine endogenous retroviruses in an immortalized porcine cell line (Yang et al., 2015). In a follow-up study, the same technology was used to generate porcine endogenous retrovirus-free piglets, a strategy that would increase safe and effective organs for pig-to-human xenotransplantations (Niu et al., 2017). However, the in vivo application of CRISPR-Cas9 in humans was impeded after a report in 2015, in which CRISPR-Cas9 gene editing in human embryos showed low rates of homology directed repair (HDR) and apparent off-target effects (i.e., alterations in genes unrelated to the target genes) (Liang et al., 2015). Thus, there are crucial safety issues that have to be solved before broader in vivo applications become a reality. Despite this setback, there are ongoing clinical trials involving CRISPR-Cas9 [https://clinicaltrials.gov/ (accessed on May 20, 2018)]. The first of these focuses on T-cells engineering for treatment of metastatic small cell lung cancer. This study started in 2016 and is expected to be completed during 2018. The engineered T-cells were given to the first patient in October 2016 [https://newatlas.com/crispr-gene-editing-first-human-trial/46453/ (accessed on May 20, 2018)], and the results are not yet published.

The number of publications regarding CRISPR-Cas9 has increased rapidly (Fig. 1A), from single articles by the originators of the technology in 2011 and 2012, to more than 2000 articles published in 2017. CRISPR-Cas9 is now also being applied within the ADME field (Fig. 1B; Table 1). In this context, it has been mainly used for the generation of in vitro and in vivo knockout models, in parallel with other approaches. Importantly, technologies such as CRISPR-Cas9 also give the opportunity to deorphanize and characterize *ADME* gene families that are currently underexplored, such as the solute carrier transporter family (César-Razquin et al., 2015). In this mini-review, we give an overview of the application of CRISPR-Cas9 gene editing for ADME studies of drugs and xenobiotics.

How Does CRISPR-Cas9 Work?

In nature, CRISPR-Cas9 is an adaptive, acquired immune system, mediated by RNA, which protects eubacteria and archaea from viruses or plasmids (Barrangou et al., 2007). In bacteria, two RNA molecules, together with the endonuclease Cas9, bind to a target sequence. One of the RNA molecules (*trans*-activating CRISPR RNA) is species conserved and acts as a scaffold that binds Cas9 itself. The other RNA molecule (CRISPR RNA) is variable and confers specificity to a target genome DNA. For gene-editing purposes the technology was simplified and made more efficient by combining both RNAs into a single guide RNA (sgRNA) (Jinek et al., 2012). The sgRNA and Cas9 form a sgRNA-Cas9 complex that binds to its target sequence (Fig. 2, A and B). An additional requirement of the target sequence is the presence of a protospacer adjacent motif (PAM) (Mojica et al., 2009). If the sgRNA hybridize to DNA immediately 5' to an adjacent PAM domain the Cas9 endonuclease will create a DSB (Fig. 2B). This DSB can be repaired by the mechanism of nonhomologous end joining (NHEJ) (Overballe-Petersen et al., 2013). This, in turn, may result in small deletions or insertions (indels) that disrupt the targeted gene, resulting in loss of its function (Fig. 2C). Alternatively, if there is a donor DNA template to the targeted region the DSB can be repaired by HDR, thereby allowing for precise replacement of nucleotide(s), introduction of mutations, or insertion of sequences in the targeted region (Gong et al., 2005) (Fig. 2C). The

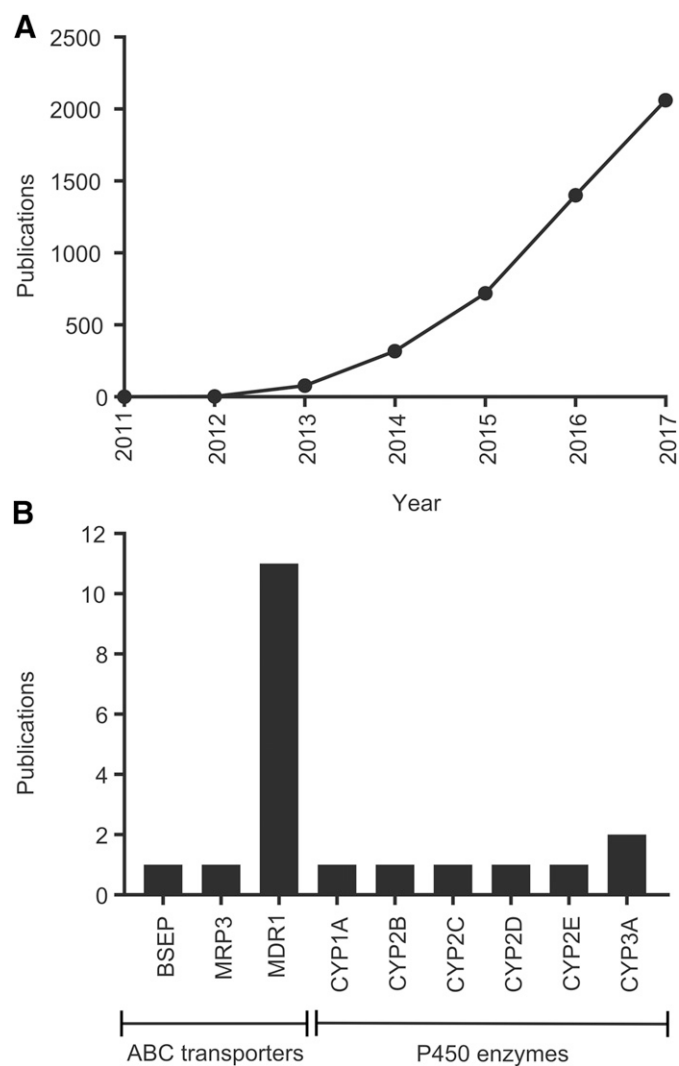


Fig. 1. (A) Number of publications per year mentioning CRISPR-Cas9. Data were generated by PubMed search using the terms “CRISPR” and “Cas9.” (B) Total number of publications for CRISPR-Cas9 gene editing of *ADME* genes/proteins. Data were generated by PubMed search using the terms “CRISPR” and “Cas9” and the respective human gene or protein name. All hits were screened and publications were excluded if CRISPR-Cas9 had not been used to edit an *ADME* gene. If homologous genes from other species had been edited, those publications were included in this compilation. The *ADME* genes included in the analysis are the solute carrier and ATP-binding cassette (ABC) transporters identified as involved in drug ADME by the International Transporter Consortium (Giacomini et al., 2010) and the most important cytochrome P450 (P450) and uridine diphosphate-glucuronyltransferase enzymes (Evans and Relling, 1999; Williams et al., 2004). The PubMed search was performed May 18, 2018, i.e., articles published after that are not included in this compilation.

frequency of NHEJ indel formation or successful HDR varies, depending on the model cell line/organism and methods used; however, in general HDR is a rarer event than NHEJ indel formation (Mali et al., 2013). For an overview of CRISPR discovery and history, see Lander (2016).

The CRISPR-Cas9 system primarily used for gene editing comes from *Streptococcus pyogenes*. However, Cas9 proteins from other organisms are being explored to increase the flexibility and expansion of application areas (as discussed subsequently). CRISPR-Cas9 has developed from in-house design to predesigned sgRNAs and premade CRISPR kits available from multiple vendors. In addition, software is available, e.g., for sgRNA design, analysis of off-target effects, etc. [e.g., <http://crispr.mit.edu/>, <http://www.e-crisp.org/E-CRISP/>, <https://crispr.dbcls.jp/>, <http://www.rgenome.net/cas-offfinder/>, and <http://www.biootools.com/> (accessed on May 23, 2018)]. The commercial availability of kits and

