Adeno-Associated Virus-Mediated RNAi against Mutant Alleles Attenuates Abnormal Calvarial Phenotypes in an Apert Syndrome Mouse Model

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INTRODUCTION

Apert syndrome (AS), the most severe form of craniosynostosis, is caused by missense mutations including Pro253Arg(P253R) of fibroblast growth factor receptor 2 (FGFR2), which leads to enhanced FGF/FGFR2-signaling activity. Surgical correction of the deformed skull is the typical treatment for AS. Because of constant maldevelopment of sutures, the corrective surgery is often executed several times, resulting in increased patient challenge and complications. Biological therapies targeting the signaling of mutant FGFR2 allele, in combination with surgery, may bring better outcome. Here we screened and found a small interfering RNA (siRNA) specifically targeting the Fgfr2-P253R allele, and we revealed that it inhibited osteoblastic differentiation and matrix mineralization by reducing the signaling of ERK1/2 and P38, the main downstream pathways of FGFR2 in AS mice. Results demonstrate that the levels of phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) and P38, the main downstream pathways of FGF/FGFR signaling, were enhanced in AS mice.8,9,16 These findings demonstrate that these AS mouse models are good models for evaluating the therapeutic effect for AS.

To further dissect the underlying mechanisms and find novel cures for AS, we and Wang et al.5,10 have established two mouse models independently, Fgfr2+/S252W and Fgfr2+/P253R mice, mimicking human AS using a knockin approach. These two mouse models exhibit abnormal phenotypes similar to those of Apert patients, including smaller body size, premature fusion of cranial base synchondroses and coronal suture (CS), dome-shaped skulls, midfacial hypoplasia, and syndactyly of hands or feet. We have reported that the AS mice showed rostrocaudally shortened skull dimensions and mediolaterally broadened width of the distance between the two orbits.10 The calvarias, facial bone, cranial base, and brain are all abnormally developed in AS mice.12–14 Nagata and colleagues15 found that the shortening of cranial base resulting from accelerated chondrocytic differentiation was the primary reason for the acrocephalic feature in AS mice. For molecular mechanisms, several studies have revealed that the levels of phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) and P38, the main downstream pathways of FGF/FGFR signaling, were enhanced in AS mice.8,9,16 These findings demonstrate that these AS mouse models are good models for evaluating the therapeutic effect for AS.

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Surgical correction of the deformed skull is the current treatment of AS. Because of the progressive skull maldevelopment, the surgery for AS is often executed several times, resulting in increased patient challenge and complications. Biological therapies targeting the FGFR signaling may bring better outcome. In fact, several studies have explored the effects of biological inhibition of the molecular pathways activated in AS on skull phenotypes. Yokota et al. found a soluble form of FGFR2 with Ser252Trp mutation can partially alleviate the phenotype of Apert mouse model by alleviating the premature closure of coronal suture in cultured calvarias and transgenic mice. PD98059, an MEK1 inhibitor, was found to reduce coronal suture fusion in cultured calvarias of Fgfr2+/P253R mice. Inhibiting the MEK1/2 by U0126 alleviates the craniosynostosis phenotypes in vivo. The above-described molecules do not specifically antagonize the mutant FGFR2 itself, which may bring undesired effects. RNAi, in contrast, could be a powerful tool to specifically inhibit the expression of mutant alleles at the transcriptional level. By RNAi, we could specifically silence the mutant allele to treat human diseases caused by gain-of-function mutations while leaving the expression of the wild-type (WT) allele undisturbed. As we previously expressed a short hairpin RNA (shRNA) specifically targeting the Fgfr2-P253R allele genetically in mice, and we found that the shRNA effectively alleviated the abnormal skeletal phenotypes in vivo. However, the constitutive transgenic expression of RNAi in mice cannot be used in the clinic setting. To date, the therapeutic effect of exogenously applied RNAi on Apert phenotypes remains unknown.

Safety and efficiency are the major concerns for the application of RNAi-related therapeutics. The delivery of naked small interfering RNA (siRNA) into the living body is challenging, mainly because of its rapid degradation. Viral delivery of the interested genes has been proven to be an efficient approach for gene therapy in vivo. Adeno-associated virus (AAV) has unique advantages over other viral vectors, i.e., low pathogenicity, reliable safety, and high efficiency. Besides, AAV has been proven to be the effective means to deliver genes to bone tissue. Here we screened and found a siRNA specifically targeting the Fgfr2-P253R allele, and we confirmed its effects on cultured primary calvarial osteoblasts and calvarial explants from Apert mice (Fgfr2+/P253R). Furthermore, AAV-mediated shRNA was delivered to AS mice by local injection to evaluate its effects in vivo on the calvarial phenotype. Our results show that the shRNA against mutant Fgfr2 attenuated the premature fusion of coronal suture and the decreased bone volume (BV) of parietal bone in AS mice.

