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Craniosynostosis caused by Axin2 deficiency is mediated through distinct functions of β-catenin in proliferation and differentiation

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Abstract

Targeted disruption of Axin2 in mice induces skeletal defects, a phenotype resembling craniosynostosis in humans. Premature fusion of cranial sutures, caused by deficiency in intramembranous ossification, occurs at early postnatal stages. Axin2 negatively regulates both expansion of osteoprogenitors and maturation of osteoblasts through its modulation on Wnt/β-catenin signaling. We investigate the dual role of β-catenin to gain further insights into the skull morphogenetic circuitry. We show that as a transcriptional co-activator, β-catenin promotes cell division by stimulating its target cyclin D1 in osteoprogenitors. Upon differentiation of osteoprogenitors, BMP signaling is elevated to accelerate the process in a positive feedback mechanism. This Wnt-dependent BMP signal dictates cellular distribution of β-catenin. As an adhesion molecule, β-catenin promotes cell–cell interaction mediated by adherens junctions in mature osteoblasts. Finally, haploid deficiency of β-catenin alleviates the Axin2-null skeletal phenotypes. These findings support a model for disparate roles of β-catenin in osteoblast proliferation and differentiation.
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Keywords: Axin; Wnt; β-catenin; BMP; Craniosynostosis; Skull; Suture; Craniofacial morphogenesis; Development abnormalities

Introduction

Craniofacial morphogenesis, mediated by a complex regulatory mechanism, is highly dependent on the patterning information of emigrant cranial neural crest (CNC) cells (Francis-West et al., 1998). As a result, the majority of craniofacial malformations are caused by defects in CNC (Wilkie and Morriss-Kay, 2001). CNC cells give rise to a wide variety of tissues and structures, including skull bones (Le Douarin and Kalcheim, 1999). Lineage tracing/fate mapping analysis showed that the anterior skull derives mainly from CNC (Jiang et al., 2002). During skull development, cranial sutures serve as growth centers for skeletogenesis that is mediated through intramembranous ossification (Hall, 1990). This process differs from endochondral ossification in the appendicular and axial skeletons, where prior formation of cartilage templates is required.

Axin2, highly expressed in CNC cells (Jho et al., 2002 and Yu et al., in press) and developing sutures (Yu et al., 2005a), is required for skull morphogenesis (Yu et al., 2005a). We previously demonstrated that targeted disruption of Axin2 in mice induces premature suture closure (Yu et al., 2005a). This developmental defect resembles craniosynostosis in humans, affecting 1 in approximately 2500 individuals (Cohen and MacLean, 2000). Neural crest (nasal and frontal bones), but not mesoderm (parietal bones), dependent osteogenesis is particularly sensitive to the loss of Axin2 (Yu et al., 2005a). The Axin2-null mutant exhibits enhanced expansion of osteoprogenitors and accelerated intramembranous ossification (Yu et al., 2005a). Axin2, a negative regulator for the canonical Wnt pathway (Behrens et al., 1998; Zeng et al., 1997), is required for down-regulating β-catenin at different stages of osteoblast development (Yu et al., 2005a). The lack of Axin2 causes a stimulation of β-catenin signaling that is necessary and sufficient to promote intramembranous ossification (Yu et al.,

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2005a). Consistent with our findings, recent studies showed that β-catenin is required for osteoblast development (Day et al., 2005; Hill et al., 2005). Coordinate regulation of β-catenin and Sox9 determines the fate of osteochondroprogenitors (Akiyama et al., 2004; Kolpakova and Olsen, 2005). In addition to transducing Wnt signals (Logan and Nusse, 2004; Moon et al., 2004), β-catenin plays a critical role in cell adhesion (Bienz, 2005; Gumbiner, 2005; Harris and Peifer, 2005). Cell–cell interactions mediated by adherens junctions have been shown to modulate osteoblast function (Stains and Civitelli, 2005). Previous reports suggested that cadherin–catenin mediated cell adhesion are involved in musculoskeletal development (Bilezikian et al., 2002; Ferrari et al., 2000; Kawaguchi et al., 2001). Nevertheless, the exact roles of β-catenin in both the proliferation and differentiation processes that control calvarial osteoblast development remain to be determined.

Bone morphogenetic protein (BMP) (Bilezikian et al., 2002), which belongs to the transforming growth factor β (TGFβ) superfamily (Massague, 1998), regulates skeletogenesis through a cascade of signaling events. Binding of BMP to its receptors activates the downstream signal transducer called Smad (Massague et al., 2005). Activated Smad proteins then interact with transcription factors to control gene expression (Feng and Derynck, 2005; Whitman and Raftery, 2005). The effects of BMP can be limited by a group of secreted polypeptides that prevent BMP signaling by precluding ligand–receptor interactions (Canalis et al., 2003). One of these extracellular antagonists, Noggin, is induced by BMP to restrict its signaling activities in a negative feedback fashion (Gazzerro et al., 1998; Warren et al., 2003). It has been suggested that BMP and Wnt signaling interact to regulate the development of limb (Soshnikova et al., 2003), kidney (Hu and Rosenblum, 2005), tooth and postnatal hair follicles (Andl et al., 2004). Although BMP and Wnt pathways play important roles in skeletal development, the underlying mechanism coordinated by these two pathways remains elusive.

In this study, we have investigated the involvement of β-catenin in expansion of osteoprogenitors and maturation of osteoblasts using the Axin2-null model. In this mutant, only binding of β-catenin to the Axin-dependent degradation complex in the cytosol is disrupted. The interaction of β-catenin with the LEF/TCF transcription and the cadherin-mediated adhesion complexes remains intact at the Axin2-null nucleus and plasma membrane, respectively. Our findings suggest that cellular localization of β-catenin is extremely important in mediating calvarial osteoblast development. β-catenin functions as a transcription co-activator and cell adhesion molecule to promote the proliferation and differentiation processes, respectively. A Wnt dependent BMP signal is critical to control the activity of β-catenin in a positive feedback loop. Genetic analysis further reveals that haploid deficiency of β-catenin alleviates the skull abnormalities caused by the loss of Axin2. Our findings not only lead to a model of calvarial osteoblast development mediated by Wnt and BMP, but also provide new insights into how a single molecule with multiple functions can dictate complex processes of lineage-specific development.

Materials and methods

Mouse strains

The Axin2 deficient mice and genotyping method were reported previously (Yu et al., 2005a). For generating the Axin2 deficient mice, genomic DNA fragments isolated from a P1-phage 129 library were introduced into the pTV0 derived targeting vector (Riethmacher et al., 1995). The 5′ arm contained sequences from the first intron to the beginning of the second exon, which encodes the first 2 amino acids of Axin2. The 3′ arm included the end of the second exon and the third intron. A β-galactosidase cDNA with a nuclear localization signal was fused to the first codon after the translational initiation site of Axin2. The $\text{Axin2}^{\text{lacZ/+}}$ mutant embryonic stem (ES) cell lines were generated by electroporation of the targeting vector into E14.1 ES cells. Correct homologous recombination at the Axin2 locus was confirmed by Southern blotting. Two independent heterozygous ES cell clones were used to generate chimeric mice by blastocyst injection as described (Riethmacher et al., 1995). To ensure the targeted disruption, RT-PCR analyses were used to examine the expression of Axin2 and lacZ in the Axin+/+ and Axin2−/− calvarial osteoblast precursors. PCR assays were performed using primers 5′-agtagcgccgtgttagtg-3′ and 5′-atgccatctcgtatgtaggt-3′ for the Axin2 exon2, and primers 5′-cctatcccat-tacggtcaa-3′ and 5′-taaagcgagtggcaacat-3′ for the lacZ reporter. To generate the β-catenin-null allele, the $\text{Catnb}^{\text{tm2Kem}}$/J strain (Brault et al., 2001) carrying a floxed allele of β-catenin was crossed with the Zp3-Cre strain (de Vries et al., 2000). PCR genotyping for the β-catenin allele was performed as described (Brault et al., 2001). 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.

