INTRODUCTION

The number of patients with acute or chronic renal failure is rising each year, making kidney diseases a major worldwide problem. At present, kidney transplantation is the only option for restoring all aspects of normal kidney function. However, the gap between the number of patients with end-stage organ failure and the number of available donor organs is rapidly expanding. One possible approach to resolve the donor limitations is to use interspecies blastocyst complementation. This approach is based on emptying a “developmental organ niche” in one species by knocking out a specific gene or genes that are necessary for the formation of a particular organ and then using pluripotent stem cells (PSCs) from a different species to populate the empty niches and generate the desired organ. The demonstration of the feasibility of intra- and interspecies blastocyst complementation using rodent models now raises the intriguing possibility of generating human organs using easily accessible host animals, such as pigs, that are similar to humans in genetics, organ...
size and physiology. However, application of this principle for the generation of human organs first requires animal embryos, foetuses or neonates that lack the ability to generate the desired organs.

The development of the mammalian kidney includes three successive steps: pronephros, mesonephros and metanephros. Kidney development begins with the formation of the nephric duct, also known as the Wolfian duct, which grows caudally down the trunk and swells at the caudal region to form the ureteric bud (UB). The UB then outgrows and invades into the metanephric mesenchyme (MM). The interactions between the UB and the MM include a series of reciprocal inductive events in which the UB undergoes complex branching morphogenesis to give rise to the extra-renal ureter and the intra-renal collecting system, while the MM condenses around the UB tips to form the cap mesenchyme. This cap mesenchyme then undergoes a mesenchymal-epithelial transition (MET) to differentiate into the renal vesicles, comma-shaped body, S-shaped body and functional nephrons. Consequently, the branching morphogenesis of the UB is a critical process in kidney development.

An intricate network of signals has been reported to control kidney development, with SIX-PAX-GDNF as the main signalling pathway regulating the mammalian metanephros genesis through a range of transcription factors including SIX1, SIX4, PAX2, PAX8, GDNF and EYA1. SIX1 and SIX4 belong to the murine homeobox SIX gene family, which is homologous to the Drosophila sine oculis (SO). Studies on mice that lack SIX1 have shown that these mice exhibit unilateral or bilateral renal hypoplasia because the UB grows out normally and elongates to differentiate into ureter but then fails to undergo branching morphogenesis. SIX1/SIX4-deficient mice exhibit a more severely disrupted kidney phenotype when compared to SIX1-deficient mice, as the ureters and bilateral kidneys fail to develop. Mice lacking both SIX1 and SIX4 fail to form a detectable MM, and UB development is not induced.

The nephric duct precursors show co-expression of the transcription factors PAX2 and PAX8, members of the "paired box" (PAX) family of homeotic genes. Deletion of PAX2 results in mouse embryos that initially form the pro/mesonephros but lack both ureteric bud and mesenchyme, resulting in renal agenesis. The mutation of PAX8 alone does not lead to any kidney abnormalities, but PAX2/PAX8 double mutants fail to form the nephric duct.

Another gene, EYA1, a homolog of Drosophila eyes absent, is initially expressed in the nephrogenic cord, caudal to the mesonephros, by the metanephric mesenchyme at embryonic day 11.5 (E11.5). EYA1 inactivation embryos do not form a morphologically distinct population of metanephric mesenchyme or a UB, which suggests that EYA1 plays an important role during the conversion of nephrogenic cord cells into MM. Its absence results in kidney agenesis caused by failure of UB invasion at E11.5. BMP signalling also plays a key role in UB branching. BMP4 acts as a negative regulator of UB outgrowth and is expressed in the stromal mesenchymal cells that envelop the main trunk and the stalk of the branching ureters. BMP4 null embryos die during early development.

These genetic observations indicate that the up-stream regulators, SIX1 and SIX4, might be good choices for establishment of a kidney-deficient pig model. However, the conservation of SIX1 and SIX4 is unknown across distantly related species, and the ability of disruption one or both of SIX1 and SIX4 genes to disable kidney development in pigs remains in question. Interestingly, the recent development of genome editing using clustered regulatory interspaced short palindromic repeats (CRISPR)-associated protein (CRISPR/Cas9) technology and its combination with somatic cell nuclear transfer (SCNT) has allowed the creation of a number of useful pig models, thereby confirming that the CRISPR/Cas9-mediated genome editing system can be used effectively in pigs. Similarly, the availability of porcine kidney PK15 epithelial cells, which have a wide range of applications in scientific research, including cell transfection, virus infection and vaccine production, makes them ideal candidates for investigating the signalling pathways of kidney development through in vitro experiments.

