CRISPR/Cas9-mediated knockin of human factor IX into swine factor IX locus effectively alleviates bleeding in hemophilia B pigs

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ABSTRACT

Hemophilia B is an X-linked recessive bleeding disorder caused by abnormalities in the coagulation factor IX gene. Without prophylactic treatment, patients experience frequent spontaneous bleeding episodes. Well-characterized animal models are valuable for determining the pathobiology of the disease and for testing novel therapeutic innovations. Here, we generated a porcine model of hemophilia B (HB) using a combination of CRISPR/Cas9 and somatic cell nuclear transfer. We also tested the possibility of HB therapy by gene insertion. Frequent spontaneous joint bleeding episodes that occurred in HB pigs allowed a thorough investigation of the pathological process of hemophilic arthropathy. In contrast to the HB pigs, which showed a severe bleeding tendency and joint damage, the transgenic pigs carrying human coagulation factor IX exhibited a partial improvement in bleeding. In summary, this study not only offers a translational HB model for exploring the pathological process of hemophilic arthropathy, but also provides a possibility for the permanent correction of hemophilia in the future by genome editing in situ.

Introduction

Factor IX (FIX) is a vitamin K-dependent plasma protein that participates in the intrinsic blood coagulation pathway by converting factor X to its active form in the presence of Ca²⁺, phospholipids, and factor VIIIa. FIX is primarily synthesized in the liver and secreted into plasma. Defects in F9 will cause hemophilia B (HB), which is an inherited X-linked bleeding disorder. Based on the residual FIX plasma levels, the disease is classified as mild (5-40%), moderate (1-5%), and severe (<1%). The morbidity of HB is 1:25,000 male live births, and according to the latest global report from the World Federation of Hemophilia there are 23,775 patients worldwide with HB. Recurrent spontaneous bleeding mainly occurs in the synovial joints, particularly in the knees, ankles, and elbows. These bleeds may lead to chronic pain, immobility and a significant reduction in quality of life. This debilitating condition is the most frequent complication of hemophilia, known as hemophilic arthropathy.

The current management for HB requires lifelong intravenous protein replacement therapy with FIX, which is costly, inconvenience and not curative. HB, which is caused by a single gene abnormality, is an attractive target for gene therapy. Gene therapy using adeno-associated viral (AAV) vectors has shown successful amelioration of severe bleeding phenotypes in animal experiments and clinical trials. However, because of the non-integrating nature of the AAV vector and the high hepatocyte cellular turnover, in some cases the expression of FIX decreases over time. Genome editing can allow stable transgene expression by site-specific gene integration, and the CRISPR/Cas9 gene editing tool shows promise for efficient correction of monogenic diseases. Animal models of hemophilia and related diseases are important for evaluating novel therapeutic strategies and understanding some pathological processes that are
hemophilia. However, studies with hemophilic mice allow only small-volume blood samples and a limited frequency of blood sampling. Moreover, spontaneous bleeding rarely occurs in hemophilic mice, and intervention is necessary in certain studies. For decades, therapeutic efficacy in hemophilic dogs has proven to be an excellent predictor of human clinical efficacy. In addition, hemophilic dogs are frequently used to validate the effects of gene therapy. They show the spontaneous bleeding phenotype; however, the relatively infrequent occurrence of clinically recognizable spontaneous joint bleeding currently hampers any in-depth study of hemophilic arthropathy.

Pigs are an excellent animal model for understanding the pathogenesis of human disease and developing therapeutic strategies. They are similar to humans in anatomy, physiology, and genome. Therefore, we decided to generate HB pigs that potentially provide their own unique study opportunities. Moreover, CRISPR/Cas9-mediated homology-directed gene targeting was used to determine whether it could ameliorate the bleeding phenotype by site-specific gene insertion.

Methods

Animal studies and ethics statements

All animal studies and the breeding process were carried out in accordance with guidelines approved by the Animal Welfare and Research Ethics Committee of Jilin University. All invasive procedures were performed under inhalation anesthesia with 1.5% isoflurane. The wild-type (WT) controls used in our study were age- and sex-matched.

