

## Mini Review

## The Progress of CRISPR/Cas9-Mediated Gene Editing in Generating Mouse/Zebrafish Models of Human Skeletal Diseases

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## ABSTRACT

Genetic factors play a substantial role in the etiology of skeletal diseases, which involve 1) defects in skeletal development, including intramembranous ossification and endochondral ossification; 2) defects in skeletal metabolism, including late bone growth and bone remodeling; 3) defects in early developmental processes related to skeletal diseases, such as neural crest cell (NCC) and cilia functions; 4) disturbance of the cellular signaling pathways which potentially affect bone growth. Efficient and high-throughput genetic methods have enabled the exploration and verification of disease-causing genes and variants. Animal models including mouse and zebrafish have been extensively used in functional mechanism studies of causal genes and variants. The conventional approaches of generating mutant animal models include spontaneous mutagenesis, random integration, and targeted integration via mouse embryonic stem cells. These approaches are costly and time-consuming. Recent development and application of gene-editing tools, especially the CRISPR/Cas9 system, has significantly accelerated the process of gene-editing in diverse organisms. Here we review both mice and zebrafish models of human skeletal diseases generated by CRISPR/Cas9 system, and their contributions to deciphering the underpins of disease mechanisms.

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## 1. Introduction

The development of human skeletal system originates from the early embryonic period, followed by highly regulated growth and remodeling along the human life [1]. The disturbance of formation or maintenance of the skeletal system leads to a number of skeletal diseases, such as osteogenesis imperfecta, congenital scoliosis and osteoporosis.

Over the past few decades, it has been recognized that genetic factors play a substantial role in the etiology of human skeletal diseases [2]. Genetic defects can lead to skeletal diseases by interfering two processes: 1) prenatal development of the skeletal system or 2) postnatal bone metabolism. Representative diseases in the first category are osteogenesis imperfecta (OI) caused by *COL1A1*/*COL1A2* etc. and achondroplasia caused by *FGFR3* mutations [3,4], and congenital scoliosis resulted from compound inheritance of a rare null mutation and a hypomorphic allele of *TBX6* in a considerable proportion of disease populations (about 10%) [5–7]. Typical skeletal diseases in the second category include osteopetrosis and osteoporosis leading to an abnormal bone density [8,9]. Osteopetrosis is mainly caused by defects of osteoclast, accompanied by a decreased bone resorption activity. Genetic variants affecting osteoclast function are often correlated with osteopetrosis, such as variants in *TCIRG1*, *CLCN7*, *OSTM1*, *TNFSF11*, and *TNFRSF11A* (*RANK*) [10–13]. On the contrary, osteoporosis often occurs with decreased bone mass and low bone mineral density (BMD) [9].

Sequencing of human genome was accomplished in 2005, which has significantly driven the development of human genetics research [14]. Exome sequencing, as an efficient and cost-effective method, has promoted identification of novel genes and variants associated with genetic diseases [15–17]. Recently, whole-genome sequencing (WGS) emerged as a more powerful sequencing technique due to its whole coverage of human genome [18]. Although exome sequencing and WGS have generated a large number of potential candidate variants, many of them actually do not contribute to the disease. Even rare homozygous loss-of-function variants identified by WGS can sometimes appear to be clinically irrelevant to the corresponding diseases [19]. Therefore, function studies are indispensable to validate uncertain results from human genetic analysis and identify the true disease-causing variants/genes [20].

For the validation of a specific genetic variant, *in vitro* gene-modification techniques, including transgenesis, knock-out, and knock-in could be used [21,22]. Besides *in vitro* studies, animal models including mouse and zebrafish are also extensively used in studies of genetic diseases. Gene-modified animal models provide approaches for researchers to functionally verify the pathogenicity of candidate variants/genes and explore the disease mechanism [23,24].

The following part will review the advantages and disadvantages of several frequently-used animal models in the studies of skeletal diseases, advances in gene manipulation methods, and specific gene-edited animal models generated by CRISPR/Cas9 system which recapitulated various human skeletal diseases.

## 2. Animal Models in the Studies of Skeletal Diseases

In the studies of genetic skeletal diseases, mouse (*Mus musculus*) and zebrafish (*Danio rerio*) represent two important model animals.

Mouse is the most frequently used model animal for biomedical research [25]. Mouse is easy and inexpensive to breed. It takes 6–8 weeks to achieve sex maturity and 19–20 days to complete gestation. A female mouse can produce several litters per year, with approximately 5 cubs per litter. The skeletal phenotypes of mice and humans are comparable

both physiologically and anatomically. Micro-CT, digital X-ray microradiography and other techniques enable the characterization of the skeletal structure of mice [26,27]. An important advantage of the mouse model is the high homology between its genome and the human genome, which enables functional studies of human genes in this model [25].

