A CRISPR-engineered swine model of COL2A1 deficiency recapitulates altered early skeletal developmental defects in humans

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Abstract

Loss-of-function mutations in the COL2A1 gene were recently described as a cause of type II collagenopathy, a major subgroup of genetic skeletal diseases. However, the pathogenic mechanisms associated with COL2A1 mutations remain unclear, and there are few large-mammal models of these diseases. In this study, we established a swine model carrying COL2A1 mutations using CRISPR/Cas9 and somatic cell nuclear transfer technologies. Animals mutant for COL2A1 exhibited severe skeletal dysplasia characterized by shortened long bones, abnormal vertebrae, depressed nasal bridge, and cleft palate. Importantly, COL2A1 mutant piglets suffered tracheal collapse, which was almost certainly the cause of their death shortly after birth. In conclusion, we have demonstrated for the first time that overt and striking skeletal dysplasia occurring in human patients can be recapitulated in large transgenic mammals. This model underscores the importance of employing large animals as models to investigate the pathogenesis and potential therapeutics of skeletal diseases.

Keywords

COL2A1 mutation, CRISPR/Cas9, swine model, genetic skeletal diseases, cartilage development, tracheal collapse

Introduction

Genetic skeletal disorders (GSDs) arise through mutations that disrupt normal skeletal development and homeostasis(C. Chen et al., 2016). Although individually rare, GSDs are of important clinical relevance as a group owing to their high overall frequency(Kornak & Mundlos, 2003). The rapid development of genetic diagnostic technology over the past few decades has led to the identification of 436 GSDs, which have been classified into 42 groups(Mortier et al., 2019). The type II collagen group, one of the major GSD groups identified, consists of 17 clinical disorders that are associated with mutations in the *COL2A1* gene, and comprise a spectrum of autosomal-dominant disorders characterized by skeletal dysplasia(Deng, Huang, & Yuan, 2016; Kannu, Bateman, & Savarirayan, 2012). Type II collagen is the most abundant protein in cartilage, and, together with additional tissue-specific collagens and proteoglycans, provides structural integrity to this tissue(Myllyharju & Kivirikko, 2004). Type II collagen, a fibrillar collagen, is a molecule composed of homotrimers of collagen alpha 1 type II ($\alpha1[II])_3$ chains containing characteristic repeating Gly-X-Y sequences that fold into a triple-helical structure. Although most *COL2A1* mutations are found in the triple-helical region, mutations have also been identified in the N- and C-propeptide domains situated at the amino- and carboxyl-terminal ends of the triple-helical region(Barat-Houari et al., 2016).

Genetic disorders resulting from *COL2A1* mutations, known as type II collagenopathies, include a spectrum of highly variable chondrodysplasias that are characterized according to severity. These include mild types like avascular necrosis of the femoral head (ANFH) and Stickler syndrome type I (STL 1); severe types like spondyloepiphyseal dysplasia congenita (SEDC) and Kniest dysplasia; and lethal types like achondrogenesis type II (ACG 2) and platyspondylic skeletal dysplasia, Torrance type (PLSD-T)(Deng et al., 2016). Although 663 independent probands previously reported with 460 different *COL2A1* variants have been identified that may be associated with different phenotypes(Zhang et al., 2020), the causes of skeletal deformities and the underlying pathomechanisms remain unclear. To address this, several genetically modified mouse models have been established and characterized. For example, pronounced short-limbed dwarfism was developed in a mouse model with deletion of *Col2a1* exon 7, as well as in transgenic mice expressing the *Col2a1* p.G574S mutation(Maddox, Garofalo, Smith, Keene, & Horton, 1997; Metsaranta et al., 1992). Mild phenotypes, such as the ocular features found in STL 1, have been observed in *Col2a1*$^{+/-}$mice(Kaarniranta et al., 2006). Moreover, a mouse model of SEDC displaying respiratory failure, short limb bones, and a short vertebral column has also been established(Barbieri et al., 2003; Donahue et al., 2003; Macdonald et al., 2013). The frequently lethal phenotypes associated with ACG 2 and PLSD-T were also seen in mouse models with p.R789C and p.D1469A *Col2a1* mutations, respectively(Furuichi et al., 2011; Gaiser et al., 2002). Although mouse models carrying *Col2a1* mutations show delayed skeletal development and abnormal cartilage composition and structure, they lack the overt and striking skeletal dysplasia observed in larger mammals owing to the limited size of their bones and joints.

