Published in final edited form as:

Sci Signal. ; 3(123): ra40. doi:10.1126/scisignal.2000727.

The Balance of WNT and FGF Signaling Influences Mesenchymal Stem Cell Fate During Skeletal Development

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Abstract

Craniosynostosis, a developmental disorder resulting from premature closure of the gaps (sutures) between skull bones, can be caused by excessive intramembranous ossification, a type of bone formation that does not involve formation of a cartilage template (chondrogenesis). Here, we show that endochondral ossification, a type of bone formation that proceeds through a cartilage intermediate, caused by switching the fate of mesenchymal stem cells to chondrocytes, can also result in craniosynostosis. Simultaneous knockout of *Axin2*, a negative regulator of the WNT- β -catenin pathway, and decreased activity of fibroblast growth factor (FGF) receptor 1 (FGFR1) in mice induced ectopic chondrogenesis, leading to abnormal suture morphogenesis and fusion. Genetic analyses revealed that activation of β -catenin cooperated with FGFR1 to alter the lineage commitment of mesenchymal stem cells to differentiate into chondrocytes, from which cartilage is formed. We showed that the WNT- β -catenin pathway directly controlled the stem cell population by regulating its renewal and proliferation, and indirectly modulated lineage specification by setting the balance of the FGF and bone morphogenetic protein pathways. This study identifies endochondral ossification as a mechanism of suture closure during development and implicates this process in craniosynostosis.

Introduction

Bone can form through two different processes. Intramembranous ossification is responsible for the final development of the skull bones, jawbones, and collarbones, whereas endochondral ossification occurs in the appendicular and axial skeleton. Craniosynostosis, a common human congenital craniofacial deformity affecting 1 in ~2500 individuals, is caused by premature suture closure (closing of the gaps in the skull bones during development) (1–3). The sutures serve as growth centers for skeletogenesis mediated mainly by intramembranous ossification, where the pluripotent mesenchyme develops directly into osteoblasts that form the skull bones (4). This differs from endochondral ossification in the appendicular and axial skeletons, for which prior formation of chondrogenic intermediates (cartilage) is required. Aberrant osteoblast (the cell that deposits bone) proliferation, differentiation, or apoptosis affects intramembranous ossification, causing suture abnormalities and craniosynostosis (5,6). During

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Author contributions: T.M. and W.H. conceived and designed the experiments. C.-X.D. provided Fgfr1F mice. T.M., A.J.M., and W.H. performed the experiments, analyzed the data, and wrote the paper.

Competing interests: Mice reported in this paper are available, but require a material transfer agreement from the University of Rochester.

embryogenesis, transient appearance of chondrocytes is evident in certain sutures, but these cells do not appear to contribute to the developmental process (7). Although aberrant localizations of chondrocytes have been described in the synostosis-related syndromes of humans and mice, it is not clear whether their presence, and thus endochondral ossification, is involved in normal suture morphogenesis and synostosis (8–13).

We previously linked the WNT– β -catenin pathway to craniosynostosis by showing that mice in which the gene encoding AXIN2, a negative regulator of WNT signaling, was knocked out exhibited skeletal defects resembling craniosynostosis in humans (14). In these *Axin2*-deficient mice, premature suture closure, caused by excessive intramembranous ossification, occurs at early postnatal stages (14). During the development of osteoblasts, β -catenin serves a dual role, promoting the expansion of the skeletal precursor cell population (14,15) and the commitment of these cells to the osteoblast lineage (16,17). Thus, genetic disruption of *Axin2* likely alters both of these bone regulatory activities of β -catenin.

Knockout of *Axin2* resulted in induction of the synostosis-related genes, including those encoding members of the fibroblast growth factor (FGF) receptor (FGFR) family (14,15,18–20). Genetic analyses in mice and humans have also demonstrated that the FGF pathway is involved in skeletal development and disease (6,11,21–23). Mutations in the genes encoding members of the FGFR family have been linked to synostosis-related syndromes in humans (18). Studies of mouse models support the notion that enhanced FGFR signaling causes excessive osteoblast activity and can lead to the development of craniosynostosis (9,11,20, 21,24). How the WNT and FGF pathways interact to orchestrate suture closure and skull development remains largely unknown.

We report that disruption of the genes encoding both AXIN2 (complete loss) and FGFR1 (heterozygous deficiency) in mice induced ectopic chondrogenesis and endochondral ossification, leading to abnormal suture closure and skull deformities. Our results indicate that the WNT– β -catenin pathway directly controls the skeletal precursor population by stimulating its renewal and proliferation, and indirectly influences lineage specification by setting the balance of the FGF and bone morphogenetic protein (BMP) pathways. Furthermore, we provide evidence that switching the fate of mesenchymal stem cells to chondrocytes is an etiologic mechanism for craniosynostosis in mice. Thus, in addition to excessive osteoblast activity, which forms bone through intramembranous ossification, enhanced bone formation through endochondral ossification as a result of chondrocyte activity can also contribute to synostosis-related syndromes.

Results

Disruption of *Axin2* and *Fgfr1* in mice results in ectopic chondrogenesis in the sagittal suture

In humans and mice, the skull vault has seven sutures, and the patency (ability to remain open and continue to form bone) of these sutures is essential for proper growth regulation of the skull, especially during early postnatal stages (3). Two of these are the sagittal (SAG) suture and the posterior frontal (PF) suture. The PF suture closes during a mouse's lifetime, whereas the SAG suture and all other cranial sutures remain patent and bone continues to form at these sites as the animals grow (25). In humans and mice, the PF suture is structurally unique and its development requires a specific mechanism (26,27). This suture, residing between the frontal bones, is formed from a single mesenchymal interface, from which new bone forms, between two adjacent skull bones. The fusion of the PF suture's endocranial layer may involve a mechanism different from that occurring at other sutures, because chondrocytes transiently appear only at the PF suture during the second week of postnatal development (28). Like all other mouse cranial sutures, the ectocranial layer remains patent throughout life.