Zebrafish is a small tropical freshwater fish, the skeleton of which is also similar to that of human. Key genes regulating skeletal development are conservative across zebrafish and human. Therefore, most human genes can be studied and validated in zebrafish models and the same bio-markers can be used. For example, *sp7* and *cathepsin K* can be used as the bio-marker of osteoblasts and osteoclasts respectively in both organisms [28,29]. Besides, easy maintenance and short life cycle of zebrafish can lower the cost and increase the efficiency of genetic research. The large brood size of zebrafish can offer a greater sample size. If properly bred, a female zebrafish can spawn every week or even at a shorter interval and generate hundreds of eggs in each clutch [30]. Further more, the zebrafish larvae are transparent and develop *in vitro*, which allows convenient observation and direct imaging of its skeletal development, making it an efficient tool to study skeletal diseases during the developmental process. Various techniques, such as live imaging, calcein staining, Alcian blue staining, micro-CT, have been developed to identify the skeletal phenotypes of both zebrafish larvae and adult fishes [31].

In comparison with mouse, zebrafish is more inexpensive and easier to breed. Its large brood size offers a large sample size for screening. In mouse genetic manipulation, labor-intensive embryo transplantation is usually required after microinjection. In contrast, the *in vitro* development of zebrafish can spare the procedure. These advantages make zebrafish especially suitable for high-throughput manipulation and screening.

Nevertheless, zebrafish genome usually has more than one orthologue for one human gene. In zebrafish knock-out models, the phenotypes of null mutants could be normal due to this genetic redundancy [32]. In contrast, mouse genome mostly has only one orthologous gene for each human gene. In addition, the skeletal structure of mouse is more similar to that of human compared to zebrafish [33].

## 3. CRISPR/Cas9 System in Animal Model Generation

Before gene manipulation became possible, the forward genetic approach was widely used to generate disease models. This approach requires random mutagenesis, which is whether spontaneous or induced by *N*-ethyl-*N*-nitrosourea (ENU), followed by high throughput forward screening. Since the mutagenesis is random and infrequent, it would take a long time to generate and validate an animal model with desired gene mutation [25,34]. In the early 1980s, scientists found that foreign DNA injected into zygotes can be integrated into the host genome via non-random microhomology-driven integration and nonhomologous end joining (NHEJ). In the following decades, microinjection of purified DNA into zygotes became the most common way to generate transgenic mouse or zebrafish models. Since the integration is unpredictable and inefficient, intensive screening is needed to select the subline with desired mutation, which is still costly and inefficient [35]. In the late 1980s, researchers made targeted integration possible via homologous recombination in mouse embryonic stem (ES) cells [36]. This approach requires microinjection of purified DNA into mouse ES cells and subsequent transplantation of ES cells into wild-type embryos. This approach increases the specificity of editing, but could only yield

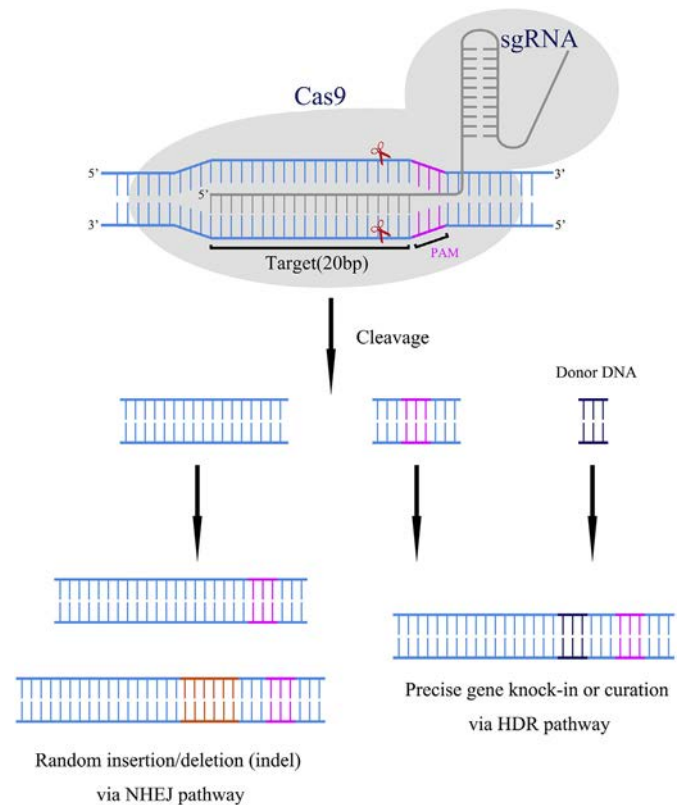
offspring with mosaic mutations. Several rounds of backcrosses are needed to yield a subline with germline transmission of the desired mutation, which makes it even more time-consuming than the random integration approach. Moreover, only a few ES cell lines are available, which limits the application of genome targeting via mouse ES cells [25].

In the last decade, the development of targeted gene editing tools, like zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and the CRISPR/Cas9 system, has revolutionized the process of model generation. Among the new tools, the CRISPR/Cas9 system has emerged as the most promising one. This system originates from the adaptive immune system of prokaryotes. Under the condition of infection, a fragment of the phage genome is taken up and integrated into the spacers of the CRISPR motif by the prokaryotes. The motif is then transcribed and processed into crRNA and tracrRNA. These transcription products can guide the Cas9 protein to the matching sequence of invading phage and cut it, generating double-strand breaks (DSBs). Researchers fused the crRNA and tracrRNA into a single guide RNA (sgRNA), which functions in the same way as the separated components do and has been widely used for its simplicity [37]. To direct the Cas9 nuclease to the specific sequence, the 20 nt at the 5' end of the sgRNA should be homologous to the target sequence and immediately followed by an NGG protospacer adjacent motif (PAM) sequence [38]. In 2013, researchers demonstrated that the CRISPR-Cas9 system can be used to edit multiple genes simultaneously in eukaryotic cells [39]. In the same year, researchers successfully generated mouse and zebrafish models with multiple mutations [40,41]. Since then, various disease models have been generated using the CRISPR/Cas9 system. Some adaptations, including using organism-specific codons in Cas9 RNA, were made to the CRISPR/Cas9 system to increase its activity in different organisms [41–45]. The RNA-guided targeting activity of the CRISPR/Cas9 system also potentiates it for other sequence-specific activities besides generating DSBs, such as transcription regulation, epigenomic modification, and base editing [46–49].

