INTRODUCTION

Human FBN1 encodes a ~320 kDa extracellular matrix (ECM) protein fibrillin-1 that is widely expressed throughout the body and constitutes the backbone of microfibrils in the ECM. FBN1 contains three cysteine-rich repeating motifs, which are EGF motifs and LTBP or TB motifs and Fib motifs. The Fib motif is a unique hybrid motif of FBN1, which is formed by the fusion of EGF and LTBP.

CRISPR/Cas9 in zebrafish: An attractive model for FBN1 genetic defects in humans

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Abstract

**Background**: Mutations in the fibrillin-1 gene (FBN1) are associated with various heritable connective tissue disorders (HCTD). The most studied HCTD is Marfan syndrome. Ninety percent of Marfan syndrome is caused by mutations in the FBN1 gene. The zebrafish share high genetic similarity to humans, representing an ideal model for genetic research of human diseases. This study aimed to generate and characterize fb1+/− mutant zebrafish using the CRISPR/Cas9 gene-editing technology.

**Methods**: CRISPR/Cas9 was applied to generate an fb1 frameshift mutation (fb1+/−) in zebrafish. F1 fb1+/− heterozygotes were crossed with transgenic fluorescent zebrafish to obtain F2 fb1+/− zebrafish. Morphological abnormalities were assessed in F2 fb1+/− zebrafish by comparing with the Tuebingen (TU) wild-type controls at different development stages.

**Results**: We successfully generated a transgenic line of fb1+/− zebrafish. Compared with TU wild-type zebrafish, F2 fb1+/− zebrafish exhibited noticeably decreased pigmentation, increased lengths, slender body shape, and abnormal cardiac blood flow from atrium to ventricle.

**Conclusion**: We generated the first fb1+/− zebrafish model using CRISPR/Cas9 gene-editing approach to mimic FBN1 genetic defects in humans, providing an attractive model of Marfan syndrome and a method to determine the pathogenicity of gene mutation sites.

**KEYWORDS**

animal model, CRISPR/Cas9, fibrillin-1, Marfan syndrome, zebrafish
motifs. Among them, there are a total of 47 EGF motifs, of which 43 are calcium-binding epidermal growth factor (cbEGF) motifs that can bind to calcium ions. The FBN1 protein contains 7 LTBP motifs. The above three motifs play an important role in maintaining the structure and protein function of FBN1 (Jondeau et al., 2011; Jones & Ikonomidou, 2010; Ramirez & Pereira, 1999; Rantamäki et al., 1997; Robinson et al., 2006). Mutations in FBN1 are associated with various heritable connective tissue disorders (HCTD), such as Marfan syndrome (MFS; Gong et al., 2019; Jondeau et al., 2017), Weill-Marchesani syndrome (WMS; Karoulias et al., 2020; Newell et al., 2017), Shprintzen-Goldberg syndrome (SGS; Bari et al., 2019), MASS phenotype (mitral valve prolapse, non-progressive aortic enlargement, skin changes and scoliosis, thoracic deformities and joint hyperactivity; Piqueras-Flores et al., 2019), and Neonatal Progeroid syndrome (Muthu & Reinhardt, 2020), accounting for ~90% of MFS cases. Mutations in the FBN1 gene can also be found in other fibrillinopathies that do not meet the diagnostic criteria of MFS (non-syndrome), such as isolated ectopia lentis or congenital ectopia lentis (Cao et al., 2019; Yang et al., 2020; Zhang et al., 2018), familial thoracic aortic aneurysm and dissection (Arnaud et al., 2019; Takeda & Komuro, 2019), isolated skeletal features (Lin, Zhao et al., 2020; Smaldone & Ramirez, 2016; Vasques et al., 2019), and so on. To date, more than 3000 mutations have been identified in FBN1 and are generally classified into missense mutations, frameshift mutations, nonsense mutations, splicing mutations, and inframe deletions or insertions (Arnaud et al., 2017; Gong et al., 2019; Lin, Liu et al., 2020; Xu et al., 2020). Most of the mutations lead to a wide range of clinical manifestations involving the integumentary, skeletal, ocular, central nervous, and cardiovascular systems, from hypopigmented or hyperpigmented scars, tall stature resulting from the overgrowth of the long bones in the limbs, to life-threatening cardiovascular abnormalities, suggesting complicate genotype-phenotype correlations in connective tissue disorders resulting from fbn1 mutation (Sakai et al., 2016). MFS is a euchromatic dominant genetic disease with incomplete penetrance. The clinical phenotype is complex and the variability is large. There are large phenotypic differences between different families and even different patients of the same family. At present, more than 1,700 FBN1 gene mutations (Bitterman & Sponseller, 2017; Groth et al., 2017; Li et al., 2017) have been discovered that can lead to MFS. Exons can also be lost during the splicing process of transcription products. The mutation sites are randomly distributed in each exon of the entire FBN1 gene, and there are no obvious mutation hotspots; 20%–35% are scattered cases caused by new mutations. Most studies (Mastromoro et al., 2021; Tognato et al., 2019) have shown that exons 24–32 of the FBN1 gene encode the 11th–18th cbEGF and the third LTBP domain and its mutations are often associated with neonatal Marfan syndrome (nMFS). nMFS is the most serious type of MFS. Patients suffer from mitral valve or tricuspid valve insufficiency at birth or shortly after birth, and usually die within one year after birth. Severe congestive heart failure is the main cause of death. In addition, some severe phenotypes of classic MFS and non-classical MFS (Damrauer et al., 2019; Milleron et al., 2020) are also associated with mutations in this region, suggesting complicate genotype-phenotype correlations in MFS.

