A Pro253Arg mutation in fibroblast growth factor receptor 2 (Fgfr2) causes skeleton malformation mimicking human Apert syndrome by affecting both chondrogenesis and osteogenesis

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Abstract

Apert syndrome is one of the most severe craniosynostosis that is mainly caused by either a Ser252Trp(S252W) or Pro253Arg(P253R) mutation in fibroblast growth factor receptor 2 (FGFR2). As an autosomal dominant disorder, Apert syndrome is mainly characterized by skull malformation resulting from premature fusion of craniofacial sutures, as well as syndactyly, etc. A P253R mutation of FGFR2 results in nearly one-thirds of the cases of Apert syndrome. The pathogenesis of Apert syndrome resulting from P253R mutation of FGFR2 is still not fully understood. Here we reported a knock-in mouse model carrying P253R mutation in Fgfr2. The mutant mice exhibit smaller body size and brachycephaly. Analysis of the mutant skulls and long bones revealed premature fusion of coronal suture, shortened cranial base and growth plates of long bones. In vitro organ culture studies further revealed that, compared with wild-type littermates, the mutant mice have prematurely fused coronal sutures and retarded long bone growth. Treatment of the cultured calvaria and femur with PD98059, an Erk1/2 inhibitor, resulted in partially alleviated coronal suture fusion and growth retardation of femur respectively. Our data indicated that the P253R mutation in Fgfr2 directly affect intramembranous and endochondral ossification, which resulted in the premature closure of coronal sutures and growth retardation of long bones and cranial base. And the Erk1/2 signaling pathway partially mediated the effects of P253R mutation of Fgfr2 on cranial sutures and long bones.

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Introduction

Skeletons are mainly formed through either intramembranous ossification or endochondral ossification [9,28,29,55]. The former occurs when mesenchymal precursor cells directly differentiate into bone-forming osteoblasts, a process by which all flat bones including calvaria are formed. For the endochondral ossification, mesenchymal cells first differentiate into chondrocytes to form a cartilaginous template, which is eventually replaced by bone tissue. Axial bone including limbs (long bones) and vertebrae are formed primarily through endochondral ossification.

Skeletogenesis is a highly coordinated process involving multiple complex interactions among a variety of genes. Disturbance of signaling pathways involved in skeleton development can lead to skeleton anomaly [9].

Fibroblast growth factor receptors (FGFRs) exist as a gene family of 4 members (FGFR1–4) that mediate signals of at least 23 fibroblast growth factors (FGF1–23) [4,17,23,57]. FGF
signaling is very essential for the skeleton development. Missense mutations of FGFR1–3 in human have been found to result in, at least, 14 congenital bone diseases that are broadly classified into two groups: chondrodysplasia syndromes and craniosynostosis syndromes [4,49,57]. The chondrodysplasia affects primarily the skeletons formed through endochondral ossification, resulting in short-limbed dwarfisms.

Craniosynostosis is a clinical condition characterized by precocious closure of one or several calvarial sutures [7,71]. Craniosynostosis has been found in over 100 distinct genetic syndromes and has an estimated overall incidence of 1 in 2500–3000 live births [25]. Besides the malformed skulls with varying severities, patients may have other anomalies including appendicular skeleton malformation, such as syndactyly of the hands and feet (Apert syndrome) or broad thumbs and big toes (Pfeiffer syndrome), and internal organ defects, etc. [10]. Depending on the sutures affected and the accompanying non-cranial complications, craniosynostosis patients have adopted clinical syndromic designations that include Apert, Crouzon, Pfeiffer and Saethre–Chozen syndromes [8], etc.

As one of the most severe forms of craniosynostosis, Apert syndrome occurs with a reported birth prevalence of 1/65,000 [15], and is mainly caused by either the Ser252Trp(S252W) or Pro253Arg(P253R) mutation in FGFR2 [58,76].

Besides a variety of abnormalities of the brain, skin and visceral organs, Apert syndrome is characterized by abnormal development and premature fusion of the cranial sutures [13,14]. Typical skeletal features of patients with Apert syndrome include premature closure of coronal sutures, metopic sutural defect, ocular hypertelorism and proptosis, midface hypoplasia, severe malocclusion and symmetric syndactyly [7,13], etc.

In addition to the abnormal osteogenesis, chondrogenesis is also affected in patients with Apert syndrome. The cranial base abnormalities and epiphyseal dysplasia were observed in Apert syndrome patients [13,70]. Consistently, the mice with Fgfr2 S252W mutation exhibited growth retardation, premature coronal suture closure and skull malformation [6,75]. In addition, cartilage abnormalities were observed in the sagittal suture, basiocciput, nasal turbinates and trachea in mice with Fgfr2 S252W mutation [75]. Moreover, other cartilage abnormalities have been reported previously in several mouse models with different mutated Fgfr2, including the small body size, retarded development of the axial and appendicular skeleton and growth arrest of the skull base [22,24,80]. Therefore, both cartilage and bone development can be significantly regulated by Fgfr2.

