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Published in:
Pharmacological research

DOI:
10.1016/j.phrs.2020.105382

Publication date:
2021

Pharmacological Research 164 (2021) 105382

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Multiple microRNAs regulate tacrolimus metabolism through CYP3A5

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ARTICLE INFO

Keywords:
Tacrolimus
MicroRNAs
Drug metabolizing enzymes
Pediatric liver transplantation
Chemical compounds studied in this article:
Tacrolimus (CID: 445643)
DMSO (CID: 679)

ABSTRACT

The CYP3A5 gene polymorphism accounts for the majority of inter-individual variability in tacrolimus pharmacokinetics. We found that the basal expression of CYP3A5 in donor grafts also played a significant role in tacrolimus metabolism under the same genetic conditions after pediatric liver transplantation. Thus, we hypothesized that some potential epigenetic factors could affect CYP3A5 expression and contributed to the variability. We used a high-throughput functional screening for miRNAs to identify miRNAs that had the most abundant expression in normal human liver and could regulate tacrolimus metabolism in HepaRG cells and HepLPCs. Four of these miRNAs (miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26-5p) were selected for testing. We found that these miRNAs inhibited tacrolimus metabolism that was dependent on CYP3A5. Putative miRNAs targeting key drug-metabolizing enzymes and transporters (DMETs) were selected using an *in silico* prediction algorithm. Luciferase reporter assays and functional studies showed that miR-26b-5p inhibited tacrolimus metabolism by directly regulating CYP3A5, while miR-29a-5p, miR-99a-5p, and miR-532-5p targeted HNF4α, NR1I3, and NR1I2, respectively, in turn regulating the downstream expression of CYP3A5; the corresponding target gene siRNAs markedly abolished the effects caused by miRNA inhibitors. Also, the expression of miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p in donor grafts were negatively correlated with tacrolimus C/D following pediatric liver transplantation. Taken together, our findings identify these miRNAs as novel regulators of tacrolimus metabolism.

1. Introduction

Tacrolimus is an immunosuppressant that has been used worldwide for the prevention of graft rejection in organ-transplant recipients. Owing to the high inter-individual variability in its pharmacokinetics and the narrow therapeutic range [1], especially in pediatric patients who routinely exhibit some 10-fold variability in pharmacokinetic parameters and blood concentration after a fixed-dose [2]. Single nucleotide polymorphisms (SNPs) in the genes encoding drug-metabolizing enzymes contribute to inter-individual variability in the metabolism of tacrolimus. SNPs in CYP3A5*3 that caused alternative splicing and protein truncation resulted in the absence of CYP3A5 from tissues of some people and accounted for 42.25 % of the Chinese population [3]. Moreover, previous studies have demonstrated that the CYP3A5*1 allele in donor genotype achieved a lower concentration/dose (C/D) ratio of tacrolimus than that with CYP3A5*3/*3 (rs776746) in living transplant patients [4–7].

However, we found that children with CYP3A5*1 showed a different coefficient of C/D value following pediatric liver transplantation under the same genetic conditions; this might be related to normal physiological conditions (basal level) of the graft and a variety of additional factors that alter the transcriptional induction of CYP3A5 gene (induced

Abbreviations: SNPs, Single nucleotide polymorphisms; HNF4α, hepatocyte nuclear factor 4a; NR1I2, nuclear receptor subfamily 1 group I member 2; NR1I3, nuclear receptor subfamily 1 group I member 3; DMEs, drug-metabolizing enzymes; HMM, epatocyte maturation medium; TEM, transition and expansion medium; DMSO, dimethyl sulfoxide; DMETs, drug-metabolizing enzymes and transporters.

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https://doi.org/10.1016/j.phrs.2020.105382
Received 14 November 2020; Received in revised form 11 December 2020; Accepted 11 December 2020
Available online 18 December 2020

Table 1
Demographics and clinical data for patients with *1*3 donor graft.

Variable	Values
Donor characteristics	
Age (year)	31.35 ± 6.51
Male/female (n)	5/21
BMI (kg/m^2)	21.85 ± 2.72
Recipient characteristics	
Age (months)	6.23 ± 1.21
Male/female (n)	6/20
BMI (kg/m^2)	15.42 ± 1.61
Primary disease	
Biliary atresia	26
GRWR (%)	3.64 ± 0.73
Dose ((mg/kg/d))	0.22 ± 0.07
Concentration (ng/mL)	9.32 ± 2.75
C/D ratio	41.84 ± 11.44

GRWR, graft to recipient weight ratio.

level) at the transcriptional level. Induction of the CYP3A5 gene is regulated by key nuclear receptors such as hepatocyte nuclear factor 4a (HNF4α), nuclear receptor subfamily 1 group I member 2 (NR1I2), and nuclear receptor subfamily 1 group I member 3 (NR1I3) [8–11]. Thus, we hypothesized that the difference in C/D value was not entirely attributable to CYP3A5 gene polymorphism and the basal expression of CYP3A5 might also play a significant role in tacrolimus metabolism. Some potential epigenetic factors such as miRNAs that regulate the expression of these nuclear receptors or CY3PA5 could contribute to the variability.

Many miRNAs have been demonstrated to participate in the regulation of drug-metabolizing enzymes (DMEs) such as CYP3A4 regulated by miR-27b, CYP2E1 by miR-378a, UGT1A by miR-298, CYP1A2 by miR-132-5p, CYP2D6 by miR-370-3p, CYP2B6 by miR-25-3p, and HNF4A by miR-449a [12–17]. Moreover, it has been shown that graft microRNAs could participate in various pathological processes after liver transplantation including the development of new-onset diabetes [18] and the downregulation of drug-metabolizing enzymes and transporters in inflammatory conditions [19]. The hepatic metabolism of drugs is altered in younger children, with diffrernt ages for the different cytochromes to reach maturity, resulting in different metabolism rates and consequent clearance rates [20]. Due to the immaturity of DMEs in infant, the P450 enzymes in the graft play a significant role in tacrolimus metabolism after pediatric living liver transplantation [21]. Hence, in this study, we aimed to evaluate the impact of graft miRNAs on tacrolimus metabolism after pediatric liver transplantation.

2. Materials and methods

2.1. Human liver cohort

A total of 26 children who underwent living-donor liver transplantation between January 2017 and July 2019 at Renji Hospital affiliated to Shanghai Jiao Tong University School of Medicine were included. There were 5 boys and 21 girls with a mean age of 6.23 ± 1.21 months. The study was approved by the hospital's ethics board and conducted in accordance with the Declaration of Helsinki. The clinical characteristics of recipients and donors are shown in Table 1. All recipients were administered the routine immunosuppressive regimen consisting of tacrolimus and steroids, with or without mycophenolate mofetil. Tacrolimus (Astella Pharma Co., Limited) was administered orally on days 2 and 3 after pediatric transplantation, divided twice daily with an initial dose of 0.15 mg/kg/day, and subsequently adjusted to achieve the target blood trough concentration (C0). The target C0 was controlled between and 10–15 ng/mL for the first month, 8–12 ng/mL up to six months, and 5–8 ng/mL thereafter. The post-transplant blood tacrolimus concentration and dosage were monitored 1 week after liver transplantation, a dose ratio was obtained as concentration/dose (C/D), and was used to estimate tacrolimus clearance. Tissue from donors was collected from reduced-size donor livers and kept frozen at −80 °C.