In the present study, we produced both SIX1−/− and SIX1−/−/SIX4−/− porcine foetuses by combining the CRISPR/Cas9 system with SCNT technology and we also obtained SIX1−/− PK15 cell lines. We then investigated the functions of the SIX1 and SIX4 genes on the tissue and organ development of porcine foetuses, and especially kidney development. The effect of SIX1 mutation on signal pathways of kidney development was also analysed. The study findings provide new insights into the function of the SIX gene family in porcine kidney development and reveal a possible strategy for the production of kidney-deficient pigs.

2 | MATERIALS AND METHODS

2.1 | CRISPR/Cas9 plasmid construction

The Cas9 expression construct pX330-U6-Chimeric-BB-CBh-hSpCas9 (Addgene plasmid 42230, Watertown, MA, USA) was a human codon-optimized SpCas9 and chimeric guide RNA expression plasmid. We used the online software (MIT CRISPR Design Tool: http://crispr.mit.edu) to design the sgRNA followed by the PAM sequence for targeting SIX1 and SIX4 genes. The sequences of sgRNAs are sgRNA1 (targeting SIX1): 5’-GCCATCGTTCGGCTTCACAC-3’ and sgRNA4 (targeting SIX4): 5’-AAGTGCGGCGGATATCAAGC-3’. The complementary oligos of the sgRNAs were synthesized, phosphorylated and annealed at 37°C for 30 minutes, and at 95°C for 5 minutes, followed by decreasing at 5°C/min to 25°C. The pX330-U6-Chimeric-BB-CBh-hSpCas9 plasmid was digested with BbsI and then ligated with the respective annealed oligos. The resulting CRISPR/Cas9 plasmids for targeting SIX1 and SIX4 were confirmed by sequencing.

2.2 | T7E1 cleavage assay

Porcine primary foetal fibroblast cells (PFFs) transfected with or without Cas9-sgRNA plasmids (as mentioned above) were cultured for 48 hours. Genomic DNA was extracted using a DNA extraction kit (TianGen, Beijing, China), and the genomic region spanning the CRISPR
target sites was PCR amplified. For sgRNA1, the forward primer was 5′-GGCGGAAAGAAGCTGGGAGTGAG-3′, and the reverse primer was 5′-TTGGGTTGGTTGAGGCCGAGAA-3′. For sgRNA4, the forward primer was 5′-GACGAAAGAGGGGAGGTGAG-3′, and the reverse primer was 5′-GAAGTTCCGAGTGGAGTTGT-3′. The PCR conditions were as follows: 95°C for 5 minute for 30 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 40 s, and a final 72°C for 7 minute. The T7E1 cleavage assay was performed using an EnGen® Mutation Detection kit (NEB, Beverly, MA) according to the manufacturer’s protocol. Briefly, a total of 200 ng of the purified PCR product was mixed with T7 endonuclease I and annealed to allow formation of heteroduplex using the following conditions: 95°C for 5 minute, 95°C to 85°C ramping at −2°C/s, 85°C to 25°C ramping at −0.1°C/s and 4°C hold. After reannealing, the products were digested with 1 μL of T7 endonuclease I at 37°C for 1 minute and then subjected to agarose gel electrophoresis. The cleavage bands were quantified with Image J (NIH, Bethesda, MD, USA).

2.3 | Cell culture, transfection and selection

Primary porcine foetal fibroblasts (PFFs) were derived from the skin of E35 Chinese Landrace pig foetuses. The PFFs were cultured in medium consisting of high glucose DMEM (Gibco, Grand Island, NY), 15% FBS (Gibco) and 1% penicillin/streptomycin solution (Gibco) at 37°C in 5% CO₂. Pairs of 1 μg targeting plasmids with 2 μg the neomycin-expression plasmid (pCMV-tdTomato) were co-transfected into 1 × 10⁶ PFFs using a basic fibroblast nucleofection kit (VPI-1002; Amaxa Biosystems/Lonza, Cologne, Germany) and nucleofection program U-023 following the manufacturer’s protocols. After 24 hour of recovery, the electroporated cells were selected with 800 μg/mL of G418 (Gibco) in 10-cm dishes for about 10 days. Individual cell colonies were picked up and cultured in 24-well plates and then passaged to 12-well plates. Approximately 10% of the single colonies were lysed in NP-40 buffer at 55°C for 30 minute and then 95°C for 10 minute; the remaining cells were used for SCNT. The lysate was used as a template for PCR screening. The primers used in amplifying the target region were as follows. For the SIX1 gene, forward: 5′-GGCGGAAAGAAGCTGGGAGTGAG-3′ and reverse: 5′-TTGGGTTGGTTGAGGCCGAGAA-3′. For the SIX4 gene, forward: 5′-CCCACCCGCGAGATTGC-3′ and reverse: 5′-GCCAGGCGGTCCAGTTG-3′. The PCR conditions were 95°C for 5 minute, followed by 35 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 40 s, and a finally 72°C for 7 minute. The PCR products were purified and then cloned into a pMD18-T vector (Takara Clontech, Tokyo, Japan) according to the manufacturer’s instructions.

