A Pro250Arg substitution in mouse Fgfr1 causes increased expression of Cbfa1 and premature fusion of calvarial sutures

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Pfeiffer syndrome is a classic form of craniosynostosis that is caused by a proline→arginine substitution at amino acid 252 (Pro252Arg) in fibroblast growth factor receptor 1 (FGFR1). Here we show that mice carrying a Pro250Arg mutation in Fgfr1, which isorthologous to the Pfeiffer syndrome mutation in humans, exhibit antero-posteriorly shortened, laterally widened and vertically heightened neurocraniums. Analysis of the posterior and anterior frontal, sagittal and coronal sutures of early post-natal mutant mice revealed premature fusion. The sutures of mutant mice had accelerated osteoblast proliferation and increased expression of genes related to osteoblast differentiation, suggesting that bone formation at the sutures is locally increased in Pfeiffer syndrome. Of note, dramatically increased expression of core-binding transcription factor α subunit type 1 (Cbfa1) accompanied premature fusion, suggesting that Cbfa1 may be a downstream target of Fgf/Fgfr1 signals. This was confirmed in vitro, where we demonstrate that transfection with wild-type or mutant Fgfr1 induces Cbfa1 expression. The induced expression was also observed using Fgf ligands (Fgf2 and Fgf8). These studies provide direct genetic evidence that the Pro252Arg mutation in FGFR1 causes human Pfeiffer syndrome and uncovers a molecular mechanism in which Fgf/Fgfr1 signals regulate intramembraneous bone formation by modulating Cbfa1 expression.

INTRODUCTION

Craniosynostosis is a clinical condition characterized by the premature fusion of one or more of the cranial sutures. As a common birth defect, craniosynostosis has been identified in >100 genetic syndromes and has an estimated incidence of 1 in 2500 live births (1). The most common craniosynostosis syndromes include Apert, Crouzon, Saethre–Chotzen and Pfeiffer syndromes (1). All are dominantly inherited, with complete penetrance and variable expressivity. Although recent investigations have linked several of these syndromes to mutations in a number of growth factors and signaling molecules, including members of the fibroblast growth factor receptor (FGFR) gene family (1), the molecular mechanisms underlying these genetic conditions remain to be determined.

FGFRs constitute a gene family of four membrane-bound tyrosine kinases that mediate signals from at least 19 ligands (2–6). These receptors share several common structural features, including a hydrophobic leader sequence, three immunoglobulin (Ig)-like domains, an acidic box, a transmembrane region and a divided tyrosine kinase domain. FGFRs normally exist in inactive monomeric forms and dimerize on binding to FGFs. This dimerization activates their tyrosine kinase activity and triggers downstream effects through as yet undefined signal transduction pathways. In situ hybridization studies showed that FGFRs are expressed in a variety of tissues and organs throughout vertebrate development with distinct expression patterns for each receptor. In general, Fgfr1 is expressed almost exclusively in mesenchyme (7–9). Fgfr2 is found predominantly in epithelium (7,8,10,11). Fgfr3 is mainly located in the developing central nervous system as well as in bone rudiment (12) and Fgfr4 is expressed in the definitive endoderm and the somatic myotome (13). This expression pattern suggests that each receptor may play important, yet distinct, roles during vertebrate development. This notion was confirmed by mutation analysis in mice using gene targeting, which revealed that Fgfr1 is essential for mesoderm patterning and cell migration during gastrulation (14–17). Fgfr2 plays a role in placenta formation and limb initiation (18,19). Fgfr3 is a negative regulator in bone growth and development (20,21) and both Fgfr3 and Fgfr4 function cooperatively to control post-natal lung development (22).

