BMP2, but not BMP4, is crucial for chondrocyte proliferation and maturation during endochondral bone development

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Summary

The BMP signaling pathway has a crucial role in chondrocyte proliferation and maturation during endochondral bone development. To investigate the specific function of the *Bmp2* and *Bmp4* genes in growth plate chondrocytes during cartilage development, we generated chondrocyte-specific *Bmp2* and *Bmp4* conditional knockout (cKO) mice and *Bmp2*,*Bmp4* double knockout (dKO) mice. We found that deletion of *Bmp2* and *Bmp4* genes or the *Bmp2* gene alone results in a severe chondrodysplasia phenotype, whereas deletion of the *Bmp4* gene alone produces a minor cartilage phenotype. Both dKO and *Bmp2* cKO mice exhibit severe disorganization of chondrocytes within the growth plate region and display profound defects in chondrocyte proliferation, differentiation and apoptosis. To understand the mechanism by which BMP2 regulates these processes, we explored the specific relationship between BMP2 and Runx2, a key regulator of chondrocyte differentiation. We found that BMP2 induces Runx2 expression at both the transcriptional and post-transcriptional levels. BMP2 enhances Runx2 protein levels through inhibition of CDK4 and subsequent prevention of Runx2 ubiquitylation and proteasomal degradation. Our studies provide novel insights into the genetic control and molecular mechanism of BMP signaling during cartilage development.

Key words: *Bmp2*, *Bmp4*, Chondrocyte, Endochondral bone formation

Introduction

During skeletal development, the majority of the bones in the body are established by the endochondral bone formation process, which is initiated by mesenchymal cell condensation and subsequent mesenchymal cell differentiation into chondrocytes and surrounding perichondrial cells. The committed chondrocytes proliferate rapidly forming the cartilage growth plate where cells are arranged in columns of proliferating, differentiating and terminally hypertrophic chondrocytes. Chondrocytes near the center of the cartilage elements exit the cell cycle initiating the process of hypertrophic differentiation to generate a calcified cartilage matrix. Eventually, the local vasculature, perichondrial osteoblasts and various other types of cells invade the calcified cartilage, replacing the terminally mature chondrocytes with marrow components and trabecular bone matrix. Primary ossification occurs with osteoblast-mediated bone formation, which initially occurs on the calcified cartilage template. Chondrocyte maturation and the endochondral bone development process is tightly regulated by a series of growth factors and transcription factors, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), indian hedgehog (Ihh), parathyroid hormone-related protein (PTHrP), Wnt signaling proteins and Runt-related transcription factor 2 (Runx2) (Yoon and Lyons, 2004; Ornitz, 2005; Kronenberg, 2003; Komori, 2003; Kolpakova and Olsen, 2005).

BMPs are multi-functional growth factors that belong to the transforming growth factor β (TGF-β) super family. In vivo evidence suggests that BMP signaling is primarily mediated through the canonical BMP–Smad pathway in chondrocytes (Yoon et al., 2005). BMPs bind the type II receptor and phosphorylate type I serine or threonine receptors, which subsequently phosphorylate Smad1, Smad5 and Smad8 (R-Smads). The activated R-Smads form a complex with Smad4 before entering the nucleus to regulate target gene transcription. Several lines of evidence suggest that the BMP–Smad pathway has a crucial role in endochondral bone development. Removal of *Bmp2* and *Bmp4* specifically from mesenchymal cells leads to defects in skeletal development (Bandyopadhyay, 2006). Deletion of the *Smad1* and *Smad5* genes or the *Bmpr1a* and *Bmpr1b* genes in cartilage results in chondrodysplasia (Yoon et al., 2005; Retting et al., 2009). In addition to the role of BMPs in early mesenchymal cell differentiation (Haas and Tuan, 1999; Hatakeyama et al., 2004), they also have crucial roles during later

stages of chondrocyte proliferation and differentiation (Shukunami et al., 2000; Leboy et al., 2001; Valcourt et al., 2002). However, as a result of overlapping and redundant functions among different BMP genes, the regulatory role of these genes during chondrocyte proliferation and maturation in vivo remains undefined.

Bmp2 and *Bmp4* mRNAs are highly expressed in pre-hypertrophic and hypertrophic chondrocytes of the growth plate (Feng et al., 2003; Nilsson et al., 2007) (supplementary material Fig. S1). *Bmpr1a* is highly expressed in pre-hypertrophic chondrocytes, and phosphorylated Smad1, Smad5 and Smad8 proteins are detected in the lower region of proliferating columnar zone and pre-hypertrophic chondrocytes (Sakou et al., 1999; Yoon et al., 2006). The specific expression patterns of these genes suggest an essential role for *Bmp2* and/or *Bmp4* in chondrocyte proliferation and maturation during endochondral bone development. In vitro studies have shown that BMP2 and BMP4 stimulate the progression of chondrocyte hypertrophy (Hatakeyama et al., 2004; Leboy et al., 1997; De Luca et al., 2001; Minina et al., 2001; Horiki et al., 2004; Clark et al., 2009). Similarly, expression of constitutively active *Bmpr1a* in chondrocytes induces the acceleration of chondrocyte differentiation into hypertrophic chondrocytes (Kobayashi et al., 2005). These findings suggest that *Bmp2* and *Bmp4* have a similar and redundant role in chondrocyte maturation. To determine which one of these BMP genes is required for chondrocyte development in vivo, we have generated chondrocyte-specific *Bmp2* and *Bmp4* cKO mice and *Bmp2,Bmp4* dKO mice. Chondrocyte-specific deletion of these BMP genes is achieved by breeding *Col2a1CreER*^{T2} transgenic mice (Chen et al., 2007) with the *Bmp2* or *Bmp4* floxed mice (*Bmp2*$^{fx/fx}$ and *Bmp4*$^{fx/fx}$). Chondrocyte-specific gene deletion is achieved by intraperitoneal injection of a single dose of tamoxifen (TM) to the pregnant female carrying embryos at embryonic day 12.5 (E12.5). We then assessed changes in chondrocyte maturation in these mutant embryos at E14.5 and E18.5. Our studies demonstrate that deletion of only *Bmp2* or both *Bmp2* and *Bmp4* genes led to severe defects in chondrocyte proliferation and maturation during endochondral bone development. By contrast, chondrocyte-specific deletion of only the *Bmp4* gene caused minor changes in chondrocyte maturation. Our findings indicate that *Bmp2* has a crucial and non-redundant role in chondrocyte proliferation and maturation during endochondral bone development.

Results

Deletion of *Bmp2* and *Bmp4* or *Bmp2* alone impairs skeletal development

To investigate the role of endogenous *Bmp2* and *Bmp4* genes in growth plate chondrocyte maturation and skeletal development, pregnant mice with embryos at E12.5 were injected with TM. E18.5 embryos were collected and whole skeletal Alizarin Red and Alcian Blue staining was performed. Whole skeletons and individual skeletal elements of *Bmp2* and *Bmp4* (*Bmp2*/*4*) dKO and *Bmp2* cKO embryos were very small compared with their Cre-negative littermate controls, suggesting impaired skeletal development in *Bmp2*/*4* dKO and *Bmp2* cKO embryos (Fig. 1A). Calvaria of these mutant embryos were smaller than those of Cre-negative littermates, and cartilaginous occipital bones were nearly absent, demonstrating that intramembranous bone formation was also impaired. Compared with Cre-negative littermates, the deformed thoracic cavities of *Bmp2*/*4* dKO and *Bmp2* cKO embryos were significantly smaller with minimal bone formation. Spines and hind limbs of *Bmp2*/*4* dKO and *Bmp2* cKO embryos were also markedly shorter than Cre-negative littermates (Fig. 1B). However, only minor differences were observed in all skeletal elements analyzed from the *Bmp4* cKO embryos compared with Cre-negative littermates, suggesting that the *Bmp4* gene has a minor role in normal embryonic skeletal growth and development or is complemented by the expression of other BMP genes (Fig. 1A,B).