## RESULTS
### Screening of a siRNA that Specifically Targets against the Mutant Fgfr2 Allele
The Fgfr2-P253R mutant allele in mice contains a guanine (G) at position 60 of the exon 7, whereas the WT Fgfr2 DNA bears a cytosine (C) at this position. To obtain a SNP-specific siRNA with only a single base difference that can distinguish between the mutant and WT Fgfr2 mRNAs, we synthesized a set of siRNAs designated S1–S11. Each siRNA fully matches the Fgfr2-P253R mRNA but contains a C:C mismatch with WT mRNA (Figure 1A). The 11 siRNAs were individually transfected into primary osteoblasts from Apert mice for assessing their silencing effects on the expressions of mutant Fgfr2. Real-time PCR revealed that the expression levels of both the total Fgfr2 and mutant Fgfr2 were reduced in S2-, S4-, S7-, S8-, S9-, S10-, and S11-treated osteoblasts. Among them, S2 showed the most remarkable silencing effect on the expression of mutant Fgfr2. There were no significant changes in the expression levels of mutant Fgfr2 in S1-, S3-, S5-, and S6-treated osteoblasts (Figures 1B–1D; the results from S4 to S11 are not shown). Western blotting was employed to further evaluate the effects of S1–S11 on FGR2 expression. Western blots revealed that S4, S7, S8, S9, S10, and S11 reduced the expression of FGR2, whereas S1, S2, S3, S5, and S6 did not downregulate the FGR2 level in WT osteoblasts (Figure 1E). Treatment of S2, S4, S7, S10, and S11 led to decreased protein levels of FGR2 in the primary osteoblasts derived from Apert mice, which contain WT and mutant Fgr2 alleles. S2 exhibited the strongest inhibitory effect on FGR2 protein level (Figures 1E and 1F; the results from S4 to S11 are not shown). Thus, S2 was employed as the mutant Fgfr2-specific siRNA for the subsequent experiments.

### S2 Treatment Decreases the Osteogenic Differentiation and Matrix Mineralization of Apert Osteoblasts by Downregulating ERK1/2 and P38 MAPK Pathways
To assess the effects of S2 on the proliferation and differentiation of osteoblasts, primary calvarial osteoblasts from Apert mice and their littermates were used to detect the changes of these parameters and related signaling pathways following S2 treatment. The expression level of FGR2 was significantly decreased in S2-treated Apert osteoblasts compared with scramble siRNA- (negative control- [NC]:) treated Apert osteoblasts. In addition, when compared with S2-treated WT osteoblasts, the expression of total FGR2 was significantly decreased in S2-treated Apert osteoblasts, but there was no significant difference in the FGR2 expression between the NC- and the S2-treated WT group, indicating that S2 discriminably knocked down the expression of Fgfr2-P253R (Figures 2A and 2B). It has been found that Fgfr2-P253R leads to the accelerated differentiation of osteoblasts through the mitogen-activated protein kinase (MAPK) pathways, including ERK1/2 and P38, which play important roles in calvarial development. We then detected the levels of phosphorylated ERK1/2 and P38 in WT and Apert osteoblasts treated with S2 or NC. In the absence of S2 treatment, Apert osteoblasts had higher phosphorylated levels of ERK1/2 and P38 than did WT osteoblasts (Figures 2A, 2C, and 2D). Although the level of phosphorylated P38 in S2-treated Apert osteoblasts was higher than that in S2-treated WT osteoblasts, S2 treatment significantly decreased the phosphorylation levels of ERK1/2 and P38 of Apert osteoblasts.
compared with NC-treated Apert osteoblasts, indicating that S2 alleviated the activation of ERK1/2 and P38 signaling caused by the FGFR2-P253R mutation (Figures 2A, 2C, and 2D). Together, these results demonstrate that S2 treatment decreased the expression of mutant FGFR2 and, subsequently, reduced the levels of phosphorylated ERK1/2 and P38.