Plasmid construction

pX330-UB6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid #42230). Two paired oligonucleotides, which were designed on the basis of porcine F9 sequences, were ligated to the pX330 plasmid to form single guide RNA (sgRNA)-expressing vectors. The coding sequences (CDS) of human F9, two homologous DNA arms of porcine F9 sequences and the plB vector (TIANGEN, VT205) were assembled together to generate the donor vector. Details of the methods used are available in the Online Supplementary Methods.

Cell culture and selection

The constructed sgRNA-expressing vectors and the donor vector were transfected via electroporation into cultured porcine fetal fibroblasts (PFF). Fibroblasts were isolated from 33-day-old fetuses of Large White pigs. The transfected cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, USA) supplemented with 15% fetal bovine serum (Gibco) at 39°C in an incubator with 5% CO2. The positive PFF were selected by the limiting dilution method. Details are given in the Online Supplementary Methods.

Somatic cell nuclear transfer and embryo transfer

The positive cells were pooled as donor cells for somatic cell nuclear transfer (SCNT). Briefly, a single donor cell was microinjected into an enucleated pig oocyte. Then, the reconstructed embryos were activated and transferred into the synchronized recipient pigs. We performed ultrasonography 30 days post transfer and obtained cloned pigs by eutocia.

Quantitative real-time polymerase chain reaction and western blot analysis

Standard protocols were used for RNA and protein isolation, quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis. Details are given in the Online Supplementary Methods.

Clinical observations

An 8-week clinical observational recording process was initiated after the piglets were born. The pigs were examined daily, and the number and type of bleeding episodes were observed, with a particular focus on joints.

Blood sampling and blood analysis

A detailed description of the blood sampling and blood analysis is provided in the Online Supplementary Methods.

Radiography

Radiographs of the pig limbs were obtained using a digital X-ray imaging system (PaxScan 4343R, Varian; Palo Alto, CA, USA).

Histological assessment

The ankle joints and livers from 2-month-old pigs were placed in 4% formaldehyde for fixation. The joints were decalcified for 3 weeks before tissue processing and paraffin embedding. The liver sections were stained with Hematoxylin & Eosin (HE). The ankle joints were stained with HE and Safranin O. After examination by light microscopy, HE-stained sections were scored for the presence of synovitis, and Safranin O stained ones were scored for cartilage degradation. The score was based on a modified grading system of hemophilic synovitis; scoring criteria are shown in Online Supplementary Table S1.

Statistical analysis

The data from the experiments were analyzed with GraphPad Prism software (t-test). P<0.05 was considered statistically significant.

Results

Generation of porcine F9 knockout pigs and human F9 knockin pigs

Two sgRNAs were specifically designed to target the porcine F9 gene using CRISPR/Cas9 (Figure 1A), considering the following: (i) a computational BLAST tool was used to analyze the selected sgRNAs to ensure that they are unique in the porcine genome, reducing the risk of off-target gene editing; (ii) to produce a non-functional coagulation factor IX protein, the target sites were selected at the 5’ end of the F9 gene; and (iii) the target sites contained mutations known to cause HB, based on the human FIX database (http://www.factorix.org/). We transfected the male and female fetal pig fibroblasts with the two Cas9/sgRNA plasmids separately, 118 fetal pig fibroblasts (88 males; 30 females) were screened by PCR and we identified 15 positive cell clones that contained 11 male clones and two female clones (Online Supplementary Figure S1). We selected some positive clones for SCNT, and the
reconstructed embryos were transferred into nine surrogate pigs (Online Supplementary Table S2). Ultrasonography was performed 30 days post transfer and revealed that four recipients had become pregnant. All pregnancies were maintained to term, and 12 founder (F0) pigs (8 males; 4 females) were delivered. Genotyping by PCR and DNA sequencing identified that eight male pigs carried an F9 deletion and four female pigs had a heterozygous genotype (Figure 1B and C). The female F0 pigs were used to mate with the male F0 pigs to generate F1 pigs. The breeding gave rise to seven F1 pigs, including two male WT pigs, two male F9-deletion pigs, and three female heterozygous pigs (Online Supplementary Figure S2).

