DNA methylation modulates allograft survival and acute rejection after renal transplantation by regulating the mTOR pathway

Chaohong Zhu1,2,3,4,5 | Wenyu Xiang1,2,3,4,5 | Bingjie Li1,2,3,4,5 | Yucheng Wang1,2,3,4,5 | Shi Feng1,2,3,4,5 | Cuili Wang1,2,3,4,5 | Ying Chen1,2,3,4,5 | Wenhong Xie1,2,3,4,5 | Lihui Qu1,2,3,4,5 | Hongfeng Huang1,2,3,4,5 | Francesco Annunziata6 | Suneetha Nunna6 | Anna Krepelova6 | Seyed Mohammad M. Rasa6 | Francesco Neri6 | Jianhua Chen1,2,3,4,5 | Hong Jiang1,2,3,4,5

1Kidney Disease Center, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, China
2Key Laboratory of Kidney Disease Prevention and Control Technology, Hangzhou, Zhejiang, China
3National Key Clinical Department of Kidney Diseases, Hangzhou, Zhejiang, China
4Institute of Nephrology, Zhejiang University, Hangzhou, Zhejiang, China
5The Third Grade Laboratory Under the National State, Administration of Traditional Chinese Medicine, Hangzhou, Zhejiang, China
6Leibniz Institute on Aging - Fritz Lipmann Institute (FLI), Jena, Thuringia, Germany

Correspondence
Hong Jiang
Email: jianghong961106@zju.edu.cn
Jianghua Chen
Email: zjkidney@zju.edu.cn

Acute rejection (AR) can lead to allograft dysfunction following renal transplantation, despite immunosuppressive treatments. Accumulating evidence points out a role for epigenetic modification in immune responses. However, the mechanism and contribution of DNA methylation in allograft survival remain unclear. In this study, we followed up patients who successively experienced end-stage renal disease, renal transplantation with allograft function or dysfunction, and hemodialysis. Peripheral blood mononuclear cells were collected at different time points for analysis of the DNA methylation. Epigenetic modifier analysis was also performed to explore its effect of methylation in a mouse model of AR. Compared with the allograft-stable cohort, patients who experienced AR-induced allograft dysfunction demonstrated more changes in methylation patterns. Pathway analysis revealed that the hypermethylated areas in the allograft dysfunction group were associated with genes related to the mechanism of target of rapamycin (mTOR) signaling pathway. Moreover, in the mouse AR model, treatment with the DNA methyltransferase inhibitor—decitabine regulated the Th1/2/17/regulatory T cell (Treg cell) immune response via its demethylating role in the suppressing the activity of the mTOR pathway, which ultimately ameliorated...
renal allograft-related inflammatory injuries. These results revealed that changes in methylation accompany AR-induced allograft dysfunction after renal transplantation. Epigenetics may provide new insights into predicting and improving allograft survival.

**KEYWORDS**

basic (laboratory) research/science, clinical research/practice, genetics, graft survival, immunosuppressant – mechanistic target of rapamycin (mTOR), immunosuppression/immune modulation, kidney (allograft) function/dysfunction, kidney transplantation/nephrology, microarray array, rejection: acute

## INTRODUCTION

Renal transplantation (RT) is a highly effective and widely used treatment for end-stage renal disease (ESRD) that, compared to dialysis, provides patients with a better quality of life and significantly longer survival time (5-year adjusted survival for patients on dialysis vs transplantation: ~45% and ~92%, respectively).\(^1\)\(^-\)\(^3\) Moreover, advances in immunosuppressive therapeutics have led to a substantial decline to <15% in the incidence of acute rejection (AR) within 1 year after RT.\(^4\) However, AR events have reportedly increased in severity and thus continue to present a substantial risk of chronic allograft nephropathy and an obstacle for graft survival, as AR reduces overall graft survival by up to 24%.\(^4\)\(^-\)\(^7\) Consequently, gaining an improved underlying AR can potentially minimize the risk and further improve the rate of overall long-term graft survival. Despite extensive effort, traditional genetic and current immunological approaches cannot thoroughly explain these anomalous findings, strongly suggesting the involvement of other biological mechanisms.