Pigs have been widely employed in biological studies owing to their genetic, anatomical, and physiological similarities with humans(Prather, Lorson, Ross, Whyte, & Walters, 2013). Many genetically engineered pig models have been developed for biomedical research into the underlying mechanisms of human diseases(Holm, Alstrup, & Luo, 2016; Yan et al., 2018). However, relatively few studies investigating GSDs have employed large mammals, including the pig. The articular structure of the major joints of pigs is more like that of humans when compared with those of smaller model organisms. Furthermore, the tools currently available for genetic manipulation, in combination with somatic cell nuclear transfer (SCNT) technology, allow for the modification of endogenous pig genes and the development of a variety of pig models of human diseases(Han et al., 2017; Yang et al., 2017; Zhou et al., 2015).

In this study, we used CRISPR/Cas 9 to modify the pig *COL2A1* gene and establish a swine model of a lethal type II collagenopathy that resembles PLSD-T. The generation of a swine model carrying *COL2A1* mutations, which displayed typical skeletal deformities and abnormal cartilage development, demonstrates that large mammals can recapitulate overt and striking skeletal dysplasia caused by genetic variants. Importantly, investigation of the trachea of *COL2A1* mutant piglets provided convincing evidence that tracheal collapse was the most likely cause of the death of the mutant piglets shortly after birth. Our findings support that genetically modified swine models are valuable tools for studying the pathogenesis of GSDs and the associated mechanisms.

Materials and Methods

Ethical statement.

All animal studies were approved by the Animal Welfare and Research Ethics Committee of Jilin University, and all procedures were conducted strictly in accordance with the Guide for the Care and Use of Laboratory Animals. All experimental pigs were obtained from the Huichang Animal Husbandry Science and Technology Co., Ltd. All animal operations were performed under anesthesia, and every effort was made to minimize animal suffering.

sgRNA design and CRISPR/Cas9 vector construction.

The targeted sgRNAs in Figure 1C were designed according to a previously described method (http://crispr.mit.edu/)(Cong et al., 2013). The sequences of the two sgRNA are provided in Figure 1C. The pX330U6-Chimeric_BB-CBh-hSpCas9 plasmid was purchased from Addgene (#42230). Two complementary sgRNA oligonucleotides with appropriate adaptors were synthesized and then annealed in standard

Taq buffer (NEB, Beijing, China). The resultant double-stranded DNA was ligated to the BbsI sites of the vector backbone to form the targeting plasmid.

Isolation of porcine fetal fibroblasts and cell culture.

Porcine fetal fibroblasts were derived from 32-day-old Bama miniature pig fetuses. Fetuses were disaggregated in culture medium containing 200 U/mL collagenase IV (type IV, 260 U/mg; Gibco, Grand Island, NY, USA), 18% fetal bovine serum (Gibco), 0.0125 mg/mL DNase I (2,000 U/mg; Sigma, St. Louis, MO, USA), and 1% penicillin/streptomycin (Gibco) for 5–6 h. Isolated PFFs were resuspended and cultured in 10-cm Petri dishes until subconfluence, and then cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 12% fetal bovine serum. First-generation cells were frozen in fetal bovine serum containing 10% dimethyl sulfoxide.

Porcine fetal fibroblast transfection and selection.

Approximately 3×10^6 PFFs were electroporated with 35 μg of the Cas9/gRNA targeting vector and ssODN template in 220 μL of Opti-MEM (Gibco) using a BTX ECM 2001 system (Harvard Bioscience, Holliston, MA, USA). The electroporated PFFs were cultured at 37°C for 72 h and then trypsinized for genomic DNA extraction. Target sequence regions were amplified by PCR using the following parameters: 94°C for 3 min, followed by 35 cycles of 94°C for 20 s, 60°C for 10 s, and 72°C for 33 s, with a final extension at 68°C for 5 min. For the selection of single-cell colonies, electroporated PFFs were plated onto 10-cm Petri dishes after 48–72 h of culture. Single-cell colonies were picked and cultured in 24-well plates for recovery, while a small part of each colony was lysed for genotyping. The regions surrounding the intended point mutations were amplified and then sequenced. Amplicons with multi-peaks were cloned into the pLB vector (Tiangen, Beijing, China) to identify the exact sequence of each allele.

Somatic cell nuclear transfer.

The SCNT protocol was conducted as described by Lai et al(Fan et al., 2013). Briefly, cumulus-oocyte complexes were matured at 39°C for 40 h in maturation medium after removing the follicles. The first polar body was then aspirated from mature oocytes using a glass pipette, and, finally, the donor cells were fused with enucleated oocytes using BTX electrofusion equipment. The reconstructed embryos were then cultured for nearly 12 h before being transferred into surrogate sows. Pregnancy was monitored by ultrasonography after 30 days. Piglet DNA was extracted from the tail and analyzed *via* PCR sequencing. The amplicons were also ligated to the pLB vector to identify the exact sequence of both alleles.