PK15 cells, purchased from iCell Bioscience Inc (Shanghai, China), were grown in MEM (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin solution (Gibco), and cultured at 37°C with 5% CO₂. The PK15 cells were seeded into 6-well plates (Corning Incorporated, Corning, NY, USA) 1 day prior to transfection at a density of 9 × 10⁵ cells per well. The PK15 cells were then transfected with 8 μg SIX1-Cas9/sgRNA plasmids with 2 μg pCMV-tdTomato vector using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. After a 24 hour transfection, the cells were selected with 1000 μg/mL G418 in 10-cm dishes for 8 days. After G418 selection, resistant cell clones for each experimental group were pooled and collected. The genomic DNAs for different experimental groups were extracted and amplified by PCR for further sequencing assays.

2.4 | SCNT and production of mutant piglets

Methods used for porcine oocyte collection, in vitro maturation and SCNT were similar to our previously described protocols. Briefly, the cumulus-oocyte complexes (COCs) were collected from ovaries and cultured for 42-44 hour at 38.5°C in an atmosphere of 5% CO₂ in air. The mature oocytes were enucleated as described by Dai et al, and a single donor cell was then injected into the perivitelline space of the enucleated oocytes. Subsequently, the donor cell and recipient cytoplast were fused and activated to form reconstructed embryos. For the production of SIX1and SIX1/SIX4 mutant foetuses, the reconstructed embryos cultured in PZM3 at 38.5°C for overnight or 2 days, respectively, and then transferred into the oviduct of an oestrus-synchronized recipient gilt. Pregnancy status of the surrogate was confirmed by B-ultrasonic at 30 days after transplantation and monitored weekly thereafter. The foetuses were removed from the euthanized recipient gilts for dissection and sampling at different pregnant stages.

2.5 | Western blotting analysis

Kidney tissues from wild-type, SIX1−/− and SIX1+/SIX4−/− foetuses were dissected, frozen immediately in liquid nitrogen and stored at −80°C until use. SIX1−/− and wild-type PK15 cells were collected by trypsinization and centrifugation. Total proteins from tissue and cells were extracted with RIPA lysis buffer (P0013B; Beyotime, Shanghai, China), and protein concentrations were measured by bicinchoninic acid assay protein assay kit (#23225; Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer’s protocol. Fifty micrograms of total proteins was used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Bedford, MA). The membranes were blocked with 5% skim milk for 1 hour at room temperature and incubated overnight with primary antibody at 4°C, followed by incubation with secondary antibody for 1.5 hour at room temperature. The protein bands were detected with ECL Western blot detection reagents (CWbio, Jiangsu, China) according to the manufacturer’s instructions. Western blotting data were quantified with Image J software. GAPDH served as the loading control. Primary antibodies used were anti-SIX1 polyclonal antibody (1:500; D4A8K, Cell Signaling Technology, Boston, MA, USA), anti-SIX4 (1:200; sc-390779; Santa Cruz, Dallas, TX, USA), anti-SALL1 (1:500; PP-K9814-00; R&D Systems, Minneapolis, MN, USA), anti-PAX2 (1:1000; ab79389; Abcam, Cambridge, MA, USA), anti-PAX8 (1:1000; ab191870; Abcam), anti-E-cadherin (1:1000; ab1416; Abcam) anti-BMP4 (1:200; sc-6896; Santa Cruz) and anti-EYA1 (1:1000; ab85009; Abcam). The secondary antibodies used were goat anti-rabbit antibody (1:2000; ab6721; Abcam) and goat anti-mouse antibody (1:2000; sc-2005; Santa Cruz).
2.6 | Histology and immunohistochemistry

Tissue samples obtained from the wild-type foetuses and knockout foetuses were prefixed with 4% paraformaldehyde overnight and then embedded in paraffin using standard procedures. Paraffin-embedded tissue sections were selected at 5 μm and mounted on glass slides. Sections were deparaffinized in xylene and rehydrated in a graded series of alcohol, followed by ddH₂O. The sections were then either stained with haematoxylin and eosin (HE) or immunohistochemically stained. Briefly, each section was incubated with primary antibody overnight at 4°C and with secondary antibody for 1 hour at room temperature. Primary antibodies were anti-SIX1 polyclonal antibody (1:200; D4ABK; Cell Signaling Technology), anti-PAX8 (1:200; ab191870; Abcam), anti-E-cadherin (1:200; ab1416; Abcam), anti-SALL1 (1:200; PP-K9814-00; RD Systems), anti-SIX2 (1:200; 11562-1-AP; Proteintech, Wuhan, China), anti-EYA1 (1:200; ab85009; Abcam) and anti-BMP4 (1:200; sc-6896; Santa Cruz). The secondary antibodies were goat anti-rabbit antibody (1:1000; ab6721; Abcam) and goat anti-mouse antibody (1:1000; sc-2005; Santa Cruz).