The proliferation of osteoblasts from Apert mice showed no significant change compared with that of WT osteoblasts (data not shown). In Apert osteoblasts, alkaline phosphatase (ALP) staining revealed that S2 treatment led to a decreased number of osteoblasts with crystal violet staining compared with that in the NC siRNA-treated group (Figure 2E). Alizarin red staining revealed that the mineralization was significantly accelerated in the Apert osteoblasts compared with that of WT osteoblasts. Following S2 treatment, WT osteoblasts showed no significant changes in mineralization compared with NC siRNA-treated osteoblasts, whereas the accelerated mineralization in the Apert osteoblasts was significantly reduced (Figure 2F), indicating that S2 treatment effectively alleviated the increased mineralization of Apert osteoblasts. The mRNA levels of several osteoblastic differentiation-related genes were detected by using real-time qPCR. We found that, compared with those of WT osteoblasts, the expression levels of *Runx2*, *Collagen 1*, *Osteocalcin*, and *Osteopontin* were increased in Apert osteoblasts in the absence of S2 treatment, and these were significantly reduced by S2 treatment. When compared with the S2-treated WT group, the expression levels of *Runx2* and *Collagen 1* were significantly decreased in the S2-treated Apert osteoblasts (Figures 2G–2J).
S2 Treatment Attenuates the Premature Closure of Coronal Sutures of the Cultured Calvarias of Apert Mice

The therapeutic effects of S2 were further evaluated in cultured calvarias. Calvarias of 5-day-old Apert and WT mice were dissected and cut into equivalent left and right parts along the midline. The left and right parts of calvarias were treated with NC or S2, respectively. After being cultured for 7 days, the premature closure of coronal sutures in Apert calvarias was accelerated compared with that of WT calvarias, while it was delayed by S2 treatment (Figure 3A). Analysis of suture fusion patterns indicated that S2-treated WT calvarias displayed similar suture fusion patterns with those of NC-treated WT calvarias. H&E staining revealed that Apert calvarias had an increased overlapping region of the osteogenic fronts of coronal sutures, which was decreased by S2 treatment (Figure 3B).

In addition, results from three-dimensionally reconstructed micro-computed tomography (micro-CT) images showed that the thickness of parietal bone was significantly decreased in Apert calvarias compared with that of WT calvarias. The thickness of parietal bone in Apert mice was 31.94% thinner than that of WT calvarias (Figure 3D). With the treatment of S2, the decreased thickness of parietal bone was rescued in Apert calvarias (Figure 3C). S2-treated Apert calvarias had a 24.78% increase in the thickness of parietal bone (Figure 3D). Further analysis revealed that S2 efficiently attenuated the decrease of parietal BV (Figure 3E). The bone mineral density...
(BMD) of Apert calvarias had no significant change between NC- and S2-treated groups (Figure 3F). These data demonstrate that S2 treatment partially rescued the maldevelopment of Apert calvarias.

Screening for AAV Serotypes with High Infection to Calvarias

The viral delivery of genes was proven to be an efficient approach for gene therapy in vivo. AAVs have important advantages over other viral vectors, i.e., low pathogenicity, high safety, and long-term transgene expression. To date, a few studies have explored the efficiency of AAV-based gene delivery to bone tissue. Considering that different serotypes of AAV have variable affinities to bone, we analyzed the infecting effect on mouse calvarias of AAV2, AAV5, AAV9, and AAV-DJ serotypes. The PBS (control), AAV2-GFP, AAV5-GFP, AAV9-GFP, and AAV-DJ-GFP were subcutaneously injected into the calvarias of mice at post-natal day 0. The calvarias of mice were harvested to detect the expression of GFP reporter at 5 days after injection.

All four of the AAV serotypes could infect cells of calvarias with variable efficacies, as evidenced by GFP fluorescence intensities. Among them, AAV2-GFP and AAV9-GFP exhibited stronger GFP signals in parietal bone, frontal bone, and sutures, whereas AAV5-GFP- and AAV-DJ-GFP-infected calvarias had milder fluorescence intensity than those infected by AAV2-GFP and AAV9-GFP (Figures 4A–4E). Then, we investigated the GFP expression of calvarias 30 days after injection with PBS (control), AAV2-GFP, and AAV9-GFP. Our results revealed that AAV9-GFP-injected calvarias had stronger GFP expression than that of AAV2-GFP-injected calvarias (Figures 4F and 4G), suggesting that the AAV9 viral serotype is suitable for delivering genes to be expressed for a long term in calvarias (Figure 4H). Thus, we employed the AAV9 viral serotype to deliver RNAi for in vivo treatment of Apert mice.