TABLE 1
Overview of ADME and ADME-related genes edited using CRISPR-Cas9

Gene/Protein	Cell Line or Organism	Modification	Reference
Family: Cytochrome P450s <i>CYP1A1/CYP1A1</i>	Induced pluripotent stem cells	Knockin of a Luciferase reporter cassette at the transcription start site	Smith et al. (2016)
<i>Cyp2b9/10/13/CYP2B9/10/13</i>	C57BL/6 mice	Knockout of <i>Cyp2b9</i> , <i>Cyp2b10</i> and <i>Cyp2b13</i>	Kumar et al. (2017)
<i>Cyp2c11/CYP2C11</i>	Sprague-Dawley rats	Knockout of <i>Cyp2c11</i>	Wei et al. (2018)
<i>Cyp2d1/2/3/4/5/CYP2D1/2/3/4/5</i>	Wistar rats	Knockout of the <i>Cyp2d1</i> cluster	Yoshimi et al. (2016)
<i>Cyp2d1/2/3/4/5/CYP2D1/2/3/4/5</i>	Wistar rats	Knockout of the rat <i>Cyp2d</i> cluster and knockin of human <i>CYP2D6</i>	Yoshimi et al. (2016)
<i>Cyp2e1/CYP2E1</i>	Sprague-Dawley rats	Knockout of <i>Cyp2e1</i>	Wang et al. (2016)
<i>cyp26a1/CYP26A1</i>	<i>Silurus meridionalis</i>	Knockout of <i>cyp26a1</i>	Li et al. (2016)
<i>Cyp3a1/2/CYP3A1/2</i>	Sprague-Dawley rats	Knockout of <i>Cyp3a1</i> and <i>Cyp3a2</i>	Lu et al. (2017)
<i>CYP3A5/CYP3A5</i>	Human hepatocyte cell line HuH-7	Editing of the <i>CYP3A5*3</i> locus to generate <i>CYP3A5*1</i>	Dorr et al. (2017)
<i>CYP90B1/CYP90B1</i> (PtoDWF4)	<i>Populus tomentosa</i>	Knockout of <i>CYP90B1</i>	Shen et al. (2018)
<i>CYP734A4/CYP734A4</i>	Japonica rice cultivar Zhonghua11	Knockout of <i>CYP734A4</i>	Qian et al. (2017)
Family: ABC transporters <i>ABCA1/ABCA1</i> <i>ABCA2/ABCA2</i>	Human pluripotent stem cells	Knockout of <i>ABCA1</i>	Gupta et al. (2016)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	<i>Helicoverpa armigera</i>	Knockout of <i>ABCA2</i>	Wang et al. (2017)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Canine kidney cell line MDCK	Knockout of <i>ABCB1</i>	Simoff et al. (2016)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Human osteosarcoma cell lines KHOSR2 and U-2OSR2	Knockout of <i>ABCB1</i>	Liu et al. (2016)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Human breast cancer cell line MCF-7/ADR	Knockout of <i>ABCB1</i>	Ha et al. (2016)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Human oral squamous carcinoma cell line KB _{v200} and human colorectal cancer cell line HCT-8/V	Knockout of <i>ABCB1</i>	Yang et al. (2016b)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Canine kidney cell line MDCK overexpressing human MDR1	Knockout of <i>ABCB1</i>	Karlgrén et al. (2017)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Human ovarian cancer cell line A2780/ADR	Knockout of <i>ABCB1</i>	Norouzi-Barough et al. (2018)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	<i>Spodoptera exigua</i>	Knockout of <i>MDR1</i>	Zuo et al. (2018)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	<i>Drosophila melanogaster</i>	Knockout of <i>mdr49</i>	Denecke et al. (2017)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	<i>Drosophila melanogaster</i>	Knockout of <i>mdr50</i>	Denecke et al. (2017)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	<i>Drosophila melanogaster</i>	Knockout of <i>mdr65</i>	Denecke et al. (2017)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	<i>Plasmodium falciparum</i>	Introduction of point mutations in <i>mdr1</i>	Ng et al. (2016)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Human acute lymphoblastic leukemia cell line HALO1	Knockout of <i>ABCB1</i>	Takahashi et al. (2017)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Human fibrosarcoma derived cells line HT-1080	Edition of two GpG islands in the <i>ABCB1</i> promoter	Grob et al. (2018)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Zebrafish	Knockout of <i>abcb1/b</i>	Ellis et al. (2018)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Zebrafish	Knockout of <i>mip1</i>	Tian et al. (2017)
<i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein <i>ABCB1/MDR1</i> , <i>ABCB1</i> or P-glycoprotein	Human keratinocyte cell line HaCaT	Deletion of a CpG island 10 kbp upstream of <i>ABCC3</i>	Takechi et al. (2018)
<i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i> <i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i>	Human colon carcinoma cell line T84	Knockout of <i>CFTR</i>	Chung et al. (2016)
<i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i> <i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i>	Human tracheal epithelial cells from a cystic fibrosis patient (CFTEs)	Analysis of NHEJ and template dependent editing of the F508del mutation	Hollywood et al. (2016)
<i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i> <i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i>	Human adult intestinal cell lines	Correction of a 3 basepair deletion in <i>CFTR</i>	Schwank et al. (2013)
<i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i> <i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i>	Human lung adenocarcinoma cell line Calu-3 cells and primary human airway epithelial cells	Knockout of <i>CFTR</i>	Bellec et al. (2015)
<i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i> <i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i>	Human pluripotent stem cells	Correction of a three base pair deletion in <i>CFTR</i>	Firth et al. (2015)
<i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i> <i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i>	Human colon cancer cell line Caco-2	Deletion of a 1.2 kb fragment/a CTCF binding site located 20.9 kb upstream of the transcription start site	Yang et al. (2016a)

(continued)

TABLE 1—Continued

Gene/Protein	Cell Line or Organism	Modification	Reference
<i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i>	Human colon cancer cell line Caco-2	Deletion of a 1.2 kb fragment/a binding site for intestinal selective enhancers located in intron 11	Yang et al. (2016a)
<i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i>	Human CFTE cell line	Editing of three rare mutations in <i>CFTR</i>	Sanz et al. (2017)
<i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i>	Human bronchial epithelial cell line 16HBE	Introduction of a <i>CFTR</i> mutation	Molinski et al. (2017)
<i>ABCC7</i> , also known as <i>CFTR/CFTR</i> or <i>ABC35</i>	Conditionally reprogrammed human bronchial epithelial cells	Knockout of <i>CFTR</i>	Peters-Hall et al. (2018)
<i>ABCC8/ABCC8</i>	Human embryonic stem cells	Knockout of <i>ABCC8</i>	Guo et al. (2016a,b, 2017)
<i>ABCG</i> family member <i>Bm-brown/Bm-brown</i>	<i>Bombyx mori</i>	Knockout of <i>Bm-brown</i>	Zhang et al. (2018)
<i>ABCG</i> family member <i>white (w)</i>	<i>Drosophila subobscura</i>	Mutating the <i>white</i> gene	Tanaka et al. (2016)
<i>ABCG</i> family member <i>white (w)</i>	<i>Drosophila melanogaster</i>	Mutating the <i>white</i> gene	Kondo and Ueda (2013)
<i>ABCG</i> family members <i>white (Ha-w)</i> , <i>scarlet (Ha-st)</i> , <i>brown (Ha-bw)</i> and <i>ok (Ha-ok)</i>	<i>Helicoverpa armigera</i>	Knockout of <i>white</i> , <i>scarlet</i> , <i>brown</i> and <i>ok</i>	Khan et al. (2017)
Family: SLC transporters <i>SLC31A1/CTR1</i>	Human embryonic kidney cell line HEK293T and human ovarian cell line OVCAR8	Knockout of <i>SLC31A1</i>	Tsai et al. (2015), Bompiani et al. (2016)
<i>SLC31A2/CTR2</i>	Human embryonic kidney cell line HEK293T and human ovarian cell line OVCAR8	Knockout of <i>SLC31A2</i>	Tsai et al. (2015), Bompiani et al. (2016)
<i>SLC35B2/SLC35B2</i> or <i>UGT14</i>	Primary human CD4+ T cells	Knockout of <i>SLC35B2</i>	Park et al. (2017)
<i>SLC35F2/SLC35F2</i>	Papillary thyroid carcinoma cell lines BCPAP and KTC-1	Knockout of <i>SLC35F2</i>	He et al. (2018)
<i>slc45a2/SLC45A2</i>	Atlantic salmon	Indel formation in <i>slc45a2</i>	Edwardsen et al. (2014)
<i>Slo2a1/OATP2A1</i>	Mouse mammary cell line C127	Knockout of <i>Slo2a1</i>	Sabirov et al. (2017)
Family: Others <i>AHR2A/B</i> /aryl hydrocarbon receptor 2A/B (<i>AHR2A/B</i>) <i>Ahr</i> /aryl hydrocarbon receptor (<i>AHR</i>) <i>Ahr</i> /aryl hydrocarbon receptor (<i>Ahr</i>) <i>Ahr</i> /aryl hydrocarbon receptor (<i>Ahr</i>)	<i>Fundulus heteroclitus</i> Human embryonic stem cells Young adult mouse colonic cells Human Mammary carcinoma cell line Hs578T	Insertion of mutations in <i>AHR2A</i> and <i>AHR2B</i> Knockout of <i>AHR</i> Knockout of <i>Ahr</i> Knockout of <i>Ahr</i>	Aluru et al. (2015) Angelos et al. (2017) Cheng et al. (2017) Narasimhan et al. (2018)
<i>ahr2</i> /aryl hydrocarbon receptor 2 (<i>Ahr2</i>) <i>POR</i> /cytochrome P450 oxidoreductase (<i>POR</i> or <i>CYPOR</i>) <i>PXR</i> or <i>NR1I2</i> /pregnane X receptor (<i>PXR</i>) or <i>NR1I2</i> <i>VKORC1</i> /vitamin K epoxide reductase complex subunit 1 and <i>VKORC1L1</i> /vitamin K epoxide reductase complex subunit 1-like protein 1	zebrafish Human embryonic kidney cell line HEK293 Induced pluripotent stem cells Human embryonic kidney cell line HEK293T	Knockout of <i>ahr2</i> Knockout of <i>POR</i> Knockin of an mCherry tag Knockout of <i>VKORC1</i> and <i>VKORC1L1</i>	Garcia et al. (2018) Rezende et al. (2017) Kim et al. (2018) Czogalla et al. (2017)

ABC, ATP-binding cassette; Ahr, aryl hydrocarbon receptor; CFTE, cystic fibrosis tracheal epithelial; SLC, solute carrier.