2.2. Cell culture

Undifferentiated HepaRG cells were purchased from Biopredic International (Rennes, France) and cultured in complete William's E medium (Gibco) for 1–3 days until complete fusion. The medium was then changed to hepatocyte maturation medium (HMM) for another 9 days as previously described [22]. Hepatocyte-derived liver progenitor-like cells (HepLPCs) were obtained from purified human primary hepatocytes; the cells expressed important DMEs at levels comparable to those in human hepatocytes. Briefly, for rapid hepatic differentiation, HepLPCs were plated on the Matrigel-coated culture dish and cultured in transition and expansion medium (TEM) for 2–4 days until 90 % confluence. The medium was then changed to HMM for another 9 days for further maturation [23]. The medium was changed every 2–3 days. Human embryonic kidney (HEK) 293 T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM (Life Technologies) containing 10 % FBS. All cell lines were cultured at 37 °C in a humidified 5 % CO_2 atmosphere.

2.3. miRNA mimic library screening

HepaRG cells and HepLPCs were seeded in 96-well cell culture plates pre-coated with collagen. A total of 302 miRNA mimics (Ribobio, Guangzhou, China) that are conserved between humans and mice were used for transfection at a final concentration of 50 nM. At 48 h post-transfection, 100 nM tacrolimus was added to the culture medium. At 12 h later, that is 60 h after plating, the culture medium was harvested to measure the tacrolimus concentration.

2.4. Luciferase reporter gene assays

The pmiRGLO constructs were designed for pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) according to the manufacturer's recommendations and the wild-type and mutant DNA fragments of 3′-UTR of HNF4α, NR1I3, NR1I2, and CYP3A5 containing the predicted sequence of miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26-5p interaction were cloned into the pmirGLO vector.

293 T cells were seeded into 96-multiwell plates and cotransfected with 50 ng luciferase reporter vector and 50 nM mimic or miRNA negative control using the Lipofectamine 3000 reagent (Invitrogen). After 48 h of transfection, luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI). Renilla luciferase activities were standardized to those of firefly luciferase.

2.5. Transfection assays and chemical treatments

The miRNA mimics (50 nM) and inhibitors (100 nM), and negative control miRNA(50 nM) were obtained from RiboBio (Guangodong, China). HNF4α, NR1I3, NR1I2, and CYP3A5 siRNAs were designed and synthesized by GenePharma (Shanghai, China). All of them were then transiently transfected into cells using Lipofectamine 3000 reagent (Invitrogen).

Tacrolimus was obtained from MedChemexpress (Monmouth Junction, USA) and dissolved in dimethyl sulfoxide (DMSO). HepaRG cells

Table 2
Sequences of primers and oligonucleotides.

Name	Sequence
hsa-miR-29a-3p forward	TAGCACCATCTGAAATCGGTTA
hsa-miR-99a-5p forward	AACCCGTAGATCCGATCTTGTG
hsa-miR-532-5p forward	CATGCCTTGAGTGTAGGACTGT
hsa-miR-26b-5p forward	TTCAAGTAATTCAGGATAGGT
U6 forward	GGAACGATACAGAGAAGATTAGC
U6 reverse	TGGAACGCTTCACGAATTTGCG
HNF4α forward	GTACTCCTGCAGATTTAGCC
HNF4α reverse	CTGTCCTCATAGCTTGACCT
NR1I2 forward	CACCAGGACTCACCACTTCAA
NR1I2 reverse	TTTGCTTCTGGGCCTCTTGG
NR1I3 forward	GGGAGCAGCTGTGGAAATCT
NR1I3 reverse	CACACGGGCTCCATCTTCAA
CYP3A5 forward	TTGTTGGGAAATGTTTTGTCCTATC
CYP3A5 reverse	ACAGGGAGTTGACCTTCATACGTT
GAPDH forward	GAAATCCCATCACCATCTTCCAGG
GAPDH reverse	GAGCCCCAGCCTTCTCCATG
HNF4α siRNA	GGGAACCAACGUCAUCGUU
NR1I2 siRNA	AAACCUUUGACACUACCUU
NR1I3 siRNA	GCAACUGAGUAAGGAGCAA
CYP3A5 siRNA	CCAGCAGUGUUCUUUCCUU
siRNA negative control	UUCUCCGAACGUGUCACGU

and HepLPCs were treated with 100 nM tacrolimus after transfection for 48 h. Then, the culture supernatants were harvested 12 h after tacrolimus treatment. Each assay was performed at least three times.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The supernatants of HepaRG cells and HepLPCs were collected to determine the concentration of tacrolimus using ELISA kits (Guduo Biotech CO., Ltd., Shanghai, China) according to the manufacturer's instructions.

2.7. RNA extraction, RT-qPCR, and genotyping

Total RNA was extracted with TRIzol reagent (Takara Corp., Tokyo, Japan) and performed with miRNA First Strand Synthesis Kits or Primescript RT Master Mix (Takara) to generate cDNA. Then, qPCR was performed on a CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA) using TB Green Premix Ex Taq (Takara) following the manufacturer's instructions. The housekeeping genes GAPDH and U6 were used as the internal reference for the normalization of mRNA and miRNA expression. The primer sequences (Sangon Biotech Co., Ltd., Shanghai, P.R. China) are listed in Table 2.

All donors were genotyped for CYP3A5 rs776746. Briefly, the genomic DNA extracted from blood samples was amplified in an ABI 7900 system (Applied Biosystems, Foster City, CA) using Taq polymerase qPCR kit (Takara). The products were purified with a QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and run on an ABI 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations.

2.8. Western blot analysis

HepaRG cells and HepLPCs were seeded in a 6-well plate. Cells were harvested after 48 h of transfection and lysed in RIPA lysis buffer (Beyotime, Shanghai, China) on ice. Antibodies against HNF4α, CYP3A5, and GAPDH were obtained from Abcam (Cambridge, MA). NR1I3 and NR1I2 antibodies were purchased from ABclonal (Woburn, MA). Quantitative analyses for Western blotting were performed using the ChemiDocTM XRS + System with Image LabTM Software (Bio-Rad).

2.9. Statistical analyses

Pearson correlation analysis was used to calculate the correlation between tacrolimus C/D ratio and the levels of CYP3A5 and miRNAs using SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Two-tailed Student's t-tests were used to compare two groups of data. $P < 0.05$ was considered statistically significant.