Primary osteoblast isolation and culture and analyses

Isolation, culture and differentiation of primary osteoprogenitors, as well as BrdU incorporation, alkaline phosphatase liquid and real-time RT-PCR assays were reported previously (Yu et al., 2005a,b). Real-time RT-PCR assays were performed using primers 5′-tggaagtggcccatttagag-3′ and 5′-gcttttctcgtttgtggagc-3′ for BMP-2, primers 5′-tgagcctttccagcaagttt-3′ and 5′-cttcccggtctcaggtatca-3′ for BMP-4, primers 5′-ctcagaagaaggttggctgg-3′ and 5′-acctcgctcaccttgaagaa-3′ for BMP-6 and primers 5′-gacgccaaagaaccaagag-3′ 5′-tcacagtagtaggcagcatag-3′ for BMP-7. Noggin (500 ng/ml, R&D systems) and ionomycin (2 μM, sigma) were added in the in vitro cultures as indicated in the text. μCT analysis was performed using the VIVA CT40 model (Scanco). Histology were performed as described (Yu et al., 2005a). In brief, skulls were fixed in formaldehyde, decalcified and paraffin embedded. Samples were sectioned and stained with hematoxylin/eosin/Orange G for histological evaluation.

Immunostaining and immunoblot

Tissue sections were subject to immunological staining with avidin: biotinlylated enzyme complex as described (Hsu et al., 2001). Protein extracts were subject to immunoblotting as described (Hsu et al., 1999). Bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies followed by ECL mediated visualization (Amersham) and autoradiography, or fluorescein conjugated avidin (Vector Lab) for fluorescent microscopy. Immunostaining of cultured cells was performed using indirect fluorescent staining technique. Briefly, cells were fixed with 4% paraformaldehyde for 10 min and treated with 0.5% Triton for 15 min and 50 mM glycine for 10 min followed by blocking with the PBS–Triton buffer containing 3% BSA for 30 min. Samples were then incubated with primary antibody for 1 h and fluorescent conjugated secondary antibody for 45 min and mounted with vectashield (Lab Vision) containing propidium iodide. Imagines were analyzed using a Leica TCS SP spectral confocal microscope. Mouse monoclonal antibodies α-OBcad (Zymed), α-Ncad (a gift of Masatake Osawa), α-Ecad (Sigma), α-CDK4 (Cell Signaling) (Lukas et al., 1999) and α-BrdU (Lab Vision); rabbit polyclonal antibodies α-Cyclin D1 (Lab Vision) (Hall et al., 1993), α–β-catenin (Lab Vision), α-FGFR1 (Santa Cruz), α-Sox9 (Santa Cruz) (Sahar et al., 2005) and α-phospho-Smad1/5/8 (Cell signaling) were used as primary antibodies.

Results

Targeted disruption of Axin2 induces nuclear localization of β-catenin in osteoprogenitors

Axin2 disruption has a direct effect on β-catenin in osteoblast development. This was demonstrated in the Axin2-null suture during skull morphogenesis at early postnatal stages (Yu et al., 2005a). Activation of β-catenin was detected in the Axin2−/− osteogenic fronts and periosteum. However, cellular levels of activated β-catenin did not increase in the isolated Axin2−/− osteoprogenitors, but their proliferations were clearly affected by the mutation (Yu et al., 2005a). The activated form of β-catenin only showed an obvious accumulation in the differentiated Axin2−/− osteoblasts. This could be due to inability of the in vitro system to recapitulate the in vivo effects. Alternatively, cellular localizations or other functions of β-catenin that are altered but not detectable by immunoblotting might be pertinent in calvarial osteoblast development.

β-catenin is also a key component of the adhesion complex, in addition to regulating the Wnt pathway. Therefore, we investigated the distribution of β-catenin since it can locate to different cellular compartments. Using immunostaining and confocal microscopy, we detected that β-catenin is mainly localized in the cytoplasm of the CNC-derived Axin2+/+ osteoprogenitors (percentage, 91%; SD=2.8; n=100) (Fig. 1A). Due to a requirement of culture for osteoprogenitors under low densities, only images of single cells were shown. However, the addition of BIO, a pharmacological compound specifically inhibiting GSK-3β activity causing the stimulation of the canonical Wnt pathway, induced accumulation of β-catenin in the nucleus (percentage, 49%; SD=7.8; n=100) (Fig. 1B). In contrast, β-catenin was predominantly localized to the nuclei of the CNC-derived Axin2−/− osteoprogenitors (percentage, 89%; SD=1.4; n=100) (Fig. 1C). These results suggested that the Axin2 deletion appeared to alter the cellular distribution of β-catenin. Furthermore, the presence of a BMP antagonist, Noggin, had no significant effect on nuclear dislocation of β-catenin in the Axin2−/− osteoprogenitors (percentage, 91%; SD=2.1; n=100) (Fig. 1D) compared to differentiated osteoblasts (see below Fig. 5 for details).

Axin2 deficiency promotes cell–cell interaction mediated by β-catenin/OB-cadherin in mature osteoblasts

To investigate the role of β-catenin during osteoblast differentiation, we examined its distribution in mature osteoblasts using immunostaining and confocal microscopy. In the differentiated Axin2+/+ osteoblasts derived from CNC, a majority of β-catenin was membrane associated (Fig. 1E). This membrane distribution of β-catenin was dramatically stimulated by the Axin2 mutation (Fig. 1F). Because the stimulation was determined by equivalent fluorescent exposure time optimized for detection in the Axin2−/− cells, only a weak staining of β-catenin was shown in the Axin2+/+ mature osteoblast membranes. This is in agreement with the expression studies of β-catenin by immunoblotting where aberrant elevations of the