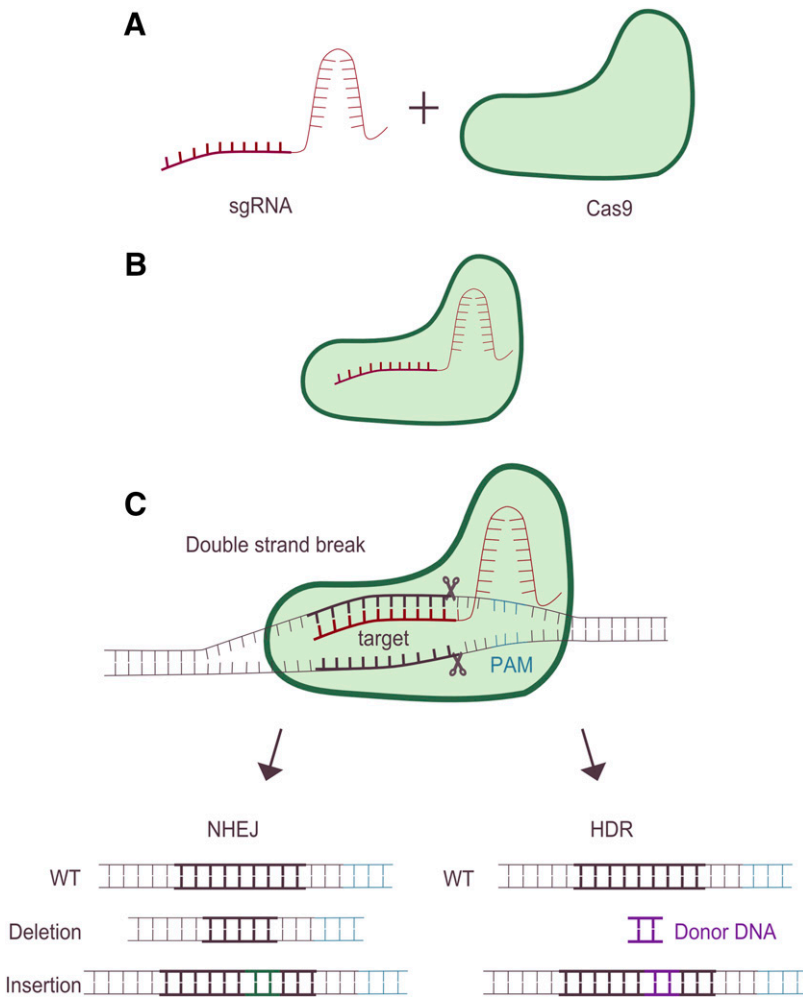


Fig. 2. Overview of the CRISPR-Cas9 gene-editing process. For CRISPR-Cas9 gene editing, a sgRNA is designed. This sgRNA consists of two parts, one that is specific for the desired target DNA and one that will interact with the second component in the CRISPR-Cas9 system, the Cas9 endonuclease (A). An important requirement of the target DNA is the presence of a PAM. After formation of a sgRNA-Cas9 complex (B), the complex will bind to the target DNA, and if an adjacent PAM is present Cas9 will cause a DNA DSB three base pairs upstream of the PAM sequence (C). Thereafter, the DSB can be repaired using two different mechanisms, NHEJ or HDR. NHEJ is an error-prone mechanism that will often result in small deletions or insertions (indels) that can lead to a disruption in the targeted locus. Alternatively, if a homology-containing donor-DNA template to the targeted region is present, the DSB can be repaired instead by HDR, thereby allowing for precise replacement, introduction of mutations or insertion of sequences in the targeted region. WT denotes wild type.

software extends the possibilities of the CRISPR-Cas9 technology to researchers within many fields.

Improved CRISPR-Cas9 Technologies. New or improved CRISPR technologies are constantly being developed, offering more opportunities and diverse applications (Sander and Joung, 2014). One limitation of CRISPR-Cas9 is set by the PAM motif—sites must contain the NGG PAM sequence for gene editing to occur (see Fig. 2C). To overcome this limitation, other species, endonucleases, and PAM recognition sequences are being explored, generating even more PAM motif variants (Fig. 3A) (see Nakade et al. (2017), and references therein). Together, these expand the precision and applicability of CRISPR as a gene-editing tool. Recently, a thermostable Cas9 was described with activity up to 70°C. This endonuclease exhibited significant stability in diluted human plasma, potentially providing a new opportunity for therapeutic applications via intravenous administration (Harrington et al., 2017).

One approach to overcome off-target effects is to use mutated Cas9 (Cas9D10A) that introduce a single-strand break (a nick) in the target DNA instead of a DSB (Fig. 3B) (Cong et al., 2013). Hence, two sgRNAs (one on the sense and one on the antisense strand) are needed to produce a DSB and give rise to a 5' overhang (Fig. 3C). This will minimize the off-target effects, whereas the on-target effects will occur at the same rates as with wild-type Cas9 (Ran et al., 2013).

A modified Cas9, called nuclease deficient Cas9 (dCas9), can be applied for other technical improvements (Gilbert et al., 2013). The dCas9 protein lacks endonuclease function, but it can be further modified by coupling it with a functional protein domain with different

activity, e.g., a transcriptional repressor (CRISPR interference) or activator (CRISPR activation) (Lo and Qi, 2017). With the ability of sgRNA to bind to a specific target sequence, the coupled dCas9 can be targeted to the specific gene of interest (Fig. 3D).

Similarly, the dCas9 protein can be coupled to epigenetic modifiers for locus-specific alteration of acetylation or methylation patterns and subsequent suppression/induction of gene expression (cf. Lo and Qi, 2017; Murovec et al., 2017). Other applications of dCas9 include fusion to fluorescent proteins for visualization of specific DNA sequences in living cells (Chen et al., 2013), or fusion to the unspecific endonuclease domain of FokI. Since FokI is only active as a dimer, combining the fused dCas9-FokI with two specific and adjacent sgRNAs increases the specificity of cleavage and reduces the risk for off-target effects (Guilinger et al., 2014).

Cas9 protein has also been modified to target single-stranded RNA (O'Connell et al., 2014). This modified Cas9 is referred to as RNA-targeting Cas9. In this approach, the PAM sequence is mismatched compared with the DNA sequence that allows RNA-exclusive targeting. It is supplied as part of the oligonucleotide that will hybridize to the target RNA (Fig. 3E). Thus far, RNA-targeting Cas9 has been used, e.g., for RNA cleavage, isolation, and tracking in living cells (O'Connell et al., 2014; Nelles et al., 2016). Similarly, a new endonuclease, Cas13, is being used to detect single molecules of RNA or DNA (Shmakov et al., 2017). This endonuclease has been applied in diagnostics to detect single-stranded RNA of Zika and Dengue viruses, as well as mutations in patient liquid samples (Gootenberg et al., 2018).

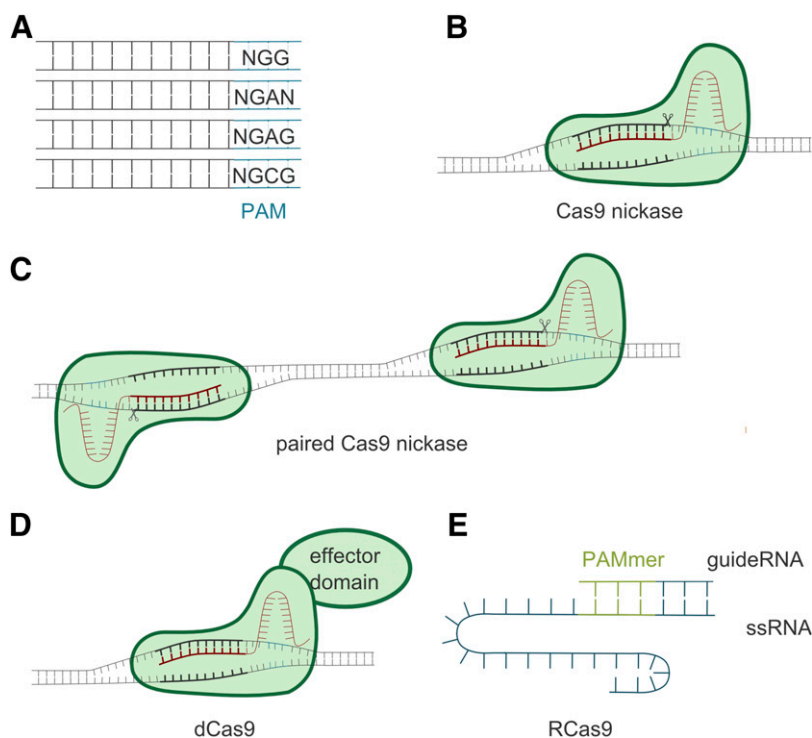


Fig. 3. The CRISPR-Cas9 technology is constantly being expanded to improve specificity and increase its versatility. Such improvements include more flexible PAM requirements by modification of the Cas9 endonuclease from *Streptococcus pyogenes*, or by use of endonucleases from other species (A). Another improvement is the engineering of Cas9 into a Cas9 nickase (Cas9D10A) that only cuts one of the DNA strands. This nickase can be used both in single (B) or paired (C) strands, thereby reducing the risk of indels at off-target sites. Another engineered Cas9 is dCas9, which does not cut the DNA, but can be coupled to an effector domain, like a transcriptional activator/repressor, epigenetic modifiers, or fluorescent proteins (D). The Cas9 endonuclease has also been modified to target single-stranded RNA (ssRNA) (RNA-targeting Cas9 or RCas9) instead of double-stranded DNA (E).

CRISPR-Cas9 for Generating ADME Models

CRISPR-Cas9, like RNA interference, has been used as a tool for target identification in drug discovery (Taylor and Woodcock, 2015; Xue et al., 2016). Since CRISPR-Cas9 is a relatively new technology, the number of CRISPR-Cas9 studies targeting *ADME* genes is limited. Nevertheless, by 2013 the first reports of CRISPR-Cas9 gene editing of ATP-binding cassette transporters were presented. In these cases, the gene edited was the *CFTR* gene (*ABCC7*) (Schwank et al., 2013) and the *white* gene (belonging to the *ABCG* family) regulating eye pigmentation in fruit fly (Kondo and Ueda, 2013), i.e., transporters not considered to be *ADME* proteins. Two years later, in 2015, the first articles about gene editing of solute carrier transporters were published. These articles described the generation of *SLC31A1* and *SLC31A2* knockouts, two copper transporters that are also involved in cisplatin and carboplatin transport (Tsai et al., 2015). A year later, in 2016, the first mammalian in vivo knockout *ADME* models were developed in Sprague-Dawley or Wistar rats. Both models focused on drug-metabolizing cytochrome P450 (P450) enzymes and the genes knocked out were *Cyp2e1* (Wang et al., 2016) or the *Cyp2d* gene cluster (Yoshimi et al., 2016). In the latter publication, a humanized rat model was also generated since the human *CYP2D6* gene was knocked in instead of the rodent *Cyp2d* genes. Since then, CRISPR-Cas9 has been applied in a number of studies focusing on drug-metabolizing enzymes, drug transporters, nuclear receptors, or elements involved in the regulation of *ADME* genes (for an overview, see Table 1).

Drug-Metabolizing Enzymes

In Vitro. To our knowledge, only one in vitro P450 model has been established using CRISPR-Cas9. It was based on the human hepatocyte cell line Huh-7 (Dorr et al., 2017). Here, CRISPR-Cas9 editing of the *CYP3A5* locus established heterozygous and homozygous in vitro models for the *CYP3A5*1* allele. The Huh-7 cell line is homozygous for the *CYP3A5*3* allele, an allele with a splice defect resulting in an inactive enzyme, which is also the most prevalent allele in Caucasian

populations (Zhou et al., 2017). However, the *CYP3A5*1* allele has an allele frequency of up to 56% in non-Caucasian populations, necessitating in vitro models that reflect other genotypes. The gene editing of *CYP3A5* was successful and resulted in elevated *CYP3A5* mRNA levels and increased metabolism of the two *CYP3A5* substrates midazolam and tacrolimus (Dorr et al., 2017).