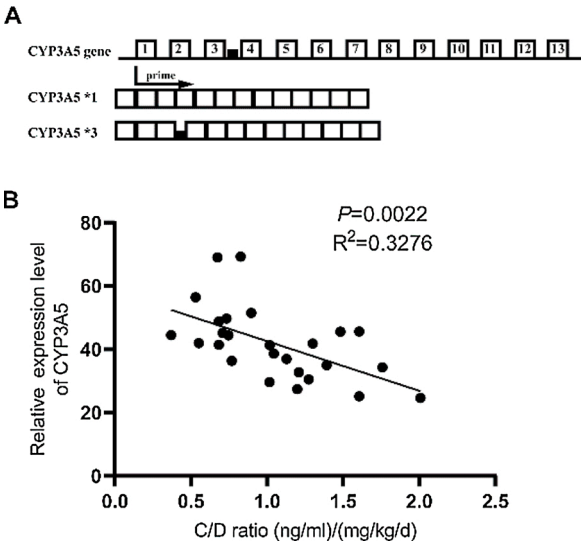

Fig. 1. Comparison of CYP3A5 expression in *1*3 donor graft. (A) Schematic drawing of the cDNA structures of various CYP3A5 alleles. Exons are numbered and boxed, and prime (exon 2-4) is indicated. Intron is indicated as filled box. (B) Pearson correlation analysis of CYP3A5 expression in *1*3 donor graft and Tacrolimus C/D following pediatric liver transplantation.

3. Results

3.1. The expression of CYP3A5 in *1*3 donor graft negatively correlated with tacrolimus C/D

To investigate the potential role of basal CYP3A5 expression in a *1*3* donor graft in tacrolimus metabolism following pediatric liver transplantation, the level of CYP3A5 in donor grafts was analyzed by PCR. The amplicon generated in the CYP3A5*1* assay contained portions of exons 2–4, excluding intron 3 (Fig. 1A). The data showed that the expression of CYP3A5 in donor grafts was negatively correlated with tacrolimus C/D (Fig. 1B). Consequently, for the heterozygote genotypes, the basal expression level of CYP3A5 in the donor graft played a leading role in tacrolimus metabolism. We hypothesized that some potential epigenetic factors such as miRNA could affect CYP3A5 expression and contributed to the tacrolimus variability.

3.2. Screening for miRNAs regulating tacrolimus metabolism

We screened for miRNAs capable of regulating tacrolimus metabolism and identified in a mimic library 302 miRNAs that were completely conserved between humans and mice. We used HepaRG cells and HepLPCs expressing key drug-metabolizing enzymes and transporters (DMETs) highly similar to those in primary hepatocytes. We transfected HepaRG cells and HepLPCs with each miRNA, added 100 nM tacrolimus into the supernatants (Fig. 2A), and measured the concentration of tacrolimus after 12 h (Fig. 2B and C). The miRNAs were selected based on the following two criteria: (a) a miRNA should inhibit tacrolimus metabolism by more than 20 % in two cell lines and (b) the miRNA should have the most abundant expression in the normal human liver [24]. Based on the screening results, we selected 4 miRNAs: miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p (Fig. 2D). We then validated the four miRNAs in three independent experiments that confirmed the increase in tacrolimus concentration by all four miRNA mimics (Fig. 2E). Therefore, based on our screening, we selected

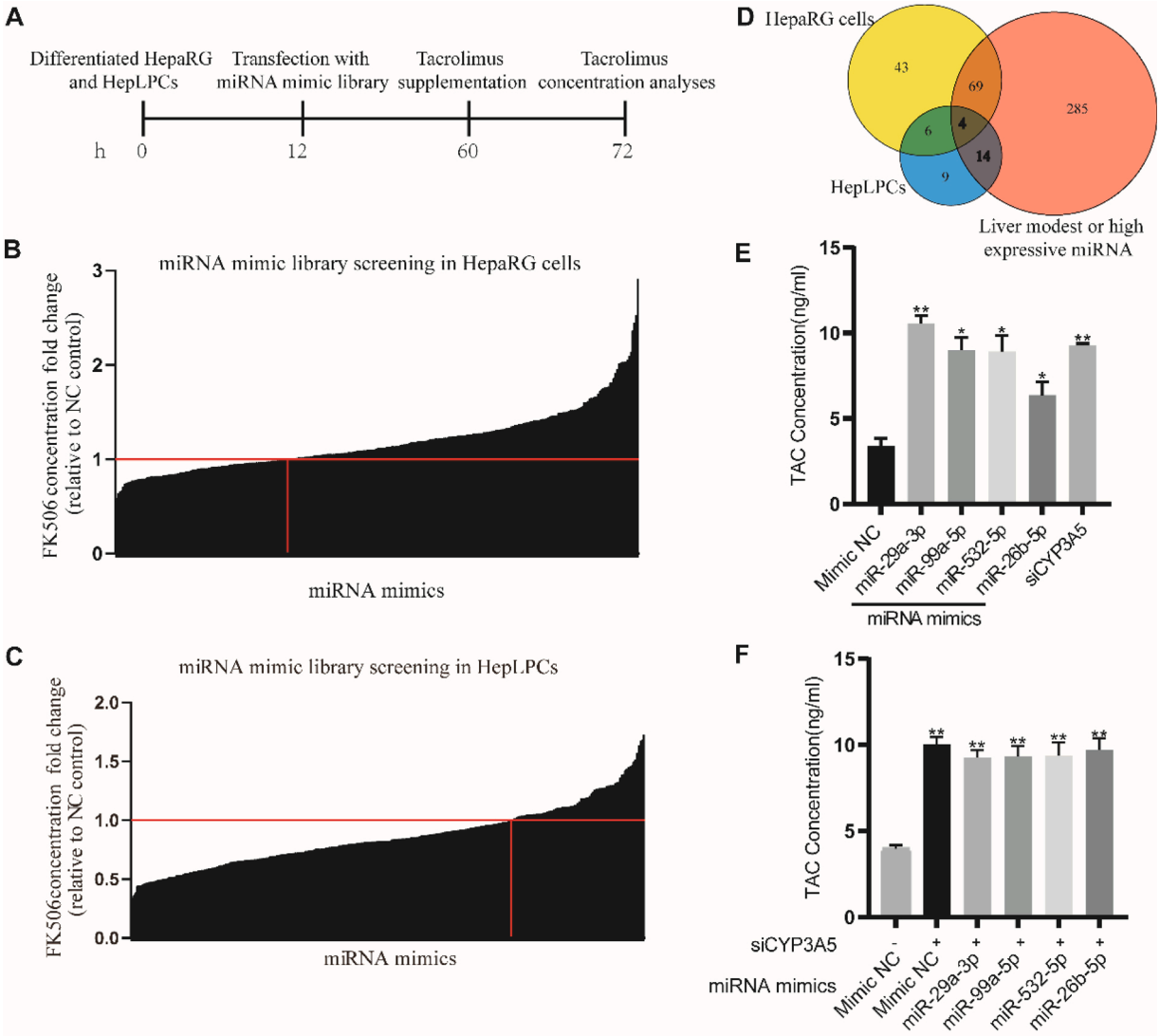

Fig. 2. Functional screening identifies miRNAs regulating tacrolimus metabolism in HepaRG cells and HepLPCs. (A) Schematic of miRNA screening in tacrolimus metabolism in HepaRG cells and HepLPCs. Tacrolimus concentration is shown as fold change of each miRNA transfection to scramble control transfection in HepaRG cells (B) and HepLPCs (C). (D) Venn diagram exhibiting miRNAs that inhibited the tacrolimus metabolism by more than 20 % in two cell lines and had most abundant expression in normal human liver. (E) ELISA analysis of tacrolimus concentration after transfection with these four miRNA mimics. (n = 3). (F) The effect of four miRNA mimics on tacrolimus concentration after transfection with CYP3A5 siRNA. Data are presented as the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with miRNA negative control.

miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p for further studies.