Micrographs were obtained using a Digital Sight DS-R1 camera attached to a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and semi-quantified by Image Pro Plus 6.0 (Media Cybernetics, MD, USA). The integrated optical density (IOD) of each micrograph was collected. Three fields for each slice (three slides per animal) were randomly selected for blinded measurements (n = 3 per group). The images were quantified using the immunoreactive area (IA) in μm² and the IOD. The staining intensity (SI) for each image was calculated as SI = IOD/IA, and the mean with standard deviation was obtained for each series.

2.7 | Off-target analysis

Potential off-target sites (OTUs) for sgRNA1 and sgRNA4 were predicted by using the CRISPR design tool (http://crispr.mit.edu).
The genomic regions flanking the OTSs were PCR amplified using genomic DNA isolated from SIX1−/− and SIX1−/−/SIX4−/− foetuses and WT controls. These PCR products were sequenced and aligned.

3 RESULTS

3.1 Generation of SIX1−/− and SIX1−/−/SIX4−/− pig foetuses

We disrupted the function of the SIX1 and SIX4 genes by choosing the first coding exon region of the two genes as the Cas9-sgRNA targeting site. The two sgRNAs targeting SIX1 and SIX4 genes were designed using online tools (http://crispr.mit.edu/) and cloned into the pX330 vector (Figure 1A). By assessing with the online tools, the rating of SIX1-sgRNA was 91 and SIX4-sgRNA was 95. The target sites are shown in Figure 1B. We then tested the targeting efficiency of the Cas9-sgRNA plasmid by transfecting the Cas9-sgRNA vector into pig primary foetal fibroblasts (PFFs) and harvesting the genomic DNA after 48 hours. The PCR amplicons that spanned the SIX1 or SIX4 target site were treated with T7E1, and the cleavage bands showed that cas9-sgRNA targeting on the SIX1 or SIX4 gene was highly efficient; the mutation efficiencies of sgRNA1 and sgRNA4 were 48.5% and 26.9%, respectively (Figure 1C).

We established SIX1 knockout cell lines by co-transfecting the Cas9-sgRNA1 vector and the TD-tomato plasmid into an early passage of primary PFFs derived from a 35-day-old male pig foetus, followed by selection with G418 for approximately 10 days and collected 23 single-cell-derived cell colonies. Genotyping analysis identified 18 SIX1 homozygous/heterozygous biallelic mutant colonies.

**FIGURE 2** Generation of SIX1−/− pig foetuses and SIX1−/−/SIX4−/− pig foetuses via SCNT. A. Compared to the age-matched wild-type foetuses, SIX1−/− pig foetuses had a normal body shape and size. However, external developmental abnormalities were observed at the region of the kidney. B. Of the SIX1−/−/SIX4−/− pig foetuses, a few foetuses had a similar body size and appearance to the wild type, but most were significantly retarded in development at embryonic day 36 (E36) and all six foetuses at E45 showed dark colour and tended to degenerate. C. Genotypes of SIX1−/− and SIX1−/−/SIX4−/− foetuses and the corresponding cell colonies used for SCNT.
The SIX1 and SIX1/SIX4 homozygous biallelic knockout cell colonies were then chosen as donor cells for SCNT (Figure 1D). To generate SIX1 knockout foetuses, a total of 1210 reconstructed embryos were transferred to three recipient gilts, and one of the three recipients was found to be pregnant (Table S5). Three foetuses were collected from the pregnant recipient pig at E75 by caesarean section surgery (Figure 2A). Genotype analysis showed that all three foetuses were biallelic SIX1 gene mutants, corresponding to the mutant colonies (Figure 2C). At the morphological level, when compared to age-matched wild-type foetuses, these three foetuses had obvious abnormalities on the superficial skin tissue, and the kidney just was under the superficial tissue skin by necroses.