Because both WNT signaling and FGF signaling have been implicated in skeletal development and synostosis, we analyzed how these pathways interact during suture development and closure by monitoring skull development in postnatal mice deficient for *Axin2* ($Ax2^{-/-}$), in mice heterozygous for *Fgfr1* ($Fgfr1^{+/-}$), or in double-mutant mice ($Ax2^{-/-}; Fgfr1^{+/-}$) (Fig. 1).

Alcian blue (AB) staining of skulls of postnatal day 7 (P7) mice revealed that the double-mutant SAG sutures displayed obvious morphological differences compared to those of the wild type, $Ax2^{-/-}$, and $Fgfr1^{+/-}$ (Fig. 1, A to D). At P7, we detected a small AB-positive region indicative of ectopic deposition of connective tissue in the SAG sutures of 37% of $Ax2^{-/-}$ skulls (Fig. 1C, C'), and all of the SAG sutures exhibited a larger area of ectopic chondrogenesis in $Ax2^{-/-}; Fgfr1^{+/-}$ animals examined (Fig. 1D, D'). Neither the skulls of the wild-type (Fig. 1A, A') nor $Fgfr1^{+/-}$ (Fig. 1B, B') animals had detectable chondrogenesis. To confirm the presence of chondrocytes, in addition to staining with AB, we stained for chondrocyte markers SOX9 and type II collagen (25). AB staining (Fig. 1, E to H and E' to H') and immunostaining of Sox9 (Fig. 1, I to L) and type II collagen (COL II, Fig. 1, M to P) in cranial sections confirmed that $Ax2^{-/-}; Fgfr1^{+/-}$ mouse skulls had ectopic chondrogenesis in the developing SAG suture. Additionally, this analysis revealed that the ectopic chondrogenic layer developed beneath the SAG suture, resembling the endocranial layer of the developing PF suture.

SAG sutures of mice with disrupted *Axin2* and *FGFR1* exhibit PF-like activity of the FGF and TGF- β pathways

The PF and SAG sutures exhibit differential activity of FGF2 and transforming growth factor- β (TGF- β) signaling, with high activity of these two pathways in the PF suture and limited activity in the SAG suture (29–31). Therefore, we examined whether the PF-like SAG suture of the $Ax2^{-/-}; Fgfr1^{+/-}$ mice exhibited the FGF2 and TGF- β profile resembling that of the PF suture by analyzing the abundance of FGF2 and phosphorylated SMAD2 (pSMAD2, a marker of TGF- β activity) (Fig. 2). Analysis of P9 wild-type mouse skulls revealed FGF2 and pSMAD2 in the intramembranous ossification regions, including the osteogenic fronts and the area surrounding the bone plates, of both the PF and the SAG sutures (Fig. 2, A, B, D, and E, black arrows). Additionally, FGF2 and TGF- β were abundant in the endocranial layer of the PF suture mesenchyme that was undergoing chondrogenesis (Fig. 2, A and D, red arrowheads). The number of FGF2- and pSMAD2-positive cells was higher in the PF suture compared to that in the SAG suture (Fig. 2, G and H). Furthermore, these chondrogenic increases in FGF2 and TGF- β signaling activities coincided with the chondrocyte-mediated abnormalities within the $Ax2^{-/-}; Fgfr1^{+/-}$ SAG sutures (Fig. 2, C and F, red arrowheads), suggesting their alterations into PF-like sutures in the double mutants. The numbers of FGF2- and pSMAD2-positive cells in the mutant SAG sutures were comparable to the numbers in the wild-type PF sutures (Fig. 2, G and H).

Alteration of WNT and FGF signaling results in craniosynostosis in mice

To determine whether disruption of *Axin2* and *Fgfr1* induces suture deformities resulting in craniosynostosis, we examined the SAG and PF sutures and skulls of mice at P50, a time when the PF sutures are normally closed. In the wild-type, $Fgfr1^{+/-}$, and $Ax2^{-/-}$ mice, the SAG sutures were open at P50 (Fig. 3, A to C), whereas in the $Ax2^{-/-}; Fgfr1^{+/-}$ mice, the SAG sutures were fused (Fig. 3D). Similar to the PF suture at this stage in the wild-type animals (Fig. 3I), the SAG suture also consisted of an ectocranial and an endocranial layer in the $Ax2^{-/-}; Fgfr1^{+/-}$ (Fig. 3H). The SAG suture was a single layer in wild-type, $Fgfr1^{+/-}$, and $Ax2^{-/-}$ mice (Fig. 3, E to G). Furthermore, the synostosis occurred in the endocranial layer of the SAG suture of the double-mutant mice. Thus, whereas individual disruption of *Axin2* or *Fgfr1* does not alter the SAG suture, combined deficiency converts the SAG suture to one resembling the PF suture, leading to craniosynostosis.

Ectopic endochondral ossification is the likely cause of synostosis due to altered *Axin2* and *Fgfr1*

To determine if the SAG suture synostosis caused by disruption of *Axin2* and *Fgfr1* is mediated through endochondral ossification, we examined three critical events in the double-mutant SAG sutures at P10, P15, and P20: chondrocyte resorption, vascular invasion, and osteoblast differentiation, which progressively lead to deposition of a bony matrix. At P10 in the double-mutant animals, the SAG suture consists of a single mesenchymal interface between two adjacent skull bones (Fig. 4A), which is also present in the SAG suture of wild-type animals (fig. S1), and a region beneath the mesenchymal interface where the ectopic chondrocytes are located (Fig. 4A), which is not present in the wild-type SAG sutures. By P10 the region of ectopic chondrocytes was comparable to that detected at P7 (Fig. 1H). No ectopic chondrocytes were detected at P15 and P20 (Fig. 4, B and C) and apoptosis, detected by TUNEL staining, in the surrounding area correlated with the reduction of chondrocytes at these stages (Fig. 4, D to F), consistent with chondrocyte resorption. Vascular invasion, detected by immunostaining for the endothelial marker laminin, was evident at the site of ectopic chondrogenesis and the area where the chondrocytes had been resorbed (Fig. 4, G to I). Whereas at P10, osteoblast differentiation (shown by alkaline phosphatase activity) was detected only at the osteogenic fronts and the periphery of the mineralized bone matrix (Fig. 4J), after chondrocyte resorption and vascular invasion, osteoblast differentiation gradually increased in the region below the osteogenic front (Fig. 4, K and L) and continued to occur at the periphery of the mineralized bony matrix (Fig. 4, M to O). These endochondral ossification events are not involved in normal SAG suture development, where only intramembranous ossification at the osteogenic front occurs (fig. S1). Therefore, ectopic endochondral ossification is a likely mechanism for craniosynostosis that occurs in the *Ax2*^{-/-}; *Fgfr1*^{+/-} mice.