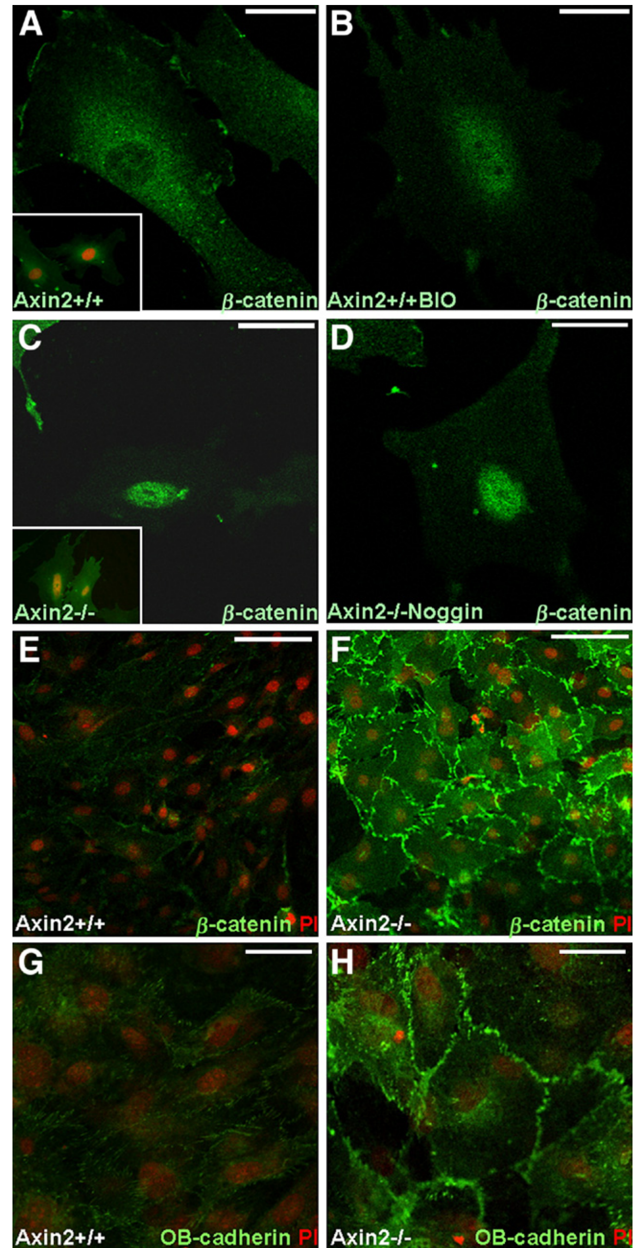

Fig. 1. The loss of Axin2 alters cellular compartmentalization of β-catenin in osteoprogenitors and stimulates formation of adhesion complex in mature osteoblasts. Nuclear localization of β-catenin in osteoprogenitors was stimulated by BIO and the Axin2 disruption. Primary osteoprogenitors isolated from the Axin2+/+ and Axin2−/− nasal and frontal bones were analyzed for β-catenin distribution. Confocal microscopy (A–D) revealed that β-catenin was localized to cytoplasm of the CNC-derived Axin2+/+ osteoprogenitors (A). The presence of BIO (B) or deletion of Axin2 (C) induced nuclear accumulation of β-catenin in the osteoprogenitors. The addition of Noggin has no effect on β-catenin nuclear localization in the Axin2−/− osteoprogenitors (D). Inset in panels A and C shows a superimposed fluorescent image for double labeling of β-catenin in green and propidium iodide (PI) in red under a low magnification. In mature osteoblasts, β-catenin is predominantly localized to the plasma membrane. The Axin2+/+ and Axin2−/− primary osteoprogenitors were induced for maturation by culturing in differentiation media for 10 days. Confocal microscopy revealed that β-catenin (E, F) and OB-cadherin (G, H) were located to plasma membrane of the Axin2+/+ (E, G) and Axin2−/− (F, H) differentiated CNC-derived osteoblasts. Note that the staining of β-catenin and OB-cadherin intensified by the ablation of Axin2. Cells were stained with β-catenin (A–F) or OB-cadherin (G, H) in green and counterstained with PI in red (E–H). Pictures were taken with the same exposure time for panels A–D and for panels E–H. Scale bars, 40 μm (A–D, G, H); 125 μm (E, F).

activated/stabilized form of β-catenin were detected in the differentiated osteoblasts of Axin2−/− (Yu et al., 2005a). The expression levels of β-catenin did not show a significant change in the Axin2+/+ and Axin2−/− osteoprogenitors, but the Axin2 deletion only causes dislocation of β-catenin in naive precursors.

Cell–cell interactions mediated by adherens junctions play a critical role in promoting osteogenesis (Stains and Civitelli, 2005). Previous reports suggested that cadherins are involved in osteoblast development (Ferrari et al., 2000; Kawaguchi et al., 2001). It has been demonstrated that the binding of OBcad to β-catenin enhances cell–cell adhesion, resulting in morphological changes of cellular aggregates and stimulating osteoblast and chondrocyte differentiation (Kii et al., 2004). These findings strongly support the importance of cell–cell interaction mediated by adherens junctions in osteoblast differentiation. To determine if Axin2 deficiency has an effect on cell adhesion in mature calvarial osteoblasts, we examined expression of cadherins, which form complexes with β-catenin. OB-cadherin/cadherin-11 (OBcad), but not N-cadherin (Ncad) and E-cadherin (Ecad), was more abundantly expressed in the differentiated primary calvarial osteoblasts (Fig. 1G and data not shown). In the Axin2−/− osteoblasts, membrane distribution of OBcad was highly elevated (Fig. 1H).

We next investigated β-catenin distribution in the osteoblast lineage during skull and suture development in vivo. In the Axin2+/+ mice at postnatal day 8, we did not observe nuclear localization of β-catenin in the developing sutures and periosteum that are enriched with osteoblast precursors. β-catenin was predominantly located to the cytoplasm of mature osteoblasts (Fig. 2A). However, the loss of Axin2 caused dislocation of β-catenin to nuclei of osteoblast precursors in the developing skull (Fig. 2B). In contrast, prominent staining of β-catenin was shown at cell–cell junctions of the Axin2−/− mature osteoblasts (Fig. 2D). At this stage, only diffused cytosolic staining of β-catenin was observed in the Axin2+/+ mature osteoblasts (Fig. 2C). Our findings suggest that β-catenin might have distinct functions in osteoprogenitors and mature osteoblasts.

To determine whether cadherin mediated adhesion has a role in the accelerated intramembranous ossification of Axin2−/−, cellular distributions of OBcad, Ncad and Ecad were examined. Diffused localizations of OBcad in the cytoplasm were detected in the Axin2+/+ calvarial bones at postnatal day 8 (Fig. 2E). Targeted disruption of Axin2 induced prominent membrane distribution of OBcad in the developing skulls (Fig. 2F). In contrast, Ncad (Figs. 2G, H) and Ecad (data not shown) showed diffused cytoplasmic staining of Axin2+/+ and Axin2−/− calvarial bones. Consistent with our studies of the in vitro cultured primary osteoblasts, these results provide evidence that Axin2 regulates cell–cell interaction through modulation of the adherens junctions during skull morphogenesis.

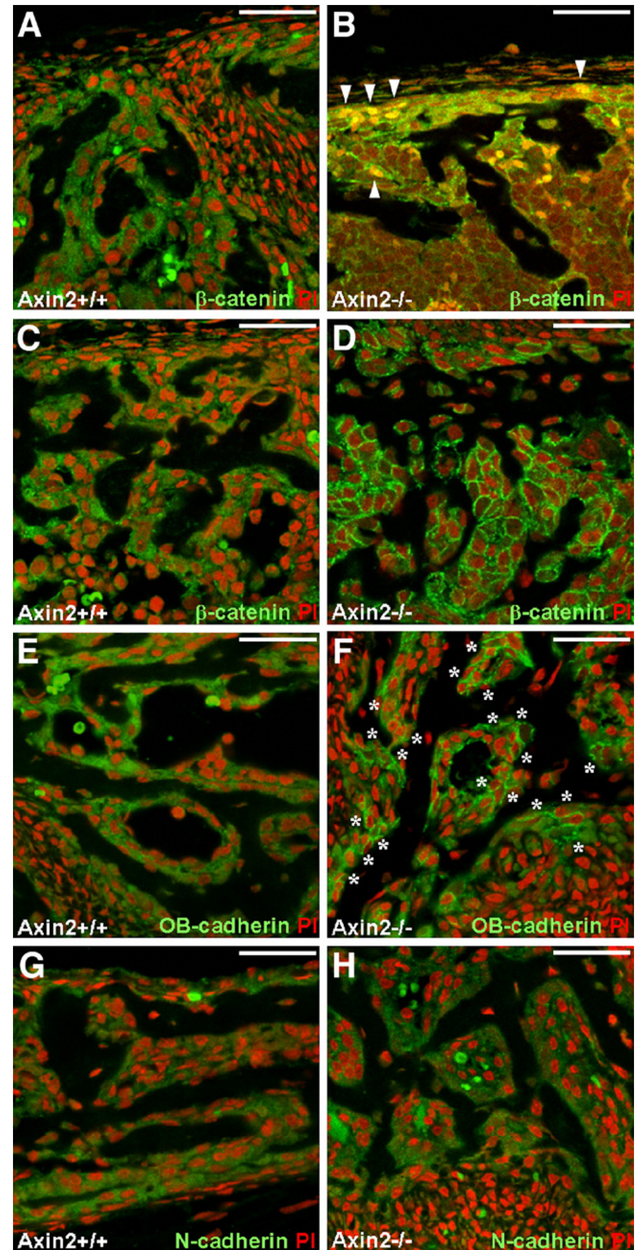

Fig. 2. Distribution of cell adhesion molecules in developing skull. Cellular localizations of adhesion molecules were analyzed by confocal microscopy. Sections of the 8-day-old Axin2+/+(A, C, E, G) and Axin2−/− (B, D, F, H) calvaria were stained with β-catenin (A, B, C, D), OB-cadherin (E, F), or N-cadherin (G, H) in green and counterstained with PI in red. Nuclear localization of β-catenin (arrowheads) was evident in calvarial osteoprogenitors located to the Axin2-null periosteum (B), but absent in the wild type counterparts (A). In the Axin2-null calvarial bones, strong membrane staining of β-catenin (D) and OB-cadherin (F, asterisks) was detected in mature osteoblasts. Diffused cytosolic staining was shown in the Axin2+/+ calvarial bones (C, E). N-cadherin showed weak expression in the cytoplasm of both Axin2+/+ and Axin2−/− (G, H). Scale bars, 40 μm (A–H).