3.3. The candidate miRNAs regulate tacrolimus metabolism through inhibiting CYP3A5 expression

CYP3A5 is the most effective drug-metabolizing enzyme of tacrolimus. Hence, to test whether the four miRNAs inhibited tacrolimus metabolism dependent on CYP3A5, we transfected HepaRG cells with CYP3A5 siRNA, then transfected them further with four miRNA mimics 48 h later, and then added 100 nM tacrolimus into the supernatant. We found that all four miRNA mimics had no impact on tacrolimus metabolism after CYP3A5 siRNA transfection (Fig. 2F).

To further confirm our findings, we detected the effects of miRNA mimics on the expression of CYP3A5 in HepaRG cells and HepLPCs. Transfection efficiencies for the mimics and inhibitors were confirmed by measuring miRNA expression levels (Fig. 3A). Overexpression of miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26-5p reduced the mRNA levels of CYP3A5 in HepaRG cells and HepLPCs compared with the negative control (Fig. 3B and C). In parallel with the observed changes in mRNA levels, the protein expression of CYP3A5 was also reduced by increasing the expression of these four miRNAs (Fig. 3D and E). Collectively, these results indicated that all four miRNAs could regulate CYP3A5 expression and thus affect tacrolimus metabolism.

3.4. miRNAs effect depends on multiple targets

To identify the relevant targets of selected miRNAs, we employed a two-step procedure to identify the potential target genes. First, we performed in *silico* analyses to identify the direct binding sites of CYP3A5 using the TargetScan algorithm. We found that the 3′ untranslated region (3′UTR) of CYP3A5 contained a binding site for miR-26b-5p. Second, TargetScan analysis suggested that HNF4α, NR1I2, and NR1I3 were potential targets of miR-29a-3p, miR-532-5p, and miR-99a-5p, respectively. All candidate genes have been demonstrated to regulate the expression of CYP3A5.

To confirm the direct interaction between miRNAs and target genes, we performed a dual luciferase reporter assay. Plasmids containing the

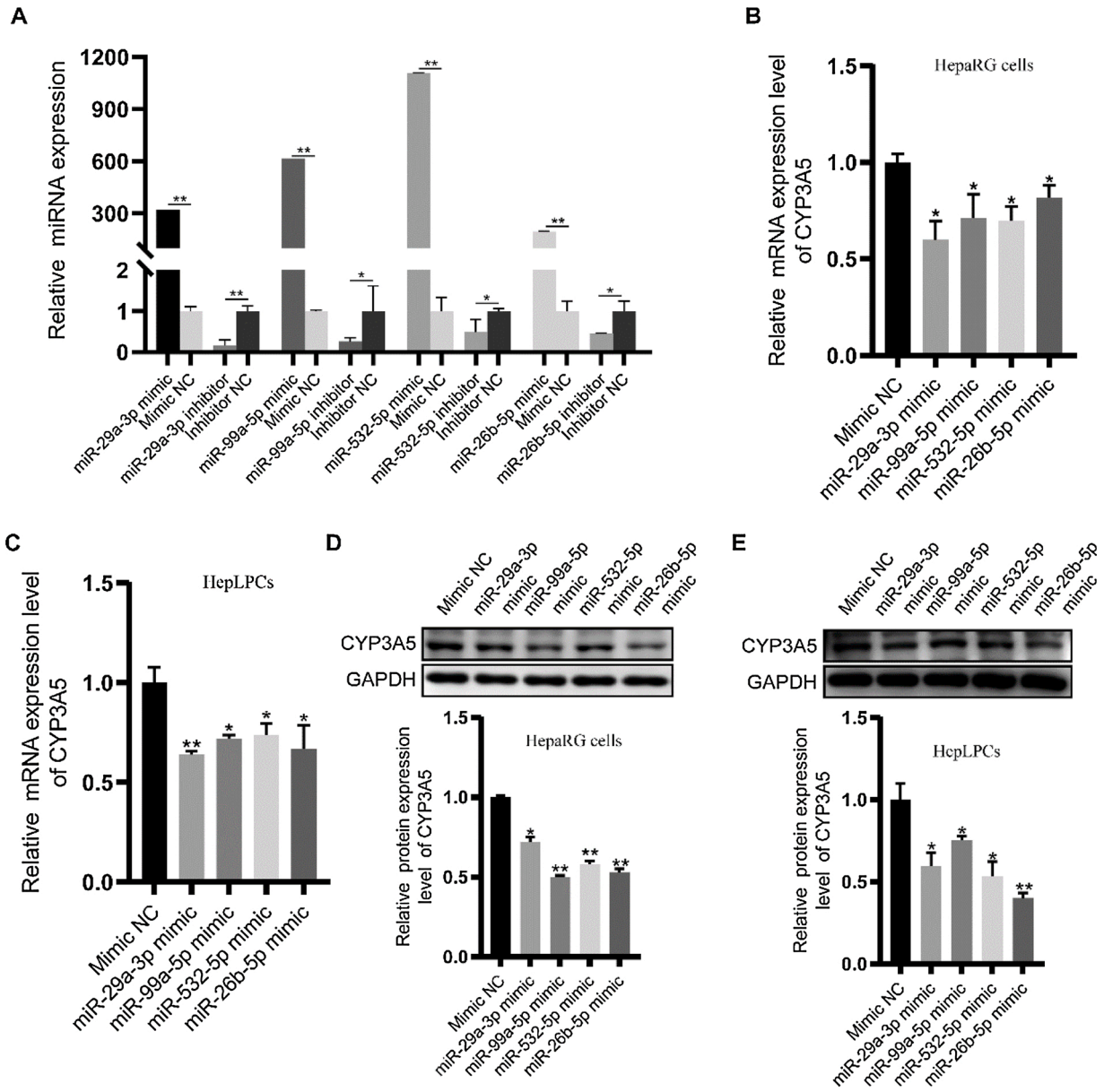

Fig. 3. miRNAs inhibit CYP3A5 expression. (A) The levels of miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p in HepaRG cells after transfection with miRNA mimics and inhibitors. (B and C) The expression levels of CYP3A5 in HepaRG cells and HepLPCs after transfection with four miRNA mimics. (D and E) The protein levels of CYP3A5 in HepaRG cells and HepLPCs after transfection with four miRNA mimics. Data are presented as the mean ± S.D. $^{*}P<0.05$, $^{**}P<0.01$ compared with miRNA negative control.

wild-type and mutated 3′UTR of HNF4α, NR1I2, NR1I3, and CYP3A5 were cotransfected with the corresponding miRNA mimics into HEK 293 T cells. Luciferase analysis showed that miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p could repress the reporter activities of pGLO-HNF4α-WT, pGLO-NR1I3-WT, pGLO-NR1I2-WT, and pGLO-CYP3A5-WT plasmids but did not affect the reporter activities of the corresponding mutant plasmids (Fig. 4A and B).