A total of 1912 reconstructed embryos derived from SIX1/SIX4 knockout cells were introduced into six recipient gilts; three gilts were found to be pregnant (Table S5). Our first attempt to retrieve an E75 foetus revealed only extremely degenerated foetal remnants. We therefore collected 10 foetuses at E45 (Figure 2B and Figure S3); one foetus had a similar body size to that of its wild-type counterparts, but the other foetuses showed significantly retarded development. All 10 foetuses showed an unusual dark colour and a tendency to degenerate when compared with their wild-type counterparts. Six foetuses were collected from a pregnant recipient pig at E36 (Figure 2B and Figure S3). A few of these foetuses had a similar body size and appearance to the wild type, but most were significantly retarded in development. However, none of the six foetuses showed the abnormal dark colour.

DNA sequencing analysis results revealed that 16 foetuses were all SIX1/SIX4 homozygous knockout mutants, corresponding to the mutant cell colonies (Figure 2C).

We determined the expression of SIX1 and SIX4 at the protein level in the gene edited foetuses and their wild-type counterparts by immunohistochemistry (IHC) and Western blotting. Unlike the expression in the wild-type controls, SIX1 protein was undetectable in the kidneys of the SIX1−/− pig foetuses (Figure 3A, B). Western blotting also confirmed the absence of both SIX1 and SIX4 protein in the SIX1−/−/SIX4−/− pig foetuses (Figure 3C). Taken together, these results confirmed that authentic SIX1−/− and SIX1−/−/SIX4−/− pig foetuses had been generated.

3.2 Phenotypic characterization of SIX1−/− porcine foetus kidneys

Necropsy of the three SIX1−/− pig foetuses revealed that the kidneys of SIX1−/− Foetus #1 were smaller than the wild type and that SIX1−/− Foetus #2 had an unilateral kidney that did not migrate to the right position in the enterocoelia (Figure 4A). Comparison of the shape and size of the kidneys, hearts and spleens revealed that although the kidneys of the SIX1−/− porcine foetuses had obvious abnormalities on the superficial skin tissue, and the kidney just was under the superficial tissue skin by necroses.

We also stained tissue sections with periodic acid–Schiff (PAS), Grocott’s methenamine-silver (GMS), Congo red (CR) and Masson’s trichrome to examine histologic changes in the glomerulus. We noted no significant differences between the SIX1−/− and the wild-type kidneys following PAS, GMS or CR staining, but Masson’s trichrome staining revealed a significant increase in glomerular collagen fibres in the SIX1−/− kidney compared with the wild type, indicating that disruption of the SIX1 gene could cause neprhion damage (Figure 6). We also performed HE staining of the heart, liver, spleen and pancreas.

**FIGURE 3** Immunohistochemistry and Western blot analysis of SIX1 and SIX4 protein in kidney. (A) Immunohistochemistry and (B) Western blot analysis of SIX1 demonstrated no detectable SIX1 protein in the kidneys of SIX1−/− porcine foetuses. (C) Western blot analyses of SIX1 and SIX4 protein demonstrated no detectable SIX1 and SIX4 protein in the kidneys of SIX1−/−/SIX4−/− porcine foetuses. Bars = 100 μm

SIX1 mutant kidneys were significantly smaller than the wild type, no obvious differences were observed in their hearts and spleens when compared to the wild type (Figure 4B).

We also examined the kidneys for histologic abnormalities by HE staining of kidney sections from the porcine E75 SIX1−/− and wild-type foetuses. The developing kidney structures, such as glomeruli, tubules, comma-shaped body and S-shaped body, were present in both the SIX1−/− and wild-type porcine foetuses, indicating that the nephrogenic progenitors were able to undergo nephrogenesis in the SIX1−/− kidney. However, the nephrogenic zone was markedly widened in the SIX1−/− kidney, indicating a possible suppression of the process of MM differentiation (Figure 5A, B). The HE staining revealed that the renal tubules of the SIX1−/− kidney appeared to be undergoing vacuolar degeneration, whereas this phenomenon was not observed in the wild-type kidney (Figure 5C, D).

We also stained tissue sections with periodic acid-Schiff (PAS), Grocott’s methenamine-silver (GMS), Congo red (CR) and Masson’s trichrome to examine histologic changes in the glomerulus. We noted no significant differences between the SIX1−/− and the wild-type kidneys following PAS, GMS or CR staining, but Masson’s trichrome staining revealed a significant increase in glomerular collagen fibres in the SIX1−/− kidney compared with the wild type, indicating that disruption of the SIX1 gene could cause neprhion damage (Figure 6). We also performed HE staining of the heart, liver, spleen and pancreas.
of the SIX1 mutant and the wild-type foetuses. HE staining revealed no obvious differences between the SIX1 mutant and the wild-type organs (Figure S1). Taken together, these results demonstrate that deletion of SIX1 gene resulted in abnormal kidney development in the porcine foetus.