According to NCBI database, 310 organisms have orthologs with human FBN1, such as mouse, rat, pig, and zebrafish (https://www.ncbi.nlm.nih.gov/gene/?term=ortholog_gene_2200 [group]. Accessed 14 February 2021). Numerous studies have used mouse models to investigate FBN1 mutation in MFS (Hibender et al., 2019; Park et al., 2019; Sato et al., 2018; Tran et al., 2019). A study has reported a heterozygous fbn1 mutant pig model that exhibits phenotypes resembling the symptoms of human MFS (Umeyama et al., 2016). However, considering the small number of offspring and relatively high cost, the application of mouse or pig model in the high-throughput genetic analysis is challenging. Recently, zebrafish has emerged as a popular vertebrate model for genetic research (Busse et al., 2020; Torraca & Mostowy, 2018). The advantages of zebrafish in human disease modeling include optical transparent embryos, high fecundity, low-cost husbandry, short life cycle, ease of experimental manipulations, as well as a high degree of homology with human genes (Fontana et al., 2018). Sequencing of zebrafish has revealed that ~70% of human genes have functional homologs in zebrafish (Howe et al., 2013), suggesting that zebrafish is an attractive model for most human genetic diseases. However, no reports are available regarding the generation of fbn1 (https://www.ncbi.nlm.nih.gov/gene/100330961, updated on June 13, 2020) mutation models in zebrafish. Recently, the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) technology has achieved tremendous progress in genome editing and has been applied in zebrafish to alter gene transcription and function (Barman et al., 2020; Cornet et al., 2018; Gupta et al., 2019). A typical CRISPR/Cas9-mediated mutagenesis pipeline in zebrafish includes small guide RNA (sgRNA) design and generation, micro-injection of a preassembled sgRNA/Cas9 complex in one-cell stage embryos (F0), and generation of F1 heterozygous carriers and F2 homozygous mutant larvae (Albadri et al., 2017; Hoshijima et al., 2019). Scientists have successfully applied this pipeline in generating mutant zebrafish models for various human diseases, such as autism spectrum disorder (Liu et al., 2018), genetic cardiovascular disorders (Tessadori et al., 2018), and liver diseases (Kim et al., 2019).
However, the application of CRISPR/Cas9 technology in generating \textit{fbn1} mutant zebrafish has not been reported yet.