To elucidate the regulatory role of miRNAs, we examined the effects of all four mimics and inhibitors on the expression of corresponding target genes in HepaRG cells and HepLPCs. As shown in Fig. 4C - J, overexpression of miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p reduced the mRNA and protein levels of HNF4α, NR1I3, NR1I2, and CYP3A5 in HepaRG cells compared with the negative control, whereas miRNA inhibitors generated opposite effects that elevated the mRNA and protein expression of target genes. The regulatory effects of miRNAs on the endogenous expression of target genes were also confirmed in HepLPCs (Supplementary Fig. S1).

To determine whether CYP3A5 was required for miRNAs to trigger tacrolimus metabolism, we first transfected HepaRG cells with HNF4α siRNA (siHNF4α), siNR1I3, siNR1I2, and siCYP3A5. Compared with the scramble control, siRNA-mediated knockdown led to a significant reduction in the protein levels of these genes (Fig. 5A). Then, the HepaRG cells and HepLPCs were transfected with scramble control, miRNA inhibitor, siRNA, or a combination of siRNA and miRNA inhibitor. As shown in Fig. 5B - I, miRNA knockdown increased mRNA and protein levels of CYP3A5. Compared to the control HepaRG cells, knockdown of target genes reduced the mRNA and protein levels of CYP3A5. Compared to HepaRG cells treated with miRNA inhibitors, additional treatment with siRNAs rescued the mRNA and protein levels of CYP3A5 induced by miRNA inhibitors. Similar results were also observed in HepLPCs (Supplementary Fig. S2). These results indicated that all four miRNAs suppressed the expression of CYP3A5 by targeting the corresponding target genes.

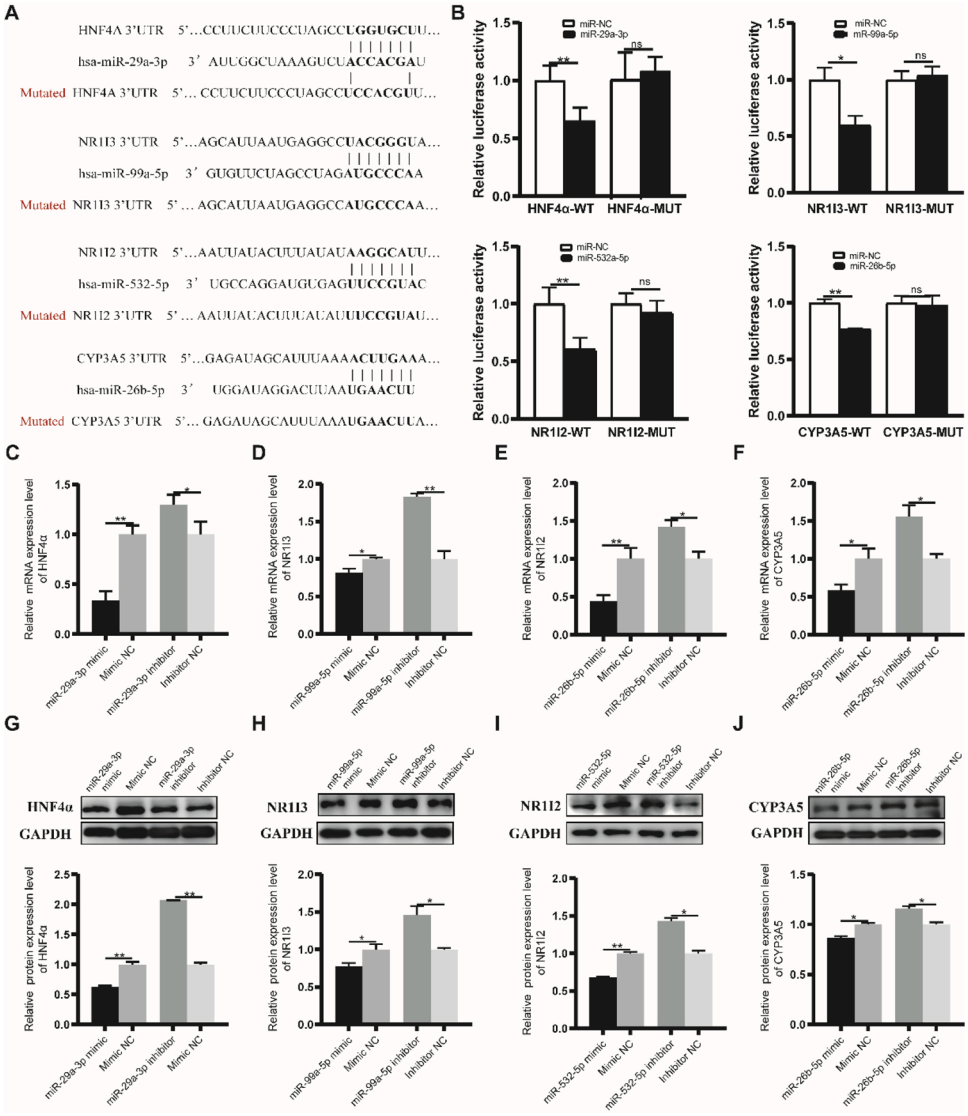

Fig. 4. miRNAs effect depends on multiple targets. (A) Construction diagram of the wild-type 3′-UTR (3′-UTR-WT) and mutated 3′-UTR (3′-UTR-MUT) of HNF4α, NR1I3, NR1I2, and CYP3A5 in dual luciferase reporter assays. (B) Luciferase reporter activity in HEK 293 T cells cotransfected with target genes 3′-UTR-WT or 3′-UTR-MUT constructs with corresponding miRNA mimics. (C-F) The expression of corresponding target genes in HepaRG cells after transfection with these four miRNA mimics and inhibitors. (G-H) The protein levels of corresponding target genes in HepaRG cells after transfection with these four miRNA mimics and inhibitors. Data are presented as the mean ± S. D. $^{*}P<0.05$, $^{**}P<0.01$ compared with miRNA negative control.