The signaling pathways related to FGFRs signaling in osteogenesis have been explored extensively in the last 10 years. FGFRs signaling is mediated mainly via the extracellular signal-regulated kinase 1/2 (Erk1/2), the p38, the PI3-kinase and the PLCγ pathway, etc. [66]. The Erk1/2 pathway has been found significantly involved in the development of osteoblast and chondrocyte lineages. Blocking of the Erk1/2 pathway suppressed the AP1 and osteopontin expression, and significantly retarded cranial suture closure induced by FGF2, suggesting that the Erk1/2 pathway may mediate FGF/FGFR-stimulated cranial suture closure [30]. The activation of Erk1/2 pathway related to FGFR signaling has also been observed in other cell types including chondrocyte lineage. Erk1/2 can act as a negative regulator of chondrogenesis. The FGF-induced growth arrest of chondrocytes was found to be mediated by activation of the Erk1/2 pathway [60]. Furthermore, activation of Erk1/2 in chondrocytes in vivo can cause an achondroplasia-like dwarfism [50]. However, there is no direct in vivo evidence showing the involvement of Erk1/2 pathway in the pathogenesis of skeletal abnormalities in Apert syndrome resulting from P253R mutation of FGFR2.

In this study, we created a new mouse model with Fgfr2 P253R mutation using knock-in approach. Using this mouse model, we found that the Fgfr2 P253R mutation led to abnormalities in both osteogenesis and chondrogenesis of mutant mice, thus resulted in premature closure of coronal suture and retarded growth of synchondrosis of cranial base and growth plates of long bones during skeletal development. Furthermore, we showed that the Erk1/2 pathway may play critical roles in the premature closure of coronal sutures as well as growth retardation of long bone and cranial base cartilage in mice with Fgfr2 P253R mutation.

Materials and methods

Site-directed mutagenesis and targeting vector construction

The P253R mutation was introduced into exon 7 of Fgfr2 using an oligonucleotide, 5′-CCATCAAGACCTCAGACCGTCCATCCTCC-3′ (′g′ represents the mutated nucleotide) and a standard protocol for site-directed mutagenesis. The introduced point mutation was confirmed by sequencing. The targeting construct was made using Fgfr2 genomic DNA isolated from a phage library [19]. A 2.4 kb Apa1–EcoRV fragment of Fgfr2 genomic DNA containing the P253R mutation was inserted into the Hpal and NotI site of the ploxPneo vector [79]. The resulting plasmid was digested with Clal and Kpnl, followed by the insertion of a 2.9 kb Kpnl–Apal fragment of Fgfr2 genomic DNA. The resulting targeting construct, pFgfr2-P253R, was shown in Fig. 1A.

Homologous recombination in embryonic stem cells and generation of germline chimeras

TC1 ES cells were transfected with Notl-digested pFgfr2-P253R and selected with G418 and FIAU [20]. Genomic DNA from G418- and FIAU-resistant ES clones was digested with EcoRV and Xhol, size-fractionated, transferred to nitrocellulose membrane and then probed with a 5′ flanking (Fig. 1A), or 3′ internal fragment specific to the Fgfr2 sequence (not shown). The targeted ES clones will have 9.4 and 6.5 kb bands. Targeted ES clones were microinjected into blastocysts harvested from C57BL/6, and implanted into the uterus of pseudopregnant mice. Germline transmission was confirmed by standard procedures [20].

Genotype analysis

Since the insertion of neo gene in intron 6 of Fgfr2 causes reduced expression of Fgfr2, mice with germline transmission of Fgfr2-P253R (Fgfr2+P253R-neo) were crossed with Ellis–Chen mice [39] to remove the neo gene to get mice with gain-of-function mutation of Fgfr2 (Fgfr2+P253R mice), which were used in our following phenotype analyzing. After the removal of the neo gene, genotypes were determined by PCR using primer A (5′-TTGATACCTGGATGTTGCGGC-3′) and primer B (5′-TAGGTAGTCCATAACTCGG-3′). This pair of primers amplifies a 275 bp fragment in wild-type and a 335 bp fragment in Fgfr2+P253R mice due to the presence of a loxP site.
Image analyses of the skulls

After removal of the soft tissue, the skulls of adult mice (n=3, per genotype) were fixed in 95% ethanol. Skull 3D reconstructions were made by West China Center of Medical Sciences of Sichuan University using Micro-computed tomography (µCT80, SCANCO). Using MarVision PMC 800 (Carl Mahr Holding GmbH, Germany), 27 locations of cranial landmarks on the skulls of 4 week-old mice (n=8, per genotype) were recorded [62,63]. The means of the three-dimensional coordinates of these landmarks were used for image analyses of the skull. These data were analyzed using Euclidean Distance Matrix Analysis (EDMA, http://www.getahead.psu.edu/) to measure the localized differences between Fgfr2<sup>+/P253R</sup> mice and their control littermates. Nonparametric statistical technique was used to evaluate the significance of difference [62].

Analysis of cell proliferation

The proliferation of growth plate chondrocytes in Fgfr2<sup>+/P253R</sup> mice and wild-type mice at P9 were checked (n=6, per genotype). It was performed using BrdU (5-bromo-2-deoxyuridine) labeling reagent (B5002, Sigma) and monoclonal anti-BrdU antibody (B2531, Sigma) as described by Chen et al. [6]. The digital microscopic images of the sections were captured, and proliferating chondrocytes were quantitated using the software (Image-Pro Plus, version 4.5.1.22, Media Cybernetics Inc.). The percentage of BrdU-positive nuclei relative to total nuclei was calculated as the proliferation index. Results were expressed as mean±SD, and evaluated with Student’s t test. Differences with P values of ≤0.05 were considered significant.

