CRISPR knockout rat cytochrome P450 3A1/2 model for advancing drug metabolism and pharmacokinetics research

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Cytochrome P450 (CYP) 3A accounts for nearly 30% of the total CYP enzymes in the human liver and participates in the metabolism of over 50% of clinical drugs. Moreover, CYP3A plays an important role in chemical metabolism, toxicity, and carcinogenicity. New animal models are needed to investigate CYP3A functions, especially for drug metabolism. In this report, Cyp3a1/2 double knockout (KO) rats were generated by CRISPR-Cas9 technology, and then were characterized for viability and physiological status. The Cyp3a1/2 double KO rats were viable and fertile, and had no obvious physiological abnormalities. Compared with the wild-type (WT) rat, Cyp3a1/2 expression was completely absent in the liver of the KO rat. In vitro and in vivo metabolic studies of the CYP3A1/2 substrates indicated that CYP3A1/2 was functionally inactive in double KO rats. The Cyp3a1/2 double KO rat model was successfully generated and characterized. The Cyp3a1/2 KO rats are a novel rodent animal model that will be a powerful tool for the study of the physiological and pharmacological roles of CYP3A, especially in drug and chemical metabolism in vivo.

The Cytochrome P450 (CYP) enzymes play an essential role in the biotransformation of endogenous molecules and xenobiotics1. Today, almost 75% of drugs on the market are bio-transformed through CYP-mediated metabolism and the main CYP isoforms, CYP3A4/5, CYP2C9/19, CYP2D6, CYP1A1/2 and CYP2E1, participate in nearly 95% of the reactions2. In particular, CYP3A4, as the most abundant CYP isoform in the human liver and intestine, is involved in the phase I transformation of toxins, carcinogens, bile acids, steroid hormones, and more than 50% of the drugs used in the clinic3,4. Moreover, CYP3A4 is either induced or inhibited by many chemicals, which influences the elimination of co-administrated drugs, leading to therapy failure or unwanted toxicity4,5.

A variety of single CYP enzyme-null mouse models, including Cyp3a, have been reported as potent and precise tools used to illustrate the potential functions of CYP isoforms on the metabolism, toxicity and carcinogenicity of chemicals6,7. Until now, however, Cyp3a gene knockout (KO) rats have not been reported due to the complexity and limitation of gene editing techniques. Compared with the Cyp3a KO mouse model, Cyp3a KO rat model is more important to pharmacological research, especially drug metabolism and pharmacokinetic (DMPK) studies. On the one hand, the rat is larger in size, and possesses more blood, compared with the mouse. Moreover, rats in some disease models such as breast cancer are physiologically more similar to humans than mice8,9. Therefore, the Cyp KO rat model could be a good supplement to the Cyp KO mouse model, overcoming some disadvantages of the mouse model. On the other hand, since many CYP isoforms expressed in different species possess different substrate affinities, it is very difficult to extrapolate the results from one specific animal species to humans4. Therefore, results from multiple animals should be taken into consideration.

Recently, application of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (CRISPR-Cas9) system from Streptococcus pyogenes has greatly reduced the difficulties of genome editing in various species including the rat10-12. The CRISPR-Cas9 system consists of a non-specific
nucleotide deletion (Fig. 2d), which was not even detected in F0-#3. This inconsistency suggested that sequencing Cyp3a2 and presented impaired metabolic ability towards selected CYP3A probe substrates. To verify that CYP3A1/2 was functionally inactive in KO rats. The chemical analysis. Further expression in rat liver and intestine was confirmed by both PCR analysis of hepatic cDNA and immunohisto -

Figure 1. The strategy for generation of the Cyp3a1/2 double knockout rat model. (a) Schematic representation of the chromosomal organization of Cyp3a1 and Cyp3a2. (b) Exon/intron structure of Cyp3a1 and Cyp3a2. Exons are represented as 'exon'. (c) Targets selected for the deletion of Cyp3a1 and Cyp3a2. (d) Resulting deletions in the animal model.