Pfeiffer syndrome (23) is characterized by the premature fusion of several cranial sutures, which result in a short tower-shaped head, widely spaced eyes, a small nose and an under-developed midface (23,24). In addition, patients with Pfeiffer syndrome also have characteristic hand and foot anomalies, including broad thumbs, varying degrees of cutaneous syndactyly, shortened fingers and medially deviated broad toes. Occasionally, brain anomalies are found, including agenesis of the corpus callosum, optic atrophy, hydrocephalus and Arnold–Chiari malformation. It was shown that a C→G conversion in exon 5 of FGFR1, which predicts a proline→arginine substitution in its extracellular domain, was identified in members of five unrelated Pfeiffer syndrome pedigrees (25). This Pro252Arg mutation in FGFR1 is associated with milder

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forms, whereas various mutations in FGFR2 have been identified in both mild and severe forms of Pfeiffer syndrome (1).

To study the functions of FGFR1 in bone growth and to create a mouse model for the FGFR1-related inherited skeletal disorder, we introduced a Pro250Arg mutation, which corresponds to the Pro252Arg mutation found in Pfeiffer syndrome, into mouse Fgfr1 using homologous recombination. The resulting mutant mice exhibited craniosynostosis and skull malformations that mimic the conditions found in Pfeiffer patients. Most importantly, our data provide in vitro and in vivo evidence that FGF/FGFR1 signals through core-binding transcription factor α subunit type 1 (CBFA1) to regulate bone formation.

RESULTS

Introduction of the Pro250Arg mutation into murine Fgfr1

To investigate the molecular mechanisms underlying Pfeiffer syndrome, we introduced an Fgfr1 Pro250Arg mutation, which corresponds to the human FGFR1 Pro252Arg mutation, into mouse embryonic stem (ES) cells using homologous recombination (Fig. 1a and c). Germline transmission was obtained from three independently targeted ES clones. The presence of pLoxneo in intron 6 was found to interfere with normal Fgfr1 splicing (data not shown); therefore, mice heterozygous for the targeted mutation (Fgfr1<sup>pro250</sup>/+) were crossed with Ella–Cre mice (26) to excise the pLoxneo sequence (Fig. 1b). After neo gene removal, northern blot analysis indicated that the mutant allele was expressed at similar levels to the wild-type allele (Fig. 1d). All data presented in this study were obtained from mice lacking the neo gene.

The Fgfr1 Pro250Arg mutation results in craniosynostosis

Fgfr1<sup>pro250</sup>/+ mice were phenotypically normal at birth. However, beginning from post-natal day 3 (P3), they gradually exhibited craniofacial malformations (Fig. 2a and b). When examined at P16–P21, one prominent feature of mutant skulls was the premature fusion of the frontal, sagittal and coronal sutures, whereas the lambdoid and occipitointerparietal sutures appeared normal (Fig. 2c–e). All mutant mice (<i>n</i> = 20) exhibited facial asymmetry characterized by anterio-posteriorly shortened, laterally widened and vertically heightened neurocrania as revealed by Alizarin Red S and Alcian Blue staining (Fig. 2c–e) and morphometric analysis of soft X-ray images (data not shown). Some (12/20) mice also exhibited ocular hypertelorism (widely spaced eyes) and an underdeveloped midface (midface hypoplasia) (Fig. 2). The mutant mice also displayed increased length of incisors (Fig. 2a), which could be a consequence of craniofacial asymmetry disrupting normal mouth closure. Other than craniofacial abnormalities, no other obvious skeletal malformations were observed. In addition, analysis of the cranial base from P20 mice revealed no premature closure of the presphenoid-sphenoidal, basiphenoid–occipital and basioccipital–exoccipital synchondroses (data not shown), suggesting that mouse Fgfr1 Pro250Arg-associated craniosynostosis can develop in the absence of cranial base abnormality. It was proposed that the cranial base can be a primary site of defects in human craniosynostosis (27). Our data do not support this hypothesis in this instance.

To date, no homozygous Pfeiffer syndrome patients have been identified. We decided to test whether Fgfr1<sup>pro250</sup>/+ homozygous mice are lethal. Our data showed that heterozygous mice were fertile and generated wild-type, Fgfr1<sup>pro250</sup>+/+ and Fgfr1<sup>pro250</sup>/250 mice in a Mendelian ratio. However, homozygous mice were much smaller than heterozygous mice and displayed similar but more severe patterns of skull malformations (Fig. 2d and e).