In situ hybridization

Plasmids used for generating DIG-labeled complementary RNA probes were: Fgfr2, coll10a1 and osteopontin [26,32,52,59]. All probes were prepared with DIG RNA Labeling Kit (1175025, Roche). Samples (n=6, per genotype per time point) were fixed overnight in fresh 4% PFA in RNase-free PBS, decalcified in 15% EDTA, and embedded in paraffin. Sections with a thickness of 6 μm were placed on slides. In situ hybridization (ISH) was applied to appropriate sections using DIG Nucleic Acid Detection Kit (1175041, Roche) as described by the manufacturer. Signals in the slides were viewed by an Olympus BX51 research microscope linked to a color video monitor, and digital photographs were taken by a Spot Insight color digital camera (model 3.2.0, Diagnostic Instruments, Inc.), using the SPOT Advanced software (version 3.4.5).

RT-PCR analysis of Fgfr2 expression in chondrocytes

To identify the expression of Fgfr2 in chondrocytes of the epiphyseal cartilage of long bones, femurs were harvested from six P2 wild-type mice and six P2 Fgfr2<sup>+/P253R</sup> mice. After removing the perichondrium by surgical dissection under a dissecting microscope, the epiphyseal cartilages in the distal femur were collected and the total RNA was isolated by using the Trizol Reagent (Gibco). Fgfr2 mRNA was detected by RT-PCR using the primer F-F (5′-AAGGTTCACCGGATGCCCA-3′) and primer F-R (5′-ACCACCATGCGGCCGATTA-3′) [16]. The following pair of primers for β-actin was used as control for RT-PCR: β-F: 5′-TGGTGGTACATCAAAAGAAG-3′ and β-R: 5′-GATGCCACAGGTCTTCA-3′. The Cells-to-cDNA II kit (Ambion, 1722) and TaKaRa PCR Amplification Kit (Takara, DR011) were used in these processes.
Max3000 PCR machine (Stratagene) and SYBR Premix Ex Taq™ kit (Takara). The reaction buffer (20.0 μl) included SYBR Premix Ex Taq™ (2×) 10.0 μl, PCR forward primer upper 0.4 μl, reverse primer 0.4 μl, ROX Reference Dye (50×) 0.4 μl, cDNA template 2.0 μl and dH2O 6.8 μl. The primers for cfos [81], osteopontin [34], osteocalcin [34], and β-actin [81] were as follows: cfos forward, 5'-GCCACACTTCCCACACTCTC-3'; cfos reverse, 5'-CAGTTTCGTTCTTCTC-3'; osteopontin forward, 5'-ACACCCGTACACTCAGGCAGCG-3'; osteopontin reverse, 5'-GGTGTCCCTCAGTTGCTCC-3'; osteocalcin forward, 5'-AAGCAGGAGGGCAATAAGGT-3'; osteocalcin reverse, 5'-TTTTGAGCGGCTTTCAACGC-3'; β-actin forward, 5'-AGATGGTGGATCGGCAAGCGA-3'; β-actin reverse, 5'-GGCAAGTTGTTGTTGTTCA-3'. β-actin was used as the internal reference gene. The mean cycle threshold (Ct) values from triplicate samples were used to calculate the relative gene expression, and the coefficient of variation was less than 5% for all replicates. Mutant type group and wild-type group consisted of 6 samples respectively. Data were expressed as mean±SD, and the significance of difference was evaluated with t test.

Bone marrow cells culture and Western blotting

Two-month-old mice were sacrificed by cervical dislocation. Femoral bones were harvested, and their ends were cut off. Bone marrow cells were flushed out by slowly injecting DMEM into the marrow cavity using a sterile 21-gauge needle. The marrow suspension was pipetted gently to get a single cell suspension, and plated at a concentration of 5×10^6 cells/ml and grown in DMEM with 15% fetal bovine serum (FBS). The culture medium was changed every other day. Once subconfluent, bone marrow cells were washed twice with phosphate-buffered saline (PBS) and cultured for 12 h in serum-free DMEM with 0.1% BSA. Then dimethyl sulfoxide (DMSO) vehicle at 0.1% final concentration, Erk1/2 inhibitor PD98059 (Calbiochem) at 10 μM final concentration, and PD98059 at 50 μM final concentration were added to the culture medium respectively. After 1 h, the culture medium was supplemented with FBS at 10% final concentration. And cells were lysed 30 min later, and subjected to Western blot analysis with antibodies either to phospho-Erk1/2 (9101, Cell Signaling) or to Erk1/2 (9102, Cell Signaling).