Results

Generation of Cyp3a1 and Cyp3a2 double KO rats using CRISPR-Cas9. To investigate the role of Cyp3a in drug metabolism, we generated rats with CRISPR-Cas9-mediated disruption in both isoforms of this gene. For targeting Cyp3a1, we selected 5'-CAAGAAACAGGGGATTCC-3' followed by TGG as the target site, and 5'-TAAGAAACAAGGAATTCC-3' followed by TGG for targeting Cyp3a2. The targeting strategy is shown in Fig. 1. A mixture of Cyp3a1 sgRNA (25 ng/μL), Cyp3a2 sgRNA (25 ng/μL) and Cas9 mRNA (50 ng/μL) was co-microniected into one-cell fertilized eggs of Sprague-Dawley (SD) rats and 14 progenies were born. To identify the gene modifications of the F1 generation, the targeted loci of Cyp3a1 and Cyp3a2 were PCR amplified and T7E1 (T7 endonuclease I) cleavages were detected in rat #3, #5, #6 (unexpected death at day 11), #8, #11 and #12 founders for Cyp3a1 and in #3, #5, #7, #9, #12 and #13 founders for Cyp3a2 (Fig. 2a), which indicated the potential for genome modification at targeted loci. We sequenced these regions and confirmed these modifications (Fig. 2b,c). For Cyp3a1, founders #3, #5, #8 and #11 contained at least one frame-shifting mutation; frame-shift mutations were also observed for Cyp3a2 in founders #3, #5, #7, #9 and #13. Thus, founders #3 and #5 were crossed with wild-type (WT) rats and the genotypes of F1 offspring were determined. For Cyp3a1, several F1 progeny generated from F0-#3, five progeny out of eleven total pups (#3, #4, #7, #8 and #11) showed the same nucleotide deletion (deletion of 22 bp) as F0-#3 (Fig. 2d). Nevertheless, the genotype with nucleotide insertion and substitution was not transmitted from F0-#3 which may be due to genetic mosaicism caused by the CRISPR/Cas9 system10,14. For Cyp3a2, in the F1 generated from F0-#3, again five pups out of eleven (#1, #3, #4, #8 and #11) possessed a 10 bp nucleotide deletion (Fig. 2d), which was not even detected in F0-#3. This inconsistency suggested that sequencing 4 clones of PCR products from F1 tail DNA may be not enough to reveal all genetic modifications14. In conclusion, progeny #3, #4, #8 and #11 were identified as Cyp3a1 and Cyp3a2 double KO F1 rats by directly sequencing of the PCR products from rat tail DNA. However, in the F1 generation of F0-#4, no Cyp3a1 and Cyp3a2 double KO F1 rat was obtained (Data not shown). To generate Cyp3a1 and Cyp3a2 double KO and homozygous rats, F1-3#(♀) was mated with F1-4#(♂), and F1-8#(♀) was crossed with F1-11#(♂) to generate the F2 generation. The genomic modification in F1 was transmitted to the F2 generation efficiently and stably with the described mutations in Cyp3a1 and Cyp3a2 gene (Fig. 2d).

Off-target analysis. Recent studies have reported that the CRISPR-Cas9 system has a much greater level of off-target cleavage than zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) because the target sequence selected by CRISPR-Cas9 can tolerate a 1~3 base pair mismatch10,15–17. Hence, we examined off-target damage in Cyp3a1 and Cyp3a2 double KO rats. A genome-wide sgRNA off-target searching
Figure 2. Genotyping of Cyp3a1/2 double KO rats generated by the CRISPR-Cas9 system. (a) Detection of the mutations in the F0 generation for Cyp3a1/2 by T7E I digestion using PCR products amplified from F0 rats tail genomic DNA by Primer No. 1 and 2 (Table 3). T7E I−, before T7E I digestion. T7E I+, after T7E I digestion. △, mutant band. DNA sequencing of (b) Cyp3a1 or (c) Cyp3a2 genomic loci in F0 rats. Four TA clones of the PCR products amplified from each F0 rat were sequenced. “,” nucleotide deletion. Lowercase letter, nucleotide insertion. △, Red box, nucleotide substitution. “X”, the number of each genotype in four clones. (d) The details of mutations in F2 generation.
tool (COD) was used to pick out off-target sites (OTS) with high potential for mutation by our targeting system. OTS with a score of no less than 0.5 was chosen for further analysis. We examined eight and five OTSs for Cyp3a1 and Cyp3a2 sgRNAs, respectively (Table 1). None of these potential OTSs was mutated in our double KO rats (Fig. 3), thus suggesting CRISPR-Cas9 is a reliable gene targeting tool in generating the Cyp3a1/2 double KO rat model.

**Physiological phenotype of Cyp3a1 and Cyp3a2 double KO rat and WT rat.** CYP3A1 and CYP3A2 enzymes were involved in the metabolism of xenobiotics, steroid bile acids and other compounds. Therefore, the deletion of Cyp3a1 and Cyp3a2 may lead to important physiological changes. To investigate the effects of Cyp3a1 and Cyp3a2 disruption on rat physiology, serum samples were collected at 8 weeks and analyzed for high-density lipoproteins-cholesterol (HDL-CHOL), low-density lipoproteins-cholesterol (LDL-CHOL), total cholesterol (T-CHOL), triglycerides (TRIG), aspartate amino transferase (AST), alanine amino transferase (ALT), prostaglandin E2 (PGE2), 25-OH vitamin D, testosterone and bile acid. None of the serum clinical chemistry and physiological indices showed obvious abnormalities except for testosterone (Fig. 4a). The concentration of testosterone (a typical substrate of CYP3A) increased by 110% in the serum of KO rats. Compared with WT, homozygous Cyp3a1 and Cyp3a2 double KO rats seemed to be normal and fertile, with regular liver weight, body weight as well as organ coefficients for both genders (Fig. 4a). Furthermore, histological analysis revealed that there were no morphological changes between the WT and KO rat liver and small intestine (Fig. 4b). In general, the absence of Cyp3a1 and Cyp3a2 may not cause any consequential abnormalities.