An inverse relation between *Axin2* expression and β -catenin activity and FGFR1 abundance exists in the PF suture

Because the SAG suture in the double-mutant mice resembled that of the PF suture in wild-type mice, we examined whether WNT- β -catenin and FGFR1 signaling cooperate in the development of the PF suture with an *Axin2*^{GFP} mouse strain (32) (fig. S2A) carrying transgenes for the transcription factor rtTA under the control of the *Axin2* promoter (*Axin2*-rtTA) (33) and a green fluorescent protein (GFP) controlled by tetracycline response elements (TRE-H2BGFP) (34). With this transgenic system that permits inducible expression of GFP in the presence of doxycycline in cells in which *Axin2* is expressed, we monitored the expression of *Axin2* in early postnatal development. The *Axin2*^{GFP} mice were treated with doxycycline for 3 days before the time of analysis to examine the *Axin2* expression pattern. We detected GFP, indicating *Axin2* expression, in all cranial sutures at P5 (Fig. 5A). In the frontal suture, the intensity of GFP diminished at P7 (Fig. 5B), eventually disappearing after P9 (Fig. 5, C and D). GFP persisted in all other cranial sutures, indicating that *Axin2* expression initially occurs in all cranial sutures and then decreases only in the frontal suture.

We investigated the temporal relation between chondrogenesis and *Axin2* expression in the PF suture and found that chondrogenesis was not detectable in the PF suture at P5, the time we detected the greatest amount of *Axin2* expression (GFP) in the skeletal precursors located in the suture mesenchyme, osteogenic fronts, and periosteum (Fig. 5, E and F). However, *Axin2* expression was lower by P9 when chondrogenesis was evident (Fig. 5, G and H). In contrast, in the SAG suture where chondrogenesis was not detected, GFP persisted at P9, indicating that *Axin2* expression continued in this patent suture (Fig. 5, I to L).

The genetic inactivation of the gene encoding β -catenin has a positive effect on chondrogenesis during craniofacial morphogenesis (16,17). Because AXIN2 is a negative regulator of WNT signaling and disruption of *Axin2* increases β -catenin signaling (14,15), areas strongly

expressing *Axin2* are likely to have low β -catenin activity. We therefore predict that as *Axin2* expression decreases in the PF suture, β -catenin signaling should increase. We rarely detected nuclear β -catenin in cells located in the region where the chondrocytes appeared in the PF suture at P5 (Fig. 5M), whereas at P7 (Fig. 5N) and P9 (Fig. 5O), β -catenin accumulated in the nuclei of skeletal precursor cells located at the mesenchyme and osteogenic fronts of the PF suture. The decrease in the number of cells expressing *Axin2* was accompanied by an increase in the number of SOX9-positive chondrogenic progenitors (Fig. 5, P to R). Cells positive for β -catenin were also positive for SOX9, suggesting that β -catenin plays a role in regulation of the SOX9-positive skeletal precursors at the PF suture mesenchyme (Fig. 5, S to U). The abundance of FGFR1 was also increased in a temporal and morphological pattern similar to that of β -catenin and SOX9 (Fig. 5, V to X), implying that coordinated regulation of these molecules is involved in programming chondrogenesis-mediated PF suture development (Fig. 5, Y and Z).

Genetic ablation of *Fgfr1* results in premature chondrogenesis in the PF suture

In the *Axin2* mutants, haploid deficiency of *Fgfr1* apparently switches the fate of mesenchymal stem cells during suture morphogenesis (Figs. 1 and 4). The changes in FGFR1 abundance that occur in the PF suture suggest that this receptor might be involved in chondrogenesis that occurs during PF suture development in early postnatal stages (Fig. 5). FGF2 binds to FGFR1 (35), and FGF2 signaling has previously been implicated in suture specification (29). To define the role of FGFR1 in suture development, we created an *Fgfr1*^{Ax2} mouse model, in which we integrated tetracycline-dependent activation of a transcription factor produced under the control of the *Axin2* promoter and Cre-mediated recombination to ablate *Fgfr1* in a spatiotemporal-specific manner in cells that express *Axin2* (Fig. 6A). With this model, we inactivated *Fgfr1* in the *Axin2*-expressing cells, including those skeletal precursors at the developing sutures, by treating the mice with doxycycline at birth. We determined the efficacy of this system by crossing mice transgenic for the R26R reporter, which indicates Cre-mediated recombination through the expression of β -galactosidase (β -Gal), into the *Fgfr1*^{Ax2} background. The sutures of the resulting *Fgfr1*^{Ax2}; R26R skulls were β -Gal-positive, indicating deletion of *Fgfr1* (Fig. 6, B to G). We confirmed the lack of FGFR1 by immunostaining, which showed the absence of the protein in the *Fgfr1*^{Ax2} mutants (Fig. 6, H to K). In 80% of the mice, postnatal inactivation of *Fgfr1* led to premature chondrogenesis in the PF suture at P7, indicating that *Fgfr1* is essential for temporal regulation of chondrocyte development (Fig. 6, L and M). However, we did not detect ectopic chondrogenesis in the SAG sutures in the *Fgfr1*^{Ax2} mice (Fig. 6, N and O). This might be attributed to the difference in *Axin2* activity and β -catenin signaling between PF and SAG sutures. In the PF suture where *Axin2* expression is low (Fig. 5), β -catenin signaling is high; thus, loss of FGFR1 activity tips the balance toward chondrogenesis. In contrast, AXIN2 is abundant in the SAG suture (Fig. 5); thus, the loss of FGFR1 is not sufficient to change the fate of the mesenchymal stem cells to chondrocytes.