Identification of genetically modified piglets.

The primers designed for amplification of the *COL2A1* -specific Cas9/gRNA plasmid are shown in Figure 1C. PCR was performed using the following parameters: 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 33 s, with a final extension of 72°C for 5 min. Genomic DNA from wild-type Bama miniature pigs was used as negative control.

Radiographic analysis.

Autoradiographs of whole-body skeletons and cartilage of piglets were taken on radiographic film (ROTANODE, Tokyo, Japan) using the YEMA Radiography System equipped with a digital camera (Varian, California, USA). The image exposure factors were 30 kV 2 mAs.

Histological and immunohistochemical analysis.

For histological analyses, femurs, humeri, and tracheas were collected from *COL2A1* mutant piglets and WT piglets after removing the surrounding muscles and fascia. Femurs, humeri, and tracheas were fixed in 4% paraformaldehyde at 4degC. The bones were subsequently decalcified in 15% EDTA for 3 weeks, dehydrated in increasing concentrations of ethanol (70% for 7 h, 80% for 2 h, 96% for 2 h, and 100% for 4 h), washed with xylene, and embedded in paraffin. Then, 5-μm thick sections were cut and used for H&E(Esapa et al., 2012a) and safranin O/fast green staining as previously described(Y. Chen et al., 2017; Schumacher, Joiner,

Less, Drewry, & Williams, 2016), and were then analyzed under a microscope (catalog #BX43, Olympus, Tokyo, Japan). For immunohistochemical analysis, sections were incubated with an anti-*COL2A1* primary antibody (catalog #BA0533; Boster Biological Technology, Wuhan, China) in 3% BSA overnight at 4°C. After washing with PBS, the sections were incubated with secondary antibody (catalog #BA1039; Boster Biological Technology, Wuhan, China) for 30 min, and the staining intensity was analyzed by microscopy.

Western blotting analysis.

Fresh cartilage and trachea samples were ground and then resolved in lysis buffer. The resultant protein samples were quantified using a BCA protein assay kit (Beyotime, Haimen, China). The isolated proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes. The blots were incubated with a primary antibody against type II collagen (#BA0533, Boster Biological Technology), washed, and then incubated with a horseradish peroxidase-labeled secondary antibody (catalog #BA1039; Boster Biological Technology). Finally, the blotting bands were detected with an ECL-Plus western blotting reagent (Beyotime).

Real-time RT-PCR.

Total RNA was extracted from femurs, humeri, and tracheas of newborn WT and *COL2A1* KO piglets using TRNzol-A+ reagent (Tiangen), treated with DNase I (Fermentas, Massachusetts, USA), and reverse-transcribed to cDNA using the BioRT cDNA First-Strand Synthesis Kit (Tiangen). qPCR was performed using the ABI PRISM 7500 FAST Real-Time PCR System (Applied Biosystems, Massachusetts, USA), and the 2^{-Ct}formula was used to determine relative gene expression, which was normalized to the level of *GAPDH* mRNA. All experiments were repeated three times, and the data are expressed as means ± SEM.

Off-target analysis.

To test for off-target mutations in the *COL2A1* mutant piglets, the top eight POTS were selected for each sgRNA using the CasOFFinder Tool (http://www.rgenome.net/cas-offinder/new). Amplicons were subjected to Sanger sequence. The primers used are listed in Table S1.

Statistical analysis.

A two-tailed Student's t -test was used for comparisons between groups, and one-way analysis of variance (ANOVA) with Tukey's*post-hoc* test was used for comparisons between multiple groups. Significance compared to untreated controls is denoted with $^{*}P< 0.05$, $^{**}P < 0.01$, and $^{***}P< 0.001$ in the figures and figure legends. Statistical analysis was performed in GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

Results

Generation of a pig model with type II collagen deficiency

CRISPR/Cas9 technology is an effective approach for the generation of double-stranded DNA breaks to facilitate homologous recombination-mediated modification of target genes. We designed two guide RNAs (gRNAs) specific for *COL2A1* exon 27 where is highly conserved among different species (Figure 1A), and one single-stranded donor oligonucleotide (ssOND) for insertion into the *COL2A1* locus*via* homology-directed repair (HDR) (Figure 1B and 1C). One of the target gRNAs was tested to verify that double-stranded DNA breaks could be effectively generated (Figure 1D). Both the Cas9/gRNA expression vector and single-stranded oligonucleotide (ssODN) were transfected into porcine fetal fibroblasts (PFFs). We screened 366 PFFs by PCR and identified 7 positive clones heterozygous for a mutation in exon 27 of the *COL2A1* gene. We selected a cell clone that contained a compound heterozygous mutation and obtained 1301 embryos following SCNT. One of the compound *COL2A1* variants, c.1744G>A, was previously reported to be associated with ANFH(Kishiya, Nakamura, Ohishi, Furukawa, & Ishibashi, 2014), while a novel variant, c.1749delA, was predicted to result in a frameshift mutation (Figure 1E). All the embryos were surgically transferred to five surrogate sows, three of which became pregnant. Seven piglets were born by natural delivery. However, the piglets all died shortly after birth, and all carried the compound heterozygous