**Double KO rats lack expression of CYP3A1 and CYP3A2.** The expression of CYP3A1 and CYP3A2 in mRNA levels in double KO and WT rat liver was checked via the specific primer pairs targeting Cyp3a1 and Cyp3a2, respectively (Table 1). None of these potential OTSs was mutated in our double KO rats (Fig. 3). To further explore the impaired function of CYP3A1/2 in Cyp3a1/2 double KO rats in vitro, we carried out CYP3A-mediated metabolism and pharmacokinetics studies in Cyp3a1/2 double KO rats. To assess whether CYP3A1/2 was functionally inactive in KO rats, the in vitro (substrate: midazolam and nifedipine) and in vivo (substrate: nifedipine) metabolic studies of CYP3A1/2 substrates were carried out. In in vitro studies, the maximum velocity ($V_{\text{max}}$) of dehydronifedipine formation in rat liver microsomes (RLM) of double KO rats was $0.20 \pm 0.01 \text{ mmol/min/mg protein}$, a significant decrease (about 50%) of that in WT controls ($0.40 \pm 0.01 \text{ mmol/min/mg protein}$) (Fig. 6a,b). The Michaelis constant ($K_{M}$) value of KO RLM was $9.97 \pm 1.13 \mu M$, which was slightly decreased by 12% compared with that in WT RLM (11.37 $\pm 1.10 \mu M$) (Fig. 6c). Meanwhile, the intrinsic clearance ($CL_{\text{int}}$) of nifedipine in the RLM of double KO rats significantly decreased by 43% compared with that in WT RLM (Fig. 6d). Moreover, the $V_{\text{max}}$ and $CL_{\text{int}}$ of midazolam in the RLM of double KO rats also significantly decreased by 75% and 70%, respectively, compared with those in WT RLM (Fig. 6f,h). In summary, the CYP3A1/2 activity of KO rats was significantly decreased in our metabolic studies of the CYP3A substrates nifedipine and midazolam in vitro.

**Table 1. Details for potential off-target sites examined.**

| Match Name | Coordinate | Spacer + PAM | Number of mismatch | off-target score |
|------------|------------|--------------|--------------------|-----------------|
| Cyp3a1 sgRNA | Chr12:12839905 to 12839886 | AAGAAACACGGGATCCCTGG | 1 | 0.70 |
| Cyp3a1-off-1 | Chr12:13740208 to 13740189 | AAGAAACAGGGGATCCCTGG | 2 | 0.56 |
| Cyp3a1-off-3 | Chr12:17502003 to 17501985 | AGAGAACGCGATCCCTGG | 2 | 0.50 |
| Cyp3a1-off-4 | Chr12:43976656 to 43976638 | AGAGACAGCGATCCCTGG | 3 | 0.50 |
| Cyp3a1-off-5 | Chr12:16851108 to 16851124 | AACGAGGGATCCCTGG | 2 | 0.50 |
| Cyp3a1-off-6 | Chr12:41792250 to 41792265 | AAGACAGCTTTCCCTGG | 1 | 0.50 |
| Cyp3a1-off-7 | Chr12:51405656 to 5140580 | ACAGTTCACTGG | 1 | 0.50 |
| Cyp3a1-off-8 | Chr13:50280364 to 50280382 | AGACACAGGGGATCCCTGG | 1 | 0.50 |
| Cyp3a2 sgRNA | Chr12:12747978 to 12747978 | TAAGAGAGGGGATCCCTGG | 1 | 0.70 |
| Cyp3a2-off-1 | Chr12:12839906 to 12839886 | TAAGAGAGGGGATCCCTGG | 1 | 0.70 |
| Cyp3a2-off-3 | Chr12:41792244 to 41792247 | AAGACAGCTTTCCCTGG | 2 | 0.63 |
| Cyp3a2-off-4 | Chr12:52934974 to 52934956 | AGACACAGCTTTCCCTGG | 3 | 0.56 |
| Cyp3a2-off-5 | Chr12:40658157 to 40658141 | AAAAGAGGGGATCCCTGG | 1 | 0.50 |
clearance (CL), compared with these in WT rats. These data reflected that the function of CYP3A1/2 in KO rats was impaired in vivo. Therefore, the pharmacokinetic results of nifedipine in vivo were in agreement with the in vitro results, indicating that CYP3A1/2 was functionally inactive in the double KO rat line.