Changing the balance of β -catenin and FGFR1 signaling induces mesenchymal cells to form chondrocytes

To confirm that the switch in mesenchymal cell fate observed in the *Ax2*^{-/-}; *Fgfr1*^{+/-} mouse skulls was due to an alteration in the balance between β -catenin signaling and FGFR1 signaling, we developed the *s β cat*^{Ax2} model (fig. S2B). In this mouse model system, we deleted exon 3 of the gene encoding β -catenin in a spatiotemporal-specific fashion by treating the mice with doxycycline at birth. The resulting truncated β -catenin lacks a phosphorylation site and cannot be targeted for proteasomal degradation. However, neither the SAG suture (fig. S3, A and B) nor the PF suture (fig. S3, J and K) of the *s β cat*^{Ax2} mice exhibited ectopic chondrogenesis. The results indicate that increased β -catenin signaling alone is insufficient to switch the mesenchymal stem cell fate to chondrocytes. Similar to the *Ax2*^{-/-} mice, when combined with

enhanced β -catenin signaling in the $s\beta cat^{Ax2}$ mice, haploid deficiency of *Fgfr1* resulted in ectopic chondrogenesis in the SAG suture at P7 (fig. S3C). The SOX9- and COL II–positive chondrogenic progenitors were also evident in the SAG sutures of $s\beta cat^{Ax2}; Fgfr1^{+/-}$ mice, but not those of control or $s\beta cat^{Ax2}$ mice (fig. S3, D to I). Thus, both increased β -catenin signaling and decreased FGFR1 signaling appear to be necessary for switching mesenchymal stem cells to the chondrocyte fate.

We explored how the balance of WNT and FGF signaling induced chondrogenesis by analyzing differentiation of primary skeletal precursors isolated from newborn wild-type or $Ax2^{-/-}$; $Fgfr1^{+/-}$ mouse parietal bones and SAG sutures in culture. Detection of chondrocytes with AB revealed that differentiation of skeletal precursors into chondrocytes was increased in cultures from the $Ax2^{-/-}; Fgfr1^{+/-}$ mice (Fig. 7, A to C). The area stained by AB increased ~18 fold in the double-mutant cultures compared to that in the wild-type cultures. In addition, stimulation of β -catenin signaling with BIO, a glycogen synthase kinase 3 (GSK-3) inhibitor, prevented the chondrocyte maturation of primary skeletal precursors isolated from the newborn skulls (Fig. 7, D, E, and G). The inhibitory effect of BIO was alleviated by SU5402, an inhibitor of FGFR (Fig. 7, F and G). To determine whether the balance of WNT and FGF signaling is a general mechanism regulating chondrogenesis, we isolated primary precursors from bone marrow and performed ex vivo differentiation analysis. The chondrogenic potential was less in the bone marrow cell cultures compared to that in the calvarial cultures. Although the presence of BIO had no significant effects, the addition of BIO and SU5402 increased the formation of AB-positive cells (Fig. 7, H to K). Although this is different from the results seen with the cultures of cells isolated from skull bones in which potentiation of β -catenin signaling prevented chondrogenesis, altering β -catenin and FGF signaling still changed the fate of the bone marrow precursors, suggesting that both β -catenin and FGF signaling are important for chondrocyte development in multiple contexts.

β -Catenin signaling controls the proliferation of skeletal precursor cells

To determine whether the balance of β -catenin and FGF signaling not only controls the differentiation of skeletal precursors, but also controls the proliferation of these precursors, we evaluated the precursor population in the SAG suture in wild-type mice and in mice with mutations that altered β -catenin signaling and FGFR1 signaling, individually or in combination. We detected proliferating cells by immunostaining for Ki67 and phosphorylated histone H3, which are both markers of dividing cells. Although *Axin2* deficiency greatly enhanced cell proliferation in the SAG suture, *Fgfr1* haploid deficiency had no significant effect (Fig. 8, A to H). The number of proliferating cells in the SAG sutures of the $Fgfr1^{Ax2}$ mice with selective loss of FGFR1 signaling in *Axin2*-expressing cells was also not different from that in wild-type animals (Fig. 8, I to N). Moreover, similar numbers of mitotic cells were detected in the $s\beta cat^{Ax2}$ and $s\beta cat^{Ax2}; Fgfr1^{+/-}$ mutants (Fig. 8, O to R), suggesting that the expansion of precursor population is mediated by the WNT pathway.

Altering the balance of β -catenin and FGF signaling alters BMP signaling in regions of chondrogenesis

To elucidate the mechanism underlying the β -catenin–FGF–mediated suture morphogenesis, we analyzed the involvement of BMP, a potent chondrogenic stimulator (36,37). BMP signaling is increased by *Axin2* deficiency (15). In $Ax2^{-/-}$ SAG sutures, we detected BMP activity in the osteogenic fronts with an antibody that recognizes phosphorylated SMAD1, 5, and 8 (pSMAD1/5/8) (Fig. 9, A and B). In the $Ax2^{-/-}; Fgfr1^{+/-}$ mutants, we detected pSMAD1/5/8 in the osteogenic fronts and in the ectopic chondrogenic area (Fig. 9C) (identified as a region with SOX9-positive cells) (Fig. 9, D to F), underlying the suture mesenchyme. These results agree with previous genetic analyses, suggesting that BMP signaling is critical for the chondrocyte activity (38).