To address this issue, recent research initiatives have focused on integrative analyses of clinical/demographic data and bioinformatics data using network-based approaches, leading to the identification of latent mechanisms underlying disease-related processes.\(^8\)\(^-\)\(^10\) Epigenetic modifications have aroused considerable attention in studies on several physiological and pathological processes owing to their intrinsic roles in normal cell development and function without altering the DNA sequence.\(^11\)\(^-\)\(^14\) DNA methylation entails the addition of methyl groups to the 5’ position of cytosines through the activity of DNA methyltransferases (DNMTs), typically in the context of CpG-dinucleotide sites that are critical for gene transcriptional regulation.\(^15\)\(^,\)\(^16\) Under specific conditions, methylation can be reprogrammed in cells, resulting in long-lasting effects even after the stimuli are removed.\(^17\)\(^,\)\(^18\) Thereby, methylation has a dynamic capacity for flexible and inducible regulation of gene expression and has thus been traditionally considered as an “epigenetic clock.”\(^16\)\(^,\)\(^19\)\(^-\)\(^22\) Recent studies have shown that DNA methylation influences the activation, proliferation, differentiation, and migration of a variety of cell types implicated in allograft survival.\(^23\)\(^,\)\(^24\) During CD4\(^+\) T cell differentiation, DNA methylation was found to fix tissue-specific transcriptional patterns, thereby maintaining the original fate of differentiation while preventing differentiation by future generations of that lineage.\(^14\) Similarly, DNMTs mediate the CpG methylation of specific regions that directly affect the differentiation of T helper (Th) 1/2 cells.\(^17\)\(^,\)\(^26\) Moreover, demethylation of the RAR-related orphan receptor C (RORC) locus was reported to influence the phenotype of regulatory T (Treg) cells.\(^27\) In contrast, demethylation events that enhance the expression of the forkhead box P3 (FOXP3) gene were associated with better allograft outcomes.\(^23\)\(^,\)\(^28\) Furthermore, aberrant methylation also indirectly resulted in inflammatory injuries in diabetic kidneys by influencing the mechanistic target of rapamycin (mTOR) signaling pathway.\(^29\) Although this preliminary work has explored the effects of epigenetic modification on the immune response, the relationship between DNA methylation and AR after renal transplantation or AR-induced allograft dysfunction remains to be clarified.

Considering that epigenetics is an emerging field of research in kidney transplantation, we hypothesized that DNA methylation patterns would be modified after organ transplantation accompanied by abnormal expression of some specific genes, which would then activate the immune-associated signaling pathway, further influence the differentiation of T cells, and finally manipulate the fate of allografts. To test this hypothesis, we used a whole-genome bisulfite sequencing approach to compare the methylation patterns in transplant recipients with and without AR. We further conducted bioinformatics to examine the genes associated with hypermethylated regions and their functions. Moreover, we established a mouse model of AR that was treated with a DNA methylation inhibitor to examine the impact of methylation on allograft survival and tolerance.

## MATERIALS AND METHODS

### 2.1 Blood sample collection and processing

All blood samples were collected from the kidney transplant and hemodialysis wards of the Kidney Disease Center, First Affiliated Hospital, College of Medicine, Zhejiang University. Written and informed consent was provided by all patients who received the donor kidney and underwent the renal transplantation. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood and then preserved in cell culture freezing medium at ~80°C in the...
Renal biopsy samples from all consenting patients were also collected and stored in the biobank. The study was approved by the local research ethics committee.

2.2 | Experimental design

The study design is schematically described in Figure 1, and clinical characteristics of patients are summarized in Table S1. Group 1 consisted of a patient cohort with AR-induced allograft dysfunction, from whom blood was collected at the following time points: (a) during ESRD but before transplantation, (b) upon loss of function of the grafted kidneys, and (c) after patients had undergone an average of 3 years of hemodialysis following allograft dysfunction. Group 2 was composed of the graft-stable cohort, from whom blood was collected at the following time points: (a) during ESRD but before transplantation, (b) following transplantation when the grafted kidneys functioned normally, and (c) at a later stage during which the grafted kidneys continued to function normally. Group 3 included samples obtained from a cohort of 13 healthy individuals. All patients in Group 1 had suffered from AR (T cell–mediated rejection) prior to allograft failure. None of the patients in Group 2 had a history of AR. The details of the recipients’ diagnosis and therapy are provided in Supplemental Methods - Additional information about the recruited patients.