Like CRISPR/Cas9, ZFN and TALEN also have a targeting domain that directs them to a specific sequence and a nuclease domain capable of generating DSBs. The difference is their recognition of the specific sequence is based on protein-DNA interaction. But all these genome-editing tools achieve genome-editing by generating DSBs. Through microinjection into zygotes, these targeted nucleases can induce DSBs in a sequence-specific manner. As shown in Fig. 1, the DSBs can trigger endogenous DNA repair systems, including the NHEJ pathway and homology-directed repair (HDR) pathway [50]. The NHEJ pathway can repair the DSBs by directly joining the ends. This process is error-prone and may induce frame-shifting insertion or deletion (indel) at the excised site, thus knocking out the targeted gene. In contrast, the HDR pathway requires DNA templates to help repair the DSBs. When the targeted nucleases are co-injected with single-stranded oligodeoxynucleotides (ssODNs) or double-strand DNA (dsDNA) as donors for HDR, precise gene knock-in or curation can be achieved via the HDR pathway.

Precise gene knock-in and curation via the HDR pathway are usually preferable to the random indels generated via the NHEJ pathway, especially in clinical application. However, the efficiency of HDR is relatively low because most DSBs are repaired via the NHEJ pathway. Therefore, much effort has been made to increase the efficiency of the HDR pathway. Recent work has achieved that through optimization of templates, modification of the Cas9 protein, stimulation of the HDR pathway, and inhibition of the NHEJ pathway [51–54].

The simplicity, relatively low cost, and high efficiency are the most important advantages of CRISPR/Cas9 system. It only takes one day to assemble the CRISPR/Cas9 system. In contrast, both ZFN and TALEN rely on DNA-protein interaction to recognize specific sequences. The design of a ZFN and TALEN requires the assembly of various protein motifs and subsequent validation, which is very complicated and requires a high level of experiment skills. In the current protocol, it takes seven days to design and assemble a TALEN system [55–58]. Although the



**Fig. 1.** Mechanism of the CRISPR-Cas9 targeting system. 1. A sgRNA matches and binds to a 20-nt DNA sequence immediately upstream of an NGG DNA motif (Protospacer-Associated Motif, PAM). 2. The Cas9 protein is guided to the loci by the sgRNA and cuts both strands 3 bp upstream of the NGG. 3. The double-stranded DNA breaks activate the cellular DNA repair machinery, resulting in nonhomologous end joining (NHEJ) or homology-directed repair (HDR).

time required might be reduced to only one day, the complexity of the process is still a major drawback [59]. The simplicity and high efficiency of the CRISPR/Cas9 system also enable simultaneous editing of multiple loci and the introduction of different types of mutations. Besides indels, other types of mutations can also be achieved by targeting two loci simultaneously, which would be very complicated and time-consuming via the ZFN or TALEN approach. Small insertions can be achieved by co-injection of a small dsDNA or ssODNs. Large deletions can be achieved via NHEJ by co-injection of two sgRNAs targeting the two ends of the deletion. Co-injection of two sgRNAs and a circular DNA vector can trigger homologous recombination and introduce large insertions [25].

Nevertheless, the CRISPR/Cas9 system also has some limitations. The major limitation that restricts its clinical application is the off-target effect [60]. A few mismatches distal to the PAM region does not affect the activity of the CRISPR/Cas9 system. Therefore, sgRNAs may guide the Cas9 nuclease to the loci with sequences similar to the targeted loci and generate undesired mutations. As possible solutions to this drawback, optimization of sgRNA sequence and modification of Cas9 protein that degrades faster can both reduce the off-target effect [61]. Recent studies have also reported variant strains of Cas9 proteins with higher specificity [62,63]. Screening and engineering of Cas protein orthologues can also generate novel Cas protein orthologues with increased specificity and targeting range [47,64–67]. Recent studies have also reported many techniques to precisely detect off-target sites *in vivo*, which makes the potential consequences of the off-target activities more manageable [68]. Another major limitation of the CRISPR/Cas9 system is the mosaicism in founders (F0). This could be caused by the delayed transcription of Cas9 RNA, the persistent activity of the CRISPR/Cas9 system after the one-cell stage, or the variety of DNA-

repair mechanisms. Mosaicism can help avoid the lethality caused by homozygous null mutations, and can also lead to the generation of various mutant strains with different nucleotide changes and different mutation copies. However, in order to generate offspring with stable germline transmission of mutations, subsequent crosses and screening are needed, which is costly and time-consuming. Multiple improvements, such as using purified Cas9 protein instead of Cas9 RNA, engineering of Cas9 protein to accelerate its degradation, and embryo splitting, have been found to reduce mosaicism [69].