To determine if FGFR1 signaling affected BMP signaling, we examined BMP activity in the *Fgfr1^{Ax2}* model that exhibited accelerated chondrogenesis at the PF suture. Indeed, in the absence of FGFR1 signaling in *Axin2*-expressing cells, we detected pSMAD1/5/8 in the same region as that containing the SOX9-positive chondrogenic precursors (Fig. 9, G to J), suggesting that FGFR1 inhibits the BMP signaling that is involved in chondrocyte differentiation.

To assess the necessity for BMP signaling in the development of mesenchymal stem cells into the chondrogenic lineage in response to altered β -catenin and FGF signaling, we performed an in vitro differentiation analysis with primary skeletal precursors isolated from the skulls of newborn *Ax2^{-/-}*; *Fgfr1^{+/-}* mice. Providing additional BMP had a small, but significant, stimulatory effect on chondrocyte maturation in the *Ax2^{-/-}*; *Fgfr1^{+/-}* cultures (Fig. 9, K, M, and N). The moderate response may be due to the presence of BMP in the cultures, such that the signal is close to maximal already. However, the chondrogenic differentiation of the *Ax2^{-/-}*; *Fgfr1^{+/-}* mesenchymal cells was reduced by the addition of the BMP inhibitor Noggin (Fig. 9, K, L, and N). Thus, BMP signaling is necessary for the chondrogenesis of mesenchymal skeletal precursors that occurs when the β -catenin–FGF signaling balance is altered.

Discussion

This study provides evidence that endochondral ossification is involved in suture morphogenesis and suggests that it may also be a mechanism underlying synostosis-related syndromes. The change of the normally patent SAG suture into a structure resembling the PF suture, which closes, caused by complete genetic loss of *Axin2* and *Fgfr1* haploid insufficiency in mice, suggests that the WNT– β -catenin and FGF pathways play important roles in suture specification. Although chondrocytes have been described in the synostosis-related syndromes of human and mouse, it is not clear whether their presence is actually involved in suture closure (8–13). We show that although increased β -catenin activity stimulated the proliferation of stem cells, alone it had no effect on their commitment to the chondrogenic lineage. Similarly, diminution of FGFR1 signaling facilitated the chondrogenesis in the PF suture, but did not induce skeletal precursors to differentiate into chondrocytes and did not induce ectopic chondrogenesis in the SAG suture. In contrast, genetic mouse models in which β -catenin signaling was increased and FGFR1 signaling was decreased resulted in the developmental reprogramming of the SAG suture mesenchyme.

Therefore, we propose a mechanism by which the interplay of WNT and FGF signaling determines the fate of mesenchymal stem cells and their subsequent differentiation during craniofacial skeletogenesis (Fig. 9O). The expansion of skeletal precursors stimulated by β -catenin activity, which is increased in the absence of *Axin2*, apparently is not affected by decreased FGFR1 signaling. FGFR1 appears to act downstream of the β -catenin pathway to serve as a key determinant in the lineage decision of these precursors. Furthermore, stimulation of BMP signaling by WNT signaling is necessary to alter the stem cell fate. FGFR1 deficiency enables BMP signaling to promote the chondrocyte maturation, leading to ectopic chondrogenesis and premature chondrogenesis in the *Axin2^{-/-}*; *Fgfr1^{+/-}* and *Fgfr1^{Ax2}* models, respectively. We propose that the intensity of FGF signaling, which determines the mesenchymal microenvironment, is crucial for a dual role of BMP signaling on skeletogenesis (Fig. 9O). BMP promotion of osteoblastogenesis is favored by the presence of strong FGF signals, whereas weak FGF signals permit BMP to induce chondrogenesis.

A short period of chondrogenesis occurs in craniofacial sutures, most commonly in the SAG and mid-palatal sutures, but disappears soon after birth in humans and rodents (7). This suggests that developmental programming prohibits the chondrogenic differentiation of the suture mesenchyme. Our findings indicate that the WNT, FGF, and BMP pathways all control the

mechanism of suture morphogenesis, either endochondral ossification or intramembranous ossification. Skull sutures, therefore, provide a model for studying the development of multiple mesenchymal lineages coordinately orchestrated by these cellular signaling networks.

Substantial progress has been made in our understanding of genes associated with craniosynostosis. However, the mechanisms underlying their interactions in suture morphogenesis remains elusive. Our findings indicate that switching the fate of mesenchymal stem cells from an osteoblast progenitor to a chondrocyte progenitor results in suture abnormalities, which implies that endochondral ossification may be a mechanism for craniosynostosis (Fig. 9P). Alterations in mesenchymal stem cell fate have been associated with skeletal dysplasia, such as osteoarthritis, fibrodysplasia ossificans progressiva, osteoporosis, and osteoponia (39–43). Cancer stem cells have been hypothesized to arise from switching of their normal fate (44). Mutations affecting either the WNT or the FGF pathways are commonly detected in these skeletal disorders and many cancers (44,45). Although mesenchymal stem cells were reportedly used for the treatment of children with osteogenesis imperfecta in 1999 (46), stem cell-based therapies have certain risks of malignant transformation (47). Our work here provides additional insight into the signals that control stem cell fate determination and thus contributes to the understanding of the complex properties of stem cells, which is necessary to ensure their safe use and maximize the benefits of regenerative medicine.