Enhanced expansion of osteoprogenitors by β-catenin/cyclin D1 signaling

Localization of β-catenin to the nucleus led us to hypothesize that it functions as a transcriptional co-activator in the osteoprogenitors. β-catenin might promote cell divisions through regulation of its transcriptional target cyclin D1, as well as to activate genes required for initiating differentiation processes in the precursor cells. The nuclear accumulation of β-catenin is accompanied by stimulated expression of its direct transcriptional target cyclin D1 in the

Axin2−/− osteoprogenitors (Figs. 3A, B). An average of 85% Axin2−/− osteoprogenitors were positive for cyclin D1 whereas only 7% for the wild type (Fig. 3L). This is consistent with our findings that β-catenin and LEF/TCF dependent transcription is elevated by the Axin2 deletion (Yu et al., 2005a). The stimulation of cyclin D1 also led to stabilization of its target CDK4, which promotes cell cycle G1/S transition, in the developing skulls (Figs. 3C, D). In the suture region, the average percentage of osteoblast precursors positive for CDK4 was 19% and 46% for Axin2+/+ and Axin2−/−, respectively (Fig. 3L). Immunoblot analyses further showed an approximately 2.5-fold and 1.5-fold induction of the steady-state level of cyclin D1 and CDK4 in the Axin2−/− calvarial osteoprogenitors, respectively (Fig. 3K). Together with our previous in vivo findings (Yu et al., 2005a), the results suggest that the β-catenin/cyclin D1 pathway mediates the enhanced expansion of osteoprogenitors of the Axin2-null mutants. Because recent studies suggest that Sox9 is expressed in a subset of precursors that give rise to osteochondroprogenitors (Akiyama et al., 2005), we then examined if the Axin2 ablation affects this cell population. Immunostaining analyses of the Axin2+/+ and Axin2−/− sutures detected an expansion of the Sox9-expressing precursors at postnatal days 0 and 8 (Figs. 3E–H). The average percentage of osteoprogenitors positive for Sox9 was 8% and 66% for Axin2+/+ and Axin2−/−, respectively (Fig. 3L). However, there was no obvious difference in Sox9 expression during chondrogenesis of the Axin2+/+ and Axin2−/− nasal cartilages (Figs. 3I, J). Immunoblot analyses further showed that the Axin2 mutation causes an approximately 2.0-fold induction of the steady-state level of Sox9 in the calvarial osteoprogenitors (Fig. 3K). These data imply that Wnt signaling regulates accumulation of this Sox9-expressing precursor cell population during calvarial osteoblast development.

Alteration of BMP signaling by Axin2 deficiency

Craniosynostosis induced by Axin2 deficiency is mediated through its effects on cell proliferation and differentiation during calvarial osteoblast development. To further elucidate the mechanism underlying the Wnt-mediated osteoblast development, it is necessary to investigate the interaction of β-catenin with important skeletal regulators, especially in the differentiation processes. The important role of BMP in skeletogenesis led us to test whether this signal transduction pathway is affected by the Axin2 deletion. We first examined if the phosphorylated/active form of Smad1/5/8, key molecules to mediating the downstream effects of BMP, accumulated in the Axin2−/− skulls. Immunostaining analyses revealed that

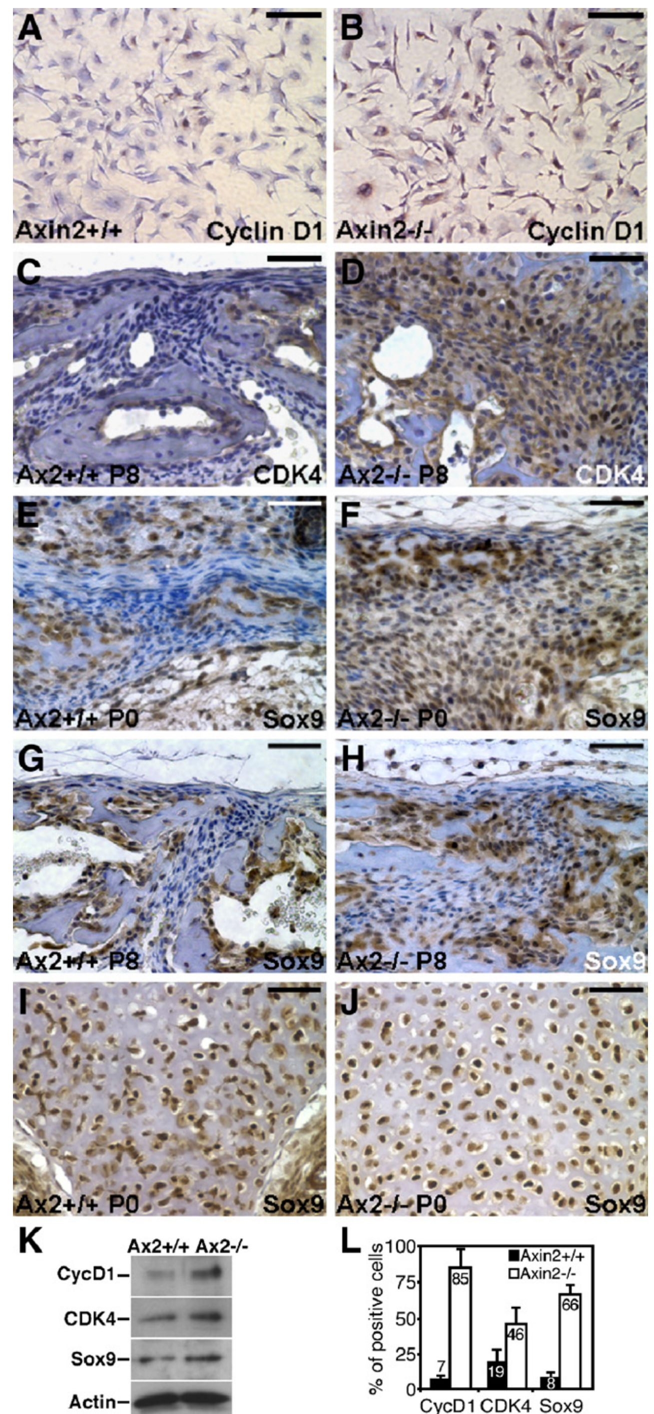

Fig. 3. Expansion of osteoblast precursors affected by Axin2 deficiency. Immunostaining of cyclin D1 (brown staining and blue counterstaining) showed an enhanced expression in the Axin2−/− osteoprogenitors (B), compared to the Axin2+/+ cells (A). Osteoblast precursors in developing mouse skull were analyzed by immunohistochemistry (C–H). Sections were immunostained with antibodies (brown) and counterstained with hematoxylin (blue). The deletion of Axin2 resulted in stimulation of CDK4 at postnatal day 8 (C, D). The Sox9-expressing precursor population was highly increased by the Axin2 inactivation at newborn (E, F) and postnatal day 8 (G, H). The Sox9 expression in the newborn nasal cartilage of Axin2+/+(I) and Axin2−/− (J) did not show any noticeable difference. The osteoblast precursors that are positive (brown) and negative (blue) for immunostaining analyses (A–D and G–H) were counted to obtain the percentage of positive cells. The graph shows the average percentage of the positive stained cells in five independent experiments (L). Immunoblot analyses revealed quantitative alterations in the expression of cyclin D1 (CycD1), CDK4 and Sox9 in the Axin2+/+ and Axin2−/− calvarial osteoprogenitors (K). The level of Actin serves as a loading control. Scale bars, 200 μm (A, B); 50 μm (C–J).