Compensatory expression of other CYP isoforms in Cyp3a1/2 KO rats. Since Cyp3a1/2 deletion may affect the expression of other CYP isoforms, the mRNA levels of the other main rat CYP enzymes were checked via quantitative real-time PCR. Compared with WT rats, no significant change was observed in the hepatic and small intestinal expression of CYP1A2, CYP2D1, CYP2D2, CYP2E1, and CYP3A9 (Fig. 7). In contrast, the hepatic mRNA expression of CYP2C11 and CYP3A18 in double KO rats was increased by nearly 290% and 300%, respectively, compared with WT controls (Fig. 7). Meanwhile, in small intestine of KO rats, the expression of CYP3A18 and CYP3A62 was up-regulated by 60% and 93%, respectively (Fig. 7).

Discussion
CYP3A as the most abundant human liver CYP enzyme is not only involved in the biotransformation of many endogenous substances, such as fatty acids, eicosanoid sterols, bile acids and vitamin D³, but also participates in the metabolism of over 75% of clinical drugs¹⁸. Traditionally, the roles of CYP3A in metabolism and metabolism-based toxicity have been investigated using specific antibodies or ‘selective’ chemical inhibitors, but in later studies most of these ‘specific’ modulators turned out to be nonspecific and even toxic under physiological conditions¹⁹,²⁰. Therefore, a novel Cyp3a KO animal model is needed to study the functions of CYP3A in vivo. To our knowledge, the present work is the first time to successfully create a Cyp3a1/2 double KO rat model using CRISPR-Cas9 system.

Traditional gene targeting via embryonic stem cells to generate specific modified alleles is a potent tool to illustrate functions of genes in mice²¹. In rats, however, this genome manipulation method in stem cells does not work because of some technical difficulties¹⁰,²². In recent years, the emergences of engineered nucleases,
Figure 4. Physiological phenotype of Cyp3a1/2 double KO rats. (a) Clinical chemistry and physiological analysis of Cyp3a1/2 double KO rat serum compared with WT rats. Serum samples were collected at nearly 8 weeks and analyzed for HDL-CHOL, LDL-CHOL, T-CHOL, TRIG, AST, ALT, AST/ALT, AP, ALB, GLB, ALB/GLB, TP, ID-BIL, D-BIL, T-BIL, GLUC, PEG2, 25-OH vitamin D, testosterone and bile acid. Organ coefficient of Cyp3a1/2 double KO rat (about 8 weeks) liver compared with WT rats. LW, liver weight; BW, body weight. The organ coefficient was defined as LW/BW ratio. Data were shown as Mean ± SEM of six rats. (b) HE staining of liver and small intestine slices from WT and Cyp3a1/2 double KO rats (n = 6). Scale bar = 100 μm.
such as ZFNs and TALENs, have been used for gene editing in rats. However, they are still limited by the time- and labor-consuming construction of engineered specific protein pairs for every target site. More recently, a state-of-the-art engineered nuclease, the CRISPR-Cas9 system, has proved a simple method to manipulate genes in rats. In particular, we have used the CRISPR-Cas9 tool to successfully knock out the Cyp2e1 gene from rats.

In rats, the high degree of nucleotide similarity between CYP3A1 and 3A2 presents a major challenge towards simultaneous disruption of these isozymes. However, compared with previous techniques such as ZFNs and TALENs, the CRISPR-Cas9 system displays distinct advantages in editing multiple genes simultaneously. In particular, we targeted the Cyp3a1 and Cyp3a2 genes simultaneously via the CRISPR-Cas9 system. In this study, the CRISPR-Cas9 tool showed great advantages again in successfully achieving precision gene targeting in the rat by co-microinjection of Cyp3a1 and Cyp3a2 sgRNA with Cas9 mRNA into zygotes.

The Cyp3a1/2 double KO rats were viable and fertile. At first, we thought simultaneous KO Cyp3a1/2 might show disruptive effects on rat physiology. However, our data indicated that Cyp3a1/2 double KO rats possessed equivalent levels of cholesterol, aminotransferase, glucose, bilirubin, PEG2, 25-hydroxy vitamin D, bile acid as well as serum proteins (Fig. 4). These results revealed that CYP3A may work as an important rather than exclusive contributor to the metabolism of endogenous substances, which is also in agreement with its role in the Cyp3a KO mouse model. Recently, it has been reported that Cyp3a KO mice showed higher levels for bile acid and testosterone compared with WT mice. In contrast, Cyp3a KO rats only presented higher level of testosterone than that of WT rats, thus suggesting different species possess different metabolic characterization. Moreover, some studies have reported that the deletion of one Cyp gene in mouse may lead to compensatory changes in other CYP metabolic enzymes. Therefore, the mRNA levels of other main CYP isoforms were quantified in this