Materials and Methods

Mouse strains

The *Axin2* mutant, *Axin2*-rtTA, *Fgfr1*Fx, TRE-H2BGFP, R26RlacZ, TRE-Cre, β -cat Δ Ex3F mouse strains and genotyping methods were reported previously (14,15,32–34,48–52). For generating the *Axin2*^{GFP} mouse strain (32), mice carrying the *Axin2*-rtTA and TRE-H2BGFP transgenes were obtained and treated with doxycycline (2 mg/ml plus 50 mg/ml sucrose) for 7 days as described (33,48,52). For generating the β cat^{Ax2} mouse strain, mice carrying *Axin2*-rtTA and TRE-Cre transgenes were first bred into the β -cat Δ Ex3F heterozygous background. The expression of a degradation-deficient β -catenin in the *Axin2*-expressing cells was induced by doxycycline treatment at birth. To generate the *Fgfr1*^{Ax2} mutant strain, mice carrying *Axin2*-rtTA and TRE-Cre transgenes were first bred into the *Fgfr1*Fx homozygous background. *Fgfr1* was inactivated in the *Axin2*-expressing cells by doxycycline treatment at birth. To simultaneously delete *Fgfr1* and monitor the Cre-mediated recombination, *Fgfr1*^{Ax2} mice were crossed into the R26R background to obtain the *Fgfr1*^{Ax2}; R26R mutants. The deletion of *Fgfr1* and expression of lacZ reporter occurred when Cre was expressed in a doxycycline-inducible manner. To generate the *Fgfr1*-null allele, the *Fgfr1*Fx strain was bred with ZP3-Cre transgene similar to the previous report (15). This deleted allele was further bred into either *Axin2* or β cat^{Ax2} mutants. Care and use of experimental animals described in this work comply with guidelines and policies of the University Committee on Animal Resources at the University of Rochester.

Cells

Isolation and culture of primary mesenchymal precursors from calvaria were performed as described (14). Briefly, isolated mesenchymal cells were cultured in minimum essential medium (α MEM) containing 10% fetal bovine serum. Only the first-passage cells were used for the study. Cells (2.5×10^5) were seeded in 24-well plates, maintained in differentiation medium containing ascorbic acid (50 μ g/ml) and 4 mM β -glycerophosphate for 3 weeks, and stained with AB as described (16). For the analysis of bone marrow mesenchymal precursors, primary bone marrow cells (5×10^6) isolated from the tibial and femoral diaphyses of 10-week-old mice were cultured in 24-well plates with α MEM containing 20% fetal bovine serum.

Nonadherent cells were removed after 1 week, and remaining cells were cultured in differentiation in medium containing ascorbic acid (50 µg/ml) and 4 mM β-glycerophosphate for 3 weeks. BIO (2 µM), SU5402 (3 µM), Noggin (9.7 nM), and BMP (2.25 nM) were added in the cultures as indicated.

Histology, β-Gal staining, TUNEL, and GFP analysis

Skulls were fixed in formaldehyde–formic acid and divided coronally at the bregma to separate the PF and SAG sutures. Samples were then embedded to obtain paraffin or frozen sections, which were stained with hematoxylin-eosin for histology, AB for chondrogenesis, alkaline phosphatase for osteoblastogenesis, or antibodies for immunological staining, which was detected with avidin–biotinylated enzyme complex as described (14,15,53–54). The immunological staining was visualized by enzymatic color reaction or fluorescence according to the manufacturer's specification (Vector Laboratories). Images were taken with a Zeiss Axio Observer microscope (Carl Zeiss). Mouse monoclonal antibodies against collagen II (Thermo Fisher; 1:50) and activated β-catenin (ABC) (Millipore; 1:200); rabbit polyclonal antibodies against FGFR1 (Santa Cruz; 1:200), laminin (Sigma; 1:50), phosphorylated histone H3 (Cell Signaling; 1:100), pSMAD1/5/8 (Cell Signaling; 1:50), and SOX9 (Santa Cruz; 1:200); rabbit monoclonal antibody against Ki67 (Thermo Fisher; 1:200) were used in these analyses. Details for β-Gal staining in whole mounts or sections were performed as described previously (33, 48). TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) staining was performed with ApopTag (Millipore) as described (33). Whole-mount GFP analysis was performed with fluorescence stereomicroscopy to visualize the skull (33). For analysis in sections, skulls were further fixed in 4% paraformaldehyde–phosphate-buffered saline, decalcified in 14% EDTA at 4°C for 3 days, processed for frozen section, and evaluated with a Zeiss Axio Observer microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. Taketo for providing reagents and H.-M.I. Yu for technical assistance.

Funding: This work was supported by NIH grant DE15654 to W.H.

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Fig. 1.

Ectopic chondrogenesis caused by loss of *Axin2* and decreased FGFR1 induces ectopic chondrogenesis. (A to D, A' to D') Skeletal staining of the P7 skulls with AB shows that ectopic chondrogenesis (arrow) occurs in the SAG suture of *Ax2*^{-/-}; *Fgfr1*^{+/-} (100%, *n* = 6) but not in the SAG suture of wild-type (WT; 100%, *n* = 4) or in *Fgfr1*^{+/-} (100%, *n* = 4) mice. An asterisk indicates faint AB reactivity observed in a few *Ax2*^{-/-} (37%, *n* = 11). (E to H, E' to H') AB staining, (I to L) immunostaining of Sox9, and (M to P) immunostaining of type II collagen (Col II) reveals that combined loss of *Axin2* and decreased FGFR1 increases the number of chondrogenic precursors and chondrocytes in the SAG suture at P7. Sections were counterstained by eosin in red. Scale bars, 2 mm (A to D); 1 mm (A' to D'); 200 μm (E to H); and 50 μm (E' to H' and I to P).

Fig. 2.

Increased abundance of FGF2 and phosphorylated Smad2 coincides with development of the normal PF suture and the mutant SAG suture that exhibits a PF suture-like structure. Sutures of P9 WT and double-mutant mice were immunostained for FGF2 (A to C) and pSmad2 (D to F). Brown color is positive for FGF2 or pSmad2 and blue indicates hematoxylin counterstaining. Black arrows indicate the osteogenic fronts, which are positive for FGF2 and pSmad2 in WT mice during PF and SAG suture morphogenesis. The areas surrounding the bone plates are also positive. In addition to these intramembranous ossification regions, red arrowheads indicate the endocranial layer of the PF suture mesenchyme that is undergoing chondrogenesis, which is also positive for FGF2 and pSmad2 in the WT mice. The PF-like SAG suture in the *Ax2*^{-/-}; *Fgfr1*^{+/-} double mutants also exhibited staining for FGF2 and pSmad2 (C and F). In the wild type, the PF suture contains higher percentage of FGF2 (G; **P* < 0.001) and pSmad2 positive cells (H; **P* < 0.002). The double-mutant SAG suture displays enhanced numbers of cells positive for FGF2 (G; ***P* < 0.008; *n* = 3) and pSmad2 (H, ***P* < 0.001; *n* = 3). Scale bars, 100 μm.