Meanwhile, we wondered whether the insertion of a single copy of the human F9 (hF9) gene could alleviate the phenotype in porcine F9 (pF9) knockout (KO) pigs. Thus, in addition to the previously identified two sgRNAs, a donor vector that carries the hF9 CDS flanked by 1-kb homology sequences on both sides was also generated (Figure 1D). By co-transfecting the Cas9/sgRNA plasmids and the donor vector into male fetal pig fibroblasts, we obtained five positive cell clones, and the subsequent sequencing results showed that the hF9 CDS was inserted correctly into the pF9 locus (Online Supplementary Figure S2).
S3). Similarly, the positive cell clones were mixed as donor cells for SCNT; the detailed records are shown in Online Supplementary Table S2. Genotyping by PCR and sequencing analysis confirmed that the five delivered pigs were genetically identical and the hF9 CDS had been inserted correctly (Figure 1E and F and Online Supplementary Figure S4). The results indicated that the pF9 KO pigs and hF9 knockin (KI) pigs developed successfully.

Blood analysis of the pF9 knockout pigs and human F9 knockin pigs

Clinically, as an X-linked recessive disorder, HB is common in men. Thus, we focused on male pigs to investigate their pathological and behavioral changes. Four pF9 KO pigs, four WT pigs, and three hF9 KI pigs were included in the blood analysis. FIX activity was severely decreased in pF9 KO pigs, while it was partly increased in hF9 KI pigs compared with the activity in pF9 KO pigs (Figure 2A). When FIX activity in porcine plasma was expressed as the percentage of coagulation factor activity in WT pig plasma, it was approximately 5.5% (range: 4.3-6.2%) in pF9 KO pigs and 13.5% (range: 12.3-15.0%) in hF9 KI pigs. Furthermore, pF9 KO pigs had a significantly prolonged activated partial thromboplastin time (APTT) compared with that of WT pigs, and the insertion of hF9 CDS partly ameliorated the condition (Figure 2B). There was no difference among the different groups with regard to the prothrombin time (PT) (Figure 2C). Apart from this, two of the pF9 KO pigs had significantly decreased red blood cell (RBC) counts and hemoglobin (Hb) levels, as well as lower levels of total proteins and albumin (Table 1). Higher white blood cell (WBC) counts were found in both pF9 KO pigs and hF9 KI pigs. There were no significant differences in platelet counts among all groups.

Porcine FIX mRNA was extremely low in all pF9 KO pigs and hF9 KI pigs, and human FIX mRNA was effectively transcribed in hF9 KI pigs (Figure 2D and E). FIX protein synthesis in livers also decreased significantly in pF9 KO pigs, and human FIX could be synthesized in hF9 KI pigs (Figure 2F). Human FIX could be successfully secreted into the blood of hF9 KI pigs, approximately 9% (approx. 90.4 ng/mL) of the FIX level in normal human plasma (Figure 2G). Based on the above analysis, the pF9 KO pigs showed lower FIX expression and exhibited coagulation abnormalities. The insertion of hF9 partly alleviated the abnormal coagulation function in the pF9 KO pigs.