AAV9-Mediated shRNA Treatment Attenuates the Premature Closure of Coronal Suture and the Decreased BV of Apert Mice

In Vivo

To evaluate the in vivo therapeutic effects of shRNA on the calvarial phenotypes of Apert mice, AAV9-Fgfr2-shRNA viruses were locally injected twice on calvarias of Apert mice at day 0 and day 4. Then 6 days later, calvarias of shRNA-treated Apert and WT mice were dissected to evaluate the gene expressions. The expression of total Fgfr2 (including WT and mutant alleles) was not significantly varied between NC- and shRNA-injected WT mice. However, there was a significant decrease of total Fgfr2 expression level in Apert calvarias after shRNA treatment compared with that of the NC-treated Apert mice (Figure 5A). Fgfr2 expression was significantly decreased in shRNA-treated Apert mice compared with shRNA-treated WT mice, indicating that shRNA preferably knocked down the expression of mutant Fgfr2 in mice (Figure 5A). Expectedly, our results showed that the expression of the Fgfr2-P253R allele was not detected in WT mice (data not shown). After shRNA injection, the expression of mutant Fgfr2 was dramatically reduced in Apert calvarias, indicating that AAV-mediated shRNA worked efficiently in calvarias (Figure 5B). Besides, the expressions of Collagen 1 and Runx 2 were significantly increased in Apert mice compared with those of WT mice, which were decreased after shRNA injection (Figures 5C and 5D).

Three-dimensional reconstruction of micro-CT images revealed that shRNA injection significantly attenuated the premature closure of coronal suture in day 20 Apert mice (Figure 5E), while
shRNA- and NC-treated WT mice displayed similar suture fusion patterns. About 53.85% of the Apert calvarias displayed fused coronal suture, while about 10.00% of coronal sutures were fused in WT calvarias. The shRNA treatment partially rescued the premature closure of coronal sutures of Apert calvarias compared with that of NC treatment (Figure 5F). Histological analysis revealed that shRNA treatment attenuated the overlapping area of osteogenic fronts in the coronal sutures of Apert skulls (Figure 5G). Without shRNA treatment, Apert mice showed reduced BV of parietal bone when compared with that of WT mice, which was partially rescued by AAV-Fgfr2-shRNA treatment (Figure 5H). Further micro-CT analysis indicated that shRNA efficiently attenuated the decreased BV of parietal bone (Figure 5I), while shRNA had no significant effect on the BMD of Apert calvarias (Figure 5J).

We further analyzed the calvarial morphology by using quantitative Euclidean distance matrix analysis (EDMA). Nine landmarks were defined on calvarias, and three-dimensional coordinates of post-natal day 20 WT and Apert mice were collected for EDMA (Table 1; Figure 6A). Along the rostrocaudal axis, the linear distances of calvarias, such as landmarks 1 to 2, 2 to 3, and 3 to 4, in NC- or AAV9-Fgfr2-shRNA-treated Apert mice, were significantly decreased when compared with NC- or AAV9-Fgfr2-shRNA-treated WT mice (Table 2; Figures 6B and 6C). The shRNA-treated Apert mice exhibited the trend of alleviation in the deformation of calvarial bone when compared with the NC-treated Apert mice, such as landmarks 1 to 3, 2 to 3, and 3 to 4, although these changes were not statistically significant (Table 2; Figures 6D and 6E).

**DISCUSSION**

Presently, the major therapy for Apert is surgery. However, AS patients have to undergo a series of surgeries throughout their lifetime and probably get serious complications. As a genetic disease, AS is caused by gain-of-function mutations of FGFR2; we thus may use a biological approach to alleviate the severity of AS. Mutations of AS result in dysregulated downstream signaling, such as enhanced...
ERK1/2, P38, PI-3K, and PLCγ pathways, etc.34 Theoretically, we can modulate the activities of mutant FGFR2 at multiple levels, such as inhibition of FGFs binding to FGFR2 and its downstream signaling and modulating other skeleton development-related-signaling pathways affected by mutant FGFR2, including BMP, Wnt, etc. Although the application of inhibitors against individual downstream signaling, for example, inhibition of ERK1/2, was found to attenuate the maldeveloped skull phenotypes of AS,16 the involvement of other downstream signaling pathways, such as PI-3K/AKT, in the pathogenesis of Apert make the treatment against just one downstream pathway not the best strategy for obtaining optimal effects. Moreover, the downstream signals, such as MAPK, also participate in various events.

Figure 5. AAV9-Mediated shRNA Treatment Attenuated the Increased Overlapping Region of Osteogenic Fronts and the Reduced Bone Volume of Parietal Bone in Apert Mice

(A) Real-time qPCR revealed that total Fgfr2 expression was decreased after AAV9-Fgfr2-shRNA treatment. (B) AAV9-Fgfr2-shRNA in vivo injection to calvarias effectively decreased the expression of Fgfr2-P253R. (C and D) The shRNA treatment also reduced mRNA expression of Collagen 1 (C) and Runx2 (D). (E) Micro-CT images revealed that the calvarias were deformed in day 20 Apert mice, which was attenuated by shRNA treatment. (F) Suture fusion patterns in four groups of mice. Four degrees of fusion were defined: patent, white; bridging, light gray; partially fused, dark gray; and fused, black. (G) Sections with H&E staining showed an increased overlapping region between parietal bone and frontal bone in Apert calvarias, which was attenuated by shRNA treatment. (H) 3D-reconstructed images revealed that shRNA treatment attenuated the bone volume (BV) of Apert calvarial bone.