Figure 5. mRNA and immunohistochemical detection of Cyp3a1 and Cyp3a2 in double KO and WT male rat liver and small intestine. (a) Hepatic mRNA of WT rats and KO rats was detected by using agarose gel electrophoresis. β-actin was set as the reference gene. (b) Liver and small intestine sections were used for immunohistochemical staining. Experiments were repeated three times independently.
Figure 6. CYP3A-mediated metabolic activity in Cyp3a1/2 double KO and WT rats in vitro. (a) The saturation curve of nifedipine metabolism in liver microsomes from both double KO and WT rats. (b) The $V_{\text{max}}$ values of nifedipine metabolism in both double KO and WT male RLM. (c) The $K_m$ values of nifedipine metabolism in both double KO and WT male RLM. (d) The $CL_{\text{int}}$ values of nifedipine metabolism in both double KO and WT male RLM. (e) The saturation curve of midazolam metabolism in liver microsomes from both double KO and WT rats. (f) The $V_{\text{max}}$ values of midazolam metabolism in both double KO and WT male RLM. (g) The $K_m$ values of midazolam metabolism in both double KO and WT male RLM. (h) The $CL_{\text{int}}$ values of midazolam metabolism in both double KO and WT male RLM. All data were expressed as mean $\pm$ SEM of six rats in each group, and $***p < 0.001$ compared to WT rats.
Table 2. Pharmacokinetic parameters of nifedipine in double KO and WT rats. t_{1/2}, half life; C_0, initial plasma concentration; AUC_{0−120min}, area under the plasma concentration−time curve during 0−120 min; AUC_{∞}, area under the plasma concentration−time curve during 0−∞; V_d, apparent volume of distribution; CL, clearance; MRT, mean residence time. All data were expressed as mean ± SEM (n = 5). ***p < 0.001 and **p < 0.01 compared with WT rats.

| Pharmacokinetic parameter | WT       | KO       |
|---------------------------|----------|----------|
| t_{1/2} (min)             | 35.52 ± 8.29 | 66.42 ± 9.23*** |
| C_0 (ng/mL)               | 926.52 ± 142.87 | 870.74 ± 204.87 |
| AUC_{0−120min} (min-ng/mL)| 26979.23 ± 1522.03 | 43338.98 ± 7259.09** |
| AUC_{∞} (min*ng/mL)       | 30118.55 ± 2436.55 | 61124.74 ± 10417.95*** |
| V_d (mL/kg)               | 341.11 ± 83.37 | 332.07 ± 80.81 |
| CL (mL/min/kg)            | 6.67 ± 0.51  | 3.36 ± 0.68*** |
| MRT (min)                 | 38.06 ± 5.41  | 47.74 ± 2.21** |

In conclusion, the CRISPR-Cas9 method was described to successfully create the Cyp3a1/2 KO rat model. The Cyp3a1/2 double KO rat model can be used in vivo to study CYP3A-mediated drug metabolism and pharmacokinetics. In this study, nifedipine and midazolam as CYP3A substrates were used to detect the CYP3A-mediated catalytic activity in WT and double KO rats. RLM from double KO rats showed a decreased in vitro metabolic activity both on nifedipine and midazolam (Fig. 6), suggesting the loss of CYP3A. However, residual metabolites of nifedipine and midazolam were still formed by RLM from double KO rats, which may be ascribed to other hepatic CYP isoforms and/or the compensatory up-regulation of other CYP isoforms. For example, previous studies have reported that midazolam is the probe substrate of CYP3A, but CYP2C also takes part in midazolam metabolism by non-CYP enzymes or other CYP isoforms in rats. In this study, there was discrepancy of midazolam between in vitro metabolic study using liver microsomes and in vivo pharmacokinetic study in WT rats. Therefore, it is difficult to decide whether midazolam has the compensatory metabolic pathways in vitro and in vivo. However, a CYP2C specific inhibitor or antibody may be effective to explore the compensatory effect of CYP2C in the metabolism of midazolam when Cyp3a1/2 KO rat model is used.

The Cyp3a1/2 double KO rat model can be used in vivo to study CYP3A-mediated drug metabolism and pharmacokinetics. In this study, nifedipine and midazolam as CYP3A substrates were used to detect the CYP3A-mediated catalytic activity in WT and double KO rats. RLM from double KO rats showed a decreased in vitro metabolic activity both on nifedipine and midazolam (Fig. 6), suggesting the loss of CYP3A. However, residual metabolites of nifedipine and midazolam were still formed by RLM from double KO rats, which may be ascribed to other hepatic CYP isoforms and/or the compensatory up-regulation of other CYP isoforms. For example, previous studies have reported that midazolam is the probe substrate of CYP3A, but CYP2C also takes part in its metabolism. In this study, we compared the pharmacokinetic profiles of midazolam between CYP3A1/2 KO and WT rats. However, there was no obvious difference of the pharmacokinetics of midazolam between KO and WT rats. Therefore, we used nifedipine instead of midazolam in the in vivo pharmacokinetic studies. The results in vivo showed that the exposure of nifedipine (AUC value) in Cyp3a1/2 KO rats significantly increased by 61%, together with increases in the t_{1/2} (93%) and MRT (25%), compared with WT rats (Table 2). Our findings demonstrated that Cyp3a1/2 double KO rats were a sensitive animal model to investigate the CYP3A-mediated metabolic pathway of chemicals, especially in in vivo studies. Given that a discrepancy in CYP3A exists between rats and humans, a CYP3A humanized rat model should be further created to overcome the species differences.