3.3 | The branching morphogenesis of the ureteric bud was incomplete in the SIX1−/− porcine foetus

The formation of a permanent kidney occurs via the interaction between the MM and the UB. The UB, a branching epithelial tube originating from the Wolffian duct, invades into the MM and then grows and branches to induce the interaction. It branches in a highly reproducible way and induces nephron formation at each of tips. Thus, the branching of the UB is critical for normal renal development. We previously determined that knockout of the SIX1 gene caused hypoplasia of the ureter in the SIX1−/− pig foetuses. We therefore investigated whether SIX1 mutation had an effect on the development of the UB in the porcine kidney by analysing the expressions of PAX8, PAX2 and E-cadherin. PAX8 and PAX2 are known to be expressed in the nephric tubules and the collecting duct. E-cadherin is the epithelial cell adhesion molecule that links the actin cytoskeleton to adjacent cells to form epithelial tissues. The immunohistochemistry images of PAX8 and E-cadherin in wild-type kidney tissues indicated an apparent branching morphogenesis and formation of a large number of collecting tubules derived from the UB and elongating from the medulla to the cortex. By contrast, this branching morphogenesis was not apparent in the SIX1−/− kidney, which also had few collecting tubes (Figure 7A). Western blots showed that the expression level of PAX2 was lower in the SIX1 mutant kidney than in the wild type (Figure 7B,C). Thus, these results suggest that SIX1 mutation can affect the development of the UB and cause the failure of branching morphogenesis of the UB.

3.4 | The expression of metanephric regulators in SIX1-deficient pigs

SALL1 and SIX2 are expressed in nephron progenitors as well as in differentiating nascent nephrons. SALL1 maintains nephron progenitors and nascent nephrons,22 while SIX2 regulates nephronic progenitor self-renewal, suppresses epithelial differentiation and promotes maintenance of the MM. During the process of kidney maturation, nephron progenitors also continue to differentiate and to decrease in number. We used IHC to show the degree of differentiation of nephron progenitors by their expression of SALL1 and SIX2 (Figure 8A,B). The expression levels of SALL1 and SIX2 were significantly increased in the SIX1-deficient kidney than in the wild-type counterpart, indicating an apparent developmental delay in the differentiation of nephron progenitors and thus, a potential suppression of the interactions between the MM and the UB.

Previous studies have shown that EYA1, SIX1 and PAX2 interact in a molecular pathway to regulate the mesenchymal production of GDNF during UB growth and branching. A requirement for SIX1 was already indicated for the expression of PAX2 in the kidney of the SIX1−/− pig foetuses (Figure 7B,C), so a SIX1-PAX2 pathway appeared to operate during metanephros in the developing pig foetuses. EYA1 expression was unaffected in the metanephric mesenchyme of the SIX1−/− mice. We examined whether the expression of EYA1 was altered in the SIX1-deficient porcine kidney by IHC (Figure 8A,B) and Western blotting (Figure 8C,D), and we found that the EYA1 expression was significantly increased when compared to expression in the wild-type pigs. This indicated that the knockout of the SIX1 gene might increase the expression of EYA1 by some type of regulation. Previous studies have identified a requirement for SIX1 for spatial restriction of BMP4 activity in the mesenchyme surrounding the nascent UB. The genetic lowering of BMP4 activity in SIX1−/− mice restored UB branching and kidney
organogenesis in vivo. In the present study, IHC (Figure 8A,B) and Western blotting (Figure 8C,D) showed that the expression of BMP4 was still reduced in the mesenchyme of the SIX1-deficient porcine kidney, indicating that other factors or other mechanisms might restrict BMP4 activity.

3.5 | The expression of metanephric regulators in SIX1-deficient PK15 cells

To obtain SIX1 knockout PK15 cell lines, we co-transfected the Cas9-sgRNA1 vector and the TD-tomato plasmid into PK15 cells, followed by selection with G418 for approximately 8 days. 55 single-cell-derived cell colonies were collected and analysed. Genotyping analysis indicated that #49 PK15 cells were biallelic mutant (Figure 9A) and were used for the subsequent experiments.

We further investigated the interaction of metanephric signalling pathways via evaluating the expression of the metanephric regulators PAX2, PAX8, SALL1 and E-cadherin by Western blotting and quantitative analysis. The transcription factors PAX2 and PAX8, which are co-expressed in nephric duct precursors, are central regulators of kidney development. Consistent with our results in the SIX1−/− porcine kidney, PAX2 and PAX8 were weakly expressed in SIX1−/− PK15 cells when compared to wild-type controls (Figure 9B,C), suggesting that PAX2 and PAX8 are essential regulators of porcine kidney organogenesis and might act as downstream regulators of the SIX1 protein.