Formation of primary ossification center is delayed in *Bmp2/4* dKO and *Bmp2* cKO embryos

To further analyze changes in skeletal development in *Bmp2*/*4* dKO and *Bmp2* cKO embryos, histological staining was performed on tibias sections of E14.5 *Bmp2*/*4* dKO, *Bmp2* and *Bmp4* cKO embryos and the Cre-negative littermates. In Cre-negative embryos, chondrocytes in the middle of tibia began the differentiation process forming a hypertrophic zone, which stained weakly with Alcian Blue or Safranin O compared with the adjacent immature chondrocytes. In *Bmp2*/*4* dKO embryos, the whole tibia was smaller than that of Cre-negative embryos and chondrocyte hypertrophy was absent, as was evidence of formation of the primary ossification center. *Bmp2* cKO embryos showed a very similar delay in the formation of the hypertrophic zone of tibia compared with *Bmp2/4* dKO embryos. By contrast, there were minimal changes in the tibiae of *Bmp4* cKO embryos when compared with the Cre-negative littermates (Fig. 2A). *Bmp4* cKO embryos had evidence of chondrocyte hypertrophy and formation of the primary ossification center. These findings demonstrated that chondrocyte hypertrophy is severely delayed by deletion of the *Bmp2* gene, but not the *Bmp4* gene, in Col2a1-positive chondrocytes during embryonic skeletal development.

Chondrocyte maturation is impaired in *Bmp2/4* dKO and *Bmp2* cKO embryos

Further histological analysis was performed on E18.5 embryos. The results demonstrated that the lengths of proliferative and hypertrophic zones of *Bmp2*/*4* dKO and *Bmp2* cKO embryos were significantly reduced with disorganized columnar chondrocyte structure (Fig. 2B). Normal hypertrophic chondrocytes were replaced with smaller number of enlarged hypertrophic chondrocytes, with expansion of both the cytoplasm and nucleus in *Bmp2*/*4* dKO and *Bmp2* cKO embryos (Fig. 2B–D). The reduced size of the growth plate was associated with less endochondral bone formation, although ectopic matrix deposition was observed at the perichondrial region surrounding the abnormal cartilage in *Bmp2*/*4* dKO and *Bmp2* cKO embryos (Fig. 2C,D). Because *Col2a1CreER*^{T2} mice do not target perichondrial cells (Chen et al., 2007), this ectopic matrix formation suggests a non-cell-autonomous effect in *Bmp2*/*4* dKO and *Bmp2* cKO embryos. To rule out the toxic effect of TM on embryonic skeletal development, we injected TM in pregnant WT mice with embryos at E12.5. E18.5 embryos were collected and histology staining was performed. No significant difference in skeletal development was found by injection of TM (supplementary material Fig. S2). In L4 vertebrae, hypertrophic chondrocyte area was reduced over 50% in *Bmp2*/*4* dKO and *Bmp2* cKO embryos compared with those in Cre-negative embryos. The reduced chondrocyte hypertrophy and decreased matrix deposition observed in the center of the vertebral body

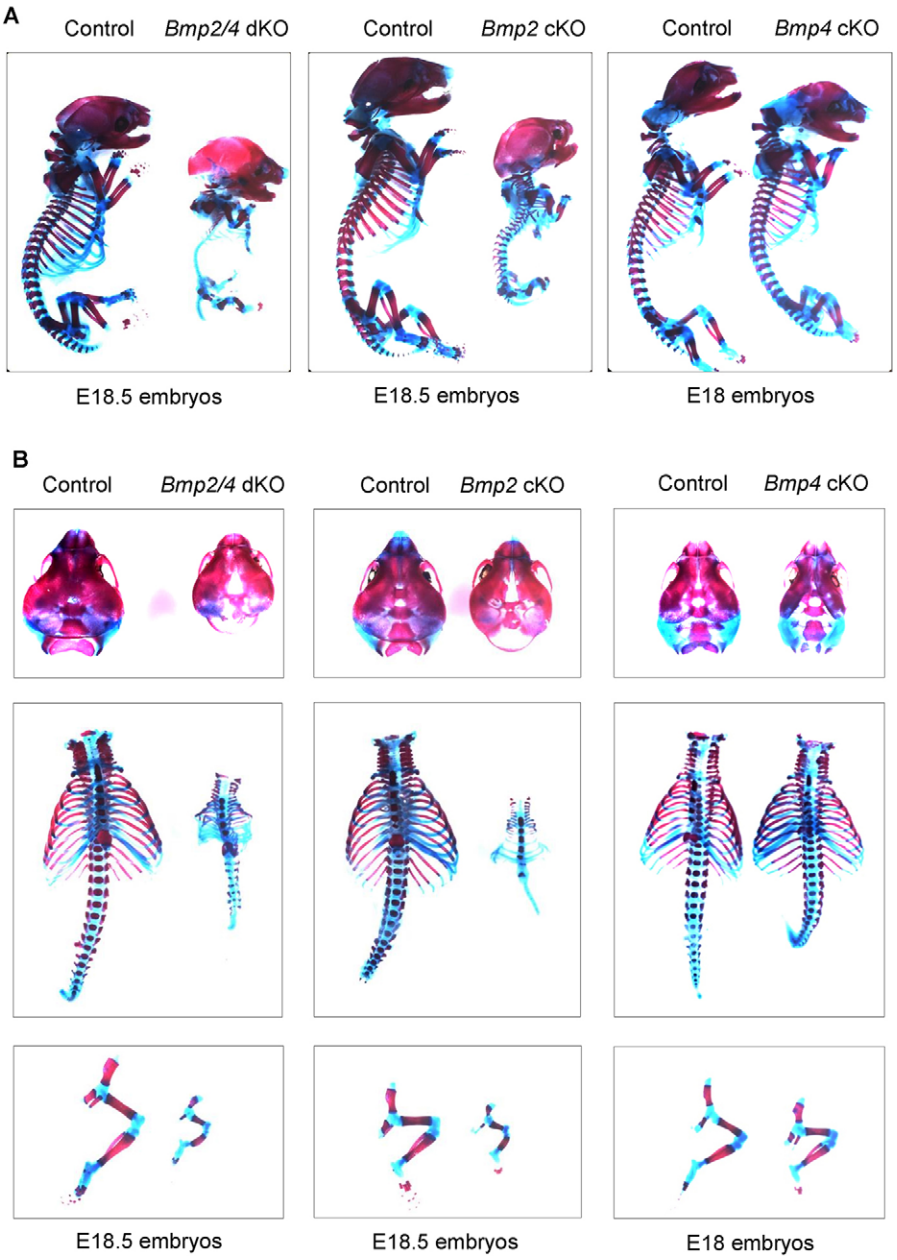


Fig. 1. Skeletal growth was impaired in *Bmp2/4* dKO, *Bmp2* cKO, but not *Bmp4* cKO embryos. (**A**) E18.5 *Bmp2/4* dKO, *Bmp2* cKO embryos and E18.0 *Bmp4* cKO embryos and their littermates were collected. The whole skeleton was stained with Alizarin Red and Alcian Blue. The body size of *Bmp2/4* dKO and *Bmp2* cKO embryos are dramatically decreased compared with their Cre-negative littermates. (**B**) In *Bmp2/4* dKO and *Bmp2* cKO embryos heads are smaller than those of Cre-negative littermates. Cartilaginous occipital bone has almost disappeared (top panel) and the thoracic cavities and spines are much smaller than Cre-negative embryos with very little bone formation. In addition, the thoracic cavities are deformed in shape (middle panel) in these KO embryos. The hind limbs are also obviously shorter (bottom panel) in *Bmp2/4* dKO and *Bmp2* cKO embryos. The whole skeletal, head and hind limb of *Bmp4* cKO embryos are relatively normal, with only minor defects in skeletal development (right panel).

(Fig. 2E, upper panel) indicates that the chondrocyte maturation process is also delayed in vertebral bones in *Bmp2/4* dKO and *Bmp2* cKO embryos. By contrast, only minor changes in growth plate chondrocyte maturation were found in E18.5 *Bmp4* cKO embryos (Fig. 2B,C,E), suggesting that the expression of the *Bmp4* gene is not absolutely required for chondrocyte maturation and cartilage development.