Based on the standards and guidelines for the interpretation of sequence variants of the College of Medical Genetics and Genomics and the Association for Molecular Pathology (2015; Richards et al., 2015), the evidence levels of mutation site analysis were as follows: PVS: very strong pathogenicity; PS: strong pathogenicity; PM: moderate pathogenicity; PP: supporting pathogenicity. The \textit{FBN1} mutation has evidence of PS2+PM2+PP1+PP3+PP4. PS2 refers to a new mutation, detected by the proband, but not detected by the parents; PM2: in the ESP database, the Thousand People database, and the EAC database. The allele frequency is 0; PP1: mutations and diseases are co-segregated in the family; PP3: multiple computer simulation calculations predict harmful; and PP4: mutation-related diseases are highly consistent with clinical phenotypes. Therefore, we conclude from the evidence supporting \textit{FBN1} mutation that its classification belongs to likely pathogenic, which can be used for prenatal diagnosis. The purpose of this study is to construct a zebrafish model with \textit{fbn1} gene knockout by CRISPR/cas9 technology, so as to confirm whether the model exhibits phenotypes resembling the symptoms of human MFS. The significance of establishing such an \textit{fbn1} gene knockout zebrafish model is to provide a model and method to determine the pathogenicity of \textit{FBN1} gene mutation sites in clinic, which is used to be the evidence supporting \textit{FBN1} mutation classification. With advantages of large number of offspring and relatively low cost, it also provides a model to investigate \textit{FBN1} mutation in MFS.

In this study, we generated and characterized the first CRISPR/Cas9-mediated loss-of-functional \textit{fbn1} mutation (\textit{fbn1}+/−) model in zebrafish.

### 2 | MATERIALS AND METHODS

#### 2.1 | Zebrafish care and husbandry

TU wild-type zebrafish and transgenic \textit{Tg(cmlc2:eGFP)} zebrafish possessing a green fluorescent heart (Siegert et al., 2018) were obtained from Model Animal Research Center of Nanjing University (Nanjing Jiangsu, China). Embryos were treated with 0.003% tyrosine inhibitor 1-phenyl-2-thio-urea (cat# P7629-10G, Sigma-Aldrich) to inhibit pigment formation. The fish were maintained at 26 ± 2°C under a 14 hr light: 10 hr dark cycle in a zebrafish circulation breeding system (ESON; Beijing Aisheng Technology Development Co., Ltd.). To generate offspring, mating was carried out at a ratio of 1:1, followed by natural spawning.

#### 2.2 | Generation of Cas9 transgenic zebrafish

\textit{fbn1} gene has not been sequenced in many databases, this design is based on NCBI database (https://www.ncbi.nlm.nih.gov/gene/XM_017351990.2), \textit{fbn1} sgRNAs (CRISPR1: TGGGAAAGAGCTTGTGCTACAGG; CRISPR2: TCCGA CAACGCCACATGTGACGG; CRISPR3: CCAGGCGCG GCCGATGTGTAGG; CRISPR4: GGGAAACGGACA CTTTCGCGAGG) were designed and synthesized by Nanjing YSY Biotech Company. The target sequences are in exon 16, near to the 1731st amino acid. A mixture (1 nl) of each sgRNA (80–100 ng/µl) and Cas9 protein (200 ng/µl; cat# P-020, Nanjing YSY Biotech) was injected into embryos (F0). The F0 embryos injected with Cas9/sgRNA were raised to sexual maturity, and four pairs of female and male zebrafish were mated to obtain F1 embryos.

#### 2.3 | Genotyping of transgenic zebrafish

Genomic DNA was obtained from F1 embryos at 24 hr post-fertilization (hpf) for genotyping using the forward (5′-GAATCCTGGCATCTGTGGTC-3′) and reverse (5′-TTGCGCAAATCTTTACTCAAA-3′) primers. The PCR protocol was 95°C for 3 min, 30 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 1 min, and 72°C for 10 min. PCR products were sequenced to identify the mutation. F0 with heritable mutations were selected to mate to generate F1 offspring, followed by genomic DNA collection at 2 months post-fertilization for genotyping. PCR reaction was performed using the primers and PCR protocol mentioned above, followed by sequencing. To confirm the genotype carried by the mutants, the PCR products of mutant 1 and mutant 2 carrying the \textit{fbn1} frameshift mutation were recombined into the pGEM-T Easy vector. After transformation, a single colony was selected for PCR identification. The confirmed F1 \textit{fbn1}+/− heterozygotes were used in the following investigation.