SALL1 is also expressed in the metanephric mesenchyme, and its absence increased apoptosis of the MM. In the present study, SALL1 expression increased in the SIX1 mutant PK15 cells (Figure 9B,C) and this increased expression of SALL1 in the SIX1−/− PK15 cells coincided with its expression in the SIX1-deficient pig foetuses. This indicates a possible increase in the number of nephron progenitors.

E-cadherin is a major protein marker of the epithelial-mesenchymal transition (EMT). A marked decrease in E-cadherin protein expression might have resulted in a blockade of the differentiation of nephron progenitors into epithelial cells (Figure 9B,C).

3.6 | Kidney phenotypic characterization of SIX1−/− SIX4−/− porcine foetuses

We used HE staining of kidney tissues to determine whether the kidney phenotypes were disrupted in SIX1/SIX4-deficient pig foetuses. The wild-type foetuses showed a normal size and morphology for the kidney (Figure 10A), whereas the kidney of SIX1/SIX4-deficient pig foetus (#1), even though they had a similar body length, showed severely retarded development and failed to develop mature kidneys (Figure 10B,C). When compared with the mature appearance of the wild-type kidney (Figure 10A), the SIX1/SIX4-deficient kidney remained at an early stage of initial metanephros formation, where the ureteric bud had invaded into the MM and branched to generate a T-like structure (Figure 10B,C). We also found glomeruli, tubules and ureters in the wild-type kidney (Figure 10A, red arrows), but not in the SIX1/SIX4-deficient kidney (Figure 10B,C). HE staining of serial sections of tissue from the whole foetuses also revealed significantly retarded development and no recognizable kidney structures in the rest of SIX1/SIX4-deficient foetuses (Figure S2). Taken together, the results demonstrated that the disruption of both the SIX1 and SIX4 genes in the pig could cause failure of kidney development and affect foetal development as a whole (Figure 10).

FIGURE 5 Haematoxylin and eosin (H&E) stained of wild-type and SIX1−/− porcine foetuses. (A, B) Glomeruli, tubules, comma-shaped bodies and S-shaped bodies are present in kidneys of both SIX1-deficient and wild-type porcine foetuses. However, the nephrogenic zone (dashed line) was markedly widened in the SIX1−/− kidney. (C, D) Renal tubules of SIX1-deficient kidney appeared to be undergoing vacuolar degeneration (red arrows) when compared with wild-type counterparts (black arrows). Bars = 100 μm.
We also found that the sizes of the testis (Figure 10B) and heart (Figure 11B) were smaller in E36 SIX1/SIX4-deficient pig foetuses than in the wild type (Figures 10A and 11A). However, unlike the kidneys of the SIX1/SIX4-deficient pig foetuses, the E36 testis and heart and E45 heart (Figure 11C) had similar internal anatomical structures when compared with the wild type. We therefore infer that the disruption of both SIX1 and SIX4 genes in the pig might affect the organ development, especially kidney development, and caused subsequent foetal lethality.

### 3.7 Off-target analysis

A certain degree of off-target cutting of the CRISPR/Cas9 system has been reported in some studies. Therefore, we attempted to test the possible off-target effects in SIX1−/− and SIX1−/−/SIX4−/− foetuses. 36 and 34 potential OTSs for sgRNA1 and sgRNA4 were predicted by online software, respectively. 20 higher scores OTSs (Table S1.) were PCR amplified using genomic DNA isolated from SIX1−/− and SIX1−/−/SIX4−/− foetuses and WT controls. Primers for amplifying the off-target sites are listed in Table S2. Sanger sequencing of the PCR products indicated that none of the sequencing reads had mutation, suggesting that no off-target occurred at these sites in the SIX1−/− and SIX1−/−/SIX4−/− foetuses.

### 4 DISCUSSION

One of the ultimate goals of regenerative medicine is to generate patient-specific organs using patient-specific pluripotent stem cells. Interspecies blastocyst complementation provides an alternative approach; however, the hosts suitable for the study of kidney regeneration are mainly rodents. Until now, large
animals with similar anatomy, size and physiology to humans but lacking the ability to generate kidney organs have not been available. Previous studies have shown that CRISPR/Cas9 can be successfully used to generate target mutations in pigs. Here, we efficiently disrupted the SIX1 and SIX4 genes in pigs by altering the protein coding sequence, and we demonstrated that both SIX1 and SIX4 gene knockout in pig foetuses result in disruption of their nephrogenesis phenotype, thereby providing an empty organ niche for the potential generation of human kidneys in pigs.