Defects in chondrocyte proliferation and apoptosis in *Bmp2/4* dKO and *Bmp2* cKO embryos

To further determine changes in cellular function in growth plate chondrocytes, we performed proliferating cell nuclear antigen (PCNA) staining and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining using tibia sections of E18.5 embryos. The results of PCNA staining demonstrated that cell proliferation was dramatically reduced in *Bmp2/4* dKO and *Bmp2* cKO embryos. By contrast, no significant reduction in PCNA-positive proliferating chondrocytes was found in *Bmp4* cKO embryos (Fig. 3A and B). The TUNEL staining images of E18.5 embryos demonstrated that chondrocyte apoptosis was significantly increased in *Bmp2/4* dKO and *Bmp2* cKO embryos (Fig. 3C).

Defects in chondrocyte differentiation in *Bmp2/4* dKO and *Bmp2* cKO embryos

To examine chondrocyte differentiation, we performed in situ hybridization assays using *Col2a1*, *Col10a1* and *Mmp13* probes. *Col2a1* is highly expressed in growth plate chondrocytes in the resting and proliferating chondrocytes in E18.5 Cre-negative embryos. Opposing *Col2a1* expression, *Col10a1* is highly expressed in pre-hypertrophic and hypertrophic chondrocytes. *Mmp13* is expressed in terminal hypertrophic chondrocytes that

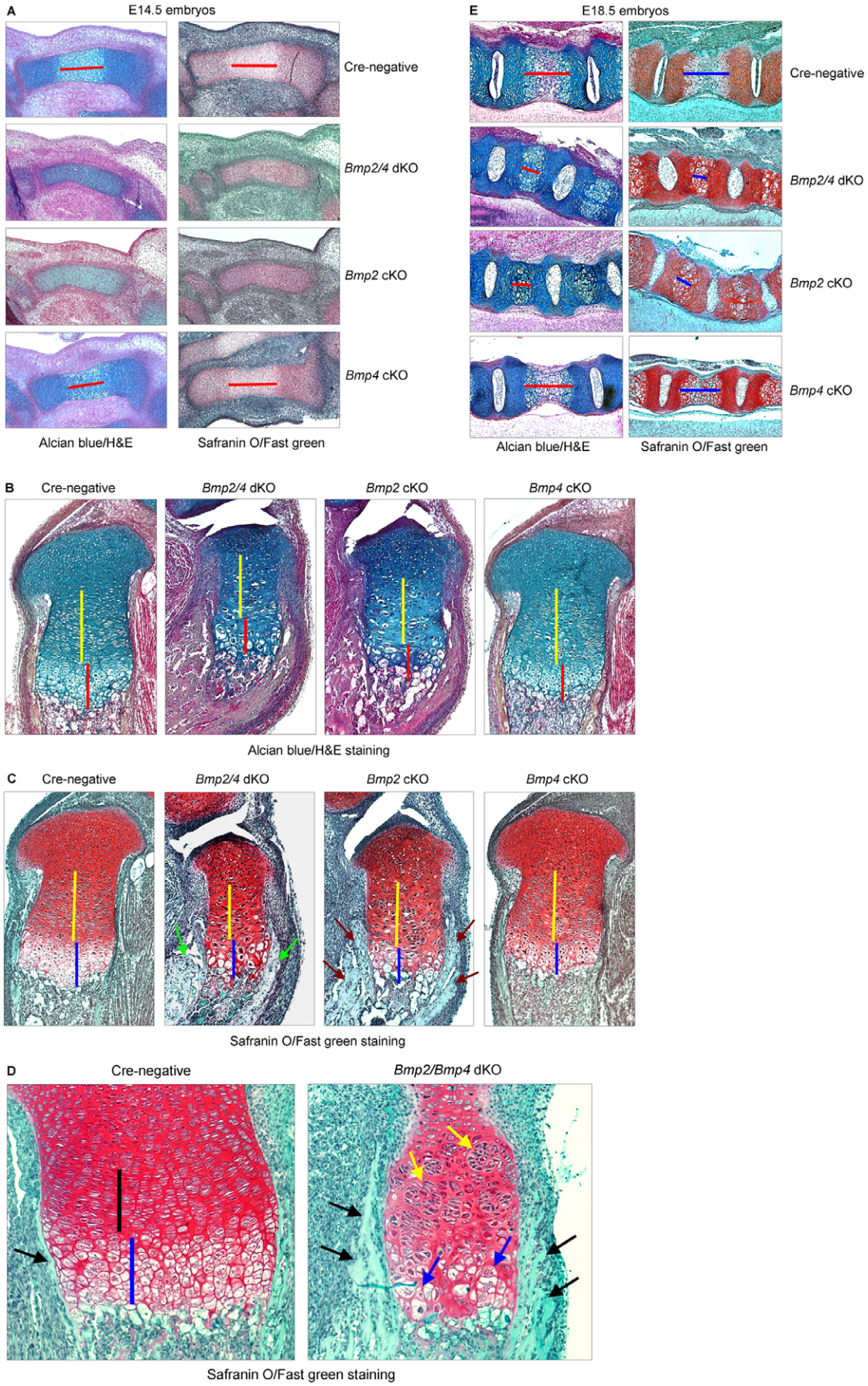


Fig. 2. See next page for legend.

are proceeding into the final apoptotic stage where cartilage matrix is degraded and replaced by bone matrix. In this study, we found that the expression of all of these chondrocyte marker genes was significantly reduced in E18.5 *Bmp2/4* dKO and *Bmp2* cKO embryos by in situ hybridization (Fig. 4A). To further determine changes in chondrocyte marker gene expression, we also performed real-time RT-PCR assays and found that expression of *Sox9*, *Acan* (aggrecan) and *Col2a1* was significantly reduced in chondrocytes in which the *Bmp2* or *Bmp2/4* genes were deleted (Fig. 4B–D). Compared with the changes in gene expression in *Bmp2/4* dKO and *Bmp2* cKO embryos, there are minor changes in the expression of these chondrocyte marker genes in *Bmp4* cKO embryos (Fig. 4B–D). In this assay, primary chondrocytes were isolated from E18.5 mutant and Cre-negative embryos. It has been reported that BMP-2 regulates itself and several other BMP genes, including *Bmp4*, *Bmp5*, *Bmp6* and *Bmp8a* (Harris et al., 1994; Ghosh-Choudhury et al., 1995; Chen et al., 1997; Edgar et al., 2007). The expression of several BMP family members was examined in *Bmp2*- and *Bmp4*-deleted chondrocytes. The expression of *Bmp5*, *Bmp7*, *Bmp8b* and *Bmp9* were significantly downregulated in the chondrocytes in which the *Bmp2* gene was deleted (Fig. 4E–I). By contrast, no significant change in the expression of these genes was found in *Bmp4*-deficient chondrocytes (Fig. 4J–N). These results suggest that these BMP genes are regulated by endogenous BMP2. In addition, we found that *Bmp4* expression was upregulated in *Bmp2*-deficient chondrocytes and *Bmp2* expression was upregulated in *Bmp4*-deficient chondrocytes (supplementary material Fig. S3), suggesting that expression of *Bmp4* and *Bmp2* genes was regulated by endogenous BMP2 and BMP4. To determine the interaction of Wnt/β-catenin and BMP signaling pathways, we isolated primary sternal chondrocytes from *Bmp2/4*$^{fx/fx}$ mice. The cells were infected with Ad-Cre or Ad-GFP (control) and treated with BIO (1 μM), a GSK-3β inhibitor, and Wnt3a (100 ng/ml). We found that BIO- and Wnt3a-induced *Alp* expression was significantly inhibited in *Bmp2/4*-deficient chondrocytes (Fig. 4O), suggesting that canonical Wnt/β-catenin signaling may stimulate chondrocyte differentiation partially through a *Bmp2/4*-dependent mechanism. Significant amounts of ectopic matrix deposition were found in perichondrial areas of *Bmp2/4* dKO and *Bmp2* cKO embryos. To determine whether bone-specific markers and key transcription factors are upregulated in these areas, we examined Runx2 and Osterix expression by immunocytochemistry. We found that the numbers of Runx2- and Osterix-positive cells, and staining intensity were significantly increased in perichondrial areas of *Bmp2/4* dKO and *Bmp2* cKO embryos (Fig. 5A,B). By contrast, Runx2 expression in the proliferating and pre-hypertrophic areas was significantly reduced in *Bmp2/4* dKO embryos (Fig. 5A). Taken together, the findings suggest that chondrocyte functions are severely impaired when the *Bmp2* gene, but not the *Bmp4* gene, is deleted in E18.5 *Bmp2/4* and *Bmp2* mutant embryos.