Because mutant mice, especially homozygous mice, were smaller and displayed shorter tail lengths than wild-type littermates, we compared their epiphysial growth plates to determine whether endochondral ossification was affected by the introduced Fgfr1 mutation. No significant differences were observed in the long bone growth plates of wild-type or mutant mice (data not shown), suggesting that the reduced size of Fgfr1<sup>pro250</sup>/+ and Fgfr1<sup>pro250</sup>/250 mice is not caused by retarded growth.
endochondral ossification as observed in mice with Fgfr3 mutations (28–31).

**The Pro250Arg mutation increases differentiation of osteoblastic cells**

The cranial sutures were examined in histological sections. Consistent with whole skull bone Alizarin Red staining, the frontal, sagittal and coronal sutures of mutant mice exhibited premature closure (Fig. 3a and b). To identify the molecular events contributing to the premature cranial fusion, cranial sections of younger mice were analyzed using bone formation markers. Staining of P7 sagittal sutures with Alizarin Red revealed that the ossification fronts in Fgfr1250/+ and Fgfr1250/250 calvarial bones overlapped significantly (Fig. 3d), whereas wild-type sutures remained open (Fig. 3c). von Kossa staining showed that bone mineralization was more advanced in heterozygous mice than in their wild-type littermates at P5 (Fig. 3e and f). The calvarial bone at the sagittal sutures of P1 mice was surrounded by numerous alkaline phosphatase (AKP)-positive osteoblasts (Fig. 3h and g). The number of AKP-positive cells was significantly higher in homozygous than in wild-type mice, and heterozygotes exhibited intermediate numbers (data not shown).

The increased number of osteoblasts in mutant mice is consistent with the faster growth of cranial sutures. During calvarial bone growth, osteoblasts undergo proliferation and differentiation. Fgfr1 is normally expressed in the cranial sutures, with higher levels at the growing front (Fig. 4a and b), suggesting involvement of Fgf/Fgfr1 signals in this process. To examine the effect of the Fgfr1 Pro250Arg mutation on osteoblast activities, we labeled mice with bromodeoxyuridine (BrdU) and found that mutant sutures had an increased number of positive cells at P5. Directly counting BrdU-positive cells on sagittal sutures indicated that there are on average 49.6 ± 14.98 cells/section in mutant (n = 3) and 27.6 ± 5.27 cells/section in wild-type (n = 3) littermates (Fig. 4c and d). This suggests that the mutation enhanced proliferation of osteoblasts in the sutures. Next, we examined the differentiation state of mutant and wild-type osteoblasts. It is known that differentiated osteoblasts produce several non-collagenous proteins, including bone sialoprotein, osteocalcin and osteopontin (32–34). We found that expression of these genes was significantly increased in mutant sutures (Fig. 4f and h) compared with wild-type controls (Fig. 4e and g). These results indicate that the Fgfr1 Pro250Arg mutation enhanced proliferation and differentiation of osteoblasts leading to accelerated bone formation.

The enhanced differentiation of osteoblast cells prompted us to search for downstream targets of Fgf/Fgfr1-mediated signals. Molecular and genetic evidence demonstrated that Cbfa1 acts as an activator of osteoblast differentiation during embryonic development in mice and humans (35–38). Moreover, recent data suggest that beads soaked with FGFs in dental epithelial explants (39) can induce Cbfa1 expression. To examine whether the Pro250Arg mutation in Fgfr1 could affect Cbfa1 expression, sections from P5 wild-type and mutant coronal (Fig. 4i and j) and sagittal (Fig. 4k and l) sutures were
hybridized with a Cbfa1 RNA probe. In the coronal and sagittal sutures, mutant osteoblasts (Fig. 4j and l) expressed Cbfa1 at much higher levels than wild-type cells (Fig. 4i and k). This observation suggests that mutated Fgfr1 causes up-regulation of Cbfa1, which subsequently activates its downstream transcription targets, including osteocalcin and bone sialoprotein (35–37), ultimately leading to accelerated differentiation of osteoblasts.