In conclusion, the CRISPR-Cas9 method was described to successfully create the Cyp3a1/2 double KO rat model. The Cyp3a1/2 double KO rats were viable, fertile, and physiological normal. The Cyp3a1/2 double KO rats are a valuable animal model to investigate the roles of CYP3A in drug and chemical metabolism, toxicity and carcinogenicity in vivo.

Methods

Materials and chemicals. Oligos (60 bp, containing Cyp3a1 or Cyp3a2 knock out target-sites) and all primers for PCR were synthesized from Biosune Biotechnology Co. LTD (Shanghai, China). In vitro Transcription T7 Kit, SYBR Premix Ex Taq, Prime Script RT Reagent Kit and TA cloning kit were bought from Takara (Dalian, China). mMessage mMACHINE SP6 kit were purchased from Thermo Scientific (Massachusetts, USA). The secondary antibody conjugated to HRP-labeled polymers was bought from Mrbiotech (Shanghai, China). Midazolam was purchased from Enhua (Nanchang, China). Mebendazole was purchased from Aladdin (Shanghai, China).

Animals. Male and female SD rats (8-week old) were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch of China. The animals were kept in a specific pathogen-free facility with access to rodent chow cubes and sterile water, with 12 h light-dark cycles. All the methods performed in animals were carried out in accordance with the National Institutes of Health standards established in the ‘Guidelines for the Care and Use of Experimental Animals’. All experimental protocols in animals were approved by the Ethics Committee on Animal Experimentation of East China Normal University (Shanghai, China).

Cyp3a1 and Cyp3a2 KO target site selection. The sequence fragments of Cyp3a1 and Cyp3a2 were submitted to an online design tool (CRISPR Design Tool, http://tools.genome-engineering.org) to generate the
potential target sites. Cyp3a1 and Cyp3a2 in rats contain 13 and 10 exons, respectively. To silence the Cyp3a1 and Cyp3a2 gene as completely as possible, target-sites were selected just downstream to the initiation codon, ATG, and followed by a protospacer adjacent motif (PAM) site (5'-NGG-3') in the 3' end.

DNA constructs and in vitro transcription. The sgRNA expression vector and Cas9-encoding plasmid were constructed according to our previous work\textsuperscript{14}. Then oligos (60 bp, containing Cyp3a1 or Cyp3a2 knock out target-sites) were cloned into the pGS3-T7-gRNA vector through overlapping PCR for the transcription of sgRNA in vitro. Cyp3a1 and Cyp3a2 sgRNA were then transcribed with the in vitro T7 Transcription Kit respectively. RNA was purified through phenol/chloroform extraction and dissolved in diethylpyrocarbonate-treated or RNase-free water.
For the transcription of Cas9 mRNA, the linearized Cas9 expression vectors were purified through phenol/chloroform extraction and ethanol precipitation followed by the transcription of Cas9 mRNA using the mMessage mMachine SP6 kit. The mRNA was recovered by lithium chloride precipitation and resuspended in Nucleos-free water. Purified Cyp3a1 and Cyp3a2 sgRNA and Cas9 mRNA products were confirmed by electrophoresis.

**Co-microinjection of sgRNA and Cas9 mRNA into zygotes.** Rat preparation and microinjection were performed as described with modifications41. Briefly, the TE buffer containing 25 ng/μL of Cyp3a1 sgRNA, 25 ng/μL of Cyp3a2 sgRNA and 50 ng/μL of Cas9 mRNA were co-injected into the cytoplasm of one-cell stage embryos. The microinjected zygotes were transferred into pseudopregnant female rats immediately after injection or after overnight culture in embryo culture medium.

**Genotyping of founders and progeny.** For the F0 generation, newborn rats were genotyped 7 to 10 days after birth. Purified genomic DNA was amplified using the primers listed in Table 3 (No. 1, 2). T7E I assay was used for a preliminary screening for the potential mutations in founders on target-sites. To identify the modification details in founders, containing potential mutations, PCR products from each founder was cloned into pMD-18T vectors for sequencing with the universal primers of the vector. For the F1 and F2 generations, the genotyping was identified by sequencing the PCR products directly. Sequence data was analyzed through DNAMAN (LynnonBiosoft, CA, USA) to identify the exact genotype (WT, heterozygote and double KO rats) of founders. Specificity of all primer pairs (No. 1 to No. 26) used in our research was checked using agarose gel electrophoresis, indicating a single and specific band for each pair of primer under our experimental conditions.