2.3 | Bisulfite-converted DNA preparation and methylation sequencing

Genomic DNA was extracted from PBMCs using an AxyPrep blood genomic DNA maxiprep kit (Axygen; Corning Inc, Tewksbury, MA). AxiPrep DNA was bisulfite-converted using an EpiTect bisulfite kit (Qiagen, Hilden, Germany) and then applied to microarray or bisulfite sequencing polymerase chain reaction (PCR) for analysis of DNA methylation. The details of sequencing procedures and data analysis are provided in Supplemental Methods - Microarray and bisulfite sequencing PCR (BSP) for DNA methylation analysis.

2.4 | Establishment of a mouse AR model of renal transplantation

Ectopic renal transplantation was performed according to previously reported microsurgical techniques.30 Kidneys from male BALB/c mice (H-2d; 8 weeks old) were transplanted into C57BL/6 mice (H-2b; 8 weeks old) mice. Half of the C57BL/6 recipients received decitabine (1.5 mg/kg per day)31 by intraperitoneal injection starting from the day after transplantation, whereas the others received stroke-physiological saline solution (SPSS; isometric). The animal experiments were performed in accordance with institutional guidelines approved by the Animal Use Committee of Zhejiang University. The grafted kidneys and spleens from recipient mice were procured on day 7 after transplantation, part of which were used for hematoxylin and eosin staining or periodic acid-Schiff staining for histopathologic assessment. The unstained sections of mouse grafted kidneys and human renal allograft biopsies were incubated with anti-CD3 antibody (Abcam, Cambridge, UK) and DNMT1 (Novus Biologicals, Littleton, CO) for immunological staining following standard protocols.

2.5 | Flow cytometry analysis

The grafted kidneys and spleens were digested and then filtered using a 40-μm cell strainer into single-cell suspensions. After enrichment, the immune cells were stimulated by Cell Stimulation Cocktail with protein transport inhibitors (eBioscience, San Diego, CA) for 6 hours in a CO2 incubator. In brief, 5 × 10^6 cells in 100 μL of staining...
buffer were incubated with CD3-PE-Cyanine7 and CD8-fluorescein isothiocyanate (FITC) antibodies, followed by fixation, permeabilization, and incubation with anti-interferon (IFN)-γ-allophycocyanin (APC), anti-interleukin (IL)-4-phycoerythrin (PE), anti-Foxp3-APC, and anti-IL-17α-PE antibodies (eBioscience). Finally, the stained cells were analyzed using a Cytoflex flow cytometer (Beckman Coulter, Brea, CA).

### 2.6 | Gene expression analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed to cDNA using a reverse transcription kit (TaKaRa, Mountain View, CA). Real-time PCR was performed using SYBR Green qPCR master mix (Vazyme Biotech, Nanjing, China) on a ViiA7 Real-Time PCR system (Applied Biosystems, Foster City, CA). Primer sequences for real-time PCR are provided in Table S7.

### 2.7 | Western blot

Proteins from the grafted kidneys were extracted and then used for separation and transfer. The membranes were blocked and then incubated with primary antibodies against T-bet, GATA3, Foxp3, RORγt (Abcam), DNMT1 (Novus Biologicals), p-Akt, p-mTOR, p-P70S6K, PTEN (Cell Signaling Technology, Danvers, MA), DDIT4, and GAPDH (Proteintech, Rosemont, IL). After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and subsequently washed again. Chemiluminescence signals were detected after exposure to Immobilon Western HRP substrate (EMD Millipore, Burlington, MA) using the ChemiDocTM XRS+System (Bio-Rad, Hercules, CA).

### 2.8 | Statistical analysis

The analytical details of methylation data are provided in the Supplemental Methods - Analysis of methylation data. Data are expressed as the mean ± SEM, unless otherwise indicated, and were analyzed with GraphPad Prism 8.0 (GraphPad Software, Inc, La Jolla, CA) using a t test, one-way analysis of variance followed by Tukey’s post hoc test, chi-square test, and Mann-Whitney U test as appropriate. A P < .05 was defined as significant.