We also checked expression of Stats (signal transcription and translation) and Msx2. Stats are known to mediate FGFR3 signals and play an important role in retarding long bone growth (30,40,41), whereas mutations of Msx2 are correlated with Boston-type craniosynostosis (42–44). Our data indicate that these proteins are unlikely to be candidates responsible for the observed craniosynostosis since no changes were found in expression or activation of Stat1, Stat5a, Stat5b or Msx2 (data not shown).

To determine whether the effect of Cbfa1 up-regulation is indeed a consequence of the Fgfr1 Pro250Arg mutation, we performed transfection assays. Transfection of C3H10T1/2 cells, which are derived from mouse fibroblasts and do not express osteoblast-specific genes (45), with mouse Fgfr1 Pro250Arg mutant cDNA caused transcription induction of Cbfa1 (Fig. 5a). Similar results were also observed using MC3T3-E1 cells (data not shown). These data confirm our in vivo observation that the Pro250Arg mutation up-regulates Cbfa1. Of note, we found that transfections with wild-type Fgfr1 also induced Cbfa1 expression, although at lower levels (Fig. 5a). This observation suggests that signals through wild-type Fgfr1 may also regulate Cbfa1 expression during the normal processes of suture development. To test this, we treated C3H10T1/2 cells, which normally express Fgfr1 (data not shown), with FGF2 and FGF8. A dramatic induction of Cbfa1 expression was observed when the cells were treated with these FGFs at a concentration of 10 ng/ml (Fig. 5b and c). Interestingly, differential responses of Cbfa1 induction were observed when the cells were treated with different FGFs. At low doses, FGF2 (10 ng/ml) induced Cbfa1 expression at much higher levels than high doses of FGF2 (200 ng/ml); however, the opposite effect was observed with FGF8 treatment (Fig. 5b). Multiple Fgfs have been detected in the cranial sutures (46,47). Therefore, it is conceivable that these ligands may

Figure 3. Histological analysis of sagittal sutures of wild-type (+/+) and mutant (250+/+) mice at different developmental stages. (a and b) Hematoxylin and eosin staining revealed premature fusion of the sagittal suture from P20 mutant mice (arrows). (c and d) Alizarin Red staining showing calcified calvarial bone (arrows) in P7 mice. (e and f) Von Kossa stain showing advanced bone mineralization in P5 mutant bone. Arrows point to von Kossa-positive signals. (g and h) P1 mutant mice showed more ALP-positive cells than their controls (arrows). Bar, 210 µm for all panels.

Figure 4. The Pro250Arg mutation results in increased proliferation and enhanced differentiation of osteoblasts of cranial sutures. All sutures were from P5 mice. (a and b) Immunohistochemical staining of wild-type (a) and 250+/+ (b) sutures with an antibody to Fgfr1. (c and d) BrdU incorporation showing that mutant sutures (d) exhibit accelerated proliferation of osteoblasts compared with wild-type suture (c). (e-l) In situ hybridization using antisense probes for bone sialoprotein in wild-type (e) and 250+/+ (f), osteocalcin in wild-type (g) and 250+/+ (h) and Cbfa1 in wild-type (i and k) and 250+/+ (j and l) sutures. Arrows point to positive signals in (e), (f), (h), (j) and (l). Bar, 230 µm for all panels.
have quite different effects on Cbfa1 activity and have distinct effects on bone formation.