Clinical observations of the porcine F9 knockout pigs and human F9 knockin pigs

All piglets were separated from their mothers after delivery, and each piglet was housed individually by artificial suckling in a cage the insides of which had cushioned buffers to protect the piglets and avoid trauma (Figure 3A). Clinical observations of ten cloned pigs, including five pF9 KO pigs, three hF9 KI pigs, and two WT pigs were performed from birth to 8 weeks of age. No bleeding episodes were recorded in WT pigs throughout the observation period. All pF9 KO pigs had multiple spontaneous bleeding episodes, while spontaneous bleeding with a lower frequency occurred in 67% of hF9 KI pigs (2 of 3); no bleeding episode was found in one hF9 KI pig (Online Supplementary Table S3). Most of the bleeding occurred in...
joints, especially the ankle joints (KO: 4 of 5; KI: 2 of 3) and the knee joints (KO: 4 of 5; KI: 0 of 3), which caused the pigs to limp (Figure 3B). Other joints, such as the elbow joints (KO: 2 of 5; KI: 1 of 3) and toe joints (KO: 1 of 5; KI: 1 of 3), also showed occasional bleeding. In addition, some bleeds were observed in other locations, including muscles (KO: 2 of 5; KI: 0 of 3), nose (KO: 2 of 5; KI: 0 of 3), and eyes (KO: 1 of 5; KI: 0 of 3). Bleeds were less common during the first week after birth, and the peak frequency of bleeding occurred at 5-6 weeks of age (Figure 3C). Among the whole population, two pF9 KO pigs died of visceral bleeding, indicating that the spontaneous bleeding frequency may have been slightly higher than our observations. In addition, histological analysis of the livers of pF9 KO pigs demonstrated erythrocyte destruction in the hepatic sinus and hemosiderin deposition; there were no significant differences between hF9 KI pigs and WT pigs (Figure 3D). According to the above-described clinical observations, pF9 KO pigs exhibited serious coagulation disorders, and the bleeding episodes were ameliorated in hF9 KI pigs.

Hemophilic arthropathy is characterized by two main...
features: chronic synovitis and cartilage destruction. An enlarged synovial membrane accompanied by inflammatory infiltrations was observed in 75% (3 of 4) of the pF9 KO pigs, and a thin synovial membrane with subsynovial adipose tissue was seen in 75% (3 of 4) of the hF9 KI pigs (Figure 4A). In addition, severe cartilage destruction was observed in 75% (3 of 4) of the pF9 KO pigs, while mild chondrocyte and proteoglycan losses were also found in 75% (3 of 4) of the hF9 KI pigs (Figure 4B). The arthropathy score was applied to the histological analysis. There was a significant difference in synovial change between pF9 KO pigs and hF9 KI pigs and almost no signs of syn-
ovitis were found in hF9 KI pigs (Figure 4C). Cartilage destruction occurred in both pF9 KO pigs and hF9 KI pigs (Figure 4D). Apart from these findings, coagulated blood was present in the joint cavity of pF9 KO pigs, creating a damaged microenvironment around the joint surfaces (Figure 4E and F).

To assess the impact of hemorrhage on bone changes, the pigs were evaluated radiographically. Compared with those of WT pigs and hF9 KI pigs, the epiphyseal plates of some joints in pF9 pigs were fused early (Figure 5A-C). Histological and radiological evaluation of arthropathic changes revealed that the arthropathic characteristics in pF9 KO pigs were similar to the findings in joints of humans with hemophilic arthropathy.

Discussion

In this study, we generated an HB translational pig model and tested the possibility of HB therapy by CRISPR/Cas9-mediated gene insertion. This large mammalian model of HB showed characteristics similar to those of hemophilic patients. Most importantly, the hemophilic pigs had a high frequency of spontaneous bleeding episodes (especially in the ankles and knees), making these animals a useful translational model for studying hemophilic arthropathy in hemophilic individuals. Compared with hemophilic pigs, the transgenic pigs with a gene insertion of human F9 exhibited significantly ameliorated bleeding symptoms. This finding highlights the potential to replace the defective gene by gene insertion in situ.

First, while designing the target sites, we found that multiple point mutations in the F9 gene were present in the 5’ untranslated region (5’ UTR) and signal peptide region based on clinically reported cases of HB.30-33 The resulting missense mutations in people with hemophilia indicates FIX:C or FIX:Ag are less than 1%; this corresponds to severe hemophilia, such as that found with point mutations occurring at -55, +8 and +19, which are related to the initiation codon (Factor IX Variant Database). Therefore, we designed two sgRNAs to target partial sequences of the 5’ UTR and signal peptide region to produce an HB pig model. In designing the donor vector for homologous recombination, except for the coding sequence of hF9, the regulatory sequences preceding the endogenous initiation codon of pF9 were complemented to avoid affecting the expression of human FIX.