**Off-target site validation.** The target sites of Cyp3a1 and Cyp3a2 were submitted to Cas9 online designer (http://cas9.wicp.net/). The degree of off-target was measured by an 'off-target score', which ranges from 1 to 0. '1' means a high probability of off target and '0' a low probability of off target events. OTS with a score ≥ 0.5 were selected for PCR analysis. The PCR product of each off-target site was subjected to T7E I digestion and then resolved on a 1.5% agarose gel to analyze the off-target effects.

**Hematoxylin and eosin (HE) staining of rat liver and small intestine slices.** About 8-week old rats were sacrificed through cervical dislocation. Livers and intestines were freshly excised and then fixed in 4% paraformaldehyde at 4°C for more than 12 h. The fixed intestines were dehydrated in 50%, 75%, 85%, 95%, and 100% ethyl alcohol for one hour for each and then immersed in ethyl alcohol and xylene mixture (v/v = 1:1) for 30 min, followed by two 15 min intervals in xylene, followed by paraffin embedding. The fixed livers similarly treated, except dehydration for 30 min per step and 15 minute immersion in the ethanol:xylene mixture. Sections (4 μm) were then deparaffinization for hematoxylin and eosin (H&E) according to normal procedures.

**Clinical-chemical and hematological analysis of Cyp3a1 and Cyp3a2 double KO and WT rats.** To further characterize the potential effects of Cyp3a1 and Cyp3a2 KO on the normal rat physiology, the serum samples were collected for clinical-chemical and hematological analysis. The serum samples were sent for analysis by ADICON Clinical Laboratories (Shanghai, China). For the analysis of PGE2, a rat PEG2 ELISA kit was bought from Shanghai MLBIO Biotechnology Co.Ltd (Shanghai, China).

**Cyp3a1 and Cyp3a2 mRNA expression in rat liver.** About 8-week old rats were sacrificed through cervical dislocation method. Livers mRNA was extracted through Trizol and the RNA was reverse-transcribed into cDNA using the Takara RR036A RT kit. The mRNA was reverse transcripted into cDNA using the Takara RR036A RT kit. The detection of cyp3a1 and cyp3a2 mRNA expression, selective primers (spanning different exons) were designed and synthesized which were listed in Table 3 (No. 24, 25). β-actin (Primer listed in Table 3, No. 26) was used as the internal reference.

**Immunohistochemical analysis.** Immunohistochemistry on double KO and WT rat liver and small intestine was conducted with a commercial rabbit anti-human CYP3A4 polyclonal antibody (1:100, ab3572, Abcam), which cross-reacts with rat CYP3, and a secondary antibody conjugated to HRP-labeled polymers (MR-SPR120, Mrbiotech).

**Quantitative reverse transcriptase PCR.** SYBR-PCR was performed using a Stratagene Mx3005P with SYBR Premix Ex Taq. The relative mRNA expression was measured by 2−ΔΔCt. The primers (spanning different exons) used for quantitative reverse transcriptase-PCR were listed in Table 3 (No. 16–23, 26). Disassociation curves of primer pairs for quantitative reverse transcriptase-PCR were monitored, showing a high specificity for our primer pairs.

**Preparation of RLMs.** The protocol of RLM preparation was modified from our previous studies32,33. The liver was separated, rinsed with ice-cold normal saline and homogenized in a 0.05 M Tris/KCl buffer (pH 7.4). The homogenate was centrifuged at 10,500 g at 4°C for 30 min. The supernatant was then centrifuged at 105,000 g at 4°C for 60 min. The precipitate was resuspended and re-centrifuged at 105,000 g at 4°C. The pellet was reconstituted with the 0.05 M Tris/KCl buffer (pH 7.4) and stored in –80°C for use.