#### 2.4 | Morphology assessment

The F1 adult \textit{fbn1}+/− zebrafish were mated with \textit{Tg(cmlc2:eGFP)} fish to generate F2 transgenics. The morphology of F2 embryos/larvae and TU control group were examined at 72 hpf, 120 hpf, 12 dpf, 19 dpf, 26 dpf, 33 dpf, and 40 dpf using a stereomicroscope (MC50-S; Mingmei). Images were acquired using an SZX7 camera (Olympus). The initial sample size was 310 in F2 \textit{fbn1}+/− zebrafish experimental group and TU control group, respectively. The death rate was about 3%. The abnormal phenotypes of the F2 and TU groups were analyzed statistically. The experiment was repeated three times.
2.5 | All reagents used in the experiment include the following

YSY buffer (cat# R-001, Nanjing YSY Biotech), Zebrafish genotyping kit (cat# K-101, Nanjing YSY Biotech), 2-Mastermix (cat# P111-01, Vazyme Biotech Co., Ltd), Ultrapure water without ribozyme pollution (cat# R-002, Nanjing YSY Biotech), 1-phenyl-2-thiourea (cat# P7629-10G, Sigma-Aldrich), T7 in vitro Transcription Kit (cat# P7629-10G, TaKaRa), MAXiScript SP6/T7 (cat# AM1314, Ambion), PCR clean up Kit (cat# AMP-MN-P-250, Axygen), DL5000 marker (cat#3428A, TaKaRa), Ampicillin (cat# A610028, Sangon Biotech Co., Ltd), DNase I (cat# 2238G2, Ambion), PCR-related reagent T4 ligase (cat# M0202L, New England Biolabs), PGM-Teasy Vector (cat# A1360, Nanjing YSY Biotech), Cas9 protein (cat# P-020, Nanjing YSY Biotech), Conventional chemical reagent (cat# 10010360, cat# 30148126, cat# 71001453, Shanghai Chemical Reagent, Co., Ltd; cat#34943-6X1L, cat#296821-1L, cat# 284505-2L, Sigma Aldrich).

2.6 | Ethical Compliance and Ethical Considerations

The animal protocol was approved by an ethics committee of the Institutional Animal Care and Use Committee of The 1st Medical Center of Chinese PLA General Hospital (Beijing, China). All animal experiments were performed following the guidelines for animal welfare in Laboratory of Translational Medicine of The 1st Medical Center of Chinese PLA General Hospital.

3 | RESULTS

3.1 | Generation of \( \text{fbn1}^{+/-} \) zebrafish

By comparative analyses using NCBI and Ensembl resources, we found that \( \text{fbn1} \) is orthologous between humans and zebrafish (Figure S1), suggesting that zebrafish is an ideal model for genetic research on \( \text{fbn1} \) mutation. To establish an \( \text{fbn1} \) mutant zebrafish
model, we designed 4 sgRNAs in exon 16 of fbn1 and tested their efficiency by co-injecting with Cas9 protein into one-cell stage zebrafish embryos (Figure S2). DNA sequencing of target-specific PCR products confirmed that the fbn1 targeted allele carried a deletion of 3 bases and an insertion of 16 bases, resulting in a frameshift mutation and truncated FBN1 protein after the mutation (Figure 1). PCR analysis showed that the wildtype allele generated a band of 338 bp, whereas the mutant allele generated two bands of 351 bp and 338 bp (Figure 2a). Then, we generated F2 transgenics fish by mating adult F1 fbn1+/− fish with Tg(cmlc2:eGFP) fish. PCR analysis and sequencing confirmed that the randomly selected F2 fbn1+/− heterozygous mutants carried the mutations (Figure 2b–e). These results suggest that we successfully generated a transgenic line of fbn1+/− zebrafish.