3.5. miRNAs attenuate the effects of target genes on tacrolimus metabolism

Based on the regulatory role of miRNAs, we next explored whether the miRNAs regulated the tacrolimus concentration *via* target genes in HepaRG cells and HepLPCs. To confirm our hypothesis, we compared HepaRG cells transfected with scramble control with those treated with miRNA inhibitor, siRNA, or a combination of miRNA inhibitor and siRNA, and then stimulated them with tacrolimus (100 nM). As shown in Fig. 6A, suppression of miRNAs led to reduced concentration of tacrolimus; compared to the scramble control, these siRNAs significantly increased the concentration of tacrolimus, whereas additional treatment with miRNA inhibitors offset the siRNA-induced upregulation of tacrolimus concentration. The functional regulatory roles of miRNAs and target genes in tacrolimus metabolism were also confirmed in HepLPCs (Fig. 6B). Based on these findings, we concluded that miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p suppressed tacrolimus concentration by targeting corresponding target genes in HepaRG cells and HepLPCs.

3.6. miRNAs validation in the donor graft supports an association between miRNAs and tacrolimus metabolism

To confirm and strengthen the association with tacrolimus metabolism of the miRNAs validated in the donor graft, we analyzed it by real-time PCR analyses for miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p. As shown in Fig.7A - D, based on Pearson's correlation analysis, the expression of miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p in the donor graft was negatively correlated with tacrolimus C/D and agreed with the *in vitro* results. Also, we tested the expression of corresponding target genes and found that target gene expression was significantly and negatively correlated with the expression of miRNAs (Fig. 7E - H). Collectively, these data strongly support the association of miRNAs in donor grafts with tacrolimus metabolism following pediatric liver transplantation.

4. Discussion

Tacrolimus-based immunosuppressive regimens are widely used after liver transplantation and are characterized by high inter-individual variability in its pharmacokinetics. Many studies have demonstrated that CYP3A5 polymorphisms contribute to the inter-individual variability in tacrolimus pharmacokinetics by causing alternative splicing

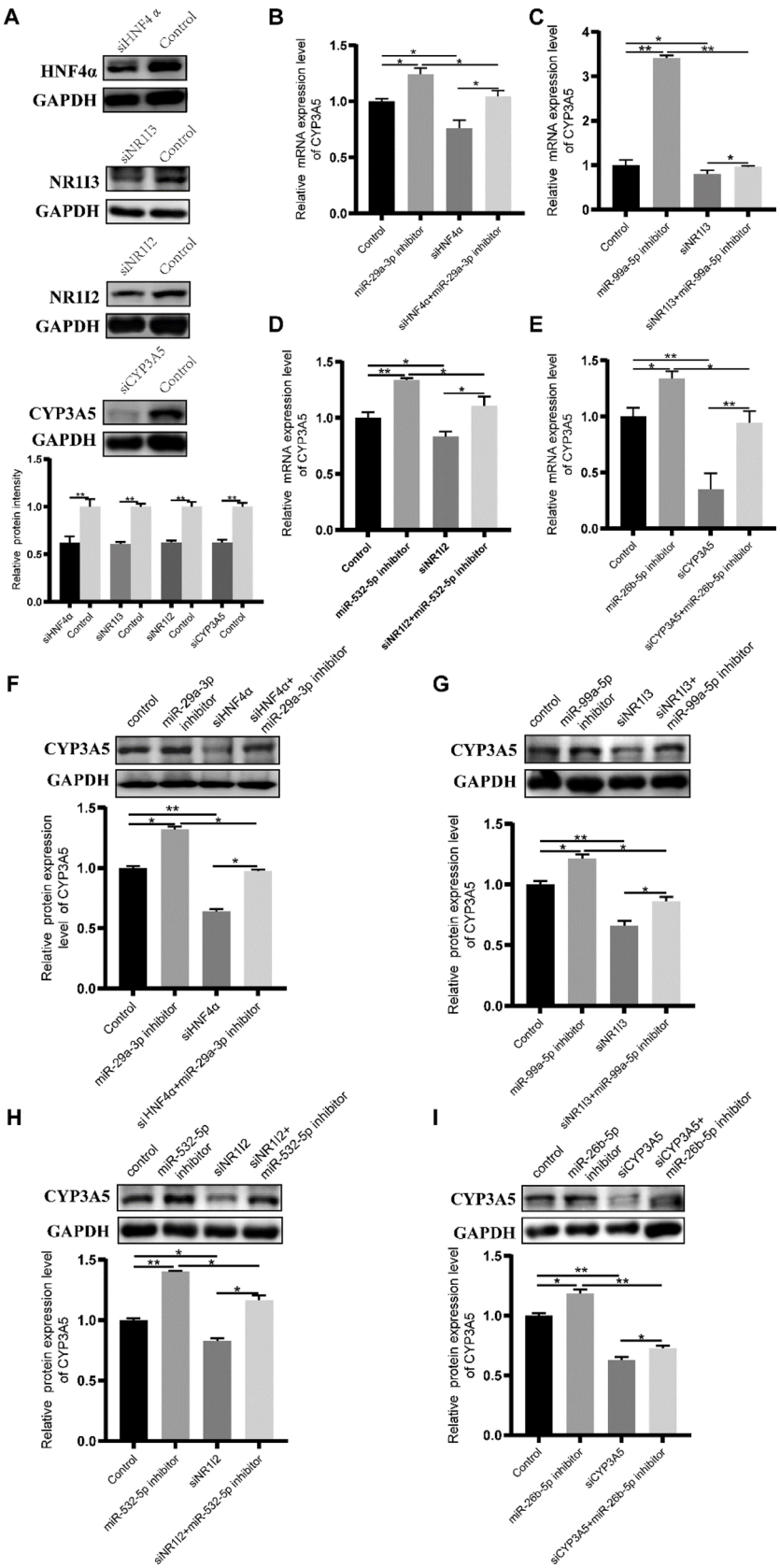

Fig. 5. miRNAs regulate CYP3A5 expression *via* target genes in HepaRG cells. (A) The protein levels of HNF4α, NR1I3, NR1I2 and CYP3A5 compared with siRNA negative control after transfection with these siRNAs. (B-E) The mRNA expression of CYP3A5 after transfection with miRNA inhibitors, siRNAs or a combination of miRNA inhibitor and siRNA in HepaRG cells. A: miR-29a-3p and siHNF4α transfection. B: miR-99a-5p and siNR1I3 transfection. C: miR-532-5p and siNR1I2 transfection. D: miR-26b-5p and siCYP3A5 transfection. (F-I) The protein levels of CY3PA5 after transfection with miRNA inhibitors, siRNAs or a combination of miRNA inhibitor and siRNA in HepaRG cells. Data are presented as the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$.