Fig. 2. Severe defects in growth plate cartilage morphology are found in *Bmp2/4* dKO and *Bmp2* cKO embryos. (**A**) Tibias of E14.5 *Bmp2/4* dKO, *Bmp2* cKO, *Bmp4* cKO embryos and Cre-negative littermate controls were collected. Alcian Blue and H&E (left panel) and Safranin O and Fast Green (right panel) staining was performed in tibias sections, respectively. In Cre-negative embryos, chondrocytes in the middle of tibia begin to differentiate into hypertrophic chondrocytes and form primary ossification center, which is lightly stained with Alcian Blue or Safranin O (top panel). In *Bmp2/4* dKO and *Bmp2* cKO embryos, the tibia is smaller than that of Cre-negative embryo and is stained dark. There was no obvious primary ossification center formation in tibia of these KO embryos (middle panels). In *Bmp4* cKO embryos, the tibia appears similar to the Cre-negative control with obvious primary ossification center formation in the middle (bottom panel). The length of the primary ossification center is marked by a red line. (**B**–**E**) E18.5 tibias of *Bmp2/4* dKO, *Bmp2* cKO, *Bmp4* cKO embryos and the Cre-negative control embryos were collected. Alcian Blue and H&E (B and E, left panel), and Safranin O and Fast green (C,D and E, right panel) staining was performed in tibia sections, respectively. Growth plate of E18.5 Cre-negative embryos shows typical columnar structure. However, growth plates of same aged *Bmp2/4* dKO and *Bmp2* cKO embryos are significantly smaller compared with their Cre-negative control embryos. The sizes of both proliferative zone (B,C, yellow bars; D, black bar) and hypertrophic zone (B, red bars and C, blue bars) were reduced. Disorganized chondrocytes with expanded cytoplasm and enlarged nucleus are found in hypertrophic chondrocyte area in *Bmp2/4* dKO (D, blue arrows) embryos. Ectopic matrix deposition was found in the perichondrium area in *Bmp2/4* dKO and *Bmp2* cKO embryos (C, green and brown arrows; D, black arrows). The length of proliferative zone and hypertrophic zone of *Bmp4* cKO embryos are similar to that of Cre-negative control embryos. No obvious disorganization of hypertrophic chondrocytes is found in *Bmp4* cKO embryos (B,C). (E) In L4 vertebrae of E18.5 Cre-negative embryos, hypertrophic chondrocytes (red bar) occupied half of the length of vertebrae. There is vascular invasion and bone matrix formation in the middle of hypertrophic zone (top panel). Limited hypertrophic chondrocytes are found in the L4 vertebrae of *Bmp2/4* dKO and *Bmp2* cKO embryos (middle panel). L4 vertebrae of *Bmp4* cKO embryo show similar chondrocyte hypertrophy to those of Cre-negative embryos (bottom panel).

BMP-2 upregulates Runx2 protein levels by downregulation of CDK4 expression

It has been well documented that BMP2 induces *Runx2* mRNA expression (Chen et al., 1998; Hassan et al., 2006). In the present studies, we examined the effects of BMP2 on *Runx2* mRNA and protein expression in chondrocytes. We found that BMP2 induced *Runx2* mRNA expression up to 3.5-fold, but enhanced Runx2 protein levels up to 10-fold (Fig. 6A,D,E). These observations suggest that, in addition to its transcriptional regulation, BMP2 also regulates Runx2 expression at the post-transcriptional level. Our previous report demonstrated that Runx2 protein levels are regulated by the ubiquitin–proteasome pathway through a cyclin-D1–CDK4-induced phosphorylation of Runx2 (Shen et al., 2006). To determine whether BMP2 regulates CDK4 expression, we performed western blot analysis and found that BMP2 significantly inhibited CDK4 expression in chondrogenic RCS cells (Fig. 6A). Similarly to BMP2, BMP4 also inhibited CDK4 expression in a time-dependent manner (supplementary material Fig. S4). The BMP2-mediated enhancement of Runx2 protein levels could be partially inhibited by expression of CDK4 in these cells (Fig. 6A). The regulatory role of BMP2 in Runx2 protein degradation was further confirmed by a Runx2 ubiquitylation assay. BMP2 inhibited Runx2 ubiquitylation whereas overexpression of CDK4 partially reversed the inhibitory effect of BMP2 on Runx2 ubiquitylation in RCS cells (Fig. 6B). To further determine the role of CDK4 in regulation of Runx2 protein expression, we transfected *Cdk4* siRNA into RCS cells and found that similar to the addition of BMP2, transfection of *Cdk4* siRNA also enhanced Runx2 protein levels. Addition of noggin blocked BMP2-induced Runx2 expression. By contrast, noggin had no effect on *Cdk4* siRNA-induced upregulation of Runx2 protein (Fig. 6C), suggesting that CDK4 works downstream of BMP2 in regulation of Runx2 protein levels. We further analyzed the dose-response effect of BMP2 on Runx2 protein levels and