**DISCUSSION**

In this study, we have introduced a Pro250Arg mutation in Fgfr1 into the mouse germline by gene targeting and generated the first animal model for FGFR1-related craniosynostosis syndromes. Molecular analysis of mutant sutures revealed that advanced ossification of calvarial bones is accompanied by enhanced expression of genes related to osteoblast differentiation. Premature closure of the coronal, sagittal and frontal sutures in mutant mice prevented further growth along the margins of these bones. This premature closure is incompatible with the relatively normal growth at other sutures and consequently results in craniofacial distortion. Of note, we found that FGF/FGFR1 signals can dramatically increase Cbfa1 expression in vivo and in vitro. These observations prompt us to propose a molecular model in which the FGF/FGFR1 signals regulate flat bone formation by modulating expression of CBFA1 (Fig. 6).

Vertebrate bones are formed through intramembranous and endochondral ossification. The former involves formation of flat bones and the latter is responsible for long bone growth. The FGF/FGFR signaling pathway has been implicated in both modes of bone formation. It has been shown that missense mutations in three FGF receptors (FGFR1–FGFR3) results in craniosynostosis syndromes, including Pfeiffer, Crouzon, Apert and Jackson–Weiss syndromes and Beare–Stevenson cutis gyrata (reviewed in ref. 1). Mutations in FGFR3 were also found to cause several skeletal dysplasias that exhibit varying degrees of long bone growth (31,48–50). To understand the mechanisms underlying these diseases, we and others have disrupted Fgfr3 by gene targeting in mice and demonstrated that Fgfr3-associated skeletal dysplasias are not caused by haploinsufficiency or loss-of-function mutations since mouse strains lacking either one or both Fgfr3 alleles do not exhibit phenotypes resembling these conditions (14,17,18,20, 21,51). Notably, loss of FGFR3 results in phenotypes opposite to those seen in achondroplasia patients, suggesting that missense mutations associated with the disease are gain-of-function mutations (20,21).

The effects of missense mutations on FGFR activity and functions have been extensively examined. To date, all mutations cause varying degrees of ligand-independent receptor activation (30,51,52). These activated receptors, in turn, activate the Stat signaling pathway, which causes up-regulation of cell cycle inhibitors and leads to decreased chondrocyte proliferation and retarded long bone growth (28,30,53). Reduced expression of IHH and/or BMP has also been observed in dwarf mice generated by gene targeting (28,30) and transgenic approaches (54).

However, most of these studies have primarily focused on the effects of Fgfr3 and its relationship to long bone growth. For flat bone formation, the mechanism of mutant receptor activation and their downstream mediators causing craniosynostosis remain to be determined. The flat bones are formed by differentiation of mesenchymal precursor cells into bone-forming osteoblasts. Their growth relies on continuous proliferation and differentiation of osteoblast cells at the growing fronts of bone. Our analysis indicates that craniosynostosis in our mutant mice is associated with increased differentiation of osteoblasts between P1 and P20, although a transient increase in proliferation was detected at P5. These observations suggest a primary role for Fgfr1 signals in promoting osteoblast differentiation. This is consistent with a recent study which showed that Fgfr1 expression was associated with osteoblast differentiation (55). It appears that excessive differentiation signals caused by the Pro250Arg mutation result in premature intramembranous ossification of the sutures, which ultimately prevents further growth of the affected bones.

Notably, we found that the Pro250Arg mutation in Fgfr1 causes significantly increased transcription of the genes for bone sialoprotein, osteocalcin, AKP and Cbfa1 in mutant

![Figure 5](image-url)

**Figure 5.** The induction of Cbfa1 expression by an Fgfr1 point mutation and Fgfs in C3H10T1/2 cells. (a) RT–PCR analysis using primers to amplify exons 1–3 of Cbfa1 (655 bp, upper band). Amplification of exon 2 of the Hprt gene was used as a control (lower band). Treatment of cells was as indicated. (b) RT–PCR analysis showing Cbfa1 expression in cells that were treated with different concentrations of FGF2 and FGF8 for 48 h.