#### 4. Skeletal Diseases and Corresponding Animals Model Generated by the CRISPR/Cas9 System

In recent years, the CRISPR/Cas9 system has been extensively used for eukaryotic genome editing and has led to the generation of numerous animal models of skeletal diseases. Existing disease animal models cover a range of skeletal diseases, revealing the pathogenic processes and factors involved in the embryonic skeletal development and bone metabolism.

Here we review both mice and zebrafish models of human skeletal diseases generated by CRISPR/Cas9 system (Table 1), the etiologies of which could be divided into several categories: 1) defects in skeletal development, including intramembranous ossification defects and endochondral ossification defects; 2) defects in skeletal metabolism, including late bone growth defects and bone remodeling defects; 3) defects in early developmental processes related to skeletal diseases, like neural crest cell (NCC) and cilia defects; 4) disturbance of the cellular signaling pathways which potentially affect bone growth. Besides, human genes and the genotypes of corresponding animal models reviewed in this article are shown in Fig. 2 which illustrates the main processes of skeleton development and remodeling.

##### 4.1. Skeletal Diseases Caused by Defects in Osteogenesis

In the early stages of skeletal development, mesenchymal cells aggregate into condensations, then differentiate into multiple cell types, including osteogenic and chondrogenic lineages which play critical roles in osteogenesis [70]. Osteogenesis occurs by one of the two processes: intramembranous ossification and endochondral ossification, both of which are accompanied by simultaneous bone resorption and remodeling [71].

##### 4.1.1. Intramembranous Ossification Defects

Cranial bones, as well as some other flat bones, are formed through intramembranous ossification. As the basis of intramembranous ossification, mesenchymal condensation and ossification center

formation first take place in embryonic mesenchymal tissue. Then, osteoprogenitor cells differentiated from the condensed mesenchymal cells proliferate and form osteoblast layers, which secrete osteoid components [71]. Osteoblasts act as the builder in intramembranous ossification. Abnormalities in osteoblast can directly lead to several human skeletal diseases. For instance, the lateral meningocele syndrome (LMS) is a rare skeletal disease associated with osteoporosis [72]. Common clinical manifestations of LMS include the thickening of the cranial vault and craniofacial defects [73,74], which are etiologically related to abnormal intramembranous ossification. In 2015, Gripp et al. identified five novel de novo *NOTCH3* mutations in six unrelated patients. All the mutations were located in exon33 of *NOTCH3*, leading to truncated proteins without the sequence required for protein degradation. Therefore, they attributed the etiology of LMS to truncating variants in exon33 of *NOTCH3* [72]. However, there was limited knowledge about the mechanisms or the actions of *NOTCH3* in the skeleton. Hence, *in vivo* functional study of those truncating variants was required to further explore the disease mechanism that how such variants affect human skeleton system.

Canalis et al. utilized CRISPR/Cas9 system to generate heterozygous *Notch3<sup>tm1.1Ecan</sup>* mice strain [75]. *Notch3* sgRNA was designed to cleave between nucleotide 6691 and 6692 of exon 33 of *Notch3* gene in mice genome. A nonsense mutation was introduced into the *Notch3* gene of progenies. This mutation resulted in a truncated protein which deleted the sequence required for *NOTCH3* degradation, leading to an upregulated *NOTCH3* level. In *Notch3<sup>tm1.1Ecan</sup>* mice, the researchers observed a 35%–60% decrease in cancellous bone volume along with a reduction in trabecular number, which mimics the skeletal manifestation of human LMS [75]. Despite the skeletal manifestations reported in LMS, the mechanisms under LMS and the actions of *NOTCH3* were also studied and discussed with those CRISPR-generated mice. Activated Notch signaling, increased cell proliferation, and enhanced *Tnfrsf11* expression were observed in osteoblasts extracted from LMS mice [75]. Those results indicated an enhanced osteoblast number and function in LMS mice. However, enhanced bone formation was not sufficient to maintain skeletal homeostasis because of the simultaneous increase in osteoclast surface/bone surface, and the bone loss still occurred. In that study, the phenotype of the *Notch3<sup>tm1.1Ecan</sup>* mutant mouse was congruent with the bone loss observed in humans with LMS. Such similarity strongly supported the pathogenicity of corresponding human variants. Additionally, CRISPR-mediated variant knock-in provided opportunities to investigate the molecular mechanism of LMS in a mice model, promoting the research of disease pathogenesis.

**Table 1**

The contribution of CRISPR and zebrafish/mice/rat model to skeletal diseases.