Calvaria organ culture

The calvaria organ culture was made according to the previously described protocol [31,56]. Briefly, calvaria were harvested from E15.5 mice. The explants of 20 mutants and 20 controls were divided into two groups equally: one group was treated with 50 μM PD98059, another was treated with 0.1% DMSO vehicle alone. The explants were subsequently cultured on a Falcon insert membrane (pore size 0.4 μm) supported by metal grids in organ culture dishes (Falcon, 3037) at 37 °C in a humidified atmosphere of 5% CO2 in air. The serum-free culture medium consisted of B27 medium (Gibco) containing 0.2% bovine serum albumin, 2 mM glutamine, 50 μg/ml ascorbic acid, 10 mM 3-glycerophosphate, and antibiotics. The culture medium with PD98059 or DMSO vehicle alone was changed every third day. After 9 days culture, the cultured calvaria were fixed overnight with 4% PFA at 4 °C, then pictures were taken under a stereomicroscope (Leica). After being decalcified, dehydrated and embedded in paraffin, coronal sutures were sectioned along the sagittal axis of the calvaria at 6 μm intervals and stained with H&E.

In vitro culture of embryonic femoral bones

The embryonic femoral bone culture was made according to the modified protocol as described before [5,79]. Femurs were harvested from E15.5 mice. The explants of 12 wild-type controls were the 1st group, the explants of 12 mutants were the 2nd, and both of them were treated with 0.1% DMSO vehicle alone. The 3rd and 4th groups both consisted of 12 mutants, and were treated with 10 μM PD98059 and 50 μM PD98059 respectively. The explants were subsequently cultured in serum-free culture medium consisted of B27 medium (Gibco) in 24 well culture plates. Calcein staining was performed to identify the ossified tissue [21].The culture medium was changed every other day. The total length (TL) and the cartilaginous tissue length (CL) were measured under a stereomicroscope (Leica) at day 0 and day 6. Changes of the cultured femurs were expressed as percentage increase (percentage increase=[length at day 0)/length at day 0]. Data were expressed as mean±SD, and the significance of difference was evaluated with t test.

Results

Generation of Fgfr2<sup>+/P253R</sup> mice

To study the effect of FGFR2 P253R mutation on skeleton development and its mechanism in vivo, we introduced the Fgfr2 P253R mutation into mouse genome using a knock-in approach [18]. Germline transmission of the targeted allele was obtained and identified by Southern blot (Fig. 1B), and the presence of the pLoxP neo gene in intron 6 was found to downregulate the expression of Fgfr2 gene (data not shown). The heterozygous mice (Fgfr2<sup>Neo253/+</sup>) were crossed with Ella-Cre mice [39] to remove the pLoxPneo gene from the germline (Fig. 1A). After deletion of the neo cassette, Northern blot analysis revealed that the expression level of Fgfr2 in the resultant Fgfr2<sup>+/P253R</sup> mice was similar to that of the wild-type mice (data not shown).

Fifty Fgfr2<sup>+/P253R</sup> mice and 100 wild-type littermates were used to observe the neonatal growth and survival. Live Fgfr2<sup>+/P253R</sup> mice were born with the expected Mendelian frequency (~25%). They exhibited severe growth retardation (Fig. 1C and D). At birth their weight was 90% of their wild-type littermates and by three weeks of age they were 40 to 50% smaller than controls. About 35% (14 in 40) mutant mice died before P20 and 45% (18 in 40) died within six months. The remaining Fgfr2<sup>+/P253R</sup> mice (20%, 8 in 40) survived to adulthood. There were no significant differences in the fertility and life span between the survivors and the controls. Since it was reported that Apert syndrome patients and Fgfr2<sup>+/S252W</sup> mice that mimic human Apert patients with FGFR2 S252W mutation have a variety of internal organ abnormalities [75], we checked the live and naturally dead mutant mice carefully, and found no significant gross and histological abnormalities in the lungs and heart. Notably, unlike the complete absence of syndactyly in Fgfr2<sup>S252W</sup> mice [6,75], syndactyly was observed in three Fgfr2<sup>+/P253R</sup> mice (Fig. 1E and F).

A P253R mutation in murine Fgfr2 results in mice with brachycephaly and premature fused coronal suture that mimicking the craniofacial feature of patients with Apert syndrome

Beginning from E16.5, Fgfr2<sup>+/P253R</sup> embryo gradually exhibited mild craniofacial malformation, mainly the dome-shaped skulls (Fig. 2A–C). With age, the mutant mice showed smaller body size than wild-type littermates and more obvious head malformation, which included wide spaced eyes and underdeveloped midface accompanied by malocclusion (Fig. 1C, 2A and D). Some mutant mice showed distorted skull. X-ray examination and skeleton preparation with staining of Alizarin and Alcian Blue revealed significant shortened anterior–posterior axis (Fig. 2A).

To quantitatively examine the craniofacial malformation, EDMA was adopted to measure the localized differences...
between Fgfr2+/P253R mice and their control littermates as described before [62]. As showed in Fig. 3, the mutant mice have significantly reduced cranial length and increased height. Compared to the neurocranium, the nasal and frontal bones were disproportionally affected. The distance between nasale and nasion was the most severely affected distance. The cranial width was increased between paired frontal bones and less increased between paired parietal bones. Compared with the normal or slightly increased head breadth in human Apert syndrome [11], Fgfr2+/P253R mice exhibited significantly increased skull breadth (Fig. 3). These multiple abnormalities of skulls in adult Fgfr2+/P253R mice resulted in significant brachycephaly, midface hypoplasia and wide spaced eyes (Fig. 3), which was consistent to human Apert syndrome patients [1,10,11,37].