mutations identified by PCR, T-cloning and Sanger sequencing. Additionally, the piglets exhibited many of the phenotypical features associated with type II collagenopathies and afforded us an opportunity to establish the cause of the premature death of the animals. As off-target effects are a major concern when using the CRISPR/Cas9 system, we performed Sanger sequencing for eight potential off-target sites (POTS). No off-target effects were found in the *COL2A1* mutant piglets (Supporting Figure S1 and Supporting Table S1).

Phenotype characterization of COL2A1 knockout pigs

As shown in Figure 2A, *COL2A1* knockout (KO) piglets were shorter than the controls. The flat face, cleft palate, and limb hypoplasia observed in the KO pig are phenotypes typically seen in patients with type II collagenopathy (Figure 2B to 2D)(Zhang et al., 2020). The cleft palate is also considered as a typical phenotype caused by *COL2A1* variants in mouse models(Barbieri et al., 2003; Vandenberg et al., 1991). Moreover, the *COL2A1* KO piglets had a short nose indicative of mid-face hypoplasia, and the ratio of abdomen circumference to chest circumference suggested that the KO piglets had a protuberant abdomen (Figure 2E).

Radiographic examinations showed that the *COL2A1* KO piglets had a markedly underdeveloped skeleton. Lateral radiographic views showed a depressed nasal bridge and short maxilla, which corresponded to the visible flat-face phenotype. Ovoid vertebral bodies could also be observed in the KO pigs (Figure 2F). Radiographic images of the *COL2A1* KO pigs further showed thoracic dysplasia, shortened long bones with ragged metaphyses, iliac hypoplasia, and brachydactyly with short metacarpals (Figure 2G to 2I). Figure 2J shows the measured length of the forelimb, hindlimb, spine, and maxilla.

Bone structure is abnormal in COL2A1 KO pigs

For analysis of bone and cartilage pathology, we isolated the long bones and ribs of both KO and wild-type (WT) piglets. We found that the long bones of KO pigs were significantly shorter than those of WT pigs (Figure 3A and 3B). In the *COL2A1* KO pigs, the heads of the humerus and femur were underdeveloped, and all the long bone epiphyses were soft and the margins, especially that of the joint structure, were unclear. Interestingly, the ribs of *COL2A1* KO pigs were also shorter than those of WT pigs, which resulted in a narrower thoracic cavity (Figure 3C). The lengths of the long bones and ribs are shown in Figure 3D.

Skeleton dysplasia in COL2A1 KO pigs restricts organ development

To explore whether the skeleton deformities affected organ development, we isolated the organs of both *COL2A1* KO and WT pigs (Figure 3E). In the mutant pigs, the absolute weights of all the organs were marginally smaller than those of the controls, and showed no significant differences in morphology (Figure 3E and 3G). However, the weight of the lungs of *COL2A1* KO pigs was lower than that of WT pigs, indicating that the deformity of the rib cage restricted lung tissue development (Figure 3F). During the process of isolating the lung, we found that the tracheas of KO pigs were soft and inelastic, a critical factor that prompted us to further explore the cause of death of the COL2A1 mutant pigs shortly after birth.

Histomorphology and pathology of the articular cartilage of the limbs of COL2A1 KO pigs

To understand the pathogenic basis for the skeletal dysplasia phenotype, we investigated the histology of the articular cartilage of the *COL2A1* KO pigs (Figure 4). The cartilage of the KO pigs presented porous areas, as well as barely defined superficial, transitional, and deep zones, the three layers that comprise normal articular cartilage. Compared with that of WT piglets (Figure 4A-α, β), the femoral head cartilage of KO piglets (Figure 4A-γ) presented vacuolized chondrocytes (arrows) and disrupted collagen formation. Similar changes could be seen in the cartilage of knee joint (Figure 4B). Besides, the lack of regular alignment of hypertrophic chondrocytes was seen in the Figure 4A-γ and Figure 4B-γ.