In Vivo. Prior to the CRISPR-Cas9 technology, in vivo knockout P450 models, humanized P450 models (where animals express the human P450 ortholog), or chimeric models (where human hepatocytes were transplanted and allowed to repopulate the host liver) were used to study *ADME*- and toxicity-related problems (Tateno et al., 2004; Cheung and Gonzalez, 2008; Hasegawa et al., 2011; Jiang and Yu, 2012; Kazuki et al., 2013; Scheer et al., 2013). However, the major applications of these models involve, e.g., studies of developmental or physiologic functions, disease mechanisms, validation of target genes, identification of off-targeted effects for drugs and drug candidates, etc.

A number of in vivo CRISPR-Cas9 knockout models have been established for P450 enzymes, including five articles on the generation of mammalian knockout and/or knockin models. These models that are based on Wistar or Sprague-Dawley rats and/or C57BL/6 mice targeted genes coding for enzymes in drug-metabolizing P450 families 2 and 3. In the first model the rat *Cyp2d* gene locus (containing *Cyp2d1-5*) was knocked out in Wistar rats and replaced with human *CYP2D6* (Yoshimi et al., 2016). Thus, this generated both a *Cyp2d* knockout rat and a humanized *CYP2D6* rat. Unfortunately, no functional characterization was done and to our knowledge no follow-up studies with these in vivo models have been published. In other models *Cyp2e1* (Wang et al., 2016) or *Cyp2c11* (Wei et al., 2018) were knocked out in Sprague-Dawley rats. The CRISPR-Cas9 technology has also been used to generate a double-knockout rat model in which *Cyp3a1* and *Cyp3a2* are knocked out (Lu et al., 2017). The Sprague-Dawley knockout models were generated to establish in vivo models for investigating physiologic functions and xenobiotic metabolism by the specific enzyme(s) of interest. In vitro (using liver microsomes from the knockout rats) and in vivo studies using prototypical P450 substrates showed that the

metabolic function of the targeted enzyme(s) was indeed impaired in all knockout models. However, all models showed compensatory upregulation or downregulation of other P450s involved in drug metabolism. Moreover, these models also showed other differences, e.g., altered serum concentrations of testosterone (Lu et al., 2017) or alkaline phosphate (Wang et al., 2016) and reduced fertility (Wei et al., 2018). Some of these differences can be attributed to the function of the knocked out gene, e.g., CYP3A and testosterone metabolism, whereas others are unexpected. This suggests that such physiologic alterations need to be taken into consideration when comparing ADME data from the knockout models with data from the wild type.

Besides the rat models, a C57BL/6 mouse model was established, with three *Cyp2b* genes—*Cyp2b9*, *Cyp2b10* and *Cyp2b13*—knocked out (Kumar et al., 2017). Interestingly, there were few compensatory changes in expression levels of other P450 enzymes in the triple knockout model. The significant changes that were observed were in female mice for which mRNA expression of *Cyp2a4*, *Cyp2c40*, and *Cyp3a13* were downregulated.

Drug Transporters

In Vitro. CRISPR-Cas9 gene editing in cell culture models for drug transport has mainly focused on *ABCB1*/MDR1 (Fig. 1B). To the best of our knowledge, the first paper regarding MDR1 and ADME was published by us in 2016 (Simoff et al., 2016) with a follow up 1 year later (Karlgrén et al., 2017). The aim in both studies was to knock out the endogenous canine *ABCB1*/MDR1 expression in two MDCK cell lines—the wild-type cell line and the cell line already overexpressing the human MDR1 transporter. The monolayer-forming MDCK cell line has been used for decades for heterologous expression of single or multiple drug transporters and subsequent transport studies, and it is favored as a predictive drug efflux model. Unfortunately, due to a large substrate overlap the endogenous background of canine MDR1 interferes with the overexpressed human *ABCB1*/MDR1 (Kuteykin-Teplyakov et al., 2010; Wang et al., 2013; Karlgrén et al., 2017). This makes it difficult to interpret and derive correct kinetic variables for the human homolog. We reasoned that selective knockout of canine *ABCB1* would provide an MDCK transport model without interference from the endogenous background. After transfection of the wild-type and overexpressing MDCK cells with plasmids containing sgRNA-Cas9 and clonal selection, two isogenic cell lines were generated with identically disrupted canine

ABCB1 genes. Sequencing analysis and liquid chromatography–tandem mass spectrometry–based targeted proteomics verified that the clones were indeed completely lacking canine MDR1 expression. The disruption was homozygous and included four nucleotide deletions that produced frame-shift and premature stop codons in both cell lines. Furthermore, functional analysis showed no efflux of prototypical MDR1 substrates in the knockout (KO) cell line based on wild-type MDCK (MDCK^{cMDR1-KO}) (Fig. 4, A and B). In comparison, the efflux ratios in the original MDCK wild-type cells were significantly higher, indicating that MDCK^{cMDR1-KO} indeed was completely lacking canine MDR1 (Fig. 4B).

A knockout cell line, MDCK-hMDR1^{cMDR1-KO}, was also developed for the corresponding MDCK cells overexpressing human MDR1. Significant differences in efflux ratios were seen between MDCK^{cMDR1-KO} and MDCK-hMDR1^{cMDR1-KO}, indicating that the homologous human *ABCB1* was largely unaffected by the CRISPR-Cas9 knockout process (Fig. 4, C and D). Recently, we used the MDCK^{cMDR1-KO} cell line, which lacks endogenous canine *ABCG2*/BCRP activity, for heterologous expression of the human drug transporter BCRP (M. Karlgrén et al., manuscript in preparation). Since BCRP and MDR1 share many substrates, the absence of interfering canine MDR1 background is an advantage of this novel model.

Five additional articles published between 2016 and 2018 applied a similar approach to knockout *ABCB1*/MDR1 in various cell lines. These investigated if the knockout of *ABCB1* could reverse resistance in human cancer derived cell lines (Ha et al., 2016; Liu et al., 2016; Yang et al., 2016b; Takahashi et al., 2017; Norouzi-Barough et al., 2018). As expected, these studies showed decreased *ABCB1*/MDR1 mRNA or protein expression levels and increased intracellular accumulation, or increased sensitivity in the MDR1 knockout cells using MDR1 substrates such as rhodamine 123, doxorubicin, and/or carfilzomib. Besides this clinically relevant drug transporter (Giacomini et al., 2010), CRISPR-Cas9 gene editing has also been used in editing of a number of other transporters (see Table 1).

In Vivo. A number of in vivo transporter models for ADME studies were developed before CRISPR-Cas9. Most of these are in vivo transporter knockout models for studies of drug transport. These models allowed studies of the role of a specific transporter in the presence of all other mechanisms influencing drug disposition in vivo, such as drug uptake, distribution, metabolism, and efflux. For overviews of in vivo knockout transporter models, see Jiang and Yu (2012) and Tang et al. (2013). Prominent examples of the applicability of single and multiple

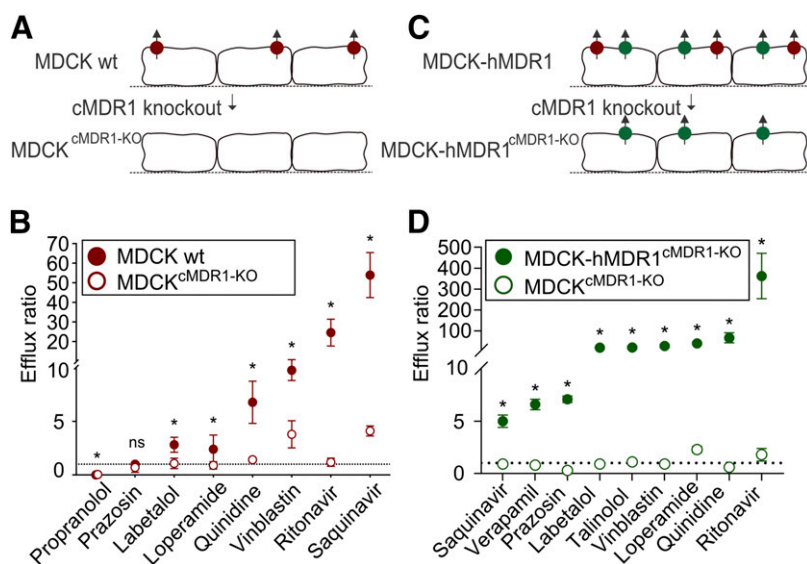


Fig. 4. Overview of MDCK cell models where the canine *ABCB1*/MDR1 has been knocked out using CRISPR-Cas9. (A) Canine MDR1 (cMDR1) was knocked out in an MDCK wild-type cell line; after clonal selection, a knockout clone was obtained that completely lacks endogenous cMDR1 expression (MDCK^{cMDR1-KO}). (B) Functional validation using Transwell studies and prototypical MDR1 substrates showed significantly lower efflux ratios for MDCK^{cMDR1-KO} compared with the parental cell line and with most efflux ratios being close to unity (dotted line). (C) cMDR1 was knocked out in an MDCK cell line already overexpressing the human MDR1 transporter (hMDR1). Here, a clone with identical gene editing as MDCK^{cMDR1-KO} and completely lacking cMDR1 expression could be identified (MDCK-hMDR1^{cMDR1-KO}). (D) Functional validation using Transwell studies and prototypical MDR1 substrates showed significantly higher efflux ratios compared with MDCK^{cMDR1-KO}. Furthermore, the hMDR1 activity was retained, indicating no editing of the human *ABCB1*. Data are presented as mean \pm standard deviation for one representative experiment performed in triplicate. ns, not significant, * $p < 0.05$; using student's t-test. Graphs (B and D) have been previously published in Simoff et al. (2016) and Karlgrén et al. (2017), respectively. These graphs were adapted and republished with kind permission from the publisher.

in vivo knockout models for studies of transport mechanisms include *ABCB1/MDR1* in the blood-brain barrier (Schinkel et al., 1995) and *SLCOs/OATPs* in Rotor-type hyperbilirubinemia (van de Steeg et al., 2012), respectively. However, a complicating factor is that knockout of a single transporter protein or several transporter proteins can lead to increased expression of other transporter proteins (e.g., Kuroda et al., 2004; Vlaming et al., 2009). Thus far, in vivo CRISPR-Cas9 gene editing of drug transporters has focused on the ATP-binding cassette transporter family (see Table 1), where it has been used to knock out *ABCB1/MDR1* homologs in insects (Denecke et al., 2017; Zuo et al., 2018). In both studies the knockout of *ABCB1/MDR1* homologs increased the susceptibility to various insecticides. The *ABCB1* homolog was also knocked out in zebrafish. The mutated zebrafish embryos showed a reduced efflux of model substrates and also increased accumulation of cadmium chloride and benzo[a]pyrene (Tian et al., 2017). In another study, *abc11b*, the ortholog of human *ABCB11/BSEP*, was knocked out in zebrafish (Ellis et al., 2018). Like humans with impaired BSEP function, the knockout of *abc11b* in zebrafish was associated with, e.g., activation of impaired bile salt excretion and autophagy. See Table 1 for a summary of CRISPR-Cas9 in vivo editing of additional transporters.