### RESULTS

#### 3.1 | Significantly different genome-wide methylation patterns between PBMCs from patients with AR-induced allograft dysfunction group

To account for potential differences in genetic background among patients in this study, PBMC samples were collected at the time of transplant (day 0), corresponding to a mean of 27.2 ± 3.68 months before allograft dysfunction. The basal methylation levels observed at this time point were then used as a basis for comparison between time points and between groups. Comparison of Groups 1a and 1b revealed 432,695 CpG sites that passed quality filtering, with 27,772 different sites (false discovery rate < 0.05) corresponding to 11,039 genes, indicating substantial differences in methylation from pre-transplant until allograft dysfunction. In contrast, no differences in methylation were found in comparative analysis of Groups 2a and 2b (Figure S1E,F), suggesting that the differences in methylation between the Group 1a and 1b were related to the dysfunction of the renal transplant. When Group 1 patients had to receive the hemodialysis after allograft dysfunction, some specifically sensitive CpG sites would be altered. Finally, 10,648 target CpG sites were screened out by the intersections of Group 1a vs 1b and Group 1b vs 1c (Figure 2A).

Among these target CpG sites, 1683 CpG sites were hypermethylated and 8965 CpG sites were hypomethylated (Figure 2B). The total annotated CpG sites with significantly different methylation between groups were distributed across the genome, with most sites located on Chr1, Chr2, and Chr6 (Figure 2C and Table S2). To focus on the potential roles of methylation in gene regulation, we screened these target CpG sites resulting in the identification of sites within TSS1500 (16.61%), TSS200 (7.22%), 5′ untranslated regions (UTRs; 9.75%), the first exon of genes (1st Exon; 2.81%), within TSS1500 (16.61%), TSS200 (7.22%), 5′ untranslated regions (UTRs; 9.75%), the first exon of genes (1st Exon; 2.81%), the gene body (Body; 36.26%), 3′ UTRs (3.9%), and intergenic regions (IGRs; 23.45%), while they were also distributed across the island (13.31%), shore (29.56%), shelf (12.13%), and open sea (44.99%) (Figure 2D and Table S3). This distinct pattern of genome-wide methylation showed that the highest methylation rates were in gene body regions, and the lowest were in 1st Exon regions. Moreover, these target CpG site were also tracked in the Groups 1 and 2. Notably, there were greater differences observed between Groups 1a and 1b than between Groups 2a and 2b, although these parallel comparisons showed similar trends in variation (Figure 2E).
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A

B

C

D

E

Hyper.MVP

Hypo.MVP

G1a  G1b  G2a  G2b

G1a  G1b  G2a  G2b

10648 MVP

G1a vs G1b

G1b vs G1c

TS9500

TSS260

3'UTR

5'UTR

Eentr

Body

3'UTR

IGR

16.6% 7.2% 9.7% 2.8% 38.3% 3.9% 23.5%

island

Shore

Shelf

Open sea

13.3% 29.6% 12.1% 45%
3.2 | Hypermethylation of immune-related signaling genes associated with differentially methylated regions (DMRs) identified in allograft dysfunction patients

We evaluated the possible biological effects of DNA methylation by examining DNA regions that are prone to methylation and represent methylation-regulated genes, using ChAMP to identify significant DMRs in each experimental group. A total of 1021 DMRs were identified between Groups 1a and 1b that mapped to 917 unique genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was then conducted to assess the potential biological relevance of the significantly hypermethylated or hypomethylated DMRs (Figure 3A). Group 1 hypermethylated genes were enriched for T cell receptor and mechanistic target of rapamycin signaling pathways, which were not found among Group 1 hypomethylated genes or among any of the Group 2 DMRs (Figure 3A; Figure S2). DAVID analysis and visualization with the enrichment map, gene ontology terms clustering for "T cell" were indicated as the major category of differentially hypermethylated genes (Figure 3B). Combined with the fact that patients in Group 1 had a history of T cell–mediated rejection prior to allograft failure, these findings suggested that hypermethylation of these regions was associated with AR-induced allograft dysfunction.