Whole skeleton preparation and Micro-CT examination revealed premature closure of coronal sutures and delayed fusion of
posterior frontal (PF) sutures in Fgfr2+/P253R mice (Fig. 4A–H). At P2, there were cartilages in the sagittal sutures of the mutants, but no cartilage was detected in the PF sutures of either the mutants or the controls (Fig. 4C–D). This was consistent with the reports by Wang et al. that there was abnormal cartilage formation at the sagittal and interfrontal sutures in Apert syndrome patients or mice mimicking Apert syndrome [35,75]. At P8, cartilage was present in the PF sutures of wild-type mice (Fig. 4E), but not in the PF sutures of mutant mice (Fig. 4F). At P17, the PF sutures were closed in wild-type mice (Fig. 4G), but remained patent in mutant mice (Fig. 4H), which was consistent with that of human Apert syndrome patients and Fgfr2+/S252W mice as described by Wang et al. [36,75].

Abnormal development of the skull of Fgfr2+/P253R mice

It has been found that Fgfr2 was involved in the regulation of calvaria development, which was further supported by the significant skull malformation found in Fgfr2+/P253R mice.

We did histological examination of the calvaria, and confirmed the premature closure of coronal sutures in mutant mice (Fig. 4I–L), which have been closed at P21 while the controls kept patent. The mutants had fewer mesenchymal cells between the osteogenic fronts of the frontal and parietal bones, and their coronal sutures closed prematurely (Fig. 4I–L).

To see if the differentiation of cranial osteoblasts was affected by Fgfr2 P253R mutation, we first did Alizarin Red S staining of the skull of Fgfr2+/P253R mice, and found accelerated intramembranous ossification in the cranium, which was apparent at the late embryonic stages (Fig. 4M and N).

We then analyzed the osteoblast differentiation by examining the expression of the osteopontin (OP) using in situ hybridization. At the late embryonic stages, compared with littermate controls, the mutant mice showed stronger intensity of OP expression in the cranium (Fig. 4O–R). Furthermore, we analyzed the osteoblastic gene expression in the cultured osteoblasts isolated from mice cranium. As shown in Fig. 4S, significant increase in cbfa1, OP and OC expression was observed in the osteoblasts isolated from Fgfr2+/P253R mice.

![Fig. 4. Abnormal osteogenesis in the skulls of Fgfr2+/P253R mice.](image-url)
These data suggested that Fgfr2 P253R mutation in mice resulted in enhanced osteoblast differentiation.

**Fgfr2 P253R mutation in mouse resulted in retarded endochondral ossification**

Skeletons are formed through either intramembranous or endochondral ossification processes. The smaller body size and shortened cranial base in Fgfr2+/P253R mice prompted us to check if Fgfr2 P253R mutation could also affect endochondral ossification in skeletons.

The Fgfr2+/P253R mice exhibited growth retardations of the synchondroses of cranial base and growth plates of long bones (Fig. 5A and B). The appearance of the first and secondary ossification centers was delayed in mutant tibial epiphysis (Fig. 5B), which was consistent with the results reported in human Apert patients by Cohen and Kreiborg, who found delayed appearance of postnatal ossification centers in patients [13]. The retarded growth of synchondroses became evident at the late embryonic period, and ultimately resulted in visible shortening of the cranial base in mutant mice (Fig. 5A). However, premature fusion of the intersphenoidal and spheno-occipital synchondroses was not observed. We then did histological examination of the long bones. We found noticeable shortened zones of proliferating and hypertrophic chondrocytes, and decreased trabecular bone areas in the growth plates of the mutant mice (Fig. 5B and C). The columnization of chondrocytes appeared normal. The number of BrdU-positive proliferating chondrocytes in the growth plates of the proximal tibia was significantly decreased in Fgfr2+/P253R mice (Fig. 6Aa n dB), indicating that the proliferation of chondrocytes was decreased in Fgfr2+/P253R mice. In situ hybridization revealed decreased Col10a1 expression in the cranial base in mutants at P1 (Fig. 6C), suggesting decreased chondrocyte differentiation. The obvious changes in chondrocyte proliferation and differentiation in mutant mice prompted us to check if Fgfr2 was expressed in chondrocytes. In situ hybridization revealed that Fgfr2 was expressed not only in osteoblasts, but also in differentiated chondrocytes in the cranial base during the late embryonic period (Fig. 6D). Fgfr2 expression in the epiphyseal growth plates of long bones was identified by RT-PCR (Fig. 6E), which was consistent with other studies [40,61].

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**Fig. 5. Retarded endochondral ossification in mutant mice.** (A) Cranial base stained with Alizarin Red S and Alcian Blue showed marked shortened synchondroses in mutant mice both in late embryonic and neonatal stages. White lines represent the total length of intersphenoidal synchondrosis, basi-occipital synchondrosis. (B) H&E staining showed the appearance of the secondary ossification center (arrow) in the tibia of wild-type mouse but not in mutant mouse at P6. (C) H&E staining showed the length of the trabecular bone (arrows) in tibia was shorter in mutant mouse.
Blocking the Erk1/2 pathway alleviated Fgfr2-mutation-induced premature closure of coronal suture as well as retarded growth of long bones

FGFs/FGFRs exert their biological effects through multiple signaling pathways. MAPK was one of the major downstream pathways of FGF signaling, and it was significantly involved in the skeletogenesis including the regulation of bone formation by FGFs/FGFRs \[33,65,78\]. To examine if the MAPK was involved in the pathogenesis of bone malformation found in \(Fgfr2^{+/P253R}\) mice, we checked and found enhanced expression level of phosphorylated Erk1/2 in bone marrow cells derived from \(Fgfr2^{+/P253R}\) mice, and their activities of Erk1/2 were dose-dependently suppressed by Erk1/2 inhibitor PD98059 (Fig. 7A).