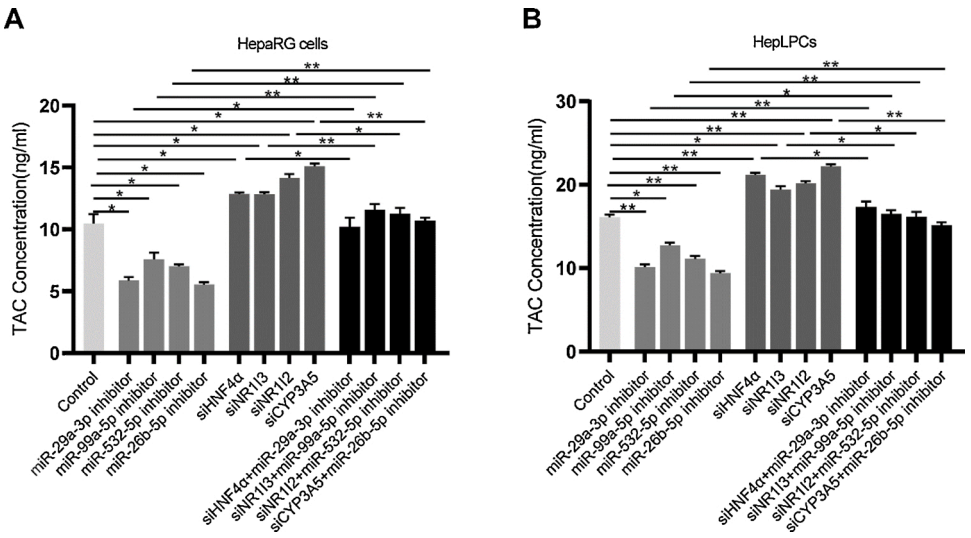

Fig. 6. miRNAs regulate tacrolimus concentration through target genes. (A and B) ELISA analysis of tacrolimus concentration after transfection with miRNA inhibitors, siRNAs or a combination of miRNA inhibitor and siRNA in HepaRG cells and HepLPCs. Data are presented as the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$.

and protein truncation of CYP3A5. CYP3A5 represented at least 50 % of the total hepatic CYP3A content in people polymorphically expressing CYP3A5 [3]. Some studies have reported that other DMETs such as CYP3A4, ABCB1, POR, and SUMO4 also had an impact on tacrolimus metabolism [25–27]. However, other studies showed that CYP3A5 dominated CYP3A4 in the metabolism of tacrolimus in individuals expressing the CYP3A5 enzyme [28–31]. The association of POR and ABCB polymorphism with the tacrolimus dose requirement was also found only in CYP3A5-expressing individuals [32]. The donor SUMO4 genetic variant was associated with a high tacrolimus C/D ratio that was related to the downregulation of the CYP3A5 enzyme [27]. Thus, CYP3A5 is the uppermost drug-metabolizing enzyme involved in tacrolimus metabolism. We found that children with CYP3A5 **1* exhibited different tacrolimus metabolism following pediatric liver transplantation under the same genetic conditions; the basal expression of CYP3A5 in **1*3* donor graft was negatively correlated with tacrolimus C/D. Thus, we thought that tacrolimus variability was not entirely attributed to CYP3A5 gene polymorphism and that CYP3A5 basal expression in donor grafts also played a significant role in tacrolimus metabolism. Moreover, it has been demonstrated that some genes and miRNAs in donor grafts could contribute to the development of metabolic disorders [18,33]. Due to the immaturity of DMEs in children [34, 35], the P450 enzymes in the donor graft play a crucial role in tacrolimus metabolism. Thus, some potential epigenetic factors such as miRNAs that regulate the expression of CY3PA5 in donor grafts could contribute to the variability.

To the best of our knowledge, this is the first demonstration that tacrolimus metabolism following pediatric liver transplantation can be regulated by miRNAs in donor grafts. High-content screening is a powerful tool for screening candidate miRNAs on a large scale [36]. First, we used HepaRG cells and HepLPCs to identify the miRNAs involved in tacrolimus metabolism selected from a large-scale miRNA library. It has been confirmed that the expression of liver function-related genes in HepLPCs was much closer to those in primary human hepatocytes and HepaRG cells, and HepLPCs were a powerful tool for accurate and efficient drug development [37]. As shown in Fig. 2, four miRNAs out of 302 miRNAs suppressed tacrolimus metabolism. Among the four miRNAs, miR-29a-3p has been demonstrated to modulate CYP2C19, ALDH5A1, and SLC22A7 in human liver cells [38, 39]. Meanwhile, the other three miRNAs have not been reported to affect DMETs. We examined whether these miRNAs inhibited tacrolimus metabolism by suppressing CYP3A5 and found that miRNAs did not change the concentration of tacrolimus after HepaRG and HepLPCs were transfected with siCYP3A5. Overexpression of miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26-5p reduced the mRNA and protein levels of CYP3A5. Thus, we identified that these miRNAs regulated tacrolimus metabolism dependent on CYP3A5.

Our present data further demonstrated that miR-26b-5p could plausibly regulate CYP3A5 activity through direct interaction with the transcript, while miR-29a-5p, miR-99a-5p, and miR-532-5p also exerted effect by regulating upstream regulators of CYP3A5 such as HNF4α, NR1I3, and NR1I2 as shown in previous studies. As demonstrated in Fig. 6, reduced levels of miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p caused tacrolimus concentration downregulation *in vitro* that could be reversed by blocking corresponding target genes using siRNA. Thus, these miRNAs could act as inhibitors of tacrolimus metabolism by inhibiting CYP3A5 directly and indirectly. To validate the association with tacrolimus metabolism of the miRNAs in the donor graft, we showed that the expression of miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p in donor graft was negatively correlated with the expression of target genes and tacrolimus C/D following pediatric liver transplantation, agreeing with the results obtained *in vitro*. Collectively, we could explain a portion of the aberrant variables of tacrolimus pharmacokinetics by testing these miRNAs in donor grafts and exclude the impact of the CYP3A5 genotype, and targeted CYP3A5 expression levels in donor grafts might be a novel way, which adjusted the tacrolimus metabolism to meet clinical needs.

We acknowledge that this study has some limitations. First, the study included a small sample size. Because the frequency of CYP3A5 **1* alleles in Chinese was only 0.35 [3], a limited number of cases with CYP3A5 **1*3* genotype were available for the study. The results need to be validated in large cohorts. Second, our results were only relevant to young children. Due to the maturation of DMEs in adults, CYP3A5 in the intestine also plays a key role in tacrolimus metabolism. Additionally, some studies implied that intestinal CYP3A5 genotype contributes more to interindividual variability than the genotype of the graft live [40,41]. Third, we selected the miRNA mimic library that included the maximum number of completely conserved miRNAs between humans and mice.