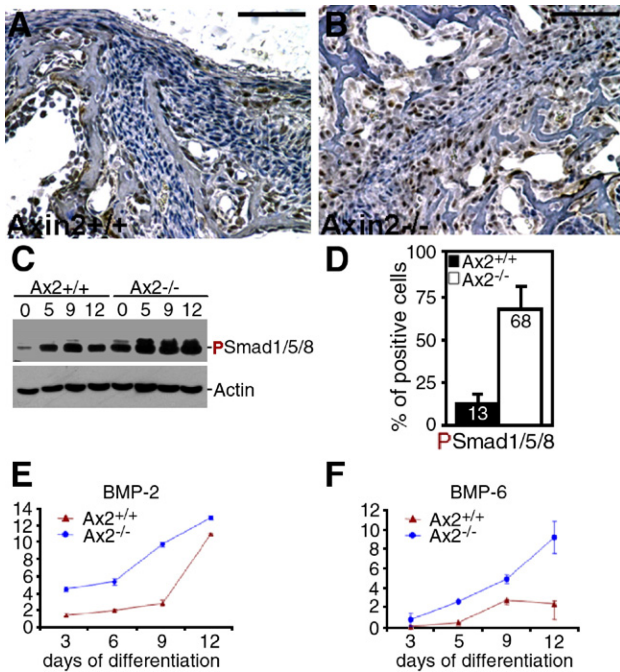

Fig. 4. BMP signaling is induced in the Axin2-null mutants during calvarial osteoblast development. Immunohistochemical staining of the 8-day-old Axin2+/+ (A) and Axin2−/− (B) sutures with the α-phospho-Smad1/5/8 antibody reveals stimulation of BMP signaling by the Axin2 deletion (brown staining and blue counterstaining). The graph shows the average percentage of the Axin2+/+ and Axin2−/− osteoblast precursors of the suture region positive for phospho-Smad1/5/8 in five independent experiments (D). (C) CNC-derived osteoblast precursors, isolated from the Axin2+/+ and Axin2−/− littermates, were cultured in differentiation media for up to 12 days. Lysates were isolated at different differentiation days as indicated. Immunoblot analysis with α-phospho-Smad1/5/8 (PSmad1/5/8) antibodies shows that BMP signaling was stimulated in the Axin2 mutants during osteoblast differentiation. The level of Actin was also analyzed as a control for protein content of the lysates. Quantitative real-time RT-PCR analyses were performed to examine expression of BMP-2 (E) and BMP-6 (F) during the course of osteoblast differentiation. The graphs represent the expression levels (in arbitrary units, Y axes) of BMP-2 and BMP-6 during the course of osteoblast differentiation. Scale bars, 50 μm (A, B).

expression of the phosphorylated Smad1/5/8 was elevated in the 8-day-old Axin2−/− sutures (Figs. 4A, B, D). During osteoblast differentiation in vitro, phosphorylated Smad1/5/8 proteins were accumulated in the CNC-derived osteoblasts in the Axin2 mutants (Fig. 4C). In three independent experiments, inactivation of Axin2 caused an abrupt activation of the BMP pathway. This result provides a mechanism by which alkaline phosphatase and osteogenic markers, the downstream targets of BMP, were stimulated in the Axin2-null mutants (Yu et al., 2005a). To examine if the Axin2 mutation also had an effect on the expression of BMP ligands, quantitative real-time RT-PCR analyses were performed. Our data suggested that BMP-2 (Fig. 4E) and BMP-6 (Fig. 4F), but not BMP-4 and BMP-7 (data not shown), were stimulated in the Axin2−/− osteoblasts. These data imply a role of BMP signaling in calvarial osteoblast development mediated by the Wnt-Axin regulatory network. Stimulation of BMP signaling might be a key step for initiating the β-catenin mediated differentiation processes.

A Wnt dependent BMP signal mediates cell–cell interaction by promoting membrane distribution of β-catenin

To determine if the adhesive role of β-catenin is required for its function in the mature osteoblasts, we used ionomycin to test this possibility. Ionomycin treatment is known to disrupt cell–cell adhesion by inducing cleavage of cadherins, leading to dislocation of the bound β-catenin (Ito et al., 1999; Marambaud et al., 2002). The addition of ionomycin altered the prominent distribution of β-catenin in the Axin2−/− membrane (Figs. 5A, B). Membrane localization of OBcad was also prevented by the presence of ionomycin (Figs. 5D, E). These data demonstrated that the presence of ionomycin has a strong effect on the formation of the OBcad/β-catenin complex in the differentiated Axin2−/− osteoblasts. The dislocation of adhesion molecules to the cytoplasm by ionomycin suggests that cell adhesion plays a role in mature osteoblasts where β-catenin functions to mediate cell–cell contacts. Because of a potential role of BMP in the Axin2 mediated osteoblast differentiation, we next examined if BMP signaling is involved in β-catenin localization. Indeed, inhibition of BMP signaling by Noggin greatly reduces the membrane accumulation of β-catenin (Fig. 5C) and OBcad (Fig. 5F), suggesting that BMP signaling might regulate cell–cell interaction during calvarial osteoblast differentiation. Note that fluorescent exposure time was optimized for the detection of β-catenin and OBcad in the Axin2−/− cells without ionomycin or Noggin in the same set of experiments. Pictures were taken under the same parameter so that a relatively weaker staining was shown in Figs. 5B, C, E and F. Our data demonstrated that the prominent membrane accumulation of β-catenin and OBcad caused by the Axin2 deletion in the differentiated cells is inhibited by Noggin. Therefore, a BMP signal, initially induced by Wnt signaling due to the loss of Axin2 (Fig. 4), dictates β-catenin localization to the plasma membrane of mature osteoblasts (Fig. 5). These findings suggest a distinct role of β-catenin as an adhesion molecule in mature osteoblasts.

The role of BMP in β-catenin mediated osteoblast differentiation

To test if the Wnt dependent BMP signal plays a role in osteoblast proliferation, Noggin was included in the culture media. Primary osteoblast precursors isolated from nasal and frontal bones of the Axin2+/+ and Axin2−/− were grown in culture media for an in vitro proliferation assay. Inhibition of BMP by Noggin has no obvious effects on expansion of the Axin2+/+ and Axin2−/− osteoprogenitors in vitro as determined by the BrdU labeling method (Fig. 6A). This is consistent with the evidence that the presence of Noggin did not interfere with β-catenin nuclear localization in the Axin2−/− osteoprogenitors (Fig. 1D). BMP signaling is not involved in expansion of osteoprogenitors mediated by Wnt. The β-catenin/cyclin D1 pathway mediates the enhanced expansion of osteoprogenitors of the Axin2-null mutants in a BMP-independent fashion.

However, cellular distribution of β-catenin in the differentiated cells was strikingly altered in the presence of Noggin (Fig. 5). We therefore hypothesized that BMP signaling plays a

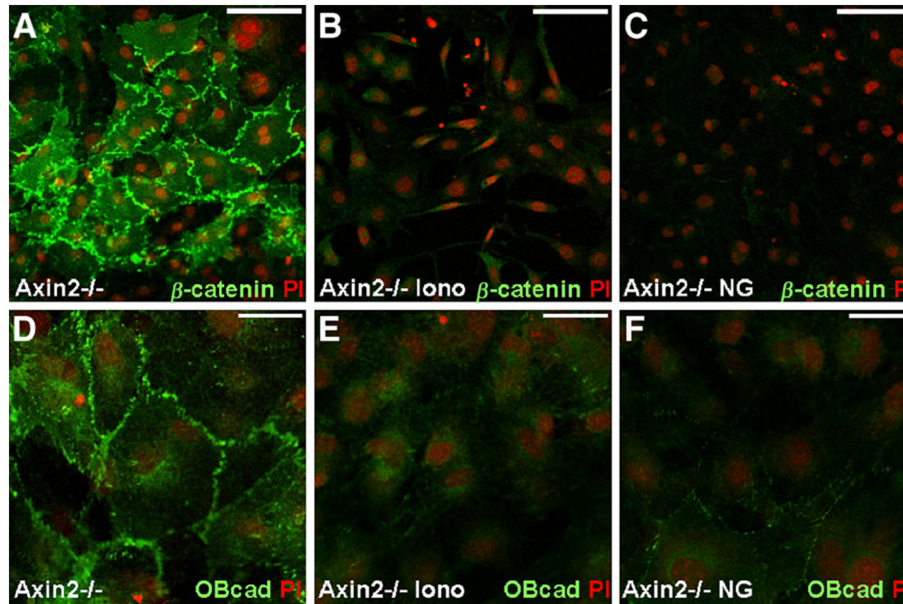

Fig. 5. Noggin inhibits membrane accumulations of β-catenin and OBcad caused by the Axin2 disruption in mature osteoblasts. Confocal microscopy revealed that β-catenin (A) and OBcad (D) were predominantly located to plasma membrane of the Axin2−/− differentiated CNC-derived osteoblasts. Cells were stained with β-catenin (A–C) or OBcad (D–F) in green and counterstained with propidium iodide (PI) in red. Pictures were taken under the same parameter optimized for detection in the Axin2−/− cells. Addition of ionomycin (Iono) or Noggin (NG) disrupts the membrane localization of β-catenin (B, C) and OB-cadherin (E, F) in the Axin2-null mutants. Scale bars, 125 μm (A–C); 40 μm (D–F).