To further confirm the direct role of MAPK in the bone development in \(Fgfr2^{+/P253R}\) mice, we cultured calvaria and femurs from both wild-type and \(Fgfr2^{+/P253R}\) mice with or without treatment of Erk1/2 inhibitor PD98059.

In the cultured calvaria, after 9 days culture, although the frontal and parietal bones either in mutant mice or in wild-type mice overlapped one another (Fig. 7B), only the mutant exhibited fusion of the coronal suture (Fig. 7C) as evidenced by the direct contact between the parietal and frontal bones. To confirm that the Erk1/2 pathway mediated the premature closure of coronal suture induced by P253R mutation of Fgfr2, E15.5 mouse calvaria explants were treated with 50 \(\mu\)M PD98059. Although there was no significant decrease in the overlapping areas of osteogenic fronts that form the coronal sutures, blocking of the Erk1/2 pathway abrogated the coronal suture obliteration and fusion (Fig. 7C). The data suggested that Erk1/2 pathway played an important role in the pathogenesis of premature fusion of the coronal sutures in mice carrying P253R mutation of Fgfr2.

We then checked if Erk1/2 was also involved in the abnormal development of long bones in \(Fgfr2^{+/P253R}\) mice. Femurs were isolated from E15.5 mutant embryos and their littermate controls were cultured in vitro. No significant difference was noted between femurs of the mutant and wild-type mice at the beginning of the study. Six days after culture, compared with wild-type bones, the mutant long bones showed significant inhibited growth \((P \leq 0.05)\) (Fig. 7D and E). Both the cartilage length and the total length growth retardation in cultured mutant embryonic femoral bones were significantly rescued by Erk1/2 inhibitor.
PD98059 \((P \leq 0.05)\). Although PD98059 dose-dependently suppressed Erk1/2 activity of mutant bone marrow cells, and dose-dependently rescued the cartilage length growth of the mutant femoral bones, no significant differences between the effects of 10 μM and 50 μM PD98059 on the total length growth of mutant femoral bones had been observed (Fig. 7E).

Histological examination showed that the zones of proliferating and hypertrophic chondrocytes were much shorter in the cultured mutant bones. The ossified bone parts were shorter in mutants as well. Inhibition of the Erk1/2 activity caused the elongation of the proliferating, hypertrophic and calcifying zones in mutant growth plates. Using of 50 μM PD98059 suppressed Erk1/2 activity in mutant cells to a level lower than wild-type cells, however, the retardation of total length growth of long bones has not been completely rescued by 50 μM PD98059 treatment, suggesting that signaling pathways other than Erk1/2 were also responsible for the retarded long bone development in \(Fgfr2^{-/-P253R}\) mice.

**Discussion**

Skeletogenesis is a highly coordinated complex process involving multiple steps and cell lineages, disturbance to any part of this process will lead to skeleton malformation. Craniosynostosis is a common skeleton dysplasia, and is mainly characterized by precocious closure of one or multiple cranial sutures. Depending on the sutures affected and the accompanied non-cranial complications, there are several types of craniosynostosis including Apert, Pfeiffer, Jackson–Weiss syndrome, etc. [7,55,71].

Apert syndrome is one of the most severe types of human craniosynostosis, and is mainly characterized by premature fusion of the coronal suture and severe syndactyly of hands or feet. Patients with Apert syndrome also have patent midline metopic and sagittal sutures in their infancy stage [36]. Apert syndrome is caused mainly by either S252W or P253R mutation of \(F\)GFR2 [58,76]. S252W more commonly occurred than P253R, and has been associated with more severe craniofacial anomalies, whereas P253R is thought to have more severe syndactyly [68,72]. We and Wang et al. have generated mouse models mimicking human Apert syndrome resulting from \(Fgfr2\) S252W mutation, both models showed skull malformation including premature fusion of coronal and sagittal sutures and defect in interfrontal sutures, which was similar to that of patients with Apert syndrome.

To study the role of \(F\)GFR2 P253R mutation in the pathogenesis of Apert syndrome, we generated a mouse model carrying the \(F\)gfr2 P253R mutation using a knock-in approach. \(Fgfr2^{-/-P253R}\) mice were born alive. Like the \(Fgfr2\) S252W