Animal model	Mutated animal gene	CRISPR/Cas9 interventions	Animal phenotype	Modeling disease	Mutated human gene
Zebrafish	<i>mapk7</i>	Knock-out	Spinal curvature	AIS	<i>MAPK7</i>
Rat	<i>Bglap</i>	Knock-out	Increased trabecular bone, increased bone strength	NA	NA
Mice	<i>Notch3</i>	Knock-out	Marked osteopenia (decreased trabecular volume)	Lateral meningocele syndrome	<i>NOTCH3</i>
Zebrafish	<i>esf1</i>	Knock-out	Missing pharyngeal cartilages	Birth defects	NA
Zebrafish	<i>atp6v1h</i>	Knock-out	Bone loss and decreased bone formation	Osteoporosis	<i>ATP6V1H</i>
Mice	<i>Atp6v1h</i>	Knock-out	Bone loss and decreased bone formation	Osteoporosis	<i>ATP6V1H</i>
Zebrafish	<i>dyx1c1</i>	Knock-out	Spinal curvature	NA	NA
Zebrafish	<i>tapt1b</i>	Knock-out	Malformation of the cranial cartilage, disorganization of chondrocytes in the ceratohyal and ceratobranchial cartilage	Osteochondrodysplastic	<i>TAPT1</i>
Mice	<i>Bril</i>	Knock-in	Hypomineralization of the skull bones, bend limbs, wavy and thin dorsal ribs and wavy and thin dorsal ribs	Osteogenesis imperfecta	<i>BRIL</i> ( <i>IFITM5</i> )
Mice	<i>Sox9</i>	Enhancer elimination	Narrower and shorter rib cage	Campomelic dysplasia	<i>SOX9</i>

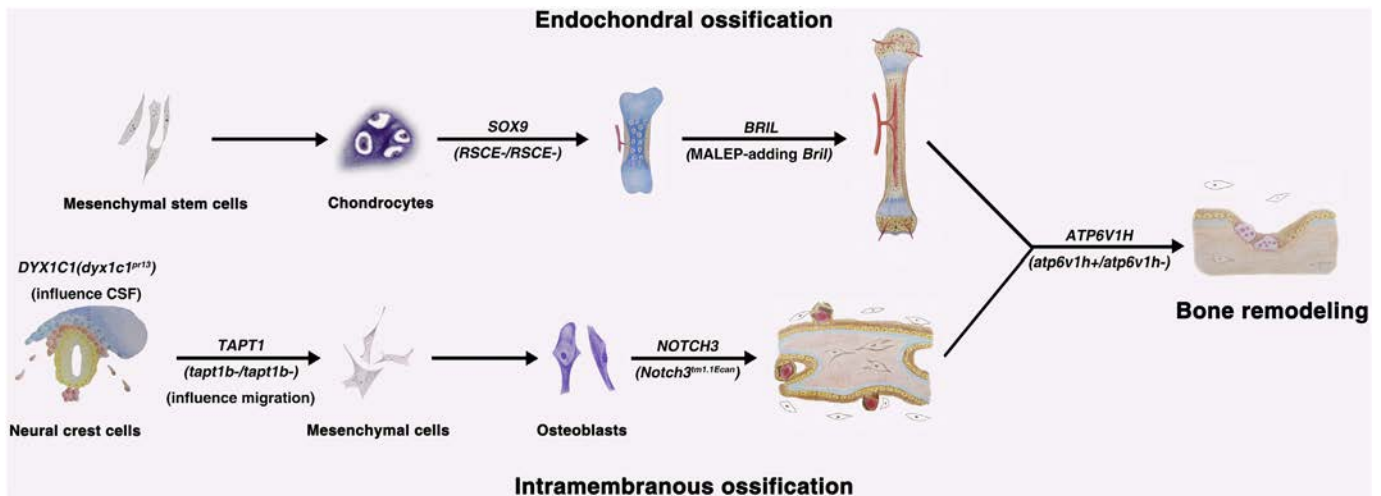


Fig. 2. Manipulation of genes participating in various physiological processes Human genes and genotypes/mutated-alleles of corresponding skeletal diseases related animal models are shown in the main physiological processes of bone development and remodeling. CRISPR/Cas9 system contributes to the establishment of those animal models. Abbreviations: CSF, cerebrospinal fluid.

#### 4.1.2. Endochondral Ossification Defects

Endochondral ossification is responsible for the initiation of osteogenesis in most components of human skeletal system, especially the long bones [76,77]. The most distinguished step of the endochondral ossification is the formation of hyaline cartilage models. After the hyaline cartilage models are formed, osteoblasts form a bone collar. The cartilage in the center starts to disintegrate, leading to a porous central region with a calcified remnant. Then, blood vessels invade and bring osteoprogenitor into the porous central region. After that, the primary and second ossification centers appear successively and promote the osteogenesis process. In the endochondral ossification process, the formation of hyaline cartilage is mostly studied [71]. Abnormalities of chondrocyte development could impede the endochondral ossification and cause various human skeletal diseases [78].

Campomelic dysplasia (CD) is a severe congenital disease characterized by impaired cartilage development. Common symptoms of CD include bowing and shortening of long bones, a bell-shaped thorax, narrow iliac wings, respiratory distress and other sexual problems [79–81]. As a subtype of CD, acampomelic campomelic dysplasia (ACD) shares all the other clinical phenotypes of CD except abnormal curvature of long bones [82]. Haploinsufficiency of *SOX9* has been identified in many ACD patients, implicating its crucial role in this disease [83]. *SOX9* is an important regulator of cartilage development [84,85], with a long enhancer region shared by different tissues [86–88]. Although the cartilage-specific *SOX9* enhancer has been expected to be 1 Mb upstream of *SOX9* based on genomic analyses of ACD patients [82,89,90]. More precise identification and characterization of the far upstream enhancer were still limited by the lack of successful and available approaches.