Figure 5 continued...

(J) The BMD showed no significant change between NC- and shRNA-treated Apert calvarias. Data are presented as mean ± SD. P, parietal bone; F, frontal bone. Black arrows indicate osteogenic fronts (*significant change compared with NC-treated Apert mice, #significant change compared with NC-treated WT mice, ※significant change when S2-treated Apert mice were compared with S2-treated WT mice; *p < 0.05, **p < 0.01, *p < 0.05, **p < 0.01, ***p < 0.05, and ******p < 0.01; WT-NC, n = 10; WT-shRNA, n = 11; Apert-NC, n = 13; Apert-shRNA, n = 11).
among 11 successive siRNAs, in which the mismatch (C:C) against allele, we compared the knocking-down effects on mutant FGFR2 (CRISPRi), exhibit promising prospects for the treatment of AS. disrupting DNA sequence, such as RNAi and CRISPR interference or mutant FGFR2 protein so far. Transcriptional regulations, without development. Indeed, there are no specifically targeting the mutant FGFR2; it also inhibits other FGFRs such as FGFR1 and 3, which are important regulators of suture development. Indeed, there are no specific antagonists for either WT or mutant FGFR2 protein so far. Transcriptional regulations, without disrupting DNA sequence, such as RNAi and CRISPR interference (CRISPRi), exhibit promising prospects for the treatment of AS. Furthermore, an RNAi approach can just target the mutant allele, which is an excellent approach for dominant genetic disorders resulting from gain-of-function mutations, including AS that contains a disease-causing allele and a normal WT copy. We are expected to biologically modulate the mutant allele while leaving the normal mRNA unaffected. Importantly, siRNA or shRNA targeting the mutant allele specifically is a readily available approach.

We previously found that transgenic expression of an shRNA targeting FGFR2 can effectively alleviate the abnormal skeletal phenotypes in AS mice. However, this transgenic approach used in mice cannot be used in a clinical setting. Local or systemic application of exogenous RNAi is more practical. Indeed, RNAi-based therapy (Patisiran) for transthyretin amyloidosis (ATTR) has recently been approved by the FDA for a phase 3 clinical trial. We thus utilized the exogenous RNAi approach to treat AS.

To find a siRNA sequence specifically targeting the mutant Fgfr2 allele, we compared the knocking-down effects on mutant Fgfr2 among 11 successive siRNAs, in which the mismatch (C:C) against the WT Fgfr2 allele is located at position 16 to 6 away from the 5’ end of the siRNA. The 5’ seed region of the siRNA is responsible for the recognition and binding to targeting mRNA; the mismatches located in this region will generally provide only moderate single-nucleotide discrimination. In contrast, mismatches in the region from the 5’ end to the seed sequence provided excellent single-nucleotide discrimination. In accordance with this theory, our results revealed that S2 (the mismatch was located in the region from the 3’ end to the seed sequence) had the optimal discrimination between WT and mutant Fgfr2 allele.

As valuable vehicles for gene delivery, AAVs are widely used in human and animal models, for their biological safety, low pathogenicity, long-term gene expression, and the ability to infect both dividing and quiescent cells. Numerous AAV serotypes have been identified with variable tissue-specific infectivity. There are very few reports in the literature about AAV-mediated gene therapies in bone tissue. The most commonly used AAV for bone tissue is the AAV2 serotype, which has an extensive infectivity in the majority of tissues. Apart from AAV2, other serotypes have not been tested in bone tissue. Here, four AAV serotypes, AAV2, AAV5, AAV9, and AAV-DJ, were tested in the calvarial bone of mice. Our results showed that the AAV5 and AAV-DJ serotypes had lower infectivity compared with the AAV2 and AAV9 serotypes at an early stage after virus injection, while AAV9 drove stronger GFP expression in calvarias than did AAV2. Furthermore, AAV9-mediated GFP expression lasted longer, as evidenced by the strongest GFP expression at day 30 after local injection. We thus demonstrate that AAV9 exhibits high infectivity in the developmental calvarial bone, suggesting that it can be used for the gene therapy of a maldeveloping skeleton. Interestingly, GFP fluorescence signals were gradually reduced with the development of calvarial bone, indicating that it is necessary to have multiple injections of AAV to obtain sustained gene expression during the development of calvarial bone.