3.2 F2 fbn1+/− zebrafish exhibits morphological and cardiac defects

Then, we examined whether our transgenic zebrafish exhibit abnormal phenotypes of connective tissue genetic diseases. Figure 3 shows the morphology of F2 fbn1+/− zebrafish in the early stage of development (72 and 120 hpf). Figure 4 shows that compared with TU wildtype zebrafish, F2 fbn1+/− zebrafish exhibited noticeably decreased pigmentation, increased body lengths, cardiac defects (abnormal cardiac blood flow from atrium to ventricle) and slender body shape. Some F2 fbn1+/− zebrafish appeared more transparent and slender at 40 dpf than those at earlier developmental stages.

The dynamic video demonstrated abnormal cardiac blood flow from atrium to ventricle in F2 fbn1+/− zebrafish throughout the developmental stages. In addition, F2 fbn1+/− zebrafish at 33 and 40 dpf showed less and slower

![Figure 2](image-url)
cardiac blood flow compared with the wild-type zebrafish. Taken together, these findings suggest that \( fb1n^{+/−} \) zebrafish exhibit typical features for connective tissue genetic diseases due to \( FBN1 \) mutations.

### 3.3 Statistical results of abnormal phenotypes in F2 and TU groups:

The initial sample size was 310 in F2 \( fb1n^{+/−} \) zebrafish experimental group and TU control group, respectively. The death rate was about 3%. The experiment was repeated three times. The abnormal phenotypes of F2 and TU groups were analyzed statistically. The body length (unit: mm), melanin reduction, and cardiac defects were observed in TU (control group) and F2 (experimental group) at 12 dpf/19 dpf/26 dpf/33 dpf/40 dpf. The two abnormal phenotypes of melanin reduction and cardiac defect were observed in three visual fields, each with about 100 samples. The statistical software was SPSS 22.0. There was statistical significance when \( p < .05 \).

T-test was used to analyze the body length data of F2 and TU control groups. The results of three experiments showed that the body length of F2 group was significantly longer than that of TU group at 12 dpf, 19 dpf, and 26 dpf (\( p < .05 \)); In the second experiment, F2 group compared with TU control group at 40 dpf, \( p = .952 \), without statistical significance; In the third experiment, F2 group compared with TU group at 33 dpf, \( p = .164 \), without statistical significance. In short, the body length of F2 \( fb1n^{+/−} \) zebrafish increased significantly throughout the developmental stages, especially at the early stage (Table 1).

Chi-square test was used to analyze the data of hypopigmentation and cardiac defects in F2 and TU control groups at 12 dpf/19 dpf/26 dpf/33 dpf/40 dpf. The results of three experiments showed that the pigmentation of F2 group was significantly lower than that of TU group at 12 dpf, 19 dpf, and 26 dpf (\( p < .05 \)) and there was no significant difference between F2 and TU groups at 40 dpf (\( p > .05 \)). According to the results of the first and second experiments, there was no significant difference at 33 dpf (\( p > .05 \)). The above results showed that the level of
pigmentation of F2 group decreased significantly at the early stage (12 dpf/19 dpf/26 dpf), but there was no significant difference between the two groups at the later stage (Table 2). The statistical results of the three experiments showed that F2 had significant heart defects compared with TU control group at 12 dpf/19 dpf/26 dpf/33 dpf/40 dpf ($p < .05$; Table 3).