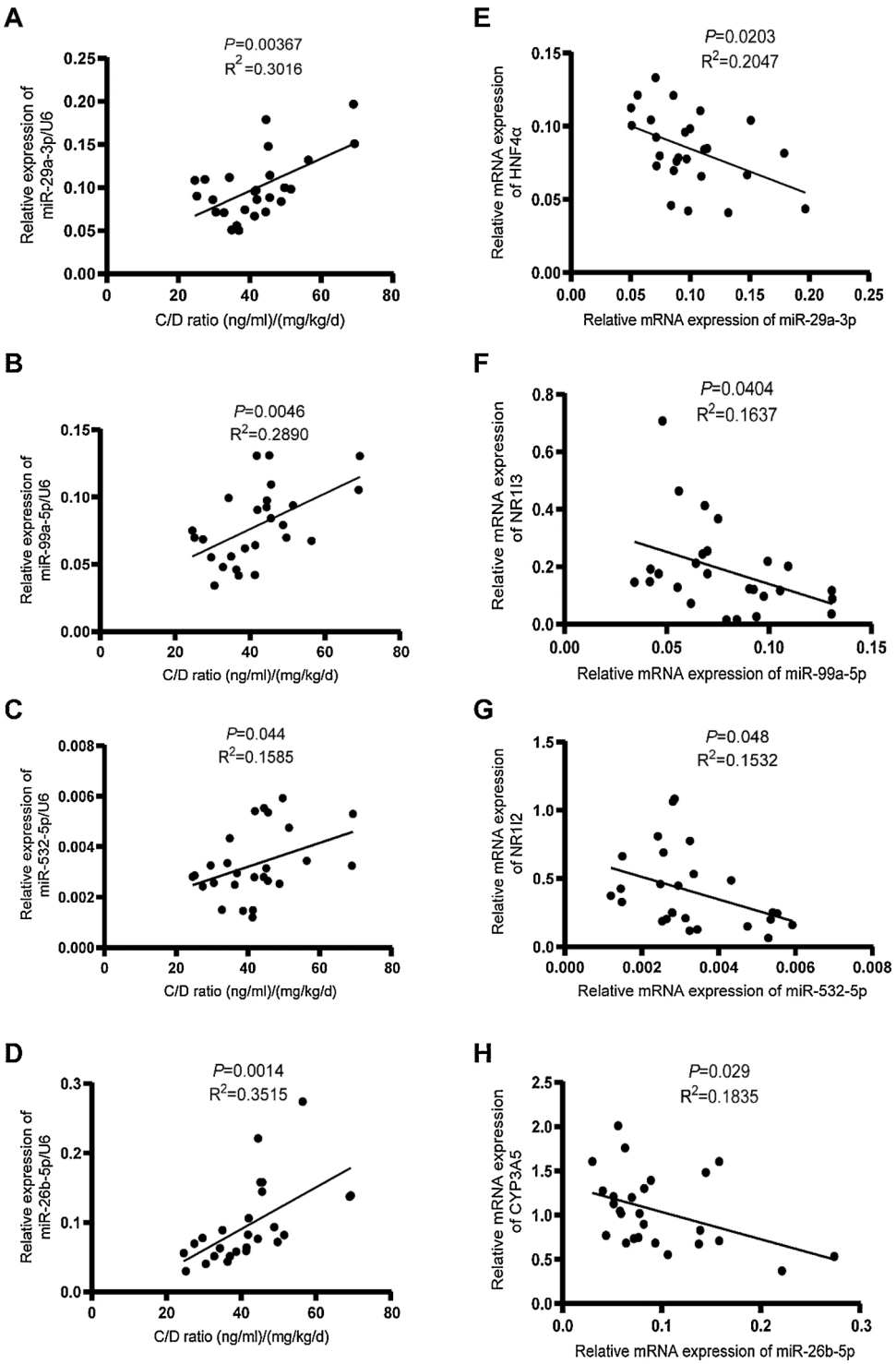

Fig. 7. Expression of miRNAs and target genes in *1*3 donor graft. (A-D) The relationship between miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p in donor graft with tacrolimus C/D following pediatric liver transplantation. (E-H) The relationship between miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p with HNF4α, NR1I3, NR1I2, and CYP3A5 respectively in donor graft.

Thus, the construction of humanized mice was performed in the following study to further verify the effects of these miRNAs.

In summary, based on the miRNA screening results, we identified miR-29a-3p, miR-99a-5p, miR-532-5p, and miR-26b-5p as novel regulators of tacrolimus metabolism by inhibiting CYP3A5 directly and indirectly. The detailed mechanism is shown in Fig. 8. Our findings indicate that miRNA-targeted therapy in donor grafts may be a novel and promising strategy for the regulation of tacrolimus metabolism to meet clinical needs.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

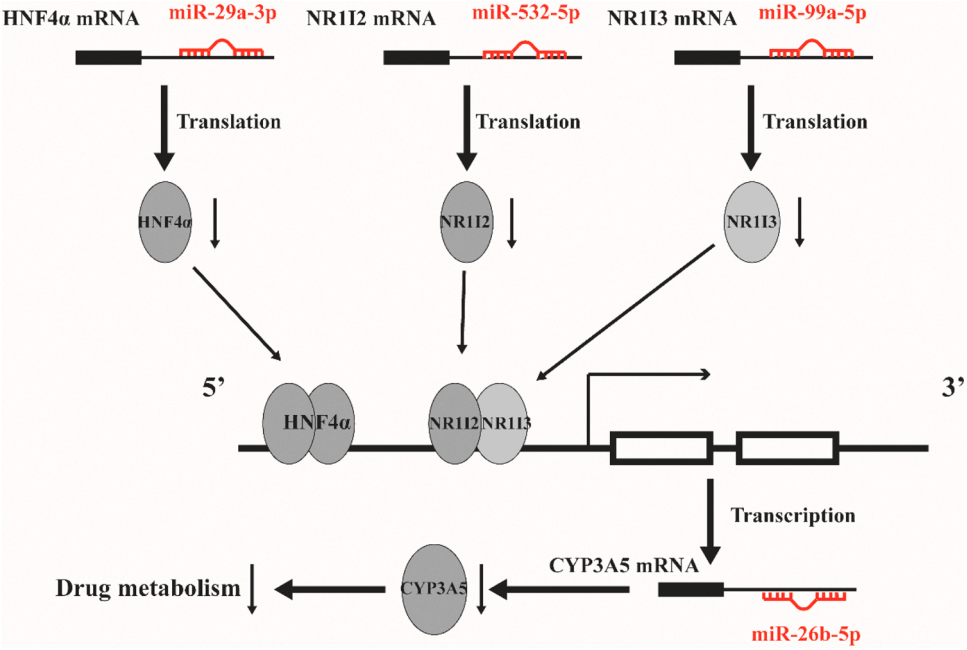

Fig. 8. Schematic of how miRNAs regulate CYP3A5-mediated tacrolimus metabolism. Overexpression of miRNA-29a-3p, miR-99a-5p, and miR-532-5p can inhibit HNF4α, NR1I3, and NR1I2 expression respectively, in turn suppressing downstream expression of CYP3A5. While miR-2b-5p directly targets CYP3A5. All of them eventually lead to inhibition of tacrolimus metabolism.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81670602, 81871287).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2020.105382.

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