In this study, we tested the therapeutic effects of AAV-mediated shRNA on the calvarial phenotypes of AS mice. Our data showed that AAV9-mediated shRNA targeting FGFR2-P253R can specifically reduce the expression of mutant FGFR2 (FGFR2-P253R), with reduced phosphorylation levels of ERK1/2 and P38, and subsequently alleviate the phenotypes of Apert calvarias by delaying the premature fusion of coronal sutures.

Since the skull phenotypes of Apert mice were just partially rescued in this study, further studies are needed to obtain a better therapeutic outcome by finding the optimal concentrations and dosage of the AAV-RNAi and optimal timing and times of injection, etc. Specifically, targeted delivering of siRNA to suture mesenchyme or osteoblasts using respective targeting peptides or aptamers may further promote the therapeutic effect of siRNA on craniosynostosis. Considering the broad effects of mutant FGFR2, systemic application of AAV-shRNA that targets mutant Fgfr2 in more tissues, such as brain and synchondrosis, may further improve outcome. Since FGFR2 is an oncogene and FGFR2-P253R has been found in gastric cancer, our

| Landmarks | Definition |
|-----------|------------|
| 1 nasion  | 3 bregma   |
| 2          | intersection of parietal and interparietal bones |
| 3          | frontal-squamosal intersection at temporal crest (bilateral) |
| 4 and 7    | joining of squamosal body to zygomatic process of squamosal (bilateral) |
| 5 and 8    | intersection of parietal, temporal, and occipital bones (bilateral) |

Table 1. The Definition of Calvarial Landmarks Used in EDMA
study also suggests that AAV-mediated Fgfr2 RNAi may serve as a therapeutic strategy for those FGFR2 mutations related to cancer.

In summary, in this study we find an RNAi sequence specifically targeting Fgfr2-P253R-causing AS. We demonstrate that AAV9 is a good tool for the gene therapies for bone-related diseases, and AAV9-mediated shRNA against Fgfr2-P253R can partially alleviate the skull maldevelopment of the Apert mouse model. So far there are few studies testing the effects of biotherapy on craniosynostosis using in vivo mouse models. Our study demonstrates that exogenous RNAi is a novel practical biological approach that will, in combination with other therapies, including surgery, help not only the treatment of patients with AS but also other skeletal diseases or regeneration.

MATERIALS AND METHODS

Animals
Fgfr2+/P253R-neo mice have been previously described. CMV-Cre mice were purchased from Jackson Laboratory. All mice were maintained on a C57BL/6J background. Apert mice (Fgfr2+/P253R) were generated by crossing male offspring of CMV-Cre and female offspring of Fgfr2+/P253R-neo mice. Mice were genotyped using the PCR method as described previously. All animal care and procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Daping Hospital (Chongqing, China).

Cell Culture and siRNA Transfection
Primary calvarial cells were obtained from 4-day-old mice as described previously. Cells were cultured in α-minimum Eagle’s medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. The culture medium was changed every 2 days. A series of siRNA (Figure 1A) and a scrambled control siRNA (NC, 5'-TTCTCCGAACGTGTTCAGTAA-3') were synthesized (Ribobio, China). Primary calvarial cells were transiently transfected with 100 mM siRNA using the Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Cell proliferation was detected using an in vitro colorimetric assay (Sigma-Aldrich). For the differentiation assay, osteoblasts were plated at 1.0 × 10^6 cells/well in a 12-well plate, with the medium supplemented with 50 μg/mL L-ascorbic acid and 4 mM Glycerol-2-phosphate. Primary calvarial cells were transfected with 100 mM siRNA and cultured for 2 weeks. Alizarin red and ALP staining was detected using a kit according to the manufacturer’s instructions (Sigma-Aldrich). mRNA and protein levels were determined by real-time PCR and western blotting after 48 hr of siRNA transfection.