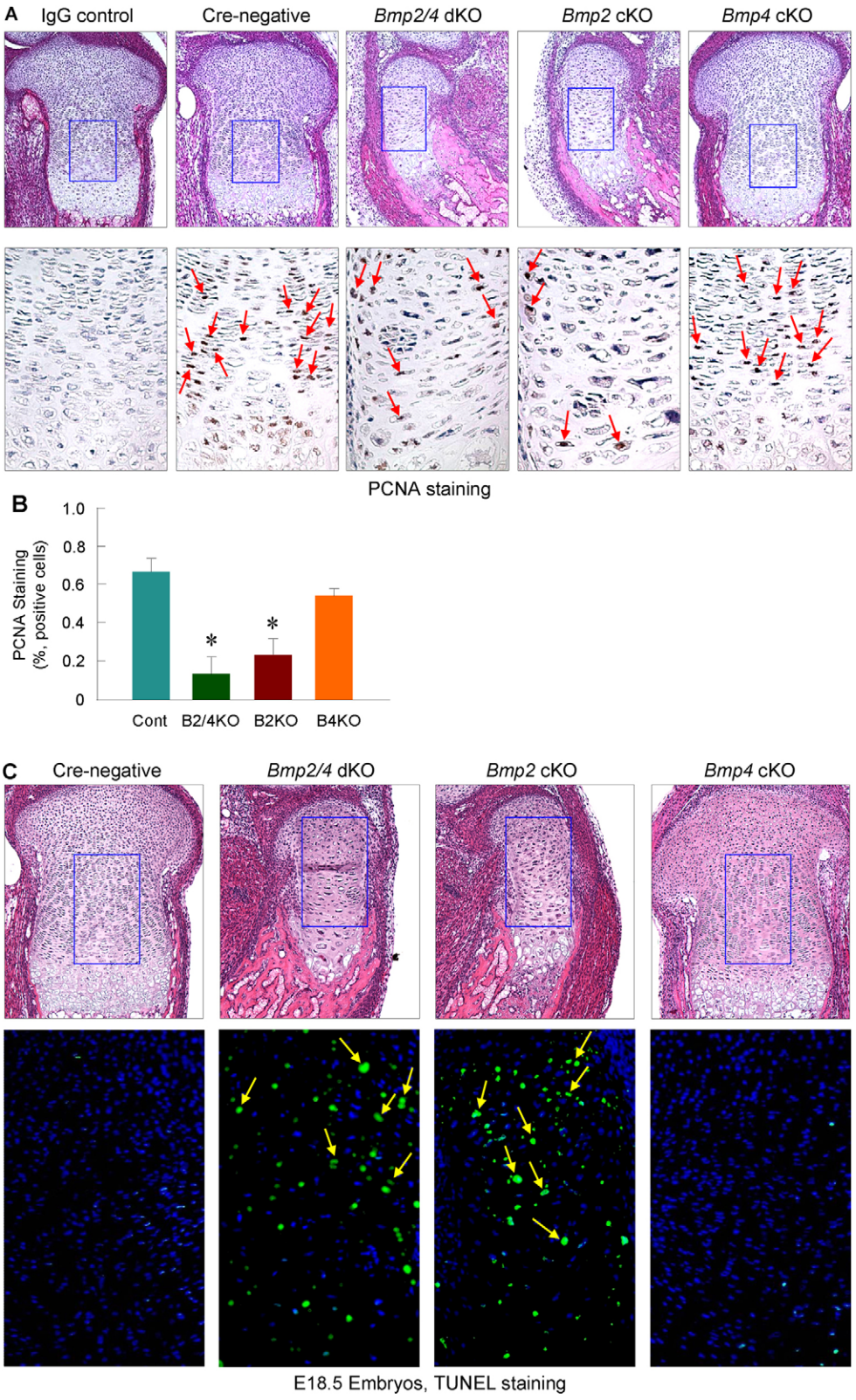
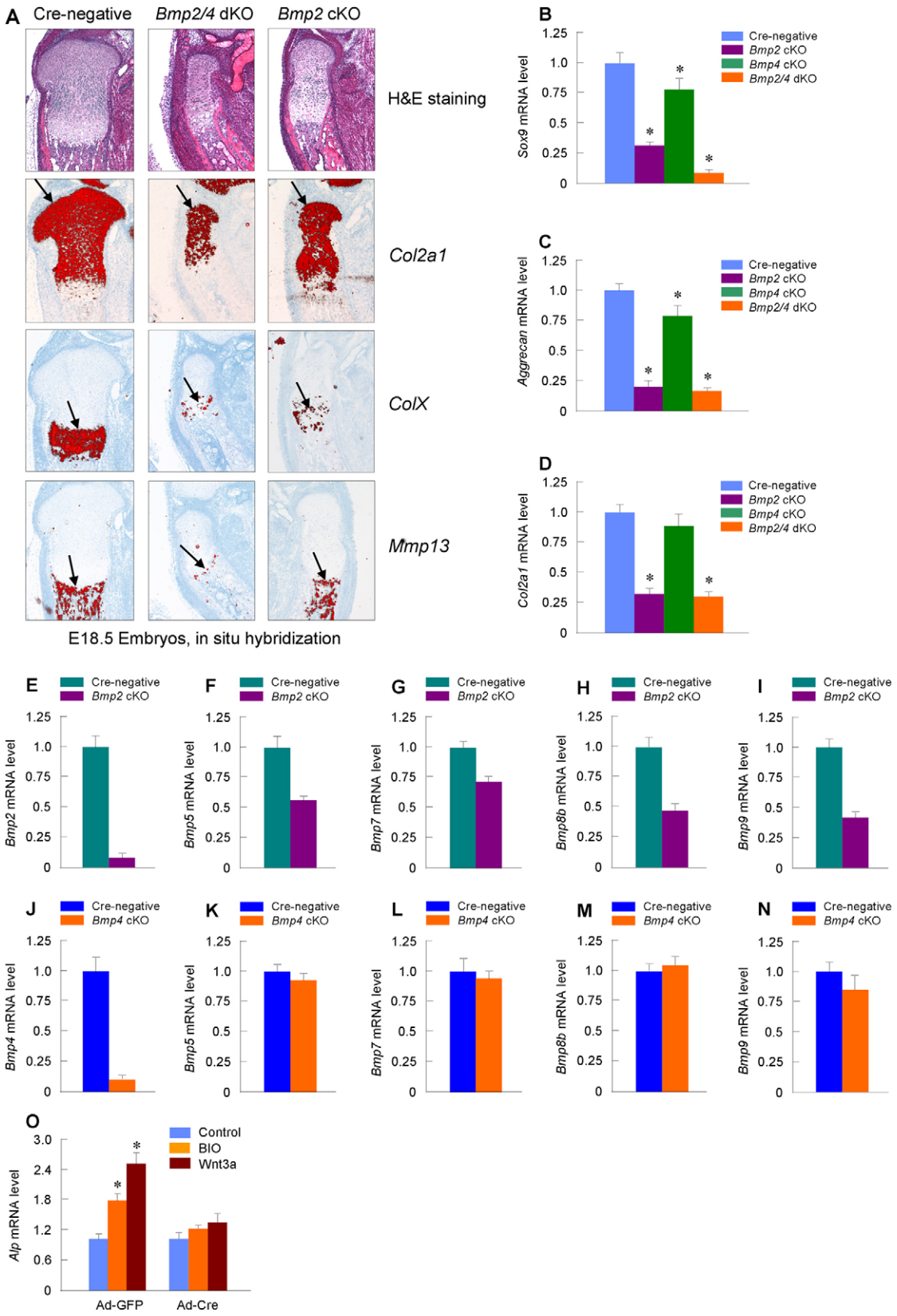


Fig. 3. Reduced chondrocyte proliferation and increased chondrocyte apoptosis in *Bmp2/4* dKO and *Bmp2* cKO embryos. (**A**,**B**) PCNA staining demonstrates that growth plate chondrocyte proliferation is reduced in E18.5 *Bmp2/4* dKO and *Bmp2* cKO embryos. The top panel shows corresponding histological sections stained with Alcian Blue and H&E and lower panel shows PCNA staining sections. Red arrows indicate the PCNA staining-positive cells (bottom panel). **P*<0.05, unpaired Student's *t*-test, *n*=4. Values are means + s.e.m. (**C**) TUNEL staining demonstrates that growth plate chondrocyte apoptosis is significantly increased in E18.5 *Bmp2/4* dKO and *Bmp2* cKO embryos (positive-stained cells are indicated by yellow arrows). Top panel shows corresponding histological sections stained with Alcian Blue and H&E.

demonstrated that BMP2 upregulated Runx2 protein levels in a dose-dependent manner. Overexpression of CDK4 significantly inhibited BMP2-induced upregulation of Runx2 protein (Fig. 6D). Interestingly, we also found that over-expression of CDK4 also inhibited BMP2-induced Runx2 mRNA expression in RCS cells (Fig. 6E). To determine if BMP2 affects cyclin-D1–CDK4 interaction, we performed immunoprecipitation assays in the absence or presence of BMP2. We found that BMP2 significantly inhibited the interaction between cyclin D1 and CDK4 in chondrocytes (Fig. 6F). Addition of noggin abolished



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the inhibitory effect of BMP2 on the cyclin-D1–CDK4 interaction (Fig. 6F). These results indicate that BMP2 might prevent Runx2 degradation through downregulation of CDK4 expression and inhibition of cyclin-D1–CDK4 interaction in chondrocytes. It has been reported that Sox9 interacts with Runx2 and inhibits Runx2 function (Akiyama et al., 2004). In this study, we examined the effect of *Sox9* siRNA on Runx2 levels in chondrogenic RCS cells. We found that transfection of *Sox9* siRNA upregulated basal and BMP2-induced Runx2 protein levels in RCS cells (supplementary material Fig. S5). These results suggest that BMP2-regulated Runx2 expression is not Sox9 dependent. Taken together, these results suggest that part of the effect of BMP2 on Runx2 upregulation is mediated through downregulation of CDK4 expression and subsequent inhibition of Runx2 ubiquitylation in chondrocytes.