3.3 | The Runx-related transcription factor 3 (RUNX3) promoter region is the most hypermethylated DMR in patients with allograft dysfunction

Among the hypermethylated DMRs identified between Group 1a and Group 1b, the DMR with the highest incidence of methylation involved the promoter of RUNX3, an essential gene involved in the immune response. Each CpG site in this methylated region (RUNX3, chr 1: 25290947-25292412) overlapped with the gene promoter (Figure 3C and Table S4). Reanalysis of this region using microarray data showed that the average of delta β value for all CpG sites in this DMR between Groups 1a and 1b (0.2147) was almost twice that between Group 2a vs Group 2b (0.0978) (Figure 3D). After viewing this methylated region in the Integrative Genomics Viewer, a smaller target methylated region was selected for closer scrutiny (RUNX3, chr 1: 25291385-25291584), which overlapped with the 1st Exon and TSS200 regions (Figure 3C; Figure S3). To verify the hypermethylation of this target region, bisulfite sequencing PCR (BSP) and additional human PBMC samples from the graft dysfunction/stable group were analyzed, revealing that this target region was indeed much more hypermethylated in the graft dysfunction cohort than in the graft-stable cohort (Figure 3E), which confirmed our previous conclusions that allograft dysfunction patients have more hypermethylation than graft-stable recipients. Therefore, methylation can be considered as an indicator for monitoring allograft function after transplantation. Recent studies have found that RUNX3 was a negative regulator in the mTOR signaling pathway. Furthermore, the mTOR signaling pathway emerged in the KEGG enrichment analysis of hypermethylated DMRs. Therefore, we next assessed the role of DNA methylation in the mTOR signaling pathway in allograft rejection.

3.4 | DNA methylation inhibitor ameliorates renal allograft inflammatory injuries

To investigate whether inhibition of DNA methylation can alleviate graft rejection, the effects of decitabine treatment on DNA methylation were explored in a mouse AR model of renal transplantation. Compared with the SPSS-treated group, histological analyses revealed the development of milder AR of the renal allograft with much less infiltration of CD3+ cells in decitabine-treated mice (Figure 4A). Moreover, the renal allografts in decitabine-treated mice showed a reduction in the severity of allograft rejection and relatively lower levels of creatinine and the proinflammatory cytokines Cxcl9, Icam1, Il6, Mcp1, Tnfa, and Tgfβ (Figure 4B,C; Figure S4A). Therefore, the affected genes modulate T cell recruitment and differentiation and ultimately lead to the infiltration of inflammatory cells and allograft pathological impairment.

3.5 | Decitabine influenced the infiltration of immune cells

In light of previous findings, the Th1 and Th2 cell immune responses to the grafted donor kidney were examined in decitabine- and SPSS-treated mice. Flow cytometry analysis revealed a 6.03% reduction in Th1 cells relative to levels in SPSS-treated mice, demonstrating that decitabine treatment suppressed the Th1-specific immune response in the grafted kidney. By contrast, the Th2-specific immune response was attenuated in the grafted kidneys from decitabine-treated mice, although only by a 1.1% decrease in Th2 cells compared to that in
Decitabine treatment significantly ameliorates renal allograft rejection. A, H&E, PAS, and immunohistochemistry (CD3) staining show the histopathological changes in renal allografts on postoperative day 7. Scale bar = 50 μm. B, Creatinine levels at 7 d after renal transplantation. C, Real-time PCR to determine the mRNA levels (Cxcl9, Icam1, Il6, Mcp1, and Tnfa) in renal allografts on postoperative day 7. Data are expressed as the mean ± SEM. BSP, bisulfite sequencing PCR; CXCL, C-X-C-motif chemokine ligand; DMR, differentially methylated region; H&E, hematoxylin and eosin; ICAM-1, intercellular cell adhesion molecule-1; IL, interleukin; MCP, monocyte chemoattractant protein; PAS, periodic acid-Schiff; PCR, polymerase chain reaction; SPSS, stroke-physiological saline solution; TGF, transforming growth factor; TNF, tumor necrosis factor [Color figure can be viewed at wileyonlinelibrary.com]
SPSS-treated mice (Figure 5A), which was further validated by quantitative PCR and western blot analysis (Figure 5B,C). These results suggested that decitabine repressed the Th1/2 immune response during acute allograft rejection, which was consistent with the observed systematic pattern of T cell differentiation, such as the similar decrease in Th1/2 levels in the spleen following decitabine treatment (Figure S5).
Likewise, we analyzed the effect of decitabine on Th17-mediated immune responses. Flow cytometry analysis showed no obvious difference of Th17 cells in the donor kidneys, and even reduction in the spleens, from decitabine-treated mice, in contrast to those of SPSS-treated mice (Figure 5A; Figure S5). However, quantitative PCR and western blot analysis revealed that the expression levels of Th17-associated cytokine (IL-17) and transcription factor (RORγt) were enhanced (Figure 5B,C). Furthermore, analysis of Treg-mediated responses displayed a significant reduction in Treg cells, which was consistent with quantitative PCR and western blot analysis (Figure 5; Figure S5). These results showed that chemical suppression of DNA methylation can mitigate the T helper cell–mediated immune response to renal allografts.