Preparation of AAV and Calvarial Injections
AAV2-GFP, AAV5-GFP, AAV9-GFP, and AAV-DJ-GFP were supplied by Hanbio (China). The S2 sequence was subjected to generate AAV9-Fgfr2-shRNA (top strand, 5'-AATTCGTCACGACACC GTCCCATCCTTCAAGAGGATGGGACGGTGTTGATTTT TT-3'; bottom strand, 5'-GATCAAAAAATCACGACACCGTCC CATCCTCTCTGAAGGATGGGACGGTGTTGACG-3'), and

Culture of Calvarias
Calvarias were cultured according to the literature. Briefly, calvarias were dissected from 5-day-old mice, then they were cut along the midline into two parts. The explants were subsequently cultured in BGJb medium (Gibco) containing 1 mg/mL BSA, 2 mM glutamine, and antibiotics supported by metal grids in a 6-well plate at 37°C in a humidified atmosphere of 5% CO₂ in air. The left parts of calvarias were treated with S2, and right parts were treated with a scrambled control siRNA. After 7 days of culture, the cultured calvarias were fixed with 4% paraformaldehyde (PFA) at 4°C, and then micro-CT scanning was taken for three-dimensional reconstruction. After being decalcified, dehydrated, and embedded in paraffin, coronal sutures were sectioned along the midline of the calvarias at 6-μm intervals and stained with H&E.

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Figure 6. Schematic Diagram for EDMA of Calvarial Morphologies of Apert and WT Mice with NC or shRNA Treatment
(A) Diagram of 9 landmarks on the mouse skull. (B) Difference of calvarial morphologies between Apert and WT mice with NC treatment. (C) Difference of calvarial morphologies between Apert and WT mice with shRNA treatment. (D) Calvarial morphologies of shRNA-treated WT mice compared to NC-treated WT mice. (E) Difference of calvarial morphologies between shRNA Apert mice and NC-treated Apert mice. Blue lines show those linear distances that have a decrease of over 5%. Broken gray lines show linear distances with no significant change. NC-treated WT mice, n = 10; shRNA-treated WT mice, n = 11; NC-treated Apert mice, n = 13; shRNA-treated Apert mice, n = 11.
a scrambled control siRNA (5'-TTCTCCGAACGTGTCGTAAT-3') was prepared to make AAV-negative control shRNA (NC shRNA top strand, 5'-AATTTGTTCTCCGAACGTGTCGTAAT-3'; bottom strand, 5'-GATCAAAAAATTCTCCGAACGTGTCGTAAT-3') and WT Calvarias Treated with NC or shRNA

| Landmarks | NC Apert shRNA Apert to WT | WT shRNA to NC | Apert shRNA to NC |
|-----------|-----------------------------|----------------|------------------|
| 1 and 2   | 0.665                       | 0.684          | 0.978            |
| 1 and 3   | 0.682                       | 0.703          | 0.988            |
| 2 and 3   | 0.761                       | 0.800          | 0.975            |
| 1 and 4   | 0.752                       | 0.766          | 0.989            |
| 2 and 4   | 1.034                       | 1.021          | 1.014            |
| 3 and 4   | 0.842                       | 0.872          | 0.977            |
| 1 and 5   | 0.752                       | 0.773          | 0.992            |
| 2 and 5   | 0.981                       | 0.983          | 1.001            |
| 3 and 5   | 0.951                       | 0.956          | 0.993            |
| 4 and 5   | 0.737                       | 0.795          | 0.953            |
| 1 and 6   | 0.732                       | 0.740          | 1.017            |
| 2 and 6   | 0.935                       | 0.920          | 1.028            |
| 3 and 6   | 0.972                       | 0.959          | 1.024            |
| 4 and 6   | 0.738                       | 0.777          | 0.989            |
| 5 and 6   | 0.780                       | 0.815          | 1.029            |
| 1 and 7   | 0.759                       | 0.772          | 0.984            |
| 2 and 7   | 0.976                       | 0.994          | 0.976            |
| 3 and 7   | 0.833                       | 0.846          | 0.999            |
| 4 and 7   | 0.998                       | 1.010          | 0.988            |
| 5 and 7   | 0.901                       | 0.920          | 0.985            |
| 6 and 7   | 0.863                       | 0.860          | 1.019            |
| 1 and 8   | 0.724                       | 0.740          | 0.984            |
| 2 and 8   | 0.906                       | 0.932          | 0.969            |
| 3 and 8   | 0.917                       | 0.911          | 1.019            |
| 4 and 8   | 0.874                       | 0.904          | 0.971            |
| 5 and 8   | 0.895                       | 0.915          | 0.982            |
| 6 and 8   | 0.887                       | 0.875          | 1.024            |
| 7 and 8   | 0.684                       | 0.711          | 0.965            |
| 1 and 9   | 0.700                       | 0.744          | 0.977            |
| 2 and 9   | 0.877                       | 0.942          | 0.950            |
| 3 and 9   | 0.976                       | 0.977          | 0.995            |
| 4 and 9   | 0.847                       | 0.906          | 0.959            |
| 5 and 9   | 0.908                       | 0.940          | 0.973            |
| 6 and 9   | 0.923                       | 0.910          | 1.014            |
| 7 and 9   | 0.674                       | 0.745          | 0.958            |
| 8 and 9   | 0.623                       | 0.764          | 0.977            |