Regulation of ADME Genes

The CRISPR-Cas9 technology has been applied to study the regulation of *ADME* genes. An example is the regulation of the drug transporter *ABCC3/MRP3* in the human keratinocyte cell line HaCaT (Takechi et al., 2018). In this study, the authors identified a CpG island, i.e., an area rich in CpG dinucleotides often found near or within the promoter or first exon of a gene, and which may be involved in the epigenetic regulation of genes. This island is located approximately 10 kb upstream of the *ABCC3* gene and its methylation status correlates negatively with *ABCC3/MRP3* mRNA expression levels in human skin samples. The CRISPR-Cas9-induced deletions in this CpG area reduced *ABCC3/MRP3* mRNA expression levels in the HaCaT cell line. This study highlights the possibility of using CRISPR-Cas9 for editing/deletion of nontranscribed DNA targets.

CRISPR-Cas9 has been used for studying expression and function of nuclear receptors involved in P450 regulation (see Table 1). The aryl hydrocarbon receptor is involved in induction of, e.g., *CYP1A1*. To generate model systems for the study of aryl hydrocarbon receptor signaling and its modulation by xenobiotics, a Luciferase reporter cassette was knocked in at the *CYP1A1* transcription start site in induced pluripotent stem cells (iPSCs) using CRISPR-Cas9 (Smith et al., 2016). The authors could monitor aryl hydrocarbon receptor activity by following *CYP1A1* expression in these cells. In another study, in human iPSC, CRISPR-Cas9 has been used to tag PXR with the fluorescent protein mCherry (Kim et al., 2018). PXR is involved in the induction of several drug-metabolizing enzymes and transporters (Kandel et al., 2016), but its expression is low in iPSC-derived hepatocytes (Kim et al., 2018). Consequently, this results in low enzyme/transporter levels. The authors reason that the established PXR-mCherry iPSC cell line forms a platform for discovering factors that can increase PXR expression levels during differentiation; however, to date, no such studies have been published. These two articles point to the possibility of following endogenous gene expression with the aid of CRISPR-Cas9. It can be speculated that once established this approach will offer an alternative to other mRNA or protein quantification technologies.

Limitations of CRISPR-Cas9

The CRISPR-Cas9 technology also has its limitations. As mentioned previously, CRISPR-Cas9 gene editing shows apparent off-target effects

as well as low efficiency (Liang et al., 2015). DSBs can occur even if a few mismatches exist between the sgRNA and the DNA target. This can result in alterations in genes unrelated to the target gene (Fu et al., 2013). Gene editing at sites with as many as five mismatches has been reported; the rate of such off-target effects can be similar to that of the gene editing at the target site (Fu et al., 2013; Lin et al., 2014).

Potential off-target effects are today often investigated using in silico analysis to avoid target sites with closely resembling sequences. If this is not possible, such off-target sites are investigated for unwanted mutations using polymerase chain reaction and/or DNA sequencing. Nevertheless, it should be noted that a recent report showed that CRISPR-Cas9 editing could also cause single-nucleotide variants in unpredictable off-target sites in vivo in mice (Schaefer et al., 2017). However, that report was retracted during the compilation of this paper since proper genetic background analysis had not been performed (Schaefer et al., 2018). Even so, this shows that—for comprehensive off-target analysis—whole genome sequencing might be the method of choice.

The low efficiency in CRISPR-Cas9 editing is a limitation, especially for in vivo applications. However, even in in vitro studies, e.g., the development of CRISPR-Cas9 knockout models by the NHEJ mechanism, the low efficiency results in the need for extensive screening to find homozygous knockout clones. In our experience, screening of clones enriched using, e.g., green fluorescent protein or antibiotic selection, can result in up to 28% of the screened clones having homozygous deletions (Simoff et al., 2016; Karlgren et al., 2017). This is in line also with other publications reporting similar efficiencies for CRISPR-Cas9 and NHEJ (Moore, 2015; Kosicki et al., 2017), but lower efficiencies for HDR (Mali et al., 2013; Shao et al., 2014). It should be noted that since the gene editing is sequence specific large variation in efficiency is seen for different targets and delivery methods (Kosicki et al., 2017).

The CRISPR-Cas9 technology is also limited by the requirement of a PAM motif in close proximity to the target site. Although endonucleases recognizing alternative PAM sequences are now available (as previously mentioned), this might still constitute a problem when targeting *ADME* genes that have high sequence identity with other gene family members. In such cases, genomic screening and validation (mRNA/protein expression or activity) might also be more difficult.

After submission of this article, results were published indicating additional problems with CRISPR-Cas9, in particular for its clinical applicability. Haapaniemi et al. (2018) showed that DSBs introduced by CRISPR-Cas9 triggered p53-dependent cell cycle arrest and that this response was independent of the targeted locus. Similarly, Ihry et al. (2018) showed that CRISPR-Cas9-induced DSBs generated a p53-dependent toxic response in human pluripotent stem cells. The targeting of a single locus was sufficient to kill most of the cells (Ihry et al., 2018). This suggests that p53-deficient cells have a growth advantage after CRISPR-Cas9 gene editing. Therefore, if CRISPR-Cas9 is to be used for therapeutic purposes, p53 function needs to be assured to avoid the risk of cancer. Another serious problem, raised by Kosicki et al. (2018), is that CRISPR-Cas9 gene editing may result in large deletions and complex gene rearrangement at the target site. Such alterations are undetectable by the short-range polymerase chain reaction assays commonly used to verify gene modifications. Lesions and crossover events were also detected outside of the targeted locus (Kosicki et al., 2018). Together, these findings highlight the importance of comprehensive genetic analysis to reveal and control unexpected gene modifications prior to a more general clinical use of CRISPR-Cas9.

Future Applications for CRISPR-Cas9 within the ADME Field

Despite the restrictions mentioned, the simplicity, programmability, versatility, and comparably low costs of CRISPR-Cas9, together with the

rapid methodological development and improvement, indicate a huge potential for a variety of applications in the ADME field. At this point in time, CRISPR-Cas9 has mainly been used to generate *in vitro* or *in vivo* knockout models of ADME genes. One major advantage with CRISPR-Cas9 knockout models is the complete elimination of background expression, which will facilitate investigations of clinically relevant drug transporters and drug-metabolizing enzymes. CRISPR-Cas9 knockout models will also be useful in studies of putative ADME genes, such as orphan solute carrier family members (César-Razquin et al., 2015; Lin et al., 2015) and other ADME genes of relevance in drug disposition and drug interactions, as drug targets, and that are associated with human disease. With CRISPR-Cas9, one has the opportunity to not just knock out the gene of interest, but also to study the role of individual nucleotides or amino acids as structural/functional constituents, or to humanize animal ADME genes under a native promoter.

An intriguing opportunity with CRISPR-Cas9 is that noncoding DNA can be targeted. Interindividual variation (expression levels, drug-drug interactions, and pharmacokinetics) of ADME proteins can be due to genetic variation in coding regions or promoter regions, but much of the variation is still unexplained. We know that the DNA encoding proteins, which are fairly well studied, represent only a minor part of the human genome. The regulatory role of noncoding DNA is mainly unexplored. Furthermore, genome-wide association studies have shown that most of the associated sites in human diseases are located in these noncoding DNA regions (Wright and Sanjana, 2016). Similar observations have been reported also for ADME proteins, e.g., regarding variation in ATP-binding cassette transporter expression levels (Matsson et al., 2012). Therefore, Wright and Sanjana (2016) proposed that a future application of CRISPR-Cas9 could be to shed light on this intriguing issue.

While many drugs and drug metabolites interact with both drug transporters and drug-metabolizing enzymes *in vivo*, transport and metabolism are often studied separately *in vitro* using recombinant proteins, cell lines overexpressing transporters, or drug-metabolizing enzymes. This is because the contribution of an individual transporter/enzyme can be difficult to determine in more complex *in vitro* models, such as human hepatocytes. As a consequence, we and others have previously developed *in vitro* models overexpressing different combinations of transporters and enzymes, in which the contribution of an individual transporter/enzyme can more easily be determined (Crespi et al., 2000; Fahrmaray et al., 2012, 2013; Kwatra et al., 2012; Neve et al., 2013). These models are useful but have drawbacks, including endogenous background expression and time-consuming experimental procedures. With CRISPR-Cas9 it is possible to both completely knock out and knock in genes; hence, with this technology we will be able to develop better and easier-to-use models than the traditional ones. For example, transport-metabolism interplay or drug metabolite interactions could be studied under more controlled conditions using such models.

The use of dCas9 for genome docking and the coupling of dCas9 to an effector domain could provide additional opportunities to generate *in vitro* models that are more predictive of the *in vivo* situation. Through dCas9 docking/coupling it could become possible (instead of knocking in or knocking out genes) to modulate expression levels of one or several endogenous genes simultaneously, thereby providing models with more *in vivo*-like expression profiles.

In summary, we have demonstrated the versatility and applicability of the CRISPR-Cas9 technology. By using examples from more basic research areas, our goal was to stimulate increased use of CRISPR-Cas9 in the ADME field. We predict that the advantages of CRISPR-Cas9 will result in a variety of more advanced and better interpretable *in vitro* and *in vivo* models for drug metabolism and disposition.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Karlgrén, Simoff, Keiser, Oswald, Artursson.