4 | DISCUSSION

MFS is characterized by pleiotropic manifestations at least partially due to the great number of mutations identified in FBN1 (Gong et al., 2019; Jondeau et al., 2017). The pathogenesis of MFS was secondary to the mutation of FBN1 gene, which resulted in the dysfunction of the structure and function of the extracellular matrix, and then the loss of connective tissue integrity (Gong et al., 2019; Jondeau et al., 2017). Many studies (De Cario et al., 2018; Siegert et al., 2018; Takeda et al., 2018) have shown that FBN1 protein is related to transforming growth factor-β (TGF-β) signal transduction function in the extracellular matrix. Mutations in the FBN1 gene can make TGF-β signal transduction dysregulated, and the pathogenesis of MFS may be related to abnormal TGF-β signaling. A large-scale genotype–phenotype mapping is required for a better understanding of the contributions of FBN1 mutations in MFS. Compared with the most popular model organism mouse that has a relatively small number of offspring and expensive husbandry, the zebrafish has become a better model organism for large-scale genetics projects (Busse et al., 2020; Torraca & Mostowy, 2018; Varshney et al., 2015). In this study, we generated the first $fbn1^{+/−}$ zebrafish model using the CRISPR/Cas9 gene-editing tool and characterized the morphological and cardiovascular abnormalities of $fbn1^{+/−}$ zebrafish at the larval stage. The TB domain truncation resulting from $fbn1^{+/−}$ mutation caused noticeable hypopigmentation, increased length, slender body shape, and cardiovascular defects in zebrafish larvae, reminiscent of human MFS.

By comparing FBN1 protein sequences between human and zebrafish, we found that human FBN1 protein is highly conserved in zebrafish, consistent with previous reports (Cetinkaya et al., 2018; Gao et al., 2019). FBN1 performs...
similar functions among different species. As the major component of microfibrils that regulate elastic fiber formation, FBN1 provides the scaffold for elastin deposition to maintain the structural integrity of the vessel wall during embryonic development and early postnatal life. The neonatal demise of fbni−/−mice due to ruptured aortic aneurysm suggests that fbni is required for the blood vessel structure and function during neonatal life (Cook et al., 2012). Chen et al. have demonstrated that injection with morpholino antisense oligomers against fbni into zebrafish embryos leads to dilated vessels around the eyes and in the head, suggesting that fbni is essential for vascular development and function in zebrafish (E. Chen et al., 2006). Thus, it is reasonable to generate a zebrafish model to mimic FBN1 genetic defects in humans.

Although zebrafish and humans share abouchomology, there are still big differences. So far, the most studied genotype–phenotype correlation is the 11–18 common sequence of cbEGF encoded by exon 24–32 and the third LTBP sequence of human FBN1 gene related to nMFS, which is the most serious form of MFS. The original intention of this study is to use CRISPR/Cas9 technology to target the cbEGF sequence and the third LTBP sequence of zebrafish fbni gene to simulate the zebrafish model of nMFS. However, we found that there are three cbEGF domains in zebrafish fbni protein during CRISPR design, which are significantly different from the cbEGF domain of human FBN1 protein. Moreover, the third LTBP domain of zebrafish fbni protein is ahead. Therefore, we plan to knockout the third LTBP domain in fbni protein, but because the blast of NCBI cannot give the genome sequence near the third LTBP domain (RID: 4yevbx78014), we change to knock out the cbEGF domain in fbni protein. However, due to our previous knockout design in cbEGF domain of fbni protein, we could not obtain sgRNA that could effectively guide Cas9 to cleave fbni, so we abandoned the idea of zebrafish model simulating nMFS and replaced the design site. According to the results of NCBI blast alignment, zebrafish fbni gene began to have genomic support in 1362aa. Considering that there are many repeats in the intron of the gene, in order to achieve gene knock out and avoid gene compensation, we designed the target sequence (CRISPR) of fbni gene to be targeted near 1731aa (after the potential start codon). By causing frameshift mutation, we can achieve the goal of targeted knock-out of fbni gene.

In our study, the fbni targeted allele carried a frameshift mutation containing a 3-base deletion and a 16-base insertion, resulting in a truncated FBN1 with a loss of a TB domain after the mutation. This frameshift mutation might affect FBN1 function by disrupting the protein structure or the binding of calcium with the cbEGF domain. It has been reported that of all the identified
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FBN1 mutations, 38.6% lead to a truncated FBN1 protein (Lin, Liu, et al., 2020). Studies have shown that truncating FBN1 mutations is strongly associated with cardiovascular events in patients with MFS (M. Chen et al., 2018; Tan et al., 2017; Wang et al., 2016). Likewise, in our study, F2 fbn1+/− zebrafish larvae showed obviously abnormal phenotype of the heart. The dynamic video of the heart showed that the cardiac blood flow from atrium to ventricle in some F2 fbn1+/− zebrafish was different from that of the control group. The statistical results of the three experiments showed that F2 had significant heart defects compared with TU group at 12 dpf/19 dpf/26 dpf/33 dpf/40 dpf (p < .05; Table 3). In addition, F2 fbn1+/- zebrafish at 33 dpf and 40 dpf had less and slower cardiac blood flow compared with the control group. These results suggest that the fbn1 mutation in our study affects cardiovascular development throughout the life span of the F2 fbn1+/- zebrafish and the abnormal cardiac phenotype gradually deteriorates with growth and development.