Fig. 3.

Craniosynostosis occurs in the SAG suture of $Ax2^{-/-}; Fgfr1^{+/-}$ at P50. (**A to D**) Ventral view of the Alizarin red-stained skulls reveals the closure of the SAG suture (arrowhead) in the $Ax2^{-/-}; Fgfr1^{+/-}$ mutant (73%, $n = 11$), but not in the WT (100%, $n = 3$), $Fgfr1^{+/-}$ (100%, $n = 4$), or $Ax2^{-/-}$ (100%, $n = 5$). (**E to H**) Histology of the SAG suture identifies both ectocranial and endocranial layers in the $Ax2^{-/-}; Fgfr1^{+/-}$ mutants, but these two layers are absent in the WT, $Fgfr1^{+/-}$, and $Ax2^{-/-}$ SAG sutures. The endocranial layer is fused in the $Ax2^{-/-}; Fgfr1^{+/-}$ mutants. (**I**) This abnormal structure resembles the WT PF suture, which normally is fused at this stage. Endo, endocranial layer; Ecto, ectocranial layer. Scale bars, 1 mm (**A to D**); 200 μm (**E to I**).

Fig. 4.

Progression of ectopic chondrogenesis to synostosis is mediated by endochondral ossification in the $Ax2^{-/-}; Fgfr1^{+/-}$ SAG suture. Time course study of endochondral ossification is analyzed by AB, TUNEL, immunostaining of laminin, alkaline phosphatase (ALP) activity, and histology at the indicated stages. (A to C) The asterisk indicates the ectopic chondrocytes found at P10 (A), which are reduced at P15 (B) and P20 (C). (D to F) White arrowheads identify apoptotic cells present during resorption of chondrocytes at P10 (D), which are not present at P15 (E) and P20 (F). (G to I) White arrows indicate the laminin-positive endothelial cells present in the P15 (H) and P20 (I), which are absent in the P10 (G) SAG suture. (J to O) Black arrowheads indicate the site of increasing osteoblastogenesis revealed by ALP staining (J to L), which is accompanied by ectopic bone formation indicated by black arrows (L and O) in histology preparations (M to O). OF, osteogenic front; EC, ectopic chondrocyte; SS, sagittal sinus. Scale bars, 100 μ m.

Fig. 5.

Decreased expression of *Axin2* precedes chondrogenesis during PF suture fusion. (A to D) *Axin2*^{GFP} mice, which are positive for GFP in *Axin2*-expressing cells, were used to examine the spatiotemporal-specific expression of *Axin2* from P5 to P28. Whole-mount GFP analysis reveals that *Axin2* is expressed in the frontal suture before P5 (A, arrow), but diminished (arrowheads) at P7 (B), P9 (C), and P28 (D). In contrast, *Axin2* is consistently present in other sutures, including SAG, coronal, and lambdoid sutures, at postnatal stages. (E to H) Chondrogenesis and down-regulation of *Axin2* are coordinated in the developing PF suture. (I to L) Strong expression of *Axin2* and no AB-stained chondrocytes are found in both P5 and P9 SAG sutures. GFP staining was counterstained by DAPI (4',6-diamidino-2-phenylindole)

in blue. (**M to O**) β -Catenin activity increases as *Axin2* expression diminishes, as determined by nuclear localization of activated β -catenin (ABC) in the PF suture. (**P to R**) Diminishing *Axin2* expression corresponds with induction of chondrocyte progenitors in the PF suture, detected by immunostaining for SOX9. (**S to U**) The activated β -catenin colocalizes with SOX9 in the skeletal precursors of PF suture at P7. (**V to X**) The abundance of FGFR1 in the PF suture is also enhanced upon chondrogenesis at P5 to P9. (**Y**) Statistical analysis of the percentage of cells positive for *Axin2*, ABC, SOX9, and FGFR1 (data represent the mean $\pm$ SEM from three animals). (**Z**) A diagram illustrates the temporal-specific regulation of *Axin2*, activated β -catenin, SOX9, and FGFR1, as well as chondrogenesis (represented as intensity of AB) in the developing PF suture. Endo, endocranial layer; Ecto, ectocranial layer; Br, brain. Scale bar, 2 mm (A to D), 100 μ m (E to L), 50 μ m (M to X).

Fig. 6.

Targeted disruption of *Fgfr1* in the *Axin2*-expressing skeletal precursors accelerates chondrogenesis in the PF suture. (A) A diagram illustrates ablation of *Fgfr1* with a system combining tetracycline-dependent activation and Cre-mediated recombination. The transcription factor rtTA is expressed under control of the *Axin2* promoter (Ax2 P). In the presence of doxycycline (Dox), rtTA drives the expression of Cre resulting in conditional deletion of *Fgfr1* in the *Axin2*-expressing cells. In the presence of the R26R allele, the Cre-mediated recombination can be monitored by β -Gal staining. (B and C) β -Gal staining of the *Fgfr1*^{Ax2}; R26R (*Axin2*-rtTA; TRE-Cre; *Fgfr1*Fx/Fx; R26R) and control (TRE-Cre; *Fgfr1*Fx/Fx; R26R) P7 skulls demonstrates the efficacy of *Fgfr1* deletion. (D to G) Sections of the β -Gal-stained skull further show that the Cre-mediated recombination occurs in the P7 PF and SAG suture mesenchyme, osteogenic fronts, and periosteum. (H to K) Immunostaining of FGFR1 indicates that its abundance was reduced in the developing PF and SAG suture of *Fgfr1*^{Ax2}; R26R at P7. The stained sections in brown were counterstained by hematoxylin in blue. (L to O) AB staining reveals that, in the *Fgfr1*^{Ax2}; R26R mutant, chondrogenesis was accelerated in the PF suture, whereas no effect was detected in the SAG suture at P7 ($n = 4$). Scale bar, 4 mm (B and C); 100 μ m (D to G); 50 μ m (H to O).