References

- Aluru N, Karchner SI, Franks DG, Nacci D, Champlin D, and Hahn ME (2015) Targeted mutagenesis of aryl hydrocarbon receptor 2a and 2b genes in Atlantic killifish (*Fundulus heteroclitus*). *Aquat Toxicol* **158**:192–201.
- Angelos MG, Ruh PN, Webber BR, Blum RH, Ryan CD, Bendzick L, Shim S, Yingst AM, Tufa DM, Verneris MR, et al. (2017) Aryl hydrocarbon receptor inhibition promotes hematolymphoid development from human pluripotent stem cells. *Blood* **129**:3428–3439.
- Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, and Horvath P (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**:1709–1712.
- Bellec J, Bacchetta M, Losa D, Anegón I, Chanson M, and Nguyen TH (2015) CFTR inactivation by lentiviral vector-mediated RNA interference and CRISPR-Cas9 genome editing in human airway epithelial cells. *Curr Gene Ther* **15**:447–459.
- Bompiani KM, Tsai CY, Achatz FP, Liebig JK, and Howell SB (2016) Copper transporters and chaperones CTR1, CTR2, ATOX1, and CCS as determinants of cisplatin sensitivity. *Metalomics* **8**:951–962.
- César-Razquin A, Snijder B, Frappier-Brinton T, Isserlin R, Gyimesi G, Bai X, Reithmeier RA, Hepworth D, Hediger MA, Edwards AM, et al. (2015) A call for systematic research on solute carriers. *Cell* **162**:478–487.
- Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW, Park J, Blackburn EH, Weissman JS, Qi LS, et al. (2013) Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* **155**:1479–1491.
- Cheng Y, Jin UH, Davidson LA, Chapkin RS, Jayaraman A, Tamamis P, Orr A, Allred C, Denison MS, Soshilov A, et al. (2017) Editor's highlight: microbial-derived 1,4-dihydroxy-2-naphthoic acid and related compounds as aryl hydrocarbon receptor agonists/antagonists: structure-activity relationships and receptor modeling. *Toxicol Sci* **155**:458–473.
- Cheung C and Gonzalez FJ (2008) Humanized mouse lines and their application for prediction of human drug metabolism and toxicological risk assessment. *J Pharmacol Exp Ther* **327**:288–299.
- Chung WY, Song M, Park J, Namkung W, Lee J, Kim H, Lee MG, and Kim JY (2016) Generation of ΔF508-CFTR T84 cell lines by CRISPR/Cas9-mediated genome editing. *Biotechnol Lett* **38**:2023–2034.
- Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**:819–823.
- Crespi CL, Fox L, Stocker P, Hu M, and Steimel DT (2000) Analysis of drug transport and metabolism in cell monolayer systems that have been modified by cytochrome P4503A4 cDNA-expression. *Eur J Pharm Sci* **12**:63–68.
- Czogalla KJ, Biswas A, Höning K, Hornung V, Liphardt K, Watzka M, and Oldenburg J (2017) Warfarin and vitamin K compete for binding to Phe55 in human VKOR. *Nat Struct Mol Biol* **24**:77–85.
- Denecke S, Fusetto R, and Batterham P (2017) Describing the role of *Drosophila melanogaster* ABC transporters in insecticide biology using CRISPR-Cas9 knockouts. *Insect Biochem Mol Biol* **91**:1–9.
- Dorr CR, Rimmel RP, Muthusamy A, Fisher J, Moriarity BS, Yasuda K, Wu B, Guan W, Schuetz EG, Oetting WS, et al. (2017) CRISPR/Cas9 genetic modification of CYP3A5*3 in HuH-7 human hepatocyte cell line leads to cell lines with increased midazolam and tacrolimus metabolism. *Drug Metab Dispos* **45**:957–965.
- Editorial (2017) CRISPR catch-up. *Nat Biotechnol* **35**:389. DOI: 10.1038/nbt.3885.
- Edvardsen RB, Leininger S, Kleppe L, Skafnesmo KO, and Wargelius A (2014) Targeted mutagenesis in Atlantic salmon (*Salmo salar* L.) using the CRISPR/Cas9 system induces complete knockout individuals in the F0 generation. *PLoS One* **9**:e108622.
- Ellis JL, Bove KE, Schuetz EG, Leino D, Valencia CA, Schuetz JD, Miethke A, and Yin C (2018) Zebrafish *abcb11b* mutant reveals strategies to restore bile excretion impaired by bile salt export pump deficiency. *Hepatology* **67**:1531–1545.
- Evans WE and Relling MV (1999) Pharmacogenomics: translating functional genomics into rational therapeutics. *Science* **286**:487–491.
- Fahrmaray C, König J, Auge D, Mieth M, and Fromm MF (2012) Identification of drugs and drug metabolites as substrates of multidrug resistance protein 2 (MRP2) using triple-transfected MDCK-OATP1B1-UGT1A1-MRP2 cells. *Br J Pharmacol* **165**:1836–1847.
- Fahrmaray C, König J, Auge D, Mieth M, Münch K, Segrestaa J, Pfeifer T, Treiber A, and Fromm M (2013) Phase I and II metabolism and MRP2-mediated export of bosentan in a MDCKII-OATP1B1-CYP3A4-UGT1A1-MRP2 quadruple-transfected cell line. *Br J Pharmacol* **169**:21–33.
- Firth AL, Menon T, Parker GS, Qualls SJ, Lewis BM, Ke E, Dargatz CT, Wright R, Khanna A, Gage FH, et al. (2015) Functional gene correction for cystic fibrosis in lung epithelial cells generated from patient iPSCs. *Cell Rep* **12**:1385–1390.
- Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, and Sander JD (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* **31**:822–826.
- Gaj T, Gersbach CA, and Barbas CF, III (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* **31**:397–405.
- Garcia GR, Bugel SM, Truong L, Spagnoli S, and Tanguay RL (2018) AHR2 required for normal behavioral responses and proper development of the skeletal and reproductive systems in zebrafish. *PLoS One* **13**:e0193484.
- Gartzke D, Delzer J, Laplanche L, Uchida Y, Hoshi Y, Tachikawa M, Terasaki T, Sydor J, and Fricker G (2015) Genomic knockout of endogenous canine P-glycoprotein in wild-type, human P-glycoprotein and human BCRP transfected MDCKII cell lines by zinc finger nucleases. *Pharm Res* **32**:2060–2071.

- Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, et al.; International Transporter Consortium (2010) Membrane transporters in drug development. *Nat Rev Drug Discov* 9:215–236.
- Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, et al. (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154:442–451.
- Gong C, Bongiorno P, Martins A, Stephanou NC, Zhu H, Shuman S, and Glickman MS (2005) Mechanism of nonhomologous end-joining in mycobacteria: a low-fidelity repair system driven by Ku, ligase D and ligase C. *Nat Struct Mol Biol* 12:304–312.
- Gootenberg JS, Abudayyeh OO, Kellner MJ, Joung J, Collins JJ, and Zhang F (2018) Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science* 360:439–444.
- Grob A, Marbiah M, and Isalan M (2018) Functional insulator scanning of CpG islands to identify regulatory regions of promoters using CRISPR. *Methods Mol Biol* 1766:285–301.
- Gullinger JP, Thompson DB, and Liu DR (2014) Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. *Nat Biotechnol* 32:577–582.
- Guo D, Liu H, Gao G, Ruzi A, Wang K, Wu H, Lai K, Liu Y, Yang F, Lai L, et al. (2016a) Generation of an Abcc8 heterozygous mutation human embryonic stem cell line using CRISPR/Cas9. *Stem Cell Res (Amst)* 17:670–672.
- Guo D, Liu H, Gao G, Ruzi A, Wang K, Wu H, Lai K, Liu Y, Yang F, Lai L, et al. (2016b) Generation of an Abcc8 homozygous mutation human embryonic stem cell line using CRISPR/Cas9. *Stem Cell Res (Amst)* 17:640–642.
- Guo D, Liu H, Ruzi A, Gao G, Nasir A, Liu Y, Yang F, Wu F, Xu G, and Li YX (2017) Modeling congenital hyperinsulinism with ABCC8-deficient human embryonic stem cells generated by CRISPR/Cas9. *Sci Rep* 7:3156.
- Gupta RM, Meissner TB, Cowan CA, and Musunuru K (2016) Genome-edited human pluripotent stem cell-derived macrophages as a model of reverse cholesterol transport—brief report. *Arterioscler Thromb Vasc Biol* 36:15–18.
- Ha JS, Byun J, and Ahn DR (2016) Overcoming doxorubicin resistance of cancer cells by Cas9-mediated gene disruption. *Sci Rep* 6:22847.
- Haapaniemi E, Botla S, Persson J, Schmierer B, and Taipale J (2018) CRISPR-Cas9 genome editing induces a p53-mediated DNA damage response. *Nat Med* 24:927–930.
- Harrington LB, Paez-Espino D, Staahl BT, Chen JS, Ma E, Kypides NC, and Doudna JA (2017) A thermostable Cas9 with increased lifetime in human plasma. *Nat Commun* 8:1424.
- Hasegawa M, Kapelyukh Y, Tahara H, Seibler J, Rode A, Krueger S, Lee DN, Wolf CR, and Scheer N (2011) Quantitative prediction of human pregnane X receptor and cytochrome P450 3A4 mediated drug-drug interaction in a novel multiple humanized mouse line. *Mol Pharmacol* 80:518–528.
- He J, Jin Y, Zhou M, Li X, Chen W, Wang Y, Gu S, Cao Y, Chu C, Liu X, et al. (2018) Solute carrier family 35 member F2 is indispensable for papillary thyroid carcinoma progression through activation of transforming growth factor- β type I receptor/apoptosis signal-regulating kinase 1/mitogen-activated protein kinase signaling axis. *Cancer Sci* 109:642–655.
- Hollywood JA, Lee CM, Scallan MF, and Harrison PT (2016) Analysis of gene repair tracts from Cas9/gRNA double-stranded breaks in the human CFTR gene. *Sci Rep* 6:32230.
- Ihry RJ, Worringer KA, Salick MR, Frias E, Ho D, Theriault K, Kommineni S, Chen J, Sondy M, Ye C, et al. (2018) p53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. *Nat Med* 24:939–946.
- Jiang XL and Yu AM (2012) Genetically modified mouse models in ADME studies, in *ADME-Enabling Technologies in Drug Design and Development* (Zang D and Surapaneni S eds) pp 437–454. John Wiley & Sons, Inc., Hoboken, NJ.
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, and Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337:816–821.
- Kandel BA, Thomas M, Winter S, Damm G, Seehofer D, Burk O, Schwab M, and Zanger UM (2016) Genomewide comparison of the inducible transcriptomes of nuclear receptors CAR, PXR and PPAR α in primary human hepatocytes. *Biochim Biophys Acta* 1859:1218–1227.
- Karlgen M, Simoff I, Backlund M, Wegler C, Keiser M, Handin N, Müller J, Lundquist P, Jareborg AC, Oswald S, et al. (2017) A CRISPR-cas9 generated MDCK cell line expressing human MDR1 without endogenous canine MDR1 (cABCB1): an improved tool for drug efflux studies. *J Pharm Sci* 106:2909–2913.
- Kazuki Y, Kobayashi K, Aueviriyavit S, Oshima T, Kuroiwa Y, Tsukazaki Y, Senda N, Kawakami H, Ohtsuki S, Abe S, et al. (2013) Trans-chromosomal mice containing a human *CYP3A* cluster for prediction of xenobiotic metabolism in humans. *Hum Mol Genet* 22:578–592.
- Khan SA, Reichelt M, and Heckel DG (2017) Functional analysis of the ABCs of eye color in *Helicoverpa armigera* with CRISPR/Cas9-induced mutations. *Sci Rep* 7:40025.
- Kim H, Kim JW, Kim SJ, Choi YJ, Kim DS, and Park HJ (2018) Generation of a PXR reporter human induced pluripotent stem cell line (PXR-mCherry hiPSC) using the CRISPR/Cas9 system. *Stem Cell Res (Amst)* 26:72–75.
- Kim JS (2016) Genome editing comes of age. *Nat Protoc* 11:1573–1578.
- Kondo S and Ueda R (2013) Highly improved gene targeting by germline-specific Cas9 expression in *Drosophila*. *Genetics* 195:715–721.
- Kosicki M, Rajan SS, Lorenzetti FC, Wandall HH, Narimatsu Y, Metzkapian E, and Bennett EP (2017) Dynamics of indel profiles induced by various CRISPR/Cas9 delivery methods. *Prog Mol Biol Transl Sci* 152:49–67.
- Kosicki M, Tomberg K, and Bradley A (2018) Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol* 36:765–771.
- Kumar R, Mota LC, Litoff EJ, Rooney JP, Boswell WT, Courter E, Henderson CM, Hernandez JP, Corton JC, Moore DD, et al. (2017) Compensatory changes in CYP expression in three different toxicology mouse models: CAR-null, Cyp3a-null, and Cyp2b9/10/13-null mice. *PLoS One* 12:e0174355.
- Kuroda M, Kobayashi Y, Tanaka Y, Itani T, Mifuji R, Araki J, Kaito M, and Adachi Y (2004) Increased hepatic and renal expressions of multidrug resistance-associated protein 3 in Eisai hyperbilirubinuria rats. *J Gastroenterol Hepatol* 19:146–153.
- Kuteykin-Teplyakov K, Luna-Tortós C, Ambroziak K, and Löscher W (2010) Differences in the expression of endogenous efflux transporters in MDR1-transfected versus wildtype cell lines affect P-glycoprotein mediated drug transport. *Br J Pharmacol* 160:1453–1463.
- Kwatra D, Budda B, Vadlapudi AD, Vadlapatla RK, Pal D, and Mitra AK (2012) Transfected MDCK cell line with enhanced expression of CYP3A4 and P-glycoprotein as a model to study their role in drug transport and metabolism. *Mol Pharm* 9:1877–1886.
- Lander ES (2016) The heroes of CRISPR. *Cell* 164:18–28.
- Li M, Feng R, Ma H, Dong R, Liu Z, Jiang W, Tao W, and Wang D (2016) Retinoic acid triggers meiosis initiation via *stra8*-dependent pathway in Southern catfish, *Silurus meridionalis*. *Gen Comp Evol* 232:191–198.
- Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z, Lv J, Xie X, Chen Y, Li Y, et al. (2015) CRISPR/Cas9-mediated gene editing in human tripuricular zygotes. *Protein Cell* 6:363–372.
- Lin L, Yee SW, Kim RB, and Giacomini KM (2015) SLC transporters as therapeutic targets: emerging opportunities. *Nat Rev Drug Discov* 14:543–560.
- Lin Y, Cradick TJ, Brown MT, Deshmukh H, Ranjan P, Sarode N, Wile BM, Vertino PM, Stewart FJ, and Bao G (2014) CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. *Nucleic Acids Res* 42:7473–7485.
- Liu T, Li Z, Zhang Q, De Amorim Bernstein K, Lozano-Calderon S, Choy E, Hornicek FJ, and Duan Z (2016) Targeting ABCB1 (MDR1) in multi-drug resistant osteosarcoma cells using the CRISPR-Cas9 system to reverse drug resistance. *Oncotarget* 7:83502–83513.
- Lo A and Qi L (2017) Genetic and epigenetic control of gene expression by CRISPR-Cas systems. *F1000 Res* 6:747.
- Lu J, Shao Y, Qin X, Liu D, Chen A, Li D, Liu M, and Wang X (2017) CRISPR knockout rat cytochrome P450 3A1/2 model for advancing drug metabolism and pharmacokinetics research. *Sci Rep* 7:42922.
- Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, and Church GM (2013) RNA-guided human genome engineering via Cas9. *Science* 339:823–826.
- Matson P, Yee SW, Markova S, Morrissey K, Jenkins G, Xuan J, Jorgensen E, Kroetz DL, and Giacomini KM (2012) Discovery of regulatory elements in human AT-binding cassette transporters through expression quantitative trait mapping. *Pharmacogenomics J* 12:214–226.
- Mojica FJ, Díez-Villaseñor C, García-Martínez J, and Almendros C (2009) Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* 155:733–740.
- Molinski SV, Ahmadi S, Ip W, Ouyang H, Villella A, Miller JP, Lee PS, Kulleperuma K, Du K, Di Paola M, et al. (2017) Orkambi® and amplifier co-therapy improves function from a rare *CFTR* mutation in gene-edited cells and patient tissue. *EMBO Mol Med* 9:1224–1243.
- Moore JD (2015) The impact of CRISPR-Cas9 on target identification and validation. *Drug Discov Today* 20:450–457.
- Murovec J, Pirž Ž, and Yang B (2017) New variants of CRISPR RNA-guided genome editing enzymes. *Plant Biotechnol J* 15:917–926.
- Nakade S, Yamamoto T, and Sakuma T (2017) Cas9, Cpf1 and C2c1/2/3—what's next? *Bio-engineered* 8:265–273.
- Narasimhan S, Stanford Zulick E, Novikov O, Parks AJ, Schlezinger JJ, Wang Z, Laroche F, Feng H, Mulas F, Monti S, et al. (2018) Towards resolving the pro- and anti-tumor effects of the aryl hydrocarbon receptor. *Int J Mol Sci* 19:EI388.
- Nelles DA, Fang MY, O'Connell MR, Xu JL, Markmiller SJ, Doudna JA, and Yeo GW (2016) Programmable RNA tracking in live cells with CRISPR/Cas9. *Cell* 165:488–496.
- Neve EP, Artursson P, Ingelman-Sundberg M, and Karlgen M (2013) An integrated in vitro model for simultaneous assessment of drug uptake, metabolism, and efflux. *Mol Pharm* 10:3152–3163.
- Ng CL, Siciliano G, Lee MC, de Almeida MJ, Corey VC, Bopp SE, Bertuccini L, Wittlin S, Kasdin RG, Le Bihan A, et al. (2016) CRISPR-Cas9-modified *pfnr1* protects *Plasmodium falciparum* asexual blood stages and gametocytes against a class of piperazine-containing compounds but potentiates artemisinin-based combination therapy partner drugs. *Mol Microbiol* 101:381–393.
- Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, Zhao HY, Wang Y, Kan Y, Shrock E, et al. (2017) Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science* 357:1303–1307.
- Norouzi-Barough L, Sarookhani M, Salehi R, Sharifi M, and Moghbelinejad S (2018) CRISPR/Cas9, a new approach to successful knockdown of ABCB1/P-glycoprotein and reversal of chemosensitivity in human epithelial ovarian cancer cell line. *Iran J Basic Med Sci* 21:181–187.
- O'Connell MR, Oakes BL, Sternberg SH, East-Seletsky A, Kaplan M, and Doudna JA (2014) Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* 516:263–266.
- Overballe-Petersen S, Harms K, Orlando LA, Mayar JV, Rasmussen S, Dahl TW, Rosing MT, Poole AM, Sicheritz-Ponten T, Brunak S, et al. (2013) Bacterial natural transformation by highly fragmented and damaged DNA. *Proc Natl Acad Sci USA* 110:19860–19865.
- Park RJ, Wang T, Koundakjian D, Hultquist JF, Lamothe-Molina P, Monel B, Schumann K, Yu H, Krupczak KM, Garcia-Beltran W, et al. (2017) A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors. *Nat Genet* 49:193–203.
- Peters-Hall JR, Coquelin ML, Torres MJ, LaRanger R, Alabi BR, Sho S, Calva-Moreno JF, Thomas PJ, and Shay JW (2018) Long-term culture and cloning of primary human bronchial basal cells that maintain multipotent differentiation capacity and CFTR channel function. *Am J Physiol Lung Cell Mol Physiol* 315:L313–L327.
- Pratt J, Venkatraman N, Brinker A, Xiao Y, Blasberg J, Thompson DC, and Bourner M (2012) Use of zinc finger nuclease technology to knock out efflux transporters in C2BBe1 cells. *Curr Protoc Toxicol* 52:2.2.2.
- Qian W, Wu C, Fu Y, Hu G, He Z, and Liu W (2017) Novel rice mutants overexpressing the brassinosteroid catabolic gene *CYP734A4*. *Plant Mol Biol* 93:197–208.
- Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, et al. (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154:1380–1389.
- Rezende F, Prior KK, Löwe O, Wittig I, Strecker V, Moll F, Helfinger V, Schnütgen F, Kurrel N, Wempe F, et al. (2017) Cytochrome P450 enzymes but not NADPH oxidases are the source of the NADPH-dependent lucigenin chemiluminescence in membrane assays. *Free Radic Biol Med* 102:57–66.
- Sabirov RZ, Merzlyak PG, Okada T, Islam MR, Uramoto H, Mori T, Makino Y, Matsuura H, Xie Y, and Okada Y (2017) The organic anion transporter SLCO2A1 constitutes the core component of the Maxi-Cl channel. *EMBO J* 36:3309–3324.
- Sampon KE, Brinker A, Pratt J, Venkatraman N, Xiao Y, Blasberg J, Steiner T, Bourner M, and Thompson DC (2015) Zinc finger nuclease-mediated gene knockout results in loss of transport activity for P-glycoprotein, BCRP, and MRP2 in Caco-2 cells. *Drug Metab Dispos* 43:199–207.
- Sander JD and Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* 32:347–355.
- Sanz DJ, Hollywood JA, Scallan MF, and Harrison PT (2017) Cas9/gRNA targeted excision of cystic fibrosis-causing deep-intronic splicing mutations restores normal splicing of CFTR mRNA. *PLoS One* 12:e0184009.
- Schaefer KA, Wu WH, Colgan DF, Tsang SH, Bassuk AG, and Mahajan VB (2017) Unexpected mutations after CRISPR-Cas9 editing in vivo. *Nat Methods* 14:547–548.
- Schaefer KA, Wu WH, Colgan DF, Tsang SH, Bassuk AG, and Mahajan VB (2018) Retraction: unexpected mutations after CRISPR-Cas9 editing in vivo. *Nat Methods* 15:394.