role in osteoblast differentiation processes mediated by Wnt signaling. To determine if antagonizing BMP interfered with osteoblast differentiation of the Axin2 mutants, Noggin was added to the differentiation media at day 5. The inhibitory effect of Noggin on BMP signaling drastically reduced the accelerated osteoblast differentiation in primary cultures of the CNC-derived Axin2-null osteoblasts, as determined by alkaline phosphatase liquid assays (Fig. 6C). The presence of Noggin caused an approximately 3.5-fold decrease in the alkaline phosphatase activity at differentiation day 9. This was apparently specific to the presence of Noggin because the inhibitory effect worked in a dosage dependent fashion (Fig. 6D). A similar effect of Noggin on osteoblast differentiation was also observed in the Axin2+/+ cells (Fig. 6B). The antagonizing effect of Noggin might be attributed to its ability to dislocate β-catenin and to alter the cadherin mediated cell–

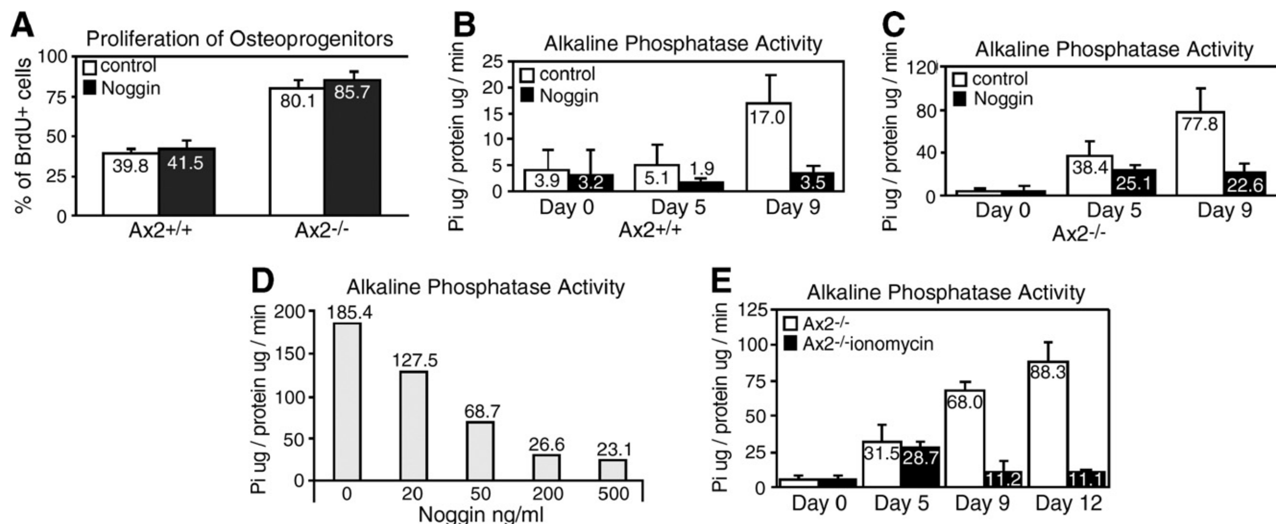

Fig. 6. Effects of Noggin on osteoblast development. Primary osteoblast precursors isolated from nasal and frontal bones of the Axin2+/+ and Axin2−/− were grown in culture media for in vitro BrdU incorporation assay (A). The percentage of proliferating osteoprogenitors was obtained by analyzing BrdU positive and negative cells in a total of >1000 cells. The addition of Noggin had no obvious effect on expansion of both the Axin2+/+ and Axin2−/− osteoprogenitors. (B–E) The CNC-derived osteoprogenitors of Axin2+/+ (B) and Axin2−/− (C–E) were analyzed for osteoblast differentiation using alkaline phosphatase liquid assay at different time points as indicated. Noggin (B–D) and Ionomycin (E) were present after day 5 of differentiation. Noggin inhibits the accelerated intramembranous ossification caused by Axin2 deficiency in a dosage dependent manner as determined by alkaline phosphatase activity at differentiation day 10 (D). Graphs, except for panel D, represent the average in three independent experiments.

cell interaction. We next tested whether the adhesion role of β-catenin is critical to mediate osteoblast differentiation. Ionomycin treatment was induced at day 5 of osteoblast differentiation in vitro. The presence of ionomycin dramatically interfered with osteoblast differentiation of the Axin2 mutants (Fig. 6E). By differentiation days 9 and 12, the addition of ionomycin resulted in approximately 6.1-fold and 8.0-fold decreases in the alkaline phosphatase activity, respectively. Our studies provide compelling evidence that cell adhesion mediated by β-catenin is required for osteoblast differentiation. In addition, BMP signaling, initially stimulated by Axin2 deficiency, appears to work in a positive feedback mechanism to control the osteoblast differentiation processes through modulation of β-catenin compartmentalization.

Haploid deficiency of β-catenin alleviates craniofacial bone defects caused by Axin2 deficiency

Our findings suggested that β-catenin plays important yet disparate roles in different stages of calvarial osteoblast development. We therefore crossed the Axin2-null mutants with mice carrying a β-catenin-null allele to definitely assess the requirement of β-catenin in skull morphogenesis mediated by Axin2. The anterior skull abnormalities induced by the targeted disruption of Axin2 were alleviated in the Axin2−/−; β-catenin+/− mutants (Fig. 7, $n=4/9$). Haploid deficiency of β-catenin was able to prevent the premature suture closure (including jugum limitans, coronal and metopic sutures) caused by the Axin2 deletion. These cranial sutures disappearing in the Axin2-null mutants are evident in the Axin2−/−; β-catenin+/− littermates (Figs. 7A–D). The metopic suture remains patent in the Axin2−/−; β-catenin+/− mice at postnatal day 28 (Figs. 7E, F). These data demonstrated a genetic interaction of Axin2 and β-catenin in skull and suture development.

Our previous results showed that Axin2 regulates both cell proliferation and differentiation during calvarial osteoblast development (Yu et al., 2005a). To determine if haploid deficiency of β-catenin affects expansion of osteoprogenitors in the Axin2−/− mice, expression of a mitotic marker Ki67 was analyzed to identify proliferating cells. Decreasing the levels of β-catenin drastically diminished the number of mitotic cells in the Axin2-null sutures at postnatal day 8 (Figs. 8A, B). The average percentage of proliferating cells was reduced by ~1.8-fold, and to a level similar to that in the wild type (Axin2+/+) mice (Fig. 8C) (Yu et al., 2005a). Therefore, haploid deficiency of β-catenin induced an inhibitory effect on enhanced expansion of the Axin2−/− osteoprogenitors. We next examined if haploid deficiency of β-catenin in the Axin2-null mice affected skeletogenesis that could be characterized by the expression of an osteogenic marker FGFR1 (Yu et al., 2005a). Indeed, the presence of the FGFR1-expressing cells was decreased from ~46% in the Axin2−/−; β-catenin+/+ sutures to ~12% in the Axin2−/−; β-catenin+/− sutures (Figs. 8D, E, H). This was also accompanied by a diminution in BMP signaling as determined by the expression of phosphorylated Smad1/5/8 (Figs. 8F, G). The average percentage of osteoprogenitors positive for phospho-Smad1/5/8 were 55% and 24% for Axin2−/−; β-catenin+/+ and Axin2−/−; β-catenin+/−, respectively (Fig. 8H). Immunoblot analysis further showed that the expression of FGFR1 and phospho-Smad1/5/8 was reduced in the Axin2−/−; β-catenin+/− osteoprogenitors (Fig. 8I). In these mutants, the Wnt dependent stimulation of BMP signaling caused by Axin2 deficiency was prevented by partial deletion of β-catenin. Our results strongly argue for a requirement of β-catenin in the Axin2-null calvarial osteoblast phenotypes. Stimulation of BMP signaling caused by the Axin2 deletion absolutely depends on β-catenin. This is consistent with the inhibitory effects of Noggin on the accelerated maturation process of the Axin2−/− osteoblasts (Fig. 6C). A positive feedback loop exists between Wnt and BMP signaling during skull morphogenesis. β-catenin with two disparate roles exerts its effects on regulating expansion of osteoprogenitors and maturation of osteoblasts.