![Fig. 7. In vitro organ culture revealed the role of Erk1/2 in skeleton malformations caused by \(Fgfr2\) P253R mutation. (A) Western blot analysis showed phosphorylated Erk1/2 was enhanced in bone marrow cells derived from \(Fgfr2^{-/-P253R}\) mice, and it was dose-dependently suppressed by Erk1/2 inhibitor PD98059. Erk1/2 was used as an internal control. (B) Top view of the cultured calvaria. Notice the overlapping areas of coronal sutures (arrowheads) had no significant difference between the mutant and control. (C) H&E staining of the cultured coronal sutures treated with PD98059 or DMSO vehicle (arrowheads point to osteogenic fronts, red lines mark the parietal and frontal bones). Notice that the mutant coronal suture was still fused with treatment of DMSO vehicle after 9d culture, but kept patent with treatment of PD98059. (D) Inhibition of the Erk1/2 pathway by PD98059 partially rescued the growth retardation in cultured mutant femoral bones. Green line represents the total length (TL) and two yellow lines represent the cartilaginous tissue length (CL). (E) Percentage increases in TL and CL of femoral bones after culture for 6 days. (Percentage increase=|length at day 6−length at day 0|/length at day 0). *, significantly different from those of the wild-type mice, \(P \leq 0.05\).
mutation-carrying mouse models [6,75], this Fgfr2+/P253R mouse model also exhibited the premature closure of coronal sutures, midface hypoplasia, severe malocclusion and dysplasia of skull base, which phenotypically mimicked that of Apert syndrome patients. Our model also had obviously increased skull breadth, which is normal or slightly increased in Apert patients [12].

Apert syndrome patients frequently exhibit symmetric syndactyly of the hands and feet, so far have found three Fgfr2+/P253R mice with syndactyly, since syndactyly have never been found in Fgfr2+/S252W mice [6,75], it suggested that there was some difference in the occurrence of syndactyly between Fgfr2+/P253R and Fgfr2+/S252W mice. In fact, Apert syndrome patients carrying Fgfr2 S252W have higher frequency of syndactyly than FGFR2 S252W-carrying patients [68]. Nevertheless, the rare occurrence of syndactyly in Fgfr2+/P253R mice still indicated that the Fgfr2 P253R mutation in mice does not affect limb development as obvious as it in Apert syndrome patients.

Although intensive studies are being carried out, the pathogenesis of craniosynostosis is still not completely clarified. There were even some controversies about the mechanisms for the craniosynostosis resulting from gain-of-function mutations of FGFR2. Lomri et al. [46] and Lemonnier et al. [42] found increased differentiation and bone matrix formation in calvarial cells derived from synostosed sutures of fetuses and infants with Apert syndrome. Mansukhani et al. [48] revealed inhibited differentiation and mineralization, and increased apoptosis in osteoblasts with the Fgfr2 S252W mutation. Lemonnier et al. also found increased apoptosis in osteoblasts harvested from Apert patients [43]. While Wang et al. and Chen et al. have found dysregulated osteoblast differentiation and increased apoptosis in knock-in mouse models carrying Fgfr2 S252W mutation respectively [6,75].

Taking the advantage of our Fgfr2+/P253R mouse model, we found that Fgfr2 P253R mutation led to increased osteoblast differentiation. Actually suture development is a highly coordinated process including mesenchymal cell condensation and proliferation, differentiation of preosteoblasts to mature osteoblasts and osteocytes, and apoptosis [55]. Suture is the growth center of calvaria. Stem cells located between the two approaching bone plates that form the sutures continuously provide osteoprogenitor cells that facilitate the calvarial expansion to accommodate the development of the underlying brain. The gain-of-function mutations in Fgfr2 could induce accelerated differentiation of osteoprogenitor cells/preosteoblasts and/or increased apoptosis in the suture areas [6,48,75], which could lead to precocious suture closure by accelerating the obliteration of these areas. Consistently, our data demonstrated that Fgfr2 P253R mutation induced accelerated differentiation of the osteoblasts, thereby caused the premature closure of coronal sutures. The obviously decreased mesenchymal cells within the coronal sutures in Fgfr2+/P253R mice than that in wild-type mice further supported the above hypothesis.

In addition to the changes of local osteogenic and osteoblast cells around sutures, several reports suggested that the synchondrosis and cartilage at sutures were also involved in the development of cranial suture [37]. In fact, craniosynostosis may involve changes in both the cranial base as well as the calvaria [7]. To check the role of cartilage formation in the pathogenesis of Apert syndrome, we checked the chondrogenesis in the mutant skulls including calvaria and cranial base.

Metopic suture defect is a characteristic skull abnormality found in Apert syndrome patient. Recently, it was identified that endochondral ossification was the major event leading to the closure of PF suture (equivalent to metopic suture in human) in mice, and haploinsufficiency of Sox9, a direct regulator of chondrogenesis, resulted in a delayed PF suture closure due to impaired cartilage formation [64]. Consistently, we found that cartilage formation in PF sutures was impaired in Fgfr2+/P253R mice, which exhibited delayed PF suture closure. The delayed closure of PF sutures in the Fgfr2+/P253R mice was consistent with the metopic or interfrontal suture defects found in Apert syndrome patients and in mouse model with Fgfr2 S252W mutation [36,75], implicating that the impaired chondrogenesis may be responsible for the metopic suture defects found in patients with Apert syndrome.

Similar to Apert syndrome patients [7], Fgfr2+/P253R mice also exhibited brachycephaly. In the late embryonic stage, the mutant mice already showed brachycephaly. However, none of the calvarial sutures was fused but the cranial base shortening was obvious, indicating that the brachycephaly observed at this stage could be caused mainly by the defect of synchondrosis. After birth, premature closure of coronal sutures further aggravated the severity of brachycephaly. On the other hand, our in vitro calvaria explant (without cranial base) experiments showed that the premature closure of coronal sutures is independent of the cranial base changes in Fgfr2+/P253R mice, indicating that both the dysregulated osteogenesis and chondrogenesis contributed to the brachycephaly found in Apert syndrome mice.