Articular cartilage formation in the hip and knee joint was assessed by safranin O/fast green staining (Figure 5A and 5C). Safranin O staining intensity was greatly reduced in the articular cartilage of *COL2A1* KO pigs compared with that of WT animals. We also compared the immunoreactivity of type II collagen in the

articular cartilage of WT and *COL2A1* KO pigs by immunohistochemical analysis using sections from the femoral head (Figure 5B) and knee articular cartilage (Figure 5D), and found that the expression of type II collagen was decreased in KO pigs.

The morphological results of hematoxylin and eosin (H&E), safranin O/fast green, and immunohistochemical staining of humerus head and trochlea sections are shown in Supporting Figure S2 and S3. Besides, the thickness of the cartilage in each joint was compared and exhibit in Supporting Figure S4.

Histomorphology and pathology of the tracheal cartilage in COL2A1 KO pigs

Based on our finding that the tracheas of KO pigs were soft and inelastic, we further investigated the pathological status of the tracheal cartilage. As can be seen in H&E-stained sections, the tracheal cartilage of *COL2A1* KO pigs was flattened and lacked the typical C-shaped curve, and the lumen was essentially obliterated (Grade IV) (Figure 6A). Moreover, the alignment of the pseudostratified ciliated columnar epithelium was lost, and the goblet cells were larger than those of WT pigs (Figure 6A-γ). Although the thickness of the tracheal cartilage was similar between *COL2A1* KO and WT piglets, the mutant piglets presented a reduced number of chondrocytes (Figure 6B). Immunohistochemical analysis further indicated that the expression of type II collagen was reduced in the tracheal cartilage of KO pigs (Figure 6C). The results of the picrosirius red staining demonstrated that there were fewer collagen fibers in the perichondrium and less hyaline cartilage in the trachea of KO pigs than in those of WT pigs (Figure 6D).

Healthy tracheal cartilage helps keep the airways open when breathing; however, lack of type II collagen expression leads to tracheal collapse and blockage of fresh air entry into the lungs, which may have been the cause of the premature death of our *COL2A1* KO piglets.

Type II collagen was decreased in the articular cartilage of COL2A1 KO pigs

To confirm that the loss of *COL2A1* expression in the KO piglets was indeed due to the mutations generated in the *COL2A1* gene, western blot and real-time qPCR was performed for the articular cartilage of the major joints (hip, knee, shoulder, and elbow). As shown in Figure 7A and 7B, the COL2A1 protein expression level was notably reduced in KO pigs compared with that of control pigs, while qPCR analysis indicated that *COL2A1* mRNA levels were significantly reduced in the articular and tracheal cartilage of KO pigs when compared with the levels in WT pigs (Figure 7C to 7E). The predicted 3D structure models suggested that the structure of type II collagen would be disrupted as a result of the c.1749delA mutation in the *COL2A1*gene (Figure 7F). The above data indicated that the level of type II collagen was decreased in the mutant piglets, which resulted in underdeveloped cartilage and skeletal deformities.

Discussion

In this study, we generated genetically modified swine carrying *COL2A1* mutations that successfully recapitulated abnormal early bone and cartilage development in humans. Importantly, our swine model provided convincing evidence that tracheal collapse was the most likely cause of the premature death of the *COL2A1* mutant piglets that occurred shortly after their birth. Additionally, this is the first genetically modified swine model generated for the investigation of GSDs, which expands the range of applications of genome editing technology in large mammals for human disease modeling.

In our study, a swine model harboring compound heterozygous mutations (c.1744G>A and c.1749delA) in the *COL2A1* gene displayed phenotypes typically observed in skeletal dysplasia. The c.1744G>A mutation was previously identified in three generations of a family that all suffered from bilateral hip joint lesions and were diagnosed with ANFH(Kishiya et al., 2014). The severe skeletal defects in our *COL2A1* mutant piglets, such as significantly shorter limbs, narrower thoracic cavity, and underdeveloped vertebral body, correspond to the clinical phenotypes of PLSD-T(Nishimura et al., 2004), a rare skeletal dysplasia characterized by extremely short limbs, platyspondyly, and severe pelvic hypoplasia(Nishimura et al., 2004; Zankl et al., 2005). This disorder is usually caused by mutations in the C-propeptide region of *COL2A1*and is lethal in the perinatal period in most reported cases(Desir et al., 2012; Nishimura et al., 2004; Okamoto et al., 2012; Zankl et al., 2005). Skeletal disorders similar to those of PLSD-T patients also occurred in a mouse

model with an ENU-induced missense mutation (c.4406A>C) in the C-propeptide coding region of *Col2a1* (Furuichi et al., 2011). In this mouse model, heterozygotes were smaller than their WT siblings and showed disproportionately short limbs and slightly hypoplastic vertebral bodies, while homozygotes died at birth and exhibited severe dwarfism with shortened limbs and snout. Other mouse models of GSDs have also been established, including for SEDC, osteoarthritis, and disproportionate micromelia(Esapa et al., 2012b; Helminen et al., 1993; Pace et al., 1997).