Discussion

In vitro studies suggest that BMP2 and BMP4 have similar functions. For example, both BMP2 and BMP4 induce mouse embryonic stem cell and human mesenchymal stem cell differentiation into chondrocytes (Kramer et al., 2000; Steinert et al., 2009). BMP2 and BMP4 also stimulate chondrocyte proliferation and hypertrophy (De Luca et al., 2001; Minina et al., 2001; Hatakeyama et al., 2004; Leboy et al., 1997). The expression of BMP2 and BMP4 proteins is detected in chondrocytes during endochondral ossification in fracture callus with the strongest expression detected in hypertrophic chondrocytes (Yu et al., 2010). During ectopic bone formation induced by implantation of Saos-2 cells into nude mice, both BMP2 and BMP4 are upregulated in mature chondrocytes (McCullough et al., 2007). Overexpression of *Bmp2* or *Bmp4* induces ectopic bone formation through a mechanism that is similar to endochondral ossification (Alden et al., 1999; Kubota et al., 2002; Jane et al., 2002). Because in vivo environments are different from in vitro studies, the in vitro findings need to be confirmed through an in vivo approach. Homozygous *Bmp2* mutant embryos (conventional deletion of the *Bmp2* gene) die between E7.5 and E10.5 and have defects in cardiac development (Zhang and Bradley, 1996); whereas homozygous *Bmp4* mutant embryos (conventional deletion of the *Bmp4* gene) die between E6.5 and E9.5, and show little or no mesodermal differentiation (Winnier et al., 1995). Because skeletal development begins around E10.5–E11.5, *Bmp2* and *Bmp4* conventional KO mouse models cannot be used to study skeletal biology. Deletion of the *Bmp2* or *Bmp4* gene specifically in the limb bud mesenchyme leads to severe chondrodysplasia, suggesting crucial roles of both *Bmp2* and *Bmp4* in early mesenchymal cell differentiation (Bandyopadhyay et al., 2006).

To determine the specific functions of *Bmp2* and *Bmp4* in chondrocyte proliferation and maturation during endochondral bone development in vivo, we have generated chondrocyte-specific *Bmp2* and *Bmp4* cKO mice and *Bmp2/4* dKO mice using *Col2a1CreER*^{T2} transgenic mice in which the expression of the CreER transgene is induced by tamoxifen and is restricted to cartilage. In our studies, deletion of the *Bmp2/4* or *Bmp2* gene in Col2a1-expressing chondrocytes resulted in severe defects in endochondral bone development, which differs from the results obtained by deletion of the *Bmp2* and *Bmp4* genes in mesenchymal progenitor cells (mediated by *Prx1Cre* transgenic mice). These findings suggest that during early mesenchymal cell differentiation, functions of *Bmp2* and *Bmp4* might be at least partially compensated by each other. However, during late stage chondrocyte maturation, *Bmp2* function cannot be compensated by *Bmp4* or other BMP genes in chondrocytes. In postnatal *Bmp2* cKO mice (mediated by *Prx1Cre*), the fracture healing process is delayed. However, the fracture healing process was not affected in *Bmp4* cKO mice (mediated by *Prx1Cre*) (Tsuji et al., 2006).

In *Bmp2/4* dKO mice and *Bmp2 cKO* embryos, both chondrocyte proliferation and maturation are impaired. Chondrocyte columns in the proliferating and hypertrophic zones are disorganized with a dramatic decrease in *Col2a1* and *Col10a1* expression. In *Bmp2/Bmp4* dKO and *Bmp2 cKO* embryos, ectopic bone formation was observed in perichondrial areas with enhanced Runx2 and Osterix expression. Because *Col2a1CreER*^{T2} mice do not target perichondrial cells, it seems that this ectopic bone formation reflects the secondary effect of deletion of the *Bmp2* gene. In contrast to the perichondrial area, Runx2 expression in the proliferating and pre-hypertrophic areas was significantly reduced in *Bmp2/4* dKO embryos. To investigate the regulatory mechanism of BMP2 on Runx2 expression, we examined the effect of BMP2 on *Runx2* mRNA and protein levels in chondrogenic RCS cells. In addition to its stimulatory effect on *Runx2* mRNA expression, BMP2 had much greater effect on Runx2 protein levels than its effect on Runx2 mRNA expression. Our in vitro studies demonstrate that BMP2 prevents Runx2 protein ubiquitylation through downregulation of CDK4 expression and inhibition of cyclin-D1–CDK4 interaction. *Sox9* is an important downstream mediator of the BMP2 and hedgehog signaling pathways in osteoblasts. A Smad responsive element responsible for BMP2 activation was identified in the *Sox9* promoter (Pan et al., 2008). *Sox9* expression is upregulated by BMP2 in mesenchymal progenitor cell line (Zehentner et al., 1999). It has also been reported that Sox9 interacts with Runx2 and inhibits Runx2 function (Akiyama et al., 2004). In the present studies, we examined the effect of *Sox9* siRNA on Runx2 protein

Fig. 4. Chondrocyte differentiation is impaired in *Bmp2/4* dKO and *Bmp2* cKO embryos. (**A**) In situ hybridization demonstrates that *Col2a1*, *Col10a1* and *Mmp13* expression is significantly reduced in E18.5 *Bmp2/4* dKO and *Bmp2* cKO embryos. (**B**–**D**) Total RNA was extracted from primary chondrocytes isolated from E18.5 Cre-negative control, *Bmp2* cKO, *Bmp4* cKO and *Bmp2/4* dKO embryos. The expression of *Sox9*, *Acan* and *Col2a1* genes was analyzed by real-time PCR. Results demonstrate that the expression of these chondrocyte marker genes is significantly reduced in *Bmp2/4* dKO and *Bmp2* cKO chondrocytes. By contrast, the expression of *Sox9* and *Acan* was slightly but significantly reduced in *Bmp4* cKO embryos. (**E**–**I**) Total RNA was extracted from primary chondrocytes derived from E18.5 *Bmp2* cKO and Cre-negative embryos. The expression of BMP genes was analyzed by real-time PCR. Results demonstrated that the expression of *Bmp5*, *Bmp7*, *Bmp8b* and *Bmp9* genes was significantly reduced in *Bmp2*-deficient chondrocytes. (**J**–**N**) Total RNA was extracted from primary chondrocytes derived from E18.5 *Bmp4* cKO and Cre-negative embryos. The expression of BMP genes was analyzed by real-time PCR. Results demonstrated that the expression of *Bmp5*, *Bmp7*, *Bmp8b* and *Bmp9* genes is not significantly changed in *Bmp4*-deficient chondrocytes. (**O**) Primary sternal chondrocytes were isolated from 3-day-old *Bmp2/4*$^{fx/fx}$ mice and were infected with Ad-Cre or Ad-GFP (control). 48 hours after infection, cells were treated with BIO (1 μM) or Wnt3a (100 ng/ml). Cell cultures were stopped 24 hours later and total RNA was extracted and expression *Alp* was examined by real-time PCR. BIO or Wnt3a-induced *Alp* upregulation is significantly inhibited in the *Bmp2/4*-deficient chondrocytes (Ad-Cre infected cells). *$P<0.05$, unpaired Student's *t*-test, $n=3$. Values are means + s.e.m.