![Figure 6](image-url)

**Figure 6.** Summary of Fgfr1 function in intramembranous ossification. Numerous studies indicate that Cbfa1 is essential for intramembranous ossification through its regulation of multiple molecules including AKP, osteocalcin and bone sialoprotein. Our data demonstrate that Fgfs/Fgfr1 signals are involved in normal flat bone development through interacting with Cbfa1. The Pro250Arg mutation in Fgfr1 results in activation of the receptor and up-regulation of Cbfa1 (thick arrows), leading to subsequent increases in transcription of the Cbfa1 downstream target genes. This causes increased bone matrix deposition, ultimately resulting in premature intramembranous ossification of the cranial sutures.
sutures. Since these genes are well known to have essential functions in bone formation (32,33,35–38), it is conceivable that their up-regulation is responsible for the observed premature craniosuture ossification. Of these genes, Cbfa1 is the only one encoding an osteoblast-specific transcription factor identified to date (35). Molecular and genetic evidence has demonstrated that Cbfa1 activates osteoblast differentiation during development. Indeed, it is expressed in cells of osteoblastic lineage during development and regulates osteoblast-specific expression of osteocalcin and osteopontin. It can also induce osteoblast differentiation of non-osteoblastic cells (35). Patients heterozygous for mutations or deletions of CBFA1 develop cleidocranial dysplasia (CCD) (38,56). Likewise, inactivation of Cbfa1 in mice leads to a total absence of osteoblasts in homozygous mutant animals and to a CCD phenotype in heterozygous mutant animals (36,37).

Taken together, these observations suggest that Cbfa1 is an indispensable regulator of osteoblast differentiation (57). However, experiments are just beginning to explore mechanisms that regulate Cbfa1 expression and activity. It was shown recently that MAPK pathways activate and phosphorylate Cbfa1 (58). Here we show that both the activation mutation of Fgfr1 and increasing FGF signals result in up-regulation of Cbfa1 in vivo and/or in vitro. Since a major pathway for FGF function is through the activation of MAPK (58), it is possible that the up-regulation of Cbfa1 by FGF signals could be mediated by MAPK. Although this notion remains to be explored, our finding that Fgfr1/Fgfr1 signals function upstream of Cbfa1 provides a connection between these two major pathways during cranial suture formation. The expression of Cbfa1, in turn, activates its downstream cascades which specify differentiation and proliferation of osteoblast cells (Fig. 6).

MATERIALS AND METHODS

Targeting vector

A co-transfer type targeting vector (59) was constructed using Fgfr1 genomic DNA (14). A 4.7 kb NdI–SpHl fragment was modified by the addition of a SaI linker. Using site-directed mutagenesis we introduced a mutation, which translates into a modified by the addition of a Sal genomic fragment (14). A 4.7 kb A co-transfer type targeting vector (59) was constructed using Targeting vector

Northern blots and in situ hybridization

Northern blots and in situ hybridization were performed using standard procedures. The Fgfr1 probe was a 500 bp EcoRI– BamHI fragment from the Fgfr1 cDNA (62). Cbfa1 and osteopontin probes were obtained from P. Ducy (Baylor College of Medicine, Houston, TX) and S. Iseki (Oxford University, Oxford, UK), respectively. The sense probes were used as controls. Slides were dipped in NTB-2 emulsion (Kodak, Rochester, NY) and exposed for 4–15 days.

Cell culture, DNA transfection and RT–PCR

MC3T3-E1 mouse calvarial cells and C3H10T1/2 fibroblasts were maintained as described (35). The Fgfr1 Pro250Arg mutation was introduced into the expression plasmid (MoFgfr1/SV) (62) by site-directed mutagenesis using a QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All PCR cloned inserts were sequenced using

Genotype analysis

Genotypes were determined by Southern blotting as described above or by PCR. For PCR analysis, the Fgfr1 allele was detected using the primer set 5′-CATGTAAACTCTGCTTGCTCG-3′ and 5′-GAGGGAGTAAAGAAGAGGAGGAG-3′, which amplifies a 508 bp wild-type or a 568 bp mutant fragment.
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