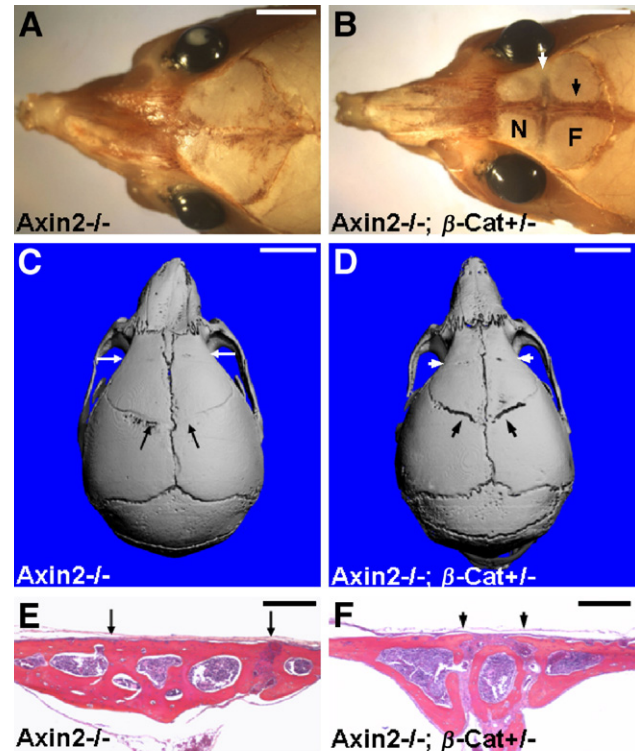

Fig. 7. Alleviation of the Axin2-null defects by haploid deficiency of β-catenin. β-catenin+/− mice were crossed into the Axin2-null background to obtain the Axin2−/−; β-catenin+/− mice. Haploid deficiency of β-catenin alleviates the Axin2−/− skull abnormalities at 4 weeks (A, B). The arrows indicate the sutures (black, metopic suture; white, jugum limitans) that lie between nasal (N) and frontal (F) bones. Theses sutures disappearing in the Axin2−/− mutants (A) are apparent in the Axin2−/−; β-catenin+/− littermates (B). μCT analyses reveal that the suture closure defects (white long and short arrows, jugum limitans; black long and short arrows, coronal suture) caused by Axin2 deficiency (C) were alleviated by haploid deficiency of β-catenin (D). Histology shows that metopic sutures, prematurely fused in the Axin2-null mutants (E, long arrows), remain patent in the Axin2−/−; β-catenin+/− mice (F, short arrows) at 4 weeks. Scale bars, 3 mm (A, B); 5 mm (C, D); 200 μm (E, F).

Discussion

Emerging evidence suggests that the canonical Wnt pathway is crucial for bone and cartilage formation (Akiyama et al.,

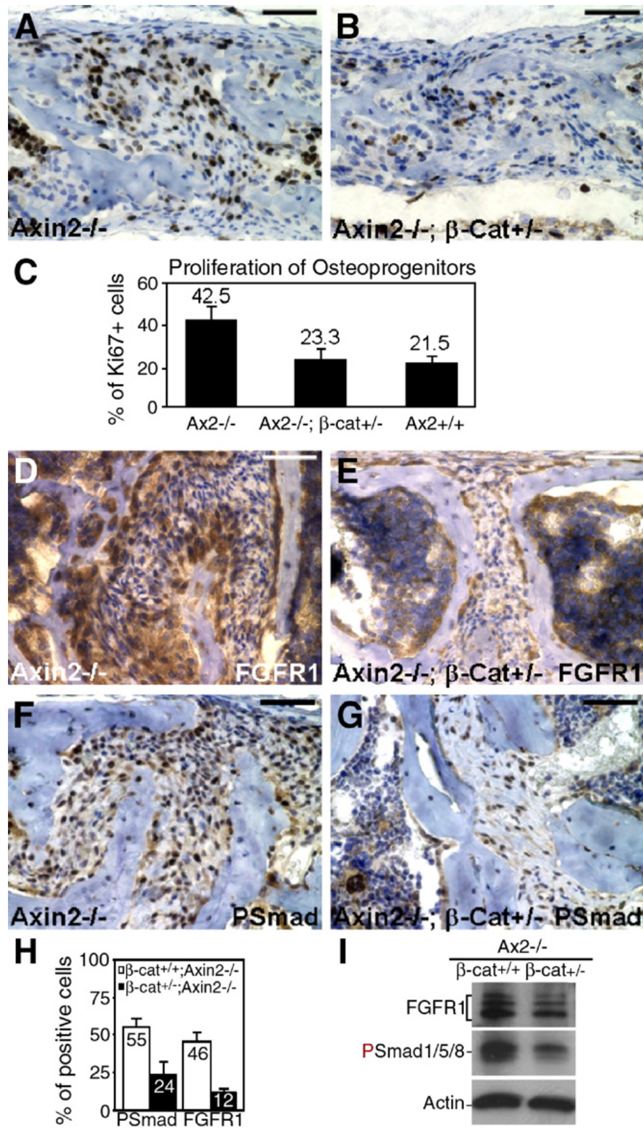

Fig. 8. Requirement of β-catenin in the Axin2 mediated osteoblast proliferation and differentiation. Immunostaining of Ki67 detects cells undergoing active divisions in the 8-day-old Axin2−/− (A) and Axin2−/−; β-catenin+/− (B) sutures. A reduced level of β-catenin causes a decrease in the presence of the mitotic osteoprogenitors stimulated by Axin2 deficiency. In the suture regions, osteoblast precursors that are positive (brown) and negative (blue) for Ki67 were counted to measure the proliferation effects. At least 8 different regions from four different mice were analyzed to obtain the average percentage of Ki67 positive (Ki67+) cells at postnatal day 8 (C). The skeletogenesis was characterized by expression of FGFR1 (D, E) and phospho-Smad1/5/8 (F, G). Sections were immunostained with α-FGFR1 or α-phospho-Smad1/5/8 antibody (brown) and counterstained with hematoxylin (blue). In the Axin2−/−; β-catenin+/− suture, a reduction of the FGFR1-expressing cells is evident at postnatal day 17 and a diminution in BMP signaling is detected at postnatal day 28. The graph shows in the suture region the average percentage of the Axin2−/−; β-catenin+/+ and Axin2−/−; β-catenin+/− osteoblast precursors positive for phospho-Smad1/5/8 (PSmad) and FGFR1 in five independent experiments (H). Immunoblot analyses revealed that haploid deficiency of β-catenin induced a quantitative change of the FGFR1 and phospho-Smad1/5/8 levels in the Axin2−/− calvarial osteoprogenitors (I). The level of Actin was also analyzed as a control for protein content. Scale bars, 50 μm (A, B, D, E, F, G).