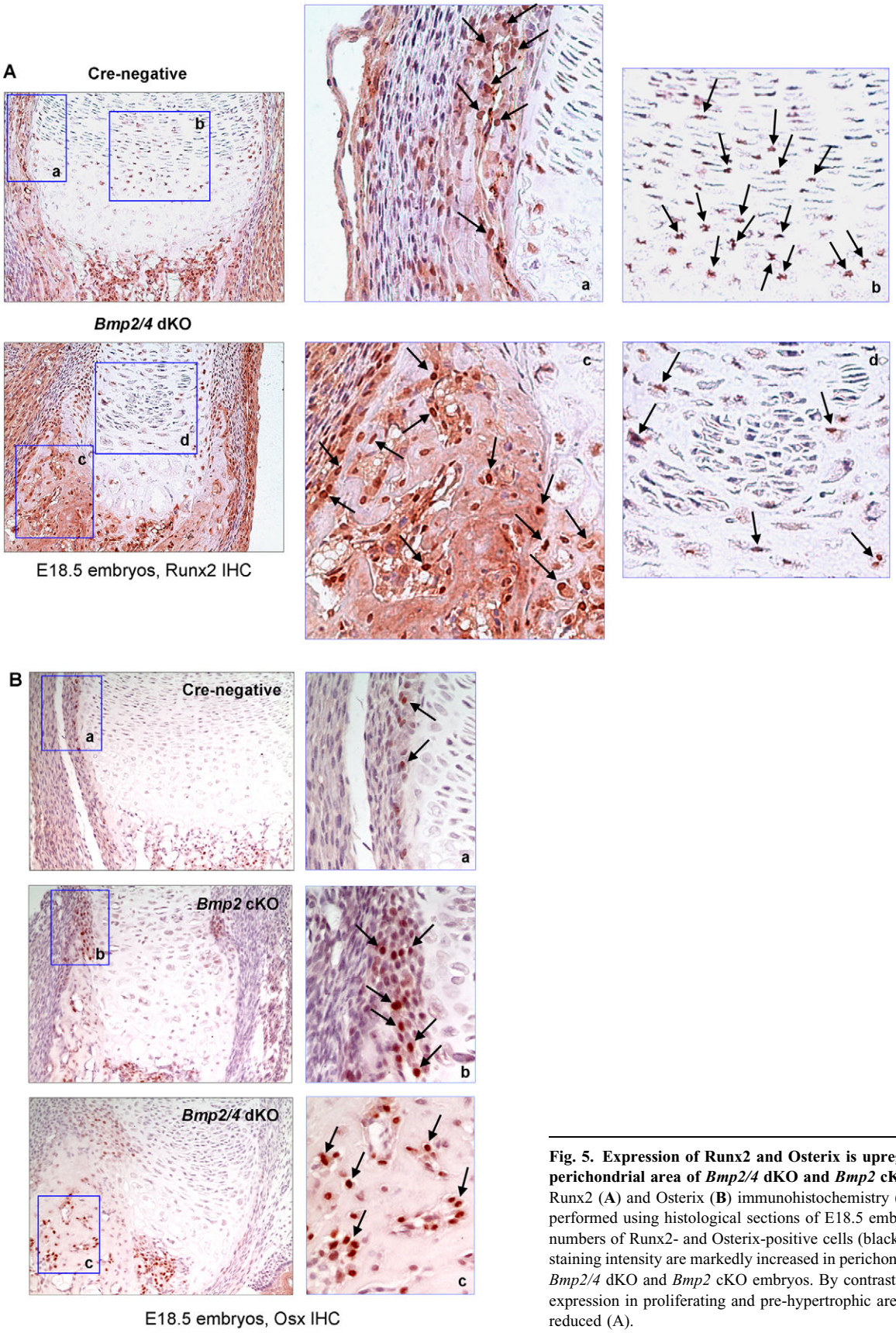

Fig. 5. Expression of Runx2 and Osterix is upregulated in perichondrial area of *Bmp2/4* dKO and *Bmp2* cKO embryos. Runx2 (**A**) and Osterix (**B**) immunohistochemistry (IHC) was performed using histological sections of E18.5 embryos. The numbers of Runx2- and Osterix-positive cells (black arrows) and staining intensity are markedly increased in perichondrial areas of *Bmp2/4* dKO and *Bmp2* cKO embryos. By contrast, Runx2 expression in proliferating and pre-hypertrophic areas is reduced (A).