A study has reported that hypopigmented or hyperpigmented scars are present in 46% of patients with MFS versus 21% of control subjects (Ledoux et al., 2011). The abnormal pigmentation may be related to SLC24A5, which is 266 kb upstream of FBN1 in the human genome and plays a key role in skin pigmentation in humans as well as in zebrafish (Lamason et al., 2005). Similar to that observed in humans, our results showed that F2 fbn1+/− zebrafish exhibited noticeably decreased pigmentation in the larval stage. Fibrillin scaffolds contribute to TGF-β/bone morphogenetic protein signaling during bone growth and metabolism. Despite the low abundance of fibrillins in skeletal tissues, mutations in FBN1 or FBN2 lead to skeletal abnormalities (Smaldone & Ramirez, 2016; Xu et al., 2019). As a result, tall, thin body habitus and long limbs are typical skeletal characteristics in patients with MFS (Sivasankari et al., 2017). To evaluate whether fbn1 mutation in this study can mimic FBN1 genetic defects in the human skeletal system, we measured the length of zebrafish and found that F2 fbn1+/- zebrafish exhibited

TABLE 2 The statistical results of decreased pigmentation of F2 and TU groups

| Decreased pigmentation | The first experiment | The second experiment | The third experiment |
|------------------------|---------------------|----------------------|---------------------|
|                        | TU      | F2      | p       | TU      | F2      | p       | TU      | F2      | p       |
| 12 dpf                 | 310     | 310     |         | 310     | 310     |         | 310     | 310     |         |
| Percentage             | 34 (11.3%) | 154 (49.7%) | <.001  | 31 (10.3%) | 155 (50.6%) | <.001  | 34 (11.3%) | 139 (44.8%) | <.001  |
| 19 dpf                 | 308     | 306     |         | 306     | 306     |         | 307     | 308     |         |
| Percentage             | 30 (9.7%) | 102 (33.3%) | <.001  | 34 (11.1%) | 111 (36.3%) | <.001  | 32 (10.4%) | 110 (35.7%) | <.001  |
| 26 dpf                 | 305     | 304     |         | 303     | 304     |         | 305     | 305     |         |
| Percentage             | 26 (8.5%) | 52 (17.1%) | .002   | 25 (8.3%) | 60 (19.7%) | <.001  | 26 (8.5%) | 57 (18.7%) | <.001  |
| 33 dpf                 | 303     | 302     |         | 301     | 302     |         | 303     | 302     |         |
| Percentage             | 24 (7.9%) | 38 (12.6%) | .059   | 23 (7.6%) | 36 (11.9%) | .077   | 22 (7.3%) | 45 (14.9%) | .003   |
| 40 dpf                 | 300     | 300     |         | 300     | 299     |         | 301     | 300     |         |
| Percentage             | 26 (8.7%) | 31 (10.3%) | .486   | 23 (7.7%) | 27 (9.0%) | .546   | 23 (7.6%) | 26 (8.7%) | .646   |