2004; Bennett et al., 2005; Day et al., 2005; Glass et al., 2005; Gong et al., 2001; Hill et al., 2005; Kolpakova and Olsen, 2005; Li et al., 2005; Little et al., 2002). A growing list of molecules, regulating β-catenin signaling, has been linked to various skeletal diseases (Hartmann, 2006). However, the exact role of β-catenin in sequential steps of bone development remains elusive. As a result of accelerated intramembranous ossification, targeted disruption of Axin2 induces premature suture closure (Yu et al., 2005a). Axin2 apparently regulates expansion of osteoprogenitors and maturation of osteoblasts through its modulation on the canonical Wnt pathway (Yu et al., 2005a). Using the Axin2-null mouse model, we investigated the exact functions and downstream signaling effects of β-catenin in osteoblast proliferation and differentiation. The results lead us to the following theory (Fig. 9). The enhanced proliferation is mediated by stimulation of a Wnt target gene cyclin D1 to promote cell divisions in osteoprogenitors. BMP signaling, elevated by Axin2 deficiency, is intimately involved in the Axin2/β-catenin-dependent osteogenesis. Inhibition of BMP by Noggin has no effects on nuclear accumulation of β-catenin as well as expansion of osteoprogenitors in the Axin2 mutants. However, Noggin strongly affects osteoblast maturation by regulating adherens junctions. During osteoblast differentiation, a BMP-dependent event, initially activated by Wnt, is required to promote cell–cell interaction by stimulating membrane accumulations of β-catenin and OBcad. Thus, it results in accelerated intramembranous ossification occurring in the Axin2−/− skulls. Disturbance of cell–cell interaction interferes with osteoblast differentiation, suggesting that adherens junctions play a significant role in the maturation processes. The presence of Noggin not only disrupts membrane distribution and stimulation of β-catenin and OBcad, but also represses osteoblast differentiation. These findings strongly support our model for distinct roles of β-catenin at different stages of calvarial osteoblast development (Fig. 9). β-catenin regulates

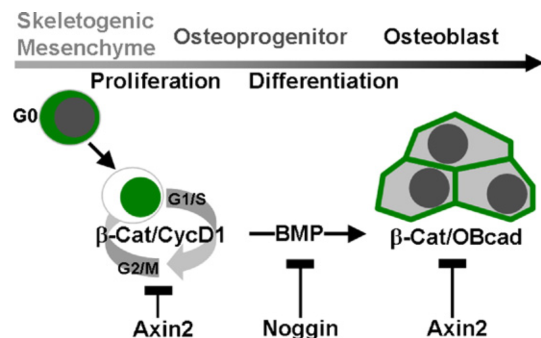

Fig. 9. Model for calvarial osteoblast development mediated by Wnt signaling. β-catenin is located to the cytoplasm of resting osteoprogenitors. An active Wnt signal promotes cell cycle entry by inducing nuclear localization of β-catenin (β-Cat) and activation of cyclin D1 (CycD1). During osteoblast differentiation, membrane distribution and stimulation of β-catenin and OBcad are mediated through a mechanism involving BMP signaling. Membrane accumulations of β-catenin and OBcad induce cell–cell interaction to promote the differentiation process. Noggin has an inhibitory effect on osteoblast differentiation, but not proliferation. Both Axin2 and Noggin regulate the Wnt and BMP pathways in a negative feedback loop, respectively. β-catenin, which functions as a transcriptional co-activator and a cell adhesion molecule, plays distinct roles in osteoblast proliferation and differentiation, respectively. Axin2, whose deficiency affects dislocation of β-catenin in osteoprogenitors and formation of adherens junctions in mature osteoblasts, negatively modulates both developmental processes.

cell cycle progression and cell–cell adhesion to control expansion of osteoprogenitors and maturation of osteoblasts, respectively.

Haploid deficiency of β-catenin alleviates the skull defects caused by Axin2 deficiency. The data reveal an absolute requirement of β-catenin in the Axin2 mediated skull morphogenesis. Haploid deficiency of β-catenin prevents premature fusion of the Axin2-null sutures by interfering with the calvarial osteoblast development. In the Axin2−/−; β-catenin+/− mutants, BMP signaling is reduced that places BMP downstream of β-catenin in osteoblast development. This is consistent with stimulation of phosphorylated Smad1/5/8 by the Axin2 deletion. On the other hand, Noggin was only able to inhibit the differentiation but not proliferation abnormalities, caused by Axin2 deficiency. Noggin interferes with membrane accumulations of β-catenin, suggesting that it is regulated by BMP during osteoblast maturation. Therefore, these two pathways appear to work in a feedback regulatory mechanism (Fig. 9). Initial β-catenin activation is responsible for stimulating the β-catenin/cyclin D1 pathway to enhance proliferation in a BMP-independent fashion. Nuclear localization of β-catenin then turns on BMP signaling that induces a second event of β-catenin activation upon osteoblast differentiation. This BMP-dependent signal facilitates the maturation processes by promoting cell–cell interaction mediated by β-catenin/OBcad.

The functions of β-catenin can also be molecularly distinct. In *C. elegans*, there are three different β-catenin gene products for adhesion and signaling functions (Korswagen et al., 2000). BAR-1 mediates Wnt signaling by forming a transcription complex with POP-1 (TCF/LEF). HMP-2 interacts exclusively with cadherin. WRM-1 is involved in a divergent Wnt pathway where it regulates POP-1 indirectly. Nevertheless, there is no evidence for multiple β-catenin gene products in vertebrates. This raises the possibility that β-catenin utilizes its distinct functions in regulating osteoblast development. Previous genetic analyses demonstrated that β-catenin is essential for different stages of skeletal development (Day et al., 2005; Hill et al., 2005). However, the nature of these studies could not distinguish which function of β-catenin is critically involved. The exact roles of β-catenin remain unclear because it is a multifunctional protein, which is involved in cellular responses including Wnt signaling (Logan and Nusse, 2004; Moon et al., 2004) and cell adhesion (Bienz, 2005; Gumbiner, 2005; Harris and Peifer, 2005). Only the binding of β-catenin to the degradation complex is disrupted in the Axin2-null mice. These mutants provide an excellent model to investigate the functional involvement of β-catenin in the adhesion and transcription complexes. Our results demonstrated that β-catenin utilizes its disparate roles to control expansion of osteoprogenitors and maturation of osteoblasts in different contexts. They also provide evidence for how one molecule with multiple cellular functions can regulate the proliferation and differentiation processes of single lineage-specific development. Although prior studies suggested that distinct molecular forms of β-catenin possess preferential binding affinity to adhesion, transcription and degradation complexes to ensure proper tissue architecture and cell-fate decisions (Brembeck et al., 2004; Gottardi and Gumbiner, 2004), details of such mechanisms regulated in these microenvironments remain to be elucidated. Further investigations of our system might gain new insights into these mechanistic regulations.

The cadherin–catenin mediated cell adhesion has been shown to promote osteogenesis (Ferrari et al., 2000; Stains and Civitelli, 2005). Our data strongly support the importance of cell–cell interaction mediated by adherens junctions in osteoblast differentiation. OBcad, but not Ncad or Ecad, is specifically stimulated in the Axin2-null mutants. Similar to the Axin2-null mutants, the loss of OBcad caused defects in skeletal development that occurred preferentially in the mouse skull (Kawaguchi et al., 2001). It has been suggested that Ncad, activated by BMP-2, is a direct target of Sox9 in chondrogenesis (Panda et al., 2001). In human craniosynostosis with FGFR2 mutation, Ncad overexpression plays a role in premature suture closure (Muenke and Schell, 1995). Therefore, promoting cell adhesion by stimulating the cadherin–catenin complex formation might be a key part of the skull morphogenetic signaling network, as well as other processes in musculoskeletal development.

FGF signaling plays a key role in skull morphogenesis and craniosynostosis (Ornitz and Marie, 2002) although the mechanism by which FGF interacts with Wnt and BMP remains to be illustrated. Expression of two potential Wnt downstream targets FGF4 and FGF18 is elevated by Axin2 deficiency during osteoblast differentiation (Yu et al., 2005a). In the Axin2-null sutures, increased numbers of the FGFR1-expressing cells are evident (Yu et al., 2005a). This process depends on β-catenin as shown in the Axin2−/−; β-catenin+/− mice. Previous studies suggested that FGF might induce BMP signaling by inhibiting the BMP-dependent activation of Noggin (Warren et al., 2003). It is possible that FGF acts in between Wnt and BMP to orchestrate skull morphogenesis. The exact role of FGF signaling in the hierarchy of skull signaling cascade and in the calvarial osteoblast developmental processes remains to be determined. Future studies focused on delineating the skull morphogenetic circuitry that regulates tissue architecture and cell-fate decisions promise important insights into the molecular and cellular bases of craniosynostosis.

Acknowledgments

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