- Scheer N, Snaith M, Wolf CR, and Seibler J (2013) Generation and utility of genetically humanized mouse models. *Drug Discov Today* **18**:1200–1211.
- Schinkel AH, Wagenaar E, van Deemter L, Mol CA, and Borst P (1995) Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96**:1698–1705.
- Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, Demircan T, Sasaki N, Boymans S, Cuppen E, van der Ent CK, et al. (2013) Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* **13**:653–658.
- Shao Y, Guan Y, Wang L, Qiu Z, Liu M, Chen Y, Wu L, Li Y, Ma X, Liu M, et al. (2014) CRISPR/Cas-mediated genome editing in the rat via direct injection of one-cell embryos. *Nat Protoc* **9**:2493–2512.
- Shen Y, Li Y, Xu D, Yang C, Li C, and Luo K (2018) Molecular cloning and characterization of a brassinosteroid biosynthesis-related gene *PtoDWF4* from *Populus tomentosa*. *Tree Physiol* DOI: 10.1093/treephys/tpy027 [published ahead of print].
- Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI, et al. (2017) Diversity and evolution of class 2 CRISPR-Cas systems. *Nat Rev Microbiol* **15**:169–182.
- Simoff I, Karlgrén M, Backlund M, Lindström AC, Gaugaz FZ, Matsson P, and Artursson P (2016) Complete knockout of endogenous Mdr1 (*Abcb1*) in MDCK cells by CRISPR-Cas9. *J Pharm Sci* **105**:1017–1021.
- Smith BW, Stanford EA, Sherr DH, and Murphy GJ (2016) Genome editing of the *CYP1A1* locus in iPSCs as a platform to map AHR expression throughout human development. *Stem Cells Int* **2016**:2574152.
- Takahashi K, Inukai T, Imamura T, Yano M, Tomoyasu C, Lucas DM, Nemoto A, Sato H, Huang M, Abe M, et al. (2017) Anti-leukemic activity of bortezomib and carfilzomib on B-cell precursor ALL cell lines. *PLoS One* **12**:e0188680.
- Takechi T, Hirota T, Sakai T, Maeda N, Kobayashi D, and Ieiri I (2018) Interindividual differences in the expression of ATP-binding cassette and solute carrier family transporters in human skin: DNA methylation regulates transcriptional activity of the human ABCB3 gene. *Drug Metab Dispos* **46**:628–635.
- Tanaka R, Murakami H, Ote M, and Yamamoto D (2016) Clustered regulatory interspaced short palindromic repeats (CRISPR)-mediated mutagenesis and phenotype rescue by *piggyBac* transgenesis in a nonmodel *Drosophila* species. *Insect Mol Biol* **25**:355–361.
- Tang SC, Hendriks JJ, Beijnen JH, and Schinkel AH (2013) Genetically modified mouse models for oral drug absorption and disposition. *Curr Opin Pharmacol* **13**:853–858.
- Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, Tachibana A, Soeno Y, Asahina K, Hino H, et al. (2004) Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* **165**:901–912.
- Taylor J and Woodcock S (2015) A perspective on the future of high-throughput RNAi screening: will CRISPR cut out the competition or can RNAi help guide the way? *J Biomol Screen* **20**:1040–1051.
- Tian J, Hu J, Chen M, Yin H, Miao P, Bai P, and Yin J (2017) The use of *mrp1*-deficient (*Danio rerio*) zebrafish embryos to investigate the role of MRP1 in the toxicity of cadmium chloride and benzo[a]pyrene. *Aquat Toxicol* **186**:123–133.
- Tsai CY, Liebig JK, Tsigelny IF, and Howell SB (2015) The copper transporter 1 (CTR1) is required to maintain the stability of copper transporter 2 (CTR2). *Metallomics* **7**:1477–1487.
- van de Steeg E, Stránecký V, Hartmannová H, Nosková L, Hřebíček M, Wagenaar E, van Esch A, de Waart DR, Oude Elferink RP, Kenworthy KE, et al. (2012) Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver. *J Clin Invest* **122**:519–528.
- Vlaming ML, van Esch A, Pala Z, Wagenaar E, van de Wetering K, van Tellingen O, and Schinkel AH (2009) *Abcc2* (Mrp2), *Abcc3* (Mrp3), and *Abcg2* (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo. *Mol Cancer Ther* **8**:3350–3359.
- Wang J, Wang H, Liu S, Liu L, Tay WT, Walsh TK, Yang Y, and Wu Y (2017) CRISPR/Cas9 mediated genome editing of *Helicoverpa armigera* with mutations of an ABC transporter gene *HaABCA2* confers resistance to *Bacillus thuringiensis* Cry2A toxins. *Insect Biochem Mol Biol* **87**:147–153.
- Wang X, Tang Y, Lu J, Shao Y, Qin X, Li Y, Wang L, Li D, and Liu M (2016) Characterization of novel cytochrome P450 2E1 knockout rat model generated by CRISPR/Cas9. *Biochem Pharmacol* **105**:80–90.
- Wang Z, Pal D, Patel A, Kwatra D, and Mitra AK (2013) Influence of overexpression of efflux proteins on the function and gene expression of endogenous peptide transporters in MDR-transfected MDCKII cell lines. *Int J Pharm* **441**:40–49.
- Wei Y, Yang L, Zhang X, Sui D, Wang C, Wang K, Shan M, Guo D, and Wang H (2018) Generation and characterization of a CYP2C11-null rat model by using the CRISPR/Cas9 method. *Drug Metab Dispos* **46**:525–531.
- Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, Peterkin V, Koup JR, and Ball SE (2004) Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUC_i/AUC) ratios. *Drug Metab Dispos* **32**:1201–1208.
- Williamson B, Lorbeer M, Mitchell MD, Brayman TG, and Riley RJ (2016) Evaluation of a novel PXR-knockout in HepaRG™ cells. *Pharmacol Res Perspect* **4**:e00264.
- Wright JB and Sanjana NE (2016) CRISPR screens to discover functional noncoding elements. *Trends Genet* **32**:526–529.
- Xue HY, Ji LJ, Gao AM, Liu P, He JD, and Lu XJ (2016) CRISPR-Cas9 for medical genetic screens: applications and future perspectives. *J Med Genet* **53**:91–97.
- Yang L, Güell M, Niu D, George H, Lesha E, Grishin D, Aach J, Shrock E, Xu W, Poci J, et al. (2015) Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science* **350**:1101–1104.
- Yang R, Kerschner JL, Gosalia N, Neems D, Gorsic LK, Safi A, Crawford GE, Kosak ST, Leir SH, and Harris A (2016a) Differential contribution of *cis*-regulatory elements to higher order chromatin structure and expression of the *CFTR* locus. *Nucleic Acids Res* **44**:3082–3094.
- Yang Y, Qiu JG, Li Y, Di JM, Zhang WJ, Jiang QW, Zheng DW, Chen Y, Wei MN, Huang JR, et al. (2016b) Targeting ABCB1-mediated tumor multidrug resistance by CRISPR/Cas9-based genome editing. *Am J Transl Res* **8**:3986–3994.
- Yoshimi K, Kunihiro Y, Kaneko T, Nagahora H, Voigt B, and Mashimo T (2016) ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. *Nat Commun* **7**:10431.
- Zhang H, Kiuchi T, Hirayama C, Katsuma S, and Shimada T (2018) *Bombyx* ortholog of the *Drosophila* eye color gene *brown* controls riboflavin transport in Malpighian tubules. *Insect Biochem Mol Biol* **92**:65–72.
- Zhou Y, Ingelman-Sundberg M, and Lauschke VM (2017) Worldwide distribution of cytochrome P450 alleles: a meta-analysis of population-scale sequencing projects. *Clin Pharmacol Ther* **102**:688–700.
- Zuo E, Huo X, Yao X, Hu X, Sun Y, Yin J, He B, Wang X, Shi L, Ping J, et al. (2017) CRISPR/Cas9-mediated targeted chromosome elimination. *Genome Biol* **18**:224.
- Zuo YY, Huang JL, Wang J, Feng Y, Han TT, Wu YD, and Yang YH (2018) Knockout of a P-glycoprotein gene increases susceptibility to abamectin and emamectin benzoate in *Spodoptera exigua*. *Insect Mol Biol* **27**:36–45.

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