**The CLint of nifedipine and midazolam in Cyp3a1 and Cyp3a2 double KO and WT RLM.** For the research of CYP3A1 and CYP3A2 enzymes activity in vitro, nifedipine and midazolam were chosen as specific substrates and dehydrofinedipine and 1'-hydroxymidazolam were monitored, respectively34–37. The incubation mixture consisted of an NADPH (β-Nicotinamide adenine dinucleotide phosphate hydrate)-regenerating system,
Cyp3a1-genotyping-S
TAGGTTTATGACTGCTGTT
Cyp3a1-genotyping-A
GCGCAGCTGCTGCTGCT
Cyp3a2-genotyping-S
TAGGTTTATGACTGCTGTT
Cyp3a2-genotyping-A
GCGCAGCTGCTGCTGCT
Cyp3a2-off-1-S
AGACGAGAAGAAGATGAG
Cyp3a2-off-1-A
AACACACGAGGTTGTT
Cyp3a2-off-2-S
GAACGAGAAGAAGATGAG
Cyp3a2-off-2-A
AACACACGAGGTTGTT
Cyp3a2-off-3-S
TGGAGGAGGTAATGAGAG
Cyp3a2-off-3-A
TGGAGGAGGTAATGAGAG
Cyp3a2-off-4-S
TGGAGGAGGTAATGAGAG
Cyp3a2-off-4-A
TGGAGGAGGTAATGAGAG
Cyp3a2-off-5-S
GTAGCTGCTGCTGCTGCT
Cyp3a2-off-5-A
GTAGCTGCTGCTGCTGCT
Cyp3a2-off-6-S
ACACCTGCTGCTGCTGCT
Cyp3a2-off-6-A
ACACCTGCTGCTGCTGCT
Cyp3a2-off-7-S
GGAGGAGGTAATGAGAG
Cyp3a2-off-7-A
GGAGGAGGTAATGAGAG
Cyp3a2-off-8-S
CAGAGAGGTAATGAGAG
Cyp3a2-off-8-A
CAGAGAGGTAATGAGAG
Cyp3a2-off-1-S
GCTGGGATGATGAGAGAG
Cyp3a2-off-1-A
GCTGGGATGATGAGAGAG
Cyp3a2-off-2-S
GCGCAGCTGCTGCTGCT
Cyp3a2-off-2-A
GCGCAGCTGCTGCTGCT
Cyp3a2-off-3-S
TGGAGGAGGTAATGAGAG
Cyp3a2-off-3-A
TGGAGGAGGTAATGAGAG

Table 3. Primer pairs used in the research.

nifedipine (2 μM to 100 μM) or midazolam (10 μM to 100 μM) and 0.5 mg/mL (for nifedipine) or 1 mg/mL (for midazolam) of RLM in 0.05 M Tris–HCl buffer (pH 7.4). The supernatant of the incubation mixture was transferred to the autosampler vial after a protein precipitation process for LC-MS/MS analysis. The enzyme kinetic data of nifedipine and midazolam metabolism in RLM was fitted according to the typical Michaelis–Menten equation by GraphPad Prism 5. The CLint was defined as the Vmax/Km ratio.

Pharmacokinetics of nifedipine in Cyp3a1 and Cyp3a2 double KO and WT rats. Nifedipine (200 μg/kg) was administered through the tail vein for all WT and KO rats and blood samples were collected into heparinized centrifuge tubes at 2, 5, 10, 20, 30, 45, 60, 90, and 120 min by orbital bleeding with capillary tubes. Blood samples were centrifuged as soon as possible at 5,500 g for 15 min, and the plasma was transferred into new tubes and frozen at 0 °C for further analysis.

LC-MS/MS analysis of 1′-hydroxymidazolam, nifedipine and dehydronifedipine. For the LC-MS/MS analysis, a 1290 HPLC-6460 triple quadrupole mass spectrometer coupled with an ESI ion source (Agilent Technologies, USA) was employed. For 1′-hydroxymidazolam detection, a Zorbax Eclipse C18 column (2.1*50 mm, 1.8 μm; Agilent Technologies, USA) was used with a mobile phase system of water (A)-acetonitrile (B). The ion transitions of 342.0 → 324.0 and 285.0 → 193.1 for 1′-hydroxymidazolam and diazepam (IS) were monitored in positive ESI mode, respectively.

For the detection of nifedipine in rat plasma, the mobile phase consisted of water (A) and acetonitrile (B). The liquid–liquid extraction method was used to isolate nifedipine and IS from plasma.

Data analysis and statistics. All data were presented as mean ± SEM. Statistical analysis between different groups was performed using two-tailed t-test and p-values less than 0.05 were considered to indicate statistical significance. The enzyme kinetic data of nifedipine and midazolam metabolism in RLM was fitted according to the typical Michaelis–Menten equation with GraphPad Prism 5.0 (GraphPad Software, CA, USA).
Pharmacokinetic parameters were calculated by WinNonlin software version 5.2.1 (Pharsight Corporation, Mountain View, USA) based on non-compartmental analysis.

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Acknowledgements

We thank Dr. Stefan Siwko from Texas A&M University Health Science Center for language editing and Ms. Yongmei Li for microinjection assistance. This work was supported in whole or part by grants from the National Natural Science Foundation of China (No. 813301908), and the Science and Technology Commission of Shanghai Municipality (Nos 15140904700, 13ZR1412600 and 14DZ2270100).
Author Contributions
J.L. conducted most of the experiments and wrote most of the manuscript. Y.S. and D.L. conducted experiments on the genotyping of founders and progenies of rats. X.Q. and A.C. conducted experiments on the pharmacokinetics and LC-MS/MS analysis. D.L., M.L. and X.W. designed experiments. D.L. and X.W. analyzed the results and wrote the manuscript. M.L. and X.W. revised and finalized this manuscript.

Additional Information
Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Lu, J. et al. CRISPR knockout rat cytochrome P450 3A1/2 model for advancing drug metabolism and pharmacokinetics research. Sci. Rep. 7, 42922; doi: 10.1038/srep42922 (2017).

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