Histological analysis showed that the *COL2A1* KO piglets displayed reduced articular cartilage thickness in the major limb joints compared with that of WT piglets, and the articular cartilage tissue exhibited porous areas and indiscernible superficial, transitional, and proliferative zones. Moreover, the regular alignment of hypertrophic chondrocytes was lost, as has also been reported for *Col2a1* mutant mice, although porous areas were not seen in the cartilage tissue of these animals(Esapa et al., 2012a; Garofalo et al., 1991; Liang et al., 2014). A previous study suggested that loss of chondrocyte columnar organization was due to the increased deposition of mutated type II collagen in cartilage, as was also seen in other transgenic mouse models of SEDC(Donahue et al., 2003; Gaiser et al., 2002). However, the mechanism involved in the loss of the hypertrophic zone in cartilage has also been reported to result from the activation of the endoplasmic reticulum (ER) stress-unfolding protein response-apoptosis cascade resulting from the accumulation of mutated type II collagen, that finally leads to chondrodysplasia(Chung, Jensen, Gawron, Steplewski, & Fertala, 2009; Liang et al., 2014). The enlarged and extended chondrocyte phenotype seen in our model is also typically observed in affected human patients with *COL2A1* mutations and *Col2a1* mutant mice(Esapa et al., 2012a; Su et al., 2010). A mutant form of type II collagen was revealed to lead to articular chondrocyte hypertrophy through the inhibition of integrin beta 1/SMAD1 interaction(Lian et al., 2019). Surprisingly, in the safranin O-/fast green-stained sections of cartilage tissue of the femoral head (Figure 4C), red safranin O staining, which represents cartilage, was absent. We suspect that this was due not only to the *COL2A1* mutations leading to reduced cartilage formation, but also the lack of the type II collagen in the cartilage. Other safranin O-/fast green-stained sections also showed reduced safranin O staining intensity (Figure 5A; Supporting Figure S3A and S3C), which has also been reported in previous studies(Fernandes, Seegmiller, Nelson, & Eyre, 2003). Notably, we did not perform transmission electron microscopy (TEM) analysis as the piglets died too abruptly to enable fresh tissue collection. However, other studies have indicated that the rough ER and Golgi bodies in mutated chondrocytes are dilated, and there is aberrant accumulation of glycogen granules, as well as fewer and thinner collagen fiber aggregates, in the cartilage matrix of *Col2a1* mutant mice(Arita et al., 2002; Garofalo et al., 1991; Li et al., 1995).

Importantly, in our study, we detected tracheal collapse in *COL2A1* mutant piglets, which was almost certainly the cause of their death shortly after birth. We found that the tracheal cartilage of *COL2A1* mutant piglets was flattened, lacking the typical C-shaped curve, and the lumen was essentially obliterated. The picrosirius red-stained sections showed a decreased density of collagen fibers in the mutant piglets (Figure 6D). Additionally, in our *COL2A1* mutant pigs, the pseudostratified ciliated columnar epithelium was not aligned, and the goblet cells were larger than those of control piglets (Figure 6A). These findings have not been previously reported. Two patients affected with PLSD-T caused by *COL2A1* mutations were reported to suffer only from respiratory failure in infancy(Nishimura et al., 2004; Okamoto et al., 2012), while a few studies have only indicated that transgenic mice with *Col2a1* mutations show severe respiratory failure(Barbieri et al., 2003; Donahue et al., 2003). Nevertheless, pulmonary hypoplasia and tracheal collapse are characteristic findings that have been presumed to be the cause of neonatal lethality in atelosteogenesis type 1 (AO1), in which some phenotypes are similar to those observed in PLSD-T(Bejjani et al., 1998).

The pathological mechanism of *COL2A1* mutation has been study by lots of research. We have known that *COL2A1* gene encodes the alpha 1 chain of procollagen type II, and three alpha 1 chains are folded together in a triple-helical configuration to form the procollagen homotrimer. After secretion into the extracellular matrix, the N- and C-terminal propeptides are removed, forming the mature type II collagen molecule(Bejjani et al., 1998; Strom & Upholt, 1984). The *COL2A1* gene comprises 54 exons, with the triple-helical region ranging from exon 8 to exon 51, flanked by N- and C-propeptide domains(Barat-Houari et al., 2016). Most mutations in the *COL2A1* gene occur in the central domain that encodes the triple-helical