3.6 | Decitabine controlled the activity of the mTOR pathway

Investigation of the regulatory effect of a DNA methylation inhibitor on the mTOR signaling pathway demonstrated that the expression of DNMT1 was downregulated in the allografted kidney (Figure 6A,C). Quantitative PCR analysis showed that mRNA levels of Akt1s1, Ddit4, Deptor, Pten, and Tsc2, negative regulators of the mTOR pathway, were increased (Figure 6B). These results were consistent with the findings in the recipient spleens (Figure 7). Similar to the finding of the hypermethylation of RUNX3 in the allograft dysfunction cohort, the sequencing data displayed that Runx3, Ddit4, and Pten showed lower methylation levels in the promoter and higher mRNA expression levels with the treatment of decitabine (Figure 6B; Figures S7-S9). However, the methylation level of the intron region of Foxp3 and mRNA levels of Akt, mTor, Rheb, Rictor, and Rptor were not significantly changed (Figures S4B and S10). Consequently, the phosphorylation of AKT was inhibited, leading to suppressed activity of the downstream signal molecules, including the p-mTOR and p-P70S6K (Figures 6C and 7C).

Considering that the mTOR signaling pathway affects the cell cycle progression, a series of cell cycle–related factors were explored in the recipient spleens. Quantitative PCR demonstrated that the mRNA levels of cell cycle inhibitors (p21, p27, but not p15) were enhanced in the decitabine-treated group. By contrast, other cell proliferation–related factors, including Cyclin A, Cyclin B, Cyclin D, and Ki-67, were repressed in recipients with the treatment of decitabine (Figure S6B).

Two-color immunofluorescence analysis revealed that the grafted kidneys with acute rejection contained large numbers of DNMT1-expressing CD3+ T cells in contrast to those with no or only a mild inflammatory response (Figure 8). In summary, mTOR pathway inhibition influenced the immune response of Th 1/2/17 and Treg cells. These results further demonstrated that decitabine regulated T cell–mediated allograft rejection via its demethylating role in the mTOR pathway.

4 | DISCUSSION

Substantial research has been undertaken to improve allograft survival; however, allograft injuries caused by immune responses remains a major challenge. Epigenetic modulation of expression of immune system-related genes can dynamically regulate innate and adaptive immune responses and ultimately introduces several vulnerabilities to allograft survival. Therefore, methylation can be an important potential marker for allograft outcome. However, gaining a better understanding of methylation activity after transplantation is necessary, especially given the current limitations of our understanding about the impact of epigenetic modification in organ transplantation.

Data generated by our comparative DNA microarray suggested that AR-induced allograft dysfunction was associated with changes of hypermethylation in allograft recipients and pathway enrichment analysis of the DMRs showed that hypermethylated genes were primarily involved in immune-related signaling pathways (including the mTOR pathway). Subsequent bisulfite sequencing PCR analysis further validated that DNA hypermethylation was indeed present in the AR-induced allograft dysfunction cohort. Thus, we hypothesized that administration of DNA methylation inhibitors could potentially ameliorate AR-induced allograft dysfunction.

Using an in vivo mouse model of AR, decitabine treatment decreased the DNMT1 expression level in recipients with a subsequent increase in the level of mTOR upstream negative regulators, ultimately repressing the activity of the mTOR signaling pathway, which was consistent with the findings of Chen et al. Delgoffe et al reported that mTOR pathway inhibition enhanced the expression of SOCS3, which negatively regulated the STAT4 signaling and inhibited Th1 differentiation. Although the repressed mTOR pathway could increase STAT4 phosphorylation and GATA3 expression to drive Th2 differentiation, Lee et al found that Th2 cell differentiation was still controlled by other vital determinants (such as protein kinase C). These findings indicated that multiple signaling pathways participated in Th2 cell differentiation.