whereas EGFP expression was driven by the cytomegalovirus (CMV) promoter in the AAV9 vector. AAV9-Fgfr2-shRNA and AAV9-NC-shRNA viruses were produced. For screening AAV serotypes, the AAV2-GFP, AAV3-GFP, AAV9-GFP, AAV-DJ-GFP, and PBS viruses were injected into the calvarias of post-natal day 0 mice, respectively. AAV9-Fgfr2-shRNA and AAV9-NC-shRNA viruses (Hanbio, China) were locally injected into mouse calvarias twice at day 0 and day 4. 5 μL virus at a concentration of 1.0 × 10^12 viral genomes (vg)/ml was used per injection.

**Micro-CT Scanning and Analysis**

The cultured calvarias and skull samples were scanned with micro-CT (VivaCT40, Scanco Medical, Switzerland), with the condition of 45 kV and 177 μA. Two-dimensional images were used to generate three-dimensional reconstructions. At the same time point, every measurement used the same filtering and segmentation values to obtain three-dimensional images. Identical areas of bone on parietal bones were selected as regions of interest (ROIs) for evaluation by micro-CT Evolution Program version (v.)5.0 software. The parameters included BV, tissue volume (TV), and BMD. Patency of coronal sutures was scored on three-dimensional (3D) skull reconstructions of mice according to the report by Heuzé et al. Four grades of patency were defined: (1) patent, (2) bridging, (3) partially fused, and (4) fused.

**Real-Time PCR**

Total RNA was extracted from cultured cells or calvarial bone using Trizol reagent (Invitrogen, 15596-026). Single-strand cDNA was synthesized from 1 μg total RNA using the reverse transcriptase kit (Takara, RR820A) as standard procedure. The sequences for the primers were as follows: Fgfr2 forward, 5'-TGCCCTACCTCAAGGTCTC-3', Fgfr2 reverse, 5'-TAGAATTACCCGCCAAGCAC-3'; Fgfr2-2P535 forward, 5'TT TCTCCATCAGAACGTTCG-3', Fgfr2-2P535 reverse, 5'-CGC TGTAACCCCTGACAGACAAC-3'; Cbfal forward, 5'-GCCACACT TCCCAACCTCCTC-3', Cbfal reverse, 5'-CACTTCTGTTCTCTGT TCTC-3'; Collagen 1 forward, 5'-CCCCGTTAAAGTGACTGTTA TTC-3', Collagen 1 reverse, 5'-ATGGCTTTTCATGGAAATG-3'; Osteopontin forward, 5'-CACTTCTCCTCAACTGTC-3'; Osteopontin reverse, 5'-GTCCTCTGTTTGTGGTC-3'; Osteocalcin forward, 5'-AACCGAGGGAGGAAATAAGGT-3' and Osteocalcin reverse, 5'-TTTGTAGGGTTTCACAGC-3'. All samples were measured in triplicate and normalized to internal control Cyclophilin A (forward, 5'-CCACCGCAAGAGGTAGAACG-3'; reverse, 5'-CCACCGCAAGTGATGAAA-3'). The annealing temperature was 56°C.

**Western Blotting**

Brieﬂy, protein was isolated from the cultured cells using radioimmunoprecipitation assay (RIPA) lysis buffer and then quantitated using a BCA protein quantitative kit (Thermo Fisher Scientiﬁc). Equal amounts of protein samples (25 μg) were resolved by 12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane.
EDMA
EDMA is a method for quantitatively analyzing the geometric morphology.45,46 According to Richtsmeier’s method,47 three-dimensional coordinate locations of 9 biologically relevant landmarks located on the calvarias were recorded from three-dimensional CT images of skulls. The collected coordinate data were subjected to WinEDMA software to calculate the FDM (form differences matrix) images of skulls. The data are presented as means ± SD. Statistical significance was ascertained by two-way ANOVA. When significant levels (p < 0.05) were achieved, Tukey’s post hoc test was performed (SPSS program version 13.0). The results were considered significant differences at *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

AUTHOR CONTRIBUTIONS
Study Design, F. Luo, Y.X., X.D., and L.C.; Study Conduct, Z.W., J.H., Q.T., X.S., C.L., N.S., D.Z., M.X., Z.N., W.J., J.C., H.C., S.C., and F. Li; Data Collection, M.L. and M.X.; Data Analysis and Interpretation, F. Luo, Y.X., C.D., and X.D.; Drafting Manuscript: F. Luo, Y.X., Z.W., and L.C.

CONFLICTS OF INTEREST
The authors have no conflicts of interest.

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