In recent years, the development of highly sequence-specific CRISPR/Cas9 system has offered researchers a better choice to localize and characterize the cartilage-specific *SOX9* enhancer. Mochizuk et al. utilized SIN3A-dCas9-mediated epigenetic silencing to further screen for the candidate enhancer range. A conserved region in 1.7 Mb *Sox9* upstream region was identified as cartilage-specific *SOX9* enhancer, named as rib cage-specific enhancer (RCSE). CRISPR/Cas9 system was then used to eliminate RCSE region and construct an ACD/CD disease model in mice. Two different sgRNAs respectively matched with the sequence upstream and downstream RCSE sequence were designed. Co-injection of those two sgRNAs and hCas9 mRNA successfully deleted the RCSE sequence, generating RCSE+/- and RCSE-/- mice strains. Distinctly defective rib cages were observed in RCSE+/- mice and RCSE-/- mice, which appear to be significantly shorter and narrower

compared to those of wild-type mice [91] phenotypes observed in RCSE null mice were consistent with the clinical phenotypes of human ACD/CD patients, providing opportunities to further study the mechanism and explore potential targeted therapies for ACD/CD.

In the former study, combinatorial analysis using the CRISPR/Cas9 system offered another approach for detailed investigation of the transcriptional mechanisms of inherited diseases. The identification of this new RCSE enhancer and its corresponding molecular functions will aid in the establishment of definitive diagnoses and identification of potential targets for the treatment of the ACD/CD. Moreover, CRISPR-generated RCSE null mouse could act as an animal model with impaired cartilage development, promoting the research focused on the skeletal diseases related to cartilage defect.

After the cartilage models are formed, during the process of endochondral ossification, bone collar and the primary ossification center play crucial roles. The formation of bone collar is accompanied by the impediment of nutrient supply and can lead to the hypertrophy and apoptosis of the chondrocytes, enlarging the lacunae and offering a porous structure for osteoblast covering. Simultaneously, calcification of cartilage matrix occurs, and the osteoblasts adhere to the cartilage remnant, forming primary ossification center. Disturbance of these normal processes will lead to serious skeletal defects [71].

Osteogenesis imperfecta (OI), clinically characterized by brittle bone [92], is mostly caused by autosomal dominant variants in *COL1A1* and *COL1A2* [3]. Those two genes code type I procollagen, which provides strength and resiliency to tissues such as bone. The pathogenic variants in *COL1A1* and *COL1A2* could decrease the expression of type I collagen or disrupt its structure, further affecting normal skeleton development [93]. The cartilage mineralization impediment is a classical phenotype of some subtypes of OI [94], which indicates abnormal endochondral ossification in such disease. As a specific type of OI, OI type V not only has common OI features like low bone mass and scoliosis, but also shows some particular features associated with ectopic mineralization such as the formation of “hyperplastic callus” and interosseous calcification of the forearm [95–97]. OI type V is caused by a single recurrent heterozygous mutation in the 5'-untranslated region (5'UTR) of *BRIL* gene. A novel translation starting site is created, which adds 5 amino acid residues (Met-Ala-Leu-Glu-Pro, denoted MALEP) on N-terminus of natural BRIL protein [98]. BRIL, almost exclusively expresses in osteoblasts, is a small 132-amino-acid transmembrane protein [99]. Although the MALEP-BRIL was involved in defective mineralization *in vitro* [99,100], its physiological functions *in vivo* still remain to be explored with the aid of animal models. Transgenic mice expressing OI type V MALEP-

BRIL were generated by conventional techniques before [100,101]. However, CRISPR/Cas9 system offered more efficient access to create knock-in mice model which perfectly mimicked the BRIL mutation of OI V patients.

Rauch et al. generated a knock-in mice strain through the CRISPR/Cas9 system. They synthesized gRNA targeted to 5'UTR of mice *Bril* gene and injected it into mouse embryos together with spCas9 mRNA. Two mosaic knock-in founders were screened through PCR genotyping, which contained the dominant MALEP-adding variant [102]. The CRISPR generated MALEP-BRIL heterogeneous mice offered a reliable animal model for OI type V. Striking skeletal anomalies such as hypomineralized skulls, short and bent long bones, and frail and wavy ribs were all observed in the MALEP-BRIL heterogeneous mice. The hypertrophic chondrocytes filling the midshaft of long bones were also observed, which indicated the abnormality of chondrocyte hypertrophy and the following cell apoptosis. MALEP-BRIL heterogeneous mice also exhibited less mineralization and excessive cartilaginitization, which were derived from the defective osteoblast differentiation and bone collar formation. Gene expression monitored in those knock-in mice embryos showed decreased osteoblast markers and overexpressed pro-inflammatory reaction related genes [102]. Such phenomena were consistent with the phenotypes observed in OI V patients, and indicated possible pathways (inflammation) which were abnormal in MALEP-BRIL heterogeneous mice.