The mRNA and protein expression results in HB pigs showed that the expression of porcine FIX was extremely weak and that the APTT was approximately four times longer. When FIX activity in porcine plasma was expressed as the percentage of coagulation factor activity in WT pig plasma, it was approximately 5.5% (range: 4.5-6.2%) in pF9 KO pigs and 13.5% (range: 12.3-15.0%) in hF9 KI pigs. Increasing plasma FIX levels as low as 1% results in the restoration of clotting activity.34-36 During the whole observation period, the frequency of spontaneous bleeding decreased markedly in hF9 KI pigs. However, there is still some way to go to achieve our ultimate objective. For patients, achieving relief from bleeding is not sufficient. Arthropathy develops progressively and this development is not interrupted once the initial hemorrhage has started. In our models, there were some differences in paraclinical parameters among the three groups. We suspected that the decreases in RBC counts, Hb, total protein and albumin levels in two pF9 KO pigs may be associated with chronic ongoing non-overt bleeding. The higher WBC counts in pF9 KO pigs and hF9 KI pigs could not be readily explained because they are not commonly seen in human hemophilic patients without acute infections. During an 8-week observation period, spontaneous bleeds occurred very frequently in the joints of pF9 KO pigs. This is a unique feature that was not found in other animal models of hemophilia. The histological examination of the ankle joints of pF9 KO pigs showed that without any intervention chronic proliferative synovitis and cartilage destruction occurred within two months. Hemophilic arthropathy is a multifactorial event, and detailed knowledge of the sequential cell and tissue responses after

Figure 5. Radiological changes in some joints of pF9 knockout (KO) pigs and hF9 knockin (KI) pigs. (A) Elbow joints, (B) shoulder joints, (C) ankle joints. Arrows indicate the epiphyseal plates that were fused in hemophilic pigs. WT: wild-type; L: left; R: right.

Figure 5. Radiological changes in some joints of pF9 knockout (KO) pigs and hF9 knockin (KI) pigs. (A) Elbow joints, (B) shoulder joints, (C) ankle joints. Arrows indicate the epiphyseal plates that were fused in hemophilic pigs. WT: wild-type; L: left; R: right.
hernia is important for an in-depth understanding of the pathological process initiated on extensive bleeding into a joint. The hemophilic pig is a suitable disease model for such studies. As a large animal model, pigs pose many challenges. It takes a long time to obtain enough individuals, and a significant research investment is required to develop species-appropriate reagents, assays and expertise.

Site-specific insertion of hF9 was achieved by using CRISPR/Cas9, which can break double-stranded DNAs to facilitate homologous recombination. The insertion of hF9 alleviated clotting disorders in hemophilic pigs, suggesting that it is feasible to replace defective genes with normal genes in situ. However, the gene replacement in our study occurred at the donor cell stage, not by directly completing gene correction using gene editing for individuals who are hemophilic. In a recent report, researchers successfully corrected the bleeding phenotype in newborn and adult factor IX KO mice through in vivo gene editing mediated by CRISPR/Cas9. The study provides convincing evidence of efficacy following in vivo genome editing in hemophilia and identifies the following points that should be considered in our next study: (i) application of the hyperactivating FIX variant; (ii) development of a recombinant vector suitable for HB patients with any mutations; (iii) a vector capable of effectively delivering the gene editing system; and (iv) the efficacy of Cas9-mediated in vivo genetic correction in newborn and adult individuals. Furthermore, the induction of neutralizing antibody (inhibitor) to the therapeutic protein sometimes precludes stable phenotypic correction following gene therapy, and the immune responses triggered by the functional protein, the gene editing system and the delivery vectors should be monitored over a long period of time.

In conclusion, our study offers an alternative HB model for exploring the pathological process of hemorrhagic arthropathy and provides a possibility for the permanent correction of hemophilia in the future by genome editing in situ.

Disclosures
No conflicts of interest to disclose.

Contributions
JHC, BYA, BY, XHP and HMJ carried out the experimental work, the data collection and interpretation. QBY, LYW, XWZ and HW participated in the coordination of experimental work. XCT, HSO and DXP participated in the study design. JHC carried out the analysis and interpretation of data and drafted the manuscript. TTY, XDZ and XC provided the technical supports.

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References