In addition to abnormal development of cranial cartilage, the Fgfr2+/P253R mice also showed retarded cartilage formation in long bone, which was formed through endochondral ossification. In Apert syndrome patients, it was reported that a two step linear growth deceleration results in rhizomelic shortness of the lower limbs [12]. Short statures have been observed previously in several mouse models with Fgfr2 mutations [22,24,80]. Yu et al. [22,24,80] found shorter statures in mice with conditional deficiency of Fgfr2 in mesenchymal condensations that give rise to both osteoblast and chondrocyte lineages, indicating that Fgfr2 is involved in cartilage development. The proliferation and differentiation of chondrocytes in growth plates were significantly decreased in Fgfr2+/P253R mice. To exclude the potential effects of malocclusion on long bone growth, overerupted incisors of Fgfr2+/P253R mice had been cut and remained in approximately the normal lengths, but the mutant mice still showed shorter statures. To further exclude the systemic effects of Fgfr2 P253R mutation on long bone development, we did femur explant culture experiment, and still found retarded femur growth of mutant mice when compared with femurs from wild-type mice, suggesting that the growth retardation of long bones was directly resulted from the inhibiting effects of Fgfr2 P253R mutation on the endochondral ossification of long bones. Since
it’s well known that Fgfr2 is expressed mainly in osteoblast lineage, the direct effect of Fgfr2 P253R mutation on cartilage development prompted us to observe if Fgfr2 is expressed in cartilage. We detected Fgfr2 expression not only in the osteoblasts in cranium and long bones, but also in the chondrocyte lineage either by in situ hybridization or by RT-PCR. This is consistent with previously reported expression of Fgfr2 in chondrocyte lineage [40,61]. These results further suggested that Fgfr2 was involved in the regulation of development of both osteoblasts and chondrocytes. The effects of Fgfr2 on chondrocyte development need to be further studied.

Bone development is a highly coordinated process involving a variety of signaling pathways [29,38,54]. FGFRs signaling has been found to play important roles in skeleton development and diseases including Apert syndrome [4,49,57]. A number of molecules including PKC, Wnt, IL-1, MAPK, IL-1alpha, Grb2, p85 beta and small GTPase RhoA, etc. were reported to be involved in the pathogenesis of Apert syndrome [2,44,45,47]. Of these molecules, MAPK is well known for its role in skeleton development and in mediating the effect of FGFs/FGFRs on bone development. The Erk1/2 pathway has been implicated to play important roles in both osteoblast and chondrocyte development [3,30,53,60,77,78]. It was shown recently that activation of the Erk1/2 pathway was involved in the FGF2-stimulated cranial suture closure, and the accelerated suture closure induced by FGF2 can be almost completely inhibited by an Erk1/2 blocker [30,41], demonstrating the pathogenesis of Apert syndrome resulting from P253R mutation. But the Erk1/2 pathway [60]. Activation of Erk1/2 in chondrocytes in vivo can cause an achondroplasia-like dwarfism [50]. But there is no direct evidence for the involvement of Erk1/2 in the pathogenesis of Apert syndrome resulting from P253R mutation in Fgfr2. Using in vivo mouse models mimicking human Apert syndrome, we examined the effects of PD98059, an Erk1/2 inhibitor, on the closure of coronal sutures of Fgfr2<sup>+/−P253R</sup> mice. Our data revealed that inhibition of Erk1/2 activity significantly prevented the premature closure of coronal sutures in Fgfr2<sup>+/−P253R</sup> mice. Moreover, inhibition of the Erk1/2 pathway also partially recovered the retarded endochondral ossification of cultured mutant femurs. Since other MAPK/ERK kinases such as P38 are also involved in the regulation of chondrocyte and osteoblast development [60,73], it’s speculated that these pathways may also be involved in the pathogenesis of the skeleton phenotypes resulting from Fgfr2 mutation. These findings, for the first time using mouse model mimicking human Apert syndrome, indicated an important role of the Erk1/2 pathway in Fgfr2 P253R mutation-induced abnormal osteogenesis and chondrogenesis.

In summary, in this study we have generated a novel mouse model mimicking human Apert syndrome resulting from Fgfr2 P253R mutation. Our phenotype analysis of this mouse model strongly suggested that the skeletal phenotypes of Apert syndrome may be caused by the collective effects of mutant Fgfr2 on both chondrocyte and osteoblast lineages. Moreover, we provided evidence that the Erk1/2 pathway is involved in the pathogenesis of skeletal abnormalities induced by the Fgfr2 P253R mutation, suggesting a potential application of Erk1/2 modulator in the treatment of craniosynostosis syndrome. Shukla et al. recently showed that in vivo application of U0126, an inhibitor of MEK1/2 significantly inhibits craniosynostosis resulting from Fgfr2 S252W mutation [67]. Like other previously generated mice with mutations in Fgfr1, Fgfr2 or Fgfr3 [6,27,51,74,75,82], the Fgfr2<sup>+/−P253R</sup> mouse model provides a useful in vivo system for further studies on the pathogenesis of FGFR-related human genetic bone diseases.

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