region(Hoornaert et al., 2006); consequently, when mutated, this domain is believed to exert a dominant-negative effect by interfering with the normal assembly of collagen chains, as shown in a *Col2a1* mutant (p.R798C) mouse model(Gaiser et al., 2002). Mutations in exon 2, encoding the N-propeptide domain, cause ocular disorders such as Stickler syndrome. *COL2A1* mRNA undergoes alternative splicing, resulting in two isoforms. The long form (type IIA isoform) is predominantly expressed by chondroprogenitor cells during early development and in the vitreous of the eye, while the shorter (IIB) form is expressed by differentiating cartilage chondrocytes in adults(McAlinden et al., 2008; Richards & Snead, 2008). Mutations in exon 2 of *COL2A1* can have dramatically different effects on pre-mRNA splicing, and lead to Stickler syndrome or other type II collagenopathies(Richards & Snead, 2008). Mutations in this exon usually cause a mild disorder, as demonstrated in a transgenic mouse model with a mutated 5' splice site of *COL2A1* exon 2; these mice with apparent expression of only the exon 2-containing IIA mRNA isoform displayed no overt phenotype and appeared healthy(Lewis et al., 2012). In contrast, the C-propeptide has been reported to play a crucial role in the formation of the collagen triple-helical structure(Doege & Fessler, 1986; Khoshnoodi, Cartailler, Alvares, Veis, & Hudson, 2006). After transfer into the ER, procollagen chains associate *via*their C-propeptides to form homotrimers, and this interaction is stabilized by disulfide bonds.

In summary, using a CRISPR/Cas 9 approach, we have established a pig model carrying compound *COL2A1* mutations. This *COL2A1*mutant model exhibits overt and typical phenotypes that resemble those of PLSD-T, and mainly involve abnormal early cartilage development. Our model paves the way for the establishment of large-animal models to simulate other hereditary skeletal diseases, such as ANFH and OA, and to develop effective prevention and treatment strategies.

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Competing interests

The authors declare no competing interests.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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Figure Legends

Figure 1. Generation of *COL2A1* knockout (KO) pigs.**(A)** The target position of amino acid for mutation are highly conserved among different species. **(B)** Schematic diagram of the strategy used to generate *COL2A1* KO pigs *via*ssODN-mediated homology-directed repair. Two gRNAs were used to target the pig *COL2A1* exon 27 to promote DNA breaks and homologous repair. **(C)** Target sequences of the two sgRNAs and complementary oligo sgRNAs. **(D)** Sequencing chromatogram showing a DNA break in exon 27. The arrow indicates the Cas9 cleavage site.**(E)** Sequencing chromatogram showing the compound heterozygous mutation of one of the cell clones selected for SCNT.

Figure 2. Phenotypic characterization and skeletal radiographs of the *COL2A1* knockout (KO) piglets. **(A)** Whole-body photographs of a symptomatic *COL2A1* KO piglet and a wild-type (WT) piglet shortly after birth. **(B)** The underdeveloped nose of the *COL2A1* KO pig compared with that of the WT pig. **(C)**Cleft palate of the KO pig compared with that of the WT pig.**(D)** Comparison of the fore- and hindlimbs between KO and WT piglets. **(E)** Comparison of average body weight, body length, length of the nose, and ratio of chest circumference to abdominal circumference. **(F)** Autoradiographs showing the abnormal systemic skeletal development in the *COL2A1* KO pig. A depressed nasal bridge and short maxilla were noted (yellow arrow). Additionally, lateral radiographic views showed the presence of ovoid vertebral bodies in the KO pigs (blue arrow). **(G)** Thinner and shorter ribs, smaller and splayed scapulae (yellow arrow), and underdeveloped vertebrae (blue arrow) in KO piglets compared with the same structures in WT piglets. **(H)** The underdeveloped skeleton of the forelimb in the KO pig compared with that in the WT pig. Shortened long bones with ragged metaphyses (yellow arrow) and brachydactyly with short metacarpals (blue arrow) can be observed in the KO pigs. **(I)**The underdeveloped skeleton of the hindlimbs of KO piglets compared with those of WT piglets. Iliac hypoplasia (yellow arrow), ragged metaphyses (blue arrow), and brachydactyly with short metacarpals (green arrow) were noted in KO piglets. **(J)** Comparison of the average length of the forelimbs, hindlimbs, maxilla, mandible, and spine between WT and KO piglets shortly after birth. White bar, 2cm. Data are presented as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 3. Abnormal gross morphology of the bones and organs in*COL2A1* knockout (KO) pigs compared with those of wild-type (WT) pigs. **(A)** Gross morphology of forelimb bones showing the greatly reduced lengths of the long bones and unclear margins of the epiphyses in the KO pigs; humerus, combined ulna and radius, and forehoof. **(B)** Gross morphology of hindlimb bones demonstrating the significantly shorter long bones and deformities in the hindlimbs of the KO pigs; femur, tibia, and combined fibula and hindhoof.**(C)** Gross morphology of the rib cage of *COL2A1* KO pigs showing a notable difference in size, indicating that the KO pigs have a narrower thoracic cavity. **(D)** Comparison of the average length of the WT and KO piglets. All the bones of the KO pigs were shorter than those of the WT pigs. **(E)** No obvious differences could be seen in the sizes and shapes of the organs (liver, heart, spleen, kidney, and stomach). **(F)** Comparison of the lungs showed that KO pigs presented smaller lungs and a thinner trachea (arrow). **(G)** The absolute weights of all the organs were marginally lower in the KO pigs. Lung volume and weight were significantly decreased in the KO pigs. White bar, 2cm. Data are presented as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant.