**FIGURE 7** UB branching morphogenesis is incomplete in SIX1-deficient embryos. (A) Representative immunohistochemical images of PAX8 and E-cadherin showing that the branching morphogenesis of UB was incomplete in the kidneys of SIX1−/− porcine foetuses compared with wild-type kidneys. Western blotting analyses (B) and quantitative analysis (C) show a decrease in PAX2 protein expression, indicating an apparent branching morphogenesis and formation of a large number of collecting tubules (black arrows). By contrast, this branching morphogenesis was not apparent in the SIX1-deficient kidney (red arrows). **P < 0.001. Bars = 100 μm**
The process of kidney development is successive and complex, and the interaction between the ureteric bud and the metanephric mesenchyme is important for kidney development. During these interactions, the UB undergoes a complex branching morphogenesis to give rise to the urinary collecting duct system, while the metanephric mesenchyme ultimately forms the nephrons and the interstitial tissue of the kidney. In this paper, we demonstrated that the UB grows out normally and elongates to differentiate into a ureter but fails to form a complete collecting system in SIX1-deficient pig foetuses, a finding that is consistent with the previous

The scale bar is 100 μm
studies in mice. The failure to form mature collecting ducts may be caused by the inhibition of GDNF and the formation of ectopic UBs. GDNF is a key regulator of UB outgrowth, branching and generation of the metanephric collecting duct system. The maintenance and/or activation of GDNF expression in the MM depends on a number of regulatory factors, including SIX1, SIX4,
PAX2 and PAX8. Downregulation of PAX2 and PAX8 expression in SIX1−/− foetuses and SIX1−/− PK15 cells suggests that the PAX2 and PAX8 genes may be the downstream targets of the SIX1 protein. SIX1 mutation may therefore suppress GDNF expression indirectly by regulating the expression of PAX2 and PAX8. In addition, the observations made by Xu et al.18 suggest the existence of an EYA1-Six-PAX2 regulatory hierarchy that controls early kidney development in mice. However, EYA1 expression is increased in the early kidney development of SIX1-deficient pig foetuses. These findings indicate that a feedback regulation might exist between EYA1 and SIX1/PAX2, so that the decrease in SIX1 and PAX2 expression might activate EYA1 expression.

BMP4 is a negative regulator of UB outgrowth and is expressed in the stromal cells surrounding the nephric duct prior to UB outgrowth.39 BMP4 null embryos die during early kidney development, whereas heterozygotes display ectopic or duplicated UBs.40 Thus, the impaired formation of collecting ducts in SIX1−/− porcine foetuses may result from the production of ectopic and duplicated UBs caused by the reduction in BMP4 expression.

The cap mesenchyme undergoes a mesenchyme-to-epithelial transition (MET) to form renal vesicles and then elongates into an S-shaped body that fuses with the collecting duct epithelium.9,41 SIX2 and SALL1 are important for the self-renewal of the progenitor population and regulating the process of MET.42 Furthermore, SIX2-deficient mice undergo exuberant MET.43 Our immunohistochemistry data showed that the upregulation of SIX2 and SALL1 expression increased the number of nephron progenitors in SIX1-deficient foetuses compared with the wild type, suggesting that MET might be blocked and that differentiation of progenitors might be delayed. The downregulated expression of E-cadherin showed the decreased number of epithelial cells, which further confirmed the block of MET and the possible inhibition of the interactions between the MM and the UB. Taken together, our data support the idea that SIX1 is a crucial regulator of early-stage kidney development and that SIX1 mutants fail to form a complete collecting system.

The kidney of the SIX1/SIX4-deficient pig foetus exhibited a more severely disrupted kidney phenotype than did the SIX1-deficient pig foetus, which is consistent with the observations in mice. Compared to wild-type controls, the ureters and bilateral kidneys failed to develop in the SIX1/SIX4-deficient pig foetus. The SIX1/SIX4-deficient mice died soon after birth and showed developmental defects in various organs.44 We also found the mutation of SIX1/SIX4 genes caused embryonic lethality. This demonstrated that the SIX1 and SIX4 homeoproteins are required for the development of the mammalian embryo.

In humans, the mutation of SIX1 causes branchio-oto-renal (BOR) syndrome,45 an autosomal-dominant disorder characterized by hearing loss and branchial and renal anomalies.46 In our pig study, we found that SIX1 deficiency can cause various kidney defects and ear deformities. Therefore, the SIX1-deficient pig foetus may be useful for elucidating the mechanism underlying this type of disease.

In summary, we successfully generated SIX1 and SIX1/SIX4 targeted pig foetuses using the CRISPR/Cas9 system combined with somatic cell nuclear transfer (SCNT) technology. Our study demonstrated that SIX1 is required for the UB growth and branching occurring during early kidney development. As in other species, suppression of SIX1 and SIX4 gene expression in the pig foetus led to the disruption of kidney development.

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ORCID

Manling Liu https://orcid.org/0000-0002-9500-7728

REFERENCES


SUPPORTING INFORMATION

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