Although inhibition of mTOR pathway could induce Treg development, Treg cells were still reduced after decitabine treatment. Our results demonstrated that transforming growth factor beta (TGF-β) expression was significantly downregulated in decitabine-treated group,
and impairment of TGF-β signaling was previously demonstrated to affect the Treg response. Kim et al. confirmed that the methylation level of the first intron of Foxp3 decreased, subsequently promoting Treg differentiation. However, we did not find a significant difference of methylation in the intron region between the SPSS- and decitabine-treated groups. On account of the lower level of p-mTOR, Th17
FIGURE 7  Activity of the mTOR signaling pathway was regulated by decitabine treatment. A, Quantitative PCR analysis of Dnmt1/3a/3b from recipient spleens on postoperative day 7. B, Quantitative PCR analysis of Akt1s1, Cab39, Ddit4, Deptor, Prkab2, Pten, Stk11, and Tsc2 mRNA expression from the spleens of recipient mice. C, Western blot analysis of DNMT1, DDIT4, PTEN, p-Akt, and p-mTOR from the spleens. Data are expressed as the mean ± SEM. DDIT4, DNA damage inducible transcript 4; DNMT, DNA methyltransferase; mTOR, mechanistic target of rapamycin; PCR, polymerase chain reaction; PTEN, phosphatase and tensin homolog; SPSS, stroke-physiological saline solution.
cells would be expected to be decreased; however, we found that the levels of Th17 cell-associated factors were elevated, which could be partially attributed to the specific microenvironment. Christian et al.\(^2\) found that naive T cells treated with decitabine induced RORC expression by demethylation of the *RORC* locus. Additionally, the increase of RORC may be due to the reduction in the *Foxp3* level, because TGF-β-induced *Foxp3* could antagonize RORC function.\(^4\) Furthermore, a suppressed mTOR pathway also affects the cell cycle by promoting cell cycle inhibitors and inhibiting cyclin expression.\(^4\)-\(^6\)

Our results indicated that methylation plays a role in the mTOR signaling pathway and the subsequent alterations influenced the allograft fate, which was consistent with the findings of hypermethylated genes involved in mTOR signaling pathways in the AR-induced graft dysfunction group. These results therefore contribute new insight into the mechanisms of allograft rejection and dysfunction. Moreover, several studies indicated that the majority of genes with differentially methylated loci identified in the peripheral blood could be reflected in the kidney tissues.\(^2\)-\(^6\) Therefore, DNA methylation can serve as
a promising biomarker for predicting the outcome of transplanted kidneys. However, further studies are needed to confirm the specific advantage of methylation, as an early warning for AR-induced dysfunction after transplantation based on DNA methylation-regulated CpG sites.

In summary, this study provides a novel discovery that methylation modification occurred following the transplantation, with higher activity in the case of AR-induced allograft dysfunction, and the DNMT inhibitor decitabine alleviated allograft rejection in a mouse model by suppressing the activity of the mTOR pathway (Figure 9). These findings highlight the potential therapeutic benefits of epigenetic modifiers and provide new insights into the mechanism of transplant rejection and dysfunction offering strong evidence for further exploration.

DISCLOSURE
The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

AUTHOR CONTRIBUTIONS
CZ and WX performed clinical data collection, the methylation microarray assay, flow cytometry, data analysis, and wrote the manuscript; BL and YW performed the mouse renal transplantation and revised the manuscript; SF analyzed flow cytometry results; CW performed the PCR and western blot assays. YC, BL, LQ, and HH collected patient blood; FA and AK prepared the library; SN and WX helped with DNA preparation and bisulfite conversion; SMMR and FN performed bioinformatics analyses; HJ conceived and supervised the study, designed and guided the experiments, and supervised the writing of the manuscript. JC offered clinical support; and all authors reviewed the manuscript.

DATA AVAILABILITY STATEMENT
All data relevant to this study are available from the corresponding author on reasonable request.

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FIGURE 9 Summary of the mechanism of allograft survival involving the mTOR pathway regulated by DNA methylation. DNMT1 inhibits the expression of the mTOR upstream negative regulators to cause the phosphorylation of AKT, mTOR, and P70S6K, and ultimately influence the immune response. The DNA methylation inhibitor decitabine restrains the expression of DNMT1 and the high activity of mTOR pathway, ultimately ameliorating T cell–mediated rejection and improving the allograft survival. DNMT, DNA methyltransferase; mTOR, mechanistic target of rapamycin [Color figure can be viewed at wileyonlinelibrary.com]
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.