Figure 4. Histomorphology and pathology of hindlimb articular cartilage in *COL2A1* knockout (KO) pigs. **(A)** Hematoxylin and eosin (H&E)-stained sections of the femoral head. The boxed regions (A-α and A-β) represent the superficial, transitional, and deep zones of the articular cartilage in wild-type (WT) pigs. The

A-γ region represents the corresponding abnormal cartilage zones of *COL2A1*KO pigs, characterized by porous areas with enlarged and vacuolized chondrocytes (arrows). **(B)** H&E-stained sections of the knee joint. The boxed regions (B-α and B-β) represent the superficial, transitional, and deep zones of the articular cartilage in WT pigs. The B-γ boxed region shows the disordered cartilage zones of the knee joint of the KO pig, characterized by porous areas with enlarged and vacuolized chondrocytes (arrows). Black bar, 500 μm; red bar, 200 μm; green bar, 100 μm; blue bar, 50 μm.

Figure 5. Safranin O- and fast green-stained and immunohistochemical sections of articular cartilage of hindlimbs.**(A)** Safranin O- and fast green-stained sections showing femoral head articular cartilage morphology, demonstrating the lack of cartilage tissue formation in *COL2A1* KO pigs. **(B)**Immunohistochemical staining showing that the expression of type II collagen was lower in the femoral head of KO pigs than in that of WT pigs. **(C)** Safranin O- and fast green-stained sections showing knee articular cartilage morphology, demonstrating the lack of cartilage tissue formation in *COL2A1* KO pigs.**(D)** Immunohistochemical staining showing the decreased expression of type II collagen in the knee joints of KO pigs. Black bar, 500 μm; red bar, 200 μm; green bar, 100 μm; blue bar, 50 μm.

Figure 6. Histomorphology and pathology of the tracheal cartilage in *COL2A1* knockout (KO) pigs. **(A)** Hematoxylin and eosin (H&E)-stained tracheal sections. The results of the H&E staining demonstrated that the trachea of the KO pigs had collapsed, and the lumen was essentially obliterated. The A-γ boxed region shows the misaligned pseudostratified ciliated columnar epithelium of the*COL2A1* KO pigs compared with that of WT pigs (A-α). Furthermore, the A-β boxed region (WT pigs) shows stronger staining of the tracheal cartilage and a more uniform texture than that of the cartilage shown in the A-δ region (KO pigs). **(B)** The thickness of the tracheal cartilage in the KO pigs was comparable with that of WT pigs; however, there were fewer chondrocytes in the tracheal cartilage of the KO pigs.**(C)** Immunohistochemical staining showing that type II collagen expression in tracheal cartilage was decreased in KO pigs compared with that in WT pigs. **(D)** Picrosirius red staining demonstrating that there were fewer collagen fibers in the perichondrium and less hyaline cartilage in the trachea of KO pigs when compared with those of WT pigs. Black bar, 500 μm; red bar, 200 μm; green bar, 100 μm; blue bar, 50 μm. Data are presented as means ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significant.

Figure 7. Western blot and qPCR analyses of *COL2A1*expression levels. **(A)** Western blots of COL2A1 in cartilage tissues of the hip, knee, and ankle joints from three KO piglets (F0-1, F0-3, and F0-6). **(B)** Western blots of COL2A1 in cartilage tissues of the shoulder, elbow, and wrist joints from three KO piglets (F0-1, F0-3, and F0-6). **(C-E)** Relative *COL2A1* mRNA expression levels in hindlimb joints, forelimb joints, and tracheal cartilage as quantified by qPCR. **(F)** Computer modeling of the COL2A1 3D structure and impact of the *COL2A1* mono-allelic targeted mutations. Data are presented as means ± SEM. $*P< 0.05$; $**P < 0.01$; $***P < 0.001$.

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