#### 4.2. Skeletal Disease Caused by Defects in Bone Growth and Remodeling

After osteogenesis and early bone growth, the newly formed bone stays in a dynamic state, in which the resorption and formation of bone tissue keep proceeding. The bone shape is generally maintained or slightly changed, while the bone mass keeps increasing [71]. During the bone remodeling and growth, osteoblasts and osteoclasts undertake the primary responsibility [103]. Defects in osteoblasts and/or osteoclasts could break the balance between osteoblastic formation and osteoclastic resorption, causing diseases with abnormality of bone growth and remodeling [71], such as osteoporosis and osteosclerosis.

Osteoporosis is characterized by lower-than-normal maximum bone mass [104], elevating the risk of fracture. The disturbance of osteoblast and osteoclast activities could result in a decrease of bone density, giving rise to a strong propensity of osteoporosis [105]. Similarly, CRISPR/Cas9 generated animal models also provided novel information about osteoporosis. In the research mentioned above, CRISPR generated *Notch3<sup>tm1.1Ecan</sup>* mice also showed BMD-related phenotype mimicking human osteopenia [75]. Furthermore, *ATP6V1H*, a subunit of V-ATPase which plays important roles in the biological and physiological functions of osteoclasts [106], is also a candidate gene associated with osteoporosis [107]. In the early years, research works on this gene were based on large-scale retroviral insertional mutagenesis screen. A variant in *ATP6V1H* was screened from hundreds of zebrafish mutants and identified as pathogenic mutation in the visual system [108–111].

However, this forward genetics approach was unsuitable in the study of variants gained from clinical patients with specific diseases. The appearance of the CRISPR/Cas9 system supplied a powerful reverse genetic method, and offered better approaches for *ATP6V1H* function study in skeletal development and metabolism. Based on deleterious variants in *ATP6V1H* from clinical patients with osteoporosis (or low spine BMD), two groups generated *Atp6v1h(atpv61h)*-deficient mice and zebrafish respectively using CRISPR/Cas9 in the past five years. Frameshift mutations were introduced into both model animals and produced truncated proteins. The *atpv61h +/-* zebrafish showed a distinct reduction of bone mineral density, bone volume and bone surface, which were basically consistent with the human osteoporosis phenotypes. *Atp6v1h +/-* mice also had decreased bone remodeling and a net bone matrix loss. Furtherly, both studies focused on the pathogenic mechanism of *Atp6v1h(atpv61h)* variants. *In vitro* analysis targeted to the osteoclast and osteoblast from *atpv61h +/-* zebrafish revealed a

high level of expression *mmp9* and *mmp13*, which played a necessary role in extracellular matrix remodeling and possibly affected bone density. In *Atp6v1h +/-* mouse model, osteoclasts showed increased bone resorption. The TGF- $\beta$ 1 activation was downregulated on account of increased intracellular pH, thereby reducing the induction of osteoblast formation. Hence, the bone formation was reduced more than bone resorption in *Atp6v1h* deficient mice, possibly leading to osteoporosis [112,113].

In these studies, high throughput gene knock-out using CRISPR/Cas9 followed by extensive phenotyping could help elucidate the genetic basis of common skeletal diseases. CRISPR/Cas9 generated animal models play critical roles in the exploration of osteoporosis pathogenesis. Osteoblasts and osteoclasts extracted from CRISPR generated model animals with specific genotypes assisted the research of osteoporosis in cellular and molecular level.

#### 4.3. NCC and Cilia-Related Skeletal Diseases

Besides osteogenesis, bone growth and remodeling, defects in earlier embryo components such as neural crest cells (NCC) could also lead to skeletal malformations. The development of NCC is the foundation of the whole craniofacial skeleton/cartilage development [114]. As a necessary component of NCC, Cranial neural crest cells have the potential to make most of the cranial bones [115]. Hence the defect of neural crest cells can lead to significant craniofacial malformations [116], which account for about 75% of all human birth defects [115]. Relevant studies have been focused on the development and migration of neural crest cells to reveal the mechanism of craniofacial defects [117,118].

In the field of craniofacial defect research, zebrafish model is extensively used as it has relative transparent larvae and efficient cartilage/skeleton stain methods [119,120]. The disturbance of NCC development and migration had been induced separately in CRISPR-generated mutated zebrafish, offering insight into the mechanism of NCC related human skeletal disease. For instance, development of NCC is impeded in CRISPR-generated *esf1* mutated zebrafish strain. As an essential nucleolar protein, *Esf1* is involved in 18sRNA biogenesis [121] and partially expressed in pharyngeal primordia and pharyngeal arch of zebrafish. Chen et al. designed gRNA to knockout two conserved sequences in C terminus of *esf1*, in order to interrupt the function of *Esf1* protein. The *esf1* mutated zebrafish model exhibited severe NCC-derived pharyngeal cartilage loss and defects in the eyes, brain and heart. Such an animal model supported a cellular requirement for *Esf1* during neural crest survival and development [122]. In *esf1/-esf1*-zebrafish, NCC development was dramatically impaired, which made this zebrafish strain a suitable animal model for the research of cranial cartilage-related human diseases.