TABLE 3 The statistical results of cardiac defects of F2 and TU groups

| Cardiac defects | The first experiment | The second experiment | The third experiment |
|-----------------|---------------------|----------------------|---------------------|
|                 | TU      | F2      | p       | TU      | F2      | p       | TU      | F2      | p       |
| 12 dpf          | 310     | 310     |         | 310     | 310     |         | 310     | 310     |         |
| Percentage      | 13 (4.2%) | 266 (85.8%) | <.001  | 13 (4.2%) | 265 (85.5%) | <.001  | 10 (3.2%) | 280 (90.3%) | <.001  |
| 19 dpf          | 308     | 306     |         | 306     | 306     |         | 307     | 308     |         |
| Percentage      | 10 (3.2%) | 255 (83.3%) | <.001  | 11 (3.6%) | 260 (85%) | <.001  | 6 (2.0%) | 280 (90.9%) | <.001  |
| 26 dpf          | 305     | 304     |         | 303     | 304     |         | 305     | 305     |         |
| Percentage      | 8 (2.6%) | 249 (81.9%) | <.001  | 8 (2.6%) | 262 (86.2%) | <.001  | 8 (2.6%) | 268 (87.9%) | <.001  |
| 33 dpf          | 303     | 302     |         | 301     | 302     |         | 303     | 302     |         |
| Percentage      | 8 (2.6%) | 262 (86.8%) | <.001  | 9 (3.0%) | 255 (84.4%) | <.001  | 8 (2.6%) | 264 (87.4%) | <.001  |
| 40 dpf          | 300     | 300     |         | 300     | 299     |         | 301     | 300     |         |
| Percentage      | 5 (1.7%) | 240 (80.0%) | <.001  | 5 (1.7%) | 254 (84.9%) | <.001  | 4 (1.3%) | 260 (86.7%) | <.001  |
noticeably increased lengths and slender body shape compared with the wildtype counterparts. The statistical results of three experiments showed that the body length of F2 fbni1+/− zebrafish increased significantly throughout the developmental stages, especially at the early stage (Table 1). The statistical results showed that the level of pigmentation of F2 group decreased significantly at the early stage (12 dpf/19 dpf/26 dpf), but there was no significant difference between the two groups at the later stage (Table 2). Some F2 fbni1+/− zebrafish looked more transparent and more slender at 40 dpf. It is suggested that there is a certain phenotypic difference between the F2 generation and the two phenotypes of “increased body lengths and cardiac defects” are widespread, and the heart abnormality gradually deteriorates. The biggest difference between the zebrafish phenotype after fbni1 knockout and human MFS patients is that there is no abnormality in the retina of F2 fbni1+/− zebrafish. Human MFS most often involves cardiovascular, skeletal, and ocular systems. The most common ocular manifestations of MFS are lens dislocation (mild eversion, accounting for about 60%), early and severe myopia, retinal detachment, early attack of cataract or glaucoma, corneal flattening, and iris hypoplasia also occur (Busse et al., 2020; Karoulias et al., 2020). The histological differences of the eyes, especially the retina between the experimental group and the control group need to be further studied. Unfortunately, due to the problem of experimental funding, no follow-up study has been carried out, which will be the focus of our next research. In short, these findings collectively suggest that our fbni1+/− zebrafish mutants could morphologically mimic the clinical features of MFS. Besides, similar to MFS, there is a certain phenotypic difference between the F2 fbni1+/− mutants and the abnormal cardiac phenotype gradually deteriorates with growth and development.

5 | CONCLUSIONS

In this study, using the CRISPR/Cas9 gene-editing tool, we successfully generated the first fbni1+/− zebrafish model that displays significant morphological and cardiovascular abnormalities reminiscent of MFS. Our findings may provide an efficient model for further study of the gene function of FBN1 and a method to determine the pathogenicity of gene mutation sites.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Yuanqing Yao designed the study. Xiaoyun Yin performed the experiments, interpreted the data, and wrote the manuscript. Xiaoyun Yin and Jianxiu Hao carried out some of the experiments and participated in data analyses and interpretation, critically revised the manuscript. All authors read and approved the final manuscript.

ETHICAL APPROVAL

The animal protocol was approved by the Institutional Animal Care and Use Committee of The 1st Medical Center of Chinese PLA General Hospital (Beijing, China). All animal experiments were performed following the guidelines for animal welfare in Laboratory of Translational Medicine of The 1st Medical Center of Chinese PLA General Hospital.

DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and in its online supplementary material.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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