Fig. 7.

Together, β -catenin and FGF signaling control induction of the chondrocyte fate. In vitro mesenchymal cultures show accelerated chondrogenesis in the Ax2^{-/-}; Fgfr1^{+/-} mutant. (**A** and **B**) Primary skeletal precursors, isolated from the WT and Ax2^{-/-}; Fgfr1^{+/-} SAG sutures and parietal bones, were cultured in differentiation media for 3 weeks and chondrocytes were identified by AB staining. (**C**) Analysis of the stained area shows an ~18-fold increase in the area positive for AB in the Ax2^{-/-}; Fgfr1^{+/-} cultures compared to that of WT cultures. (**D** to **K**) Chondrogenesis is affected by stimulation of WNT signaling (with BIO) and inhibition of FGF signaling (with SU5402). Primary skeletal precursors, isolated from skulls (**D** to **G**) or bone marrow (**H** to **K**), were induced for differentiation in culture with media only (**D** and **H**) or with the indicated inhibitors. Graphs show the quantification of the stained area (* $P < 0.022$; $n = 3$ individual experiments, data plotted are the mean $\pm$ SEM).

Fig. 8.

Expansion of skeletal precursors is affected by increased β -catenin signaling, but not by reduced FGF signaling. Sections of the SAG sutures from animals with the indicated genotypes were immunostained for Ki67 or for phosphorylated histone H3 (pHH3) and quantified. (**A** to **H**) Haploid deficiency of *Fgfr1* has no effect on the enhanced proliferation of skeletal precursors caused by *Axin2* deficiency. The mean percentage of Ki67- or pHH3-positive cells was significantly increased in the *Ax2*^{-/-} (**P* < 0.004, *n* = 4 for Ki67; *P* < 0.03; *n* = 3 for pHH3) and the *Ax2*^{-/-}; *Fgfr1*^{+/-} (***P* < 0.0006, *n* = 4 for Ki67; *P* < 0.009; *n* = 3 for pHH3), compared to the WT. (**I** to **N**) Targeted disruption of *Fgfr1* does not alter the skeletal precursor proliferation. There was no significant difference in the number of Ki67- or pHH3-positive cells in the control (TRE-Cre; *Fgfr1*Fx/Fx) and *Fgfr1*^{*Ax2*} (*Axin2*-rtTA; TRE-Cre; *Fgfr1*Fx/Fx) SAG sutures at P0 (*n* = 3). (**O** to **R**) Stimulated proliferation of skeletal precursors in the *sβcat*^{*Ax2*} SAG suture is not affected by *Fgfr1* haploid deficiency. At P0, regardless of the *Fgfr1* genetic background, compared to WT the percentage of pHH3-positive cells was increased in the SAG sutures of mice with mutant β -catenin, both the *sβcat*^{*Ax2*} (**P* < 0.007; *n* = 3) and the *sβcat*^{*Ax2*}; *Fgfr1*^{+/-} (***P* < 0.007; *n* = 3) mice.

Fig. 9.

BMP signaling is crucial for development of suture mesenchymal stem cells. (A to F) Activation of the BMP pathway and ectopic chondrogenesis coincide in the $Ax2^{-/-}; Fgfr1^{+/-}$ mutants. BMP signaling was detected by immunostaining for phosphorylated SMAD1/5/8, and chondrogenic cells were detected by immunostaining for SOX9 in sections of SAG sutures from P0 animals. Black arrows indicate the area surrounding the osteogenic fronts that contains cells that are positive for pSMAD1/5/8. Red arrowheads indicate that pSMAD1/5/8 is detected in the SOX9-positive chondrogenic progenitors found only in the $Ax2^{-/-}; Fgfr1^{+/-}$ SAG suture undergoing ectopic chondrogenesis. (G to J) BMP signaling is increased in the $Fgfr1^{Ax2}$ PF suture, which displays accelerated chondrogenesis. Sections of the P7 control (TRE-Cre; $Fgfr1^{Ff/Ff}$) and $Fgfr1^{Ax2}$ (Axin2-rtTA; TRE-Cre; $Fgfr1^{Ff/Ff}$) PF sutures were immunostained for pSMAD1/5/8 and SOX9. The chondrogenic domain with active BMP signaling, defined by the presence of SOX9 and pSMAD1/5/8, was expanded in the $Fgfr1^{Ax2}$ PF suture compared to the control. Black arrows and red arrows indicate the osteogenic fronts and the chondrogenic areas of the PF suture, respectively. Endo, endocranial layer; Ecto, ectocranial layer. (K to N) BMP signaling is required for chondrocyte

differentiation induced by altered β -catenin and FGF signaling. Primary skeletal precursors, isolated from the $Ax2^{-/-}$; $Fgfr1^{+/-}$ calvaria, were cultured in differentiation media with or without BMP or Noggin for 3 weeks. Quantification of the ABstained area shows that Noggin significantly suppressed chondrogenesis ($*P < 0.005$; $n = 3$ individual experiments, mean $\pm$ SEM) and BMP enhanced ($**P < 0.028$; $n = 3$ individual experiments, mean $\pm$ SEM) chondrogenesis. **(O)** Diagram illustrates the model for development of mesenchymal stem cells in calvarial morphogenesis. **(P)** Diagram illustrates endochondral ossification caused by switching the fate of mesenchymal stem cells resulting in craniosynostosis (CS). Scale bar, 100 μ m (A to J).