In recent years, the role of cilium in the process of NCC migration has been widely studied. Cilia are highly specialized microtubule-based organelles distributed on the cell membrane. They are responsible for signaling transduction, left-right (L-R) symmetry formation and central nervous system homeostasis [123–127]. Cilia also act as motile organelles, driving fluid movement and cell migration [122,124]. Symoens et al. analyzed two consanguineous families diagnosed as lethal osteogenesis imperfecta [126,128]. *TAPT1* homozygous variants were screened out from patients' genome. *TAPT1* encodes the evolutionary highly conserved transmembrane anterior posterior transformation 1 protein in centrosome and ciliary basal body. In the mechanism study, the *TAPT1* variants were found to disturb the Golgi morphology and trafficking and normal primary cilium formation. Therefore, the homologous gene, *tapt1b*, was knocked out in the zebrafish through CRISPR/Cas9 system. CRISPR generated *tapt1b/-* zebrafish model presented severe craniofacial cartilage malformations and delayed ossification. Such craniofacial cartilage defects were closely related to NCC migration dysfunction [126,128].

Apart from the establishment of NCC defect animal models, CRISPR/Cas9 was also used to explore the roles of cilia defect in human skeletal

diseases. As shown in the former study, cilia were involved in the craniofacial cartilage development and potentially contributed to lethal osteogenesis imperfect [128]. Aside from that, ependymal cell cilia lining brain ventricles can generate cerebrospinal fluid (CSF) by the polarized beating [129]. Multiple evidence supports a conserved role of CSF in human spine development [130–133]. In former studies, irregularities in CSF flow caused by cilia dysfunction have been demonstrated to lead to spine curvature and idiopathic scoliosis (IS) [130,134–136]. IS affects about 3% of children worldwide, causing spine deformities (scoliosis), reduced pulmonary function, and chronic pain [137–139]. Genetic factors play critical roles in the pathogenesis of IS, especially adolescent idiopathic scoliosis (AIS) [138,140]. As the etiology of IS is still unclear, the ‘cilia-CSF’ theory stands as an interesting hypothesis of IS pathogenesis [131,141]. Grimes et al. verified the critical role of cilia-driven CSF flow in spine development [130]. Besides, they also knocked out several cilia-related genes in zebrafish models through CRISPR/Cas9 system, which further proved the association between IS and cilia defect.

During the processes of mutant fish strains establishment, aberrant cilia motility caused a characteristic series of embryonic phenotypes that usually lead to death in 1 to 2 weeks of development, precluding further studies [142]. The phenomenon of lethality indicated the limitation of CRISPR, and could be restored with co-injection of WT mRNA. Transgenic cilia-gene mutants, similar phenotypes like severe three-dimensional spinal curvatures were also observed in the CRISPR-generated mutants [130]. Higher efficiency and greater convenience made CRISPR more accessible in the establishment of cilia defect zebrafish model, which benefited the research of cilia-related human skeletal diseases.

#### 4.4. Skeletal Disease and Cellular Signaling Pathway Disturbance

Genes participating in some typical signaling pathways, like the Notch pathway, BMP pathway, and FGFR pathway are often associated with skeletal system development impediment [72,143–147]. For instance, as mentioned above, variants in *SOX9* and *NOTCH3* which are located on typical signaling pathways both contribute to human skeletal diseases [75,91]. CRISPR/Cas9 system-mediated gene knock-out could disturb skeletal system related signaling pathways and generate mutated animal models with abnormal phenotypes of skeletal diseases. Analyses of transcriptome and proteome were accessible in animal cells, which help researchers explore the changes of signaling pathways in molecular level and dramatically assist the research about molecular mechanism of skeletal diseases. In recent years, some novel cellular signaling pathways have also been related to skeletal development with the help of CRISPR. In Gao and his colleagues' work, a *MAPK7* variant was screened from a three-generation Han Chinese family affected with AD-AIS. RAS/MAPK signaling pathway has been known to regulate cell proliferation and differentiation [148]. CRISPR technique was utilized for *in vivo* validation of *MAPK7* gene. Gao et al. designed a sgRNA targeted to *mapk7* and generated chimeric *mapk7*-disrupting indel zebrafish mutants. Zebrafish mutants presented severe spinal curvature and recapitulated human disease phenotypes, which indicated the important role of RAS/MAPK signaling pathway in the etiology of adolescent idiopathic scoliosis [24].

## 5. Summary and Outlook

The extensive utilization of next-generation sequencing and genomic association analysis has generated plentiful candidate genes and variants in congenital and adult skeletal diseases, which warrant function test and validation. Due to its high efficiency and specificity, CRISPR/Cas9 system has stimulated the generation of animal models for human skeletal diseases. CRISPR-generated animal models have enabled mechanism explorations of various skeletal diseases, which are mainly related to skeletal development and metabolism processes. Based on the elucidation of pathogenic mechanisms, CRISPR-

generated animal models could be coupled with drug screening and other therapeutic studies in the future [149].

However, there are still limitations to CRISPR/Cas9 system. Off-target gene knock-out effect would disturb the establishment of animal models and lead to potential false-positive results. Although the sequences and functions between human candidate genes and common model animals' orthologues are highly conserved, there are still natural genomic differences, impeding the confidence level of the proper skeletal disease model. Moreover, the cellular and molecular level changes caused by similar genomic operation can be dramatically different even opposite between human and model animals [75].

In conclusion, CRISPR/Cas9-editing is a powerful tool for genetic studies of human skeletal diseases. Extensive utilization of such efficient gene editing method will contribute to the future for mechanism exploration and therapeutic development of human skeletal diseases.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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