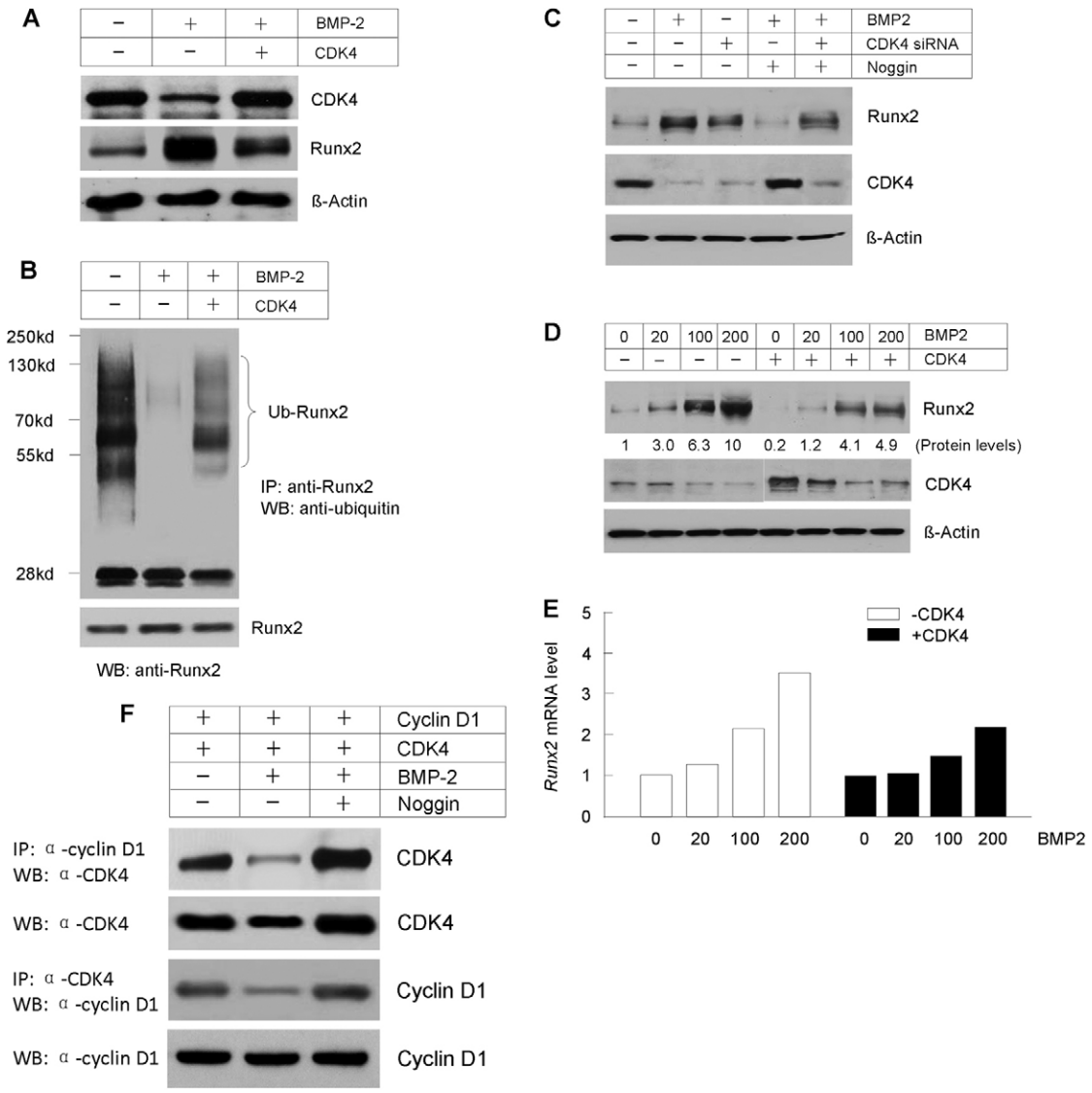

Fig. 6. BMP2 protects Runx2 protein degradation through inhibition of CDK4 expression. (**A**) CDK4 expression construct was transiently transfected into chondrogenic RCS cells. 24 hours after transfection, the cells were treated with BMP2 (100 ng/ml) for 24 hours. Runx2 and CDK4 protein expression was detected by western blotting. BMP2 inhibits CDK4 expression and enhances Runx2 protein level. Expression of CDK4 partially inhibits BMP2-induced Runx2 upregulation. (**B**) Runx2 ubiquitylation assay. CDK4 expression construct was transiently transfected into RCS cells. 24 hours after transfection, the cells were treated with BMP2 (100 ng/ml) for 24 hours. Proteasome inhibitor MG132 (10 μM) was added to the medium 4 hours before cell lysates were collected. Ubiquitylated proteins were pulled down using an UbiQapture-Q kit and polyubiquitylated Runx2 was detected using an anti-Runx2 antibody. BMP2 inhibits Runx2 ubiquitylation and expression of CDK4 partially reverses the inhibitory effect of BMP2 on Runx2 ubiquitylation. (**C**) *Cdk4* siRNA was transfected into RCS cells. Cells were treated with BMP2 (100 ng/ml) with or without noggin (300 ng/ml) 24 hours after *Cdk4* siRNA transfection. The expression of Runx2 and CDK4 protein was detected by western blotting. Silencing of CDK4 results in an upregulation of Runx2 protein levels. Noggin inhibits the effect of BMP2 on Runx2 upregulation but could not inhibit the effect of *Cdk4* siRNA on Runx2 protein upregulation. (**D**) CDK4 expression construct was transiently transfected into RCS cells. Cells were treated with BMP2 for 24 hours at different concentrations (0, 20, 100, 200 ng/ml) 24 hours after CDK4 transfection. The expression of Runx2 and CDK4 protein was detected by western blotting. BMP2 upregulates Runx2 protein levels in a dose-dependent manner. Expression of CDK4 partially inhibits BMP2-induced Runx2 protein upregulation. (**E**) RCS cells were treated with different concentrations of BMP2 with or without transfection of CDK4. *Runx2* mRNA expression was determined by real-time PCR. CDK4 partially inhibits BMP2-induced *Runx2* mRNA expression. (**F**) RCS cells were transfected with cyclin D1 and CDK4 expression plasmids and treated with BMP2 (100 ng/ml). Cell lysates were collected and subjected to IP using an anti-cyclin-D1 or anti-CDK4 antibody followed by western blotting using the anti-CDK4 or anti-cyclin-D1 antibody. Treatment of BMP2 inhibits cyclin-D1–CDK4 interaction and addition of noggin blocks the inhibitory effect of BMP2 on cyclin-D1–CDK4 interaction.

levels and found that silencing of *Sox9* upregulated Runx2 protein levels, suggesting that Sox9 has an inhibitory effect on Runx2, and that BMP2-mediated Runx2 upregulation is Sox9 independent.

BMP2 induces the expression of molecular marker genes characteristic of hypertrophic chondrocytes, such as *Col10a1* and *Alp* (Valcourt et al., 2002). When BMP signals are transduced through R-Smads, Smad1 can interact with the transcription factor Runx2 (Zhao et al., 2003). It has been reported that BMP2 promotes *Col10a1* and *Smad6* gene transcription through the conserved Runx2 binding sites (Leboy et al., 2001; Zheng et al., 2003; Wang et al., 2007). Our studies suggest that BMP2 might regulate *Col10a1* expression through Smad1–Runx2 interaction at the 5′ promoter region of the *Col10a1* gene.

Our previous observations and reports from other laboratories demonstrated that both *Bmp2* and *Bmp4* genes are expressed in chondrocytes during embryonic development and early postnatal stages at similar levels (Feng et al., 2003) (supplementary material Fig. S1). Thus, the phenotypic difference of skeletal development observed in *Bmp2* and *Bmp4* cKO embryos could not be explained by the expression patterns of these two genes in chondrocytes. Previous reports suggest that BMP2 regulates the expression of other BMP family members in mesenchymal progenitor cells, osteoblasts and chondrocytes (Harris et al., 1994; Ghosh-Choudhury et al., 1995; Chen et al., 1997; Ghosh-Choudhury et al., 2001; Edgar et al., 2007), suggesting that BMP2 serves as an upstream regulator of other BMP genes in chondrocytes. In terms of the mechanism by which BMP2 and BMP4 regulate chondrocyte maturation, the main difference between these two growth factors is that BMP2 controls *Bmp2* and expression of other BMP genes through autocrine and paracrine regulatory mechanisms. This notion is supported by several lines of evidence. (1) Our previous studies demonstrate that BMP2 upregulates *Bmp2* gene transcription through the *Bmp2* proximal promoter element (Ghosh-Choudhury et al., 2001). (2) In a fracture healing study, it has been shown that *Bmp2* expression reaches its maximal level at day 1 after fracture. *Gdf5* showed maximal expression at day 7. The expression of *Bmp4*, *Bmp7* and *Bmp8* was detected from day 14 to day 21, whereas *Bmp5*, *Bmp6* and *Gdf10* were expressed from day 3 to day 21 (Cho et al., 2002). (3) It has been reported that in a bone marrow cell culture, addition of BMP2 neutralizing antibody reduced the expression of endogenous levels of BMP2, BMP3, BMP5 and BMP8a, whereas addition of BMP2 had the opposite effect (Edgar et al., 2007). (4) In the present studies, we demonstrated that expression of *Bmp5*, *Bmp7*, *Bmp8b* and *Bmp9* was downregulated in *Bmp2*-deficient chondrocytes. By contrast, expression of these BMP genes was not significantly changed in the *Bmp4*-deficient chondrocytes.

The phenotype of *Bmp2* cKO embryos is similar to that of *Smad1/5* dKO embryos (Retting et al., 2009) and *Bmpr1a/Bmpr1b* dKO embryos (Yoon et al., 2005), including impaired skeletal development, disorganized growth plate formation, decreased cartilage matrix deposition, and decreased chondrocyte proliferation and increased chondrocyte apoptosis in the growth plate. Ectopic matrix deposition at the perichondrial area surrounding prehypertrophic and hypertrophic chondrocytes was also seen in *Smad1/5* dKO embryos, which is consistent with what we observed in *Bmp2* cKO mice. However, skeletal development was more severely impaired in *Smad1/5* dKO embryos and *Bmpr1a/Bmpr1b* dKO embryos compared with *Bmp2* cKO embryos. One possibility is that *Bmp2* cKO was induced at stage E12.5 in our studies. By contrast, the *Smad1/5* and *Bmpr1a/Bmpr1b* genes were deleted earlier than E12.5, which could have led to more severe defects in chondrogenesis. In summary, our findings indicate that Bmp2 is required for chondrocyte maturation and endochondral bone formation during embryonic development.

Materials and Methods

Generation of *Bmp2/4* dKO mice and *Bmp2* and *Bmp4* cKO mice

Col2a1-CreER^{T2} mice were generated in our lab (Chen et al., 2007). *Bmp2*$^{fx/fx}$ mice were generated in the lab of Stephen Harris at the University of Texas Health Science Center at San Antonio, TX (Singh et al., 2008) and *Bmp4*$^{fx/fx}$ mice were a gift from Brigid Hogan (Duke University, town, state) (Kulessa and Hogan, 2005; Gluhak-Heinrich et al., 2010). To generate KO embryos, *Bmp2*$^{fx/fx}$*;Bmp4*$^{fx/fx}$, *Bmp2*$^{fx/fx}$ and *Bmp4*$^{fx/fx}$ mice were crossed with *Col2a1-CreER*^{T2}*;Bmp2*$^{fx/fx}$*;Bmp4*$^{fx/fx}$, *Col2a1-CreER*^{T2}*;Bmp2*$^{fx/fx}$ and *Col2a1-CreER*^{T2}*;Bmp4*$^{fx/fx}$ mice, respectively. The pregnant mice were injected with tamoxifen (1 mg/10 g body weight, i.p.) at E12.5 and sacrificed at E14.5 and E18.5. The Cre-positive embryos were used as KO embryos and the Cre-negative littermates were used as controls.

Whole embryo Alizarin Red and Alcian Blue staining

Embryos at E18.5 were collected and the skin, viscera and adipose tissues were carefully removed. Whole skeletons were fixed in 95% ethanol for 2 days followed by fixation in acetone for an additional day, and stained with 0.015% Alcian Blue and 0.005% Alizarin Red for 3 days. Images of the skeletons were taken when most of the soft tissue was digested in 1% potassium chloride.

Histological analysis

Tibiae from E14.5 and E18.5 embryos and vertebrae from E18.5 embryos were fixed in 4% paraformaldehyde, decalcified, dehydrated and embedded in paraffin. Serial midsagittal sections (3 μm thick) of tibias and vertebrae were cut and stained with Alcian Blue and hematoxylin and eosin or Safranin O and Fast Green, respectively, for morphometric analysis.

PCNA staining

Paraffin sections (3 μm thick) of E18.5 tibias were rehydrated and blocked in 3% H_2O_2 in methanol for 15 minutes and digested in Proteinase K (10 μg/ml) for 10 minutes at room temperature. PCNA staining was performed with a PCNA staining kit (Promega, WI).

TUNEL staining

Rehydrated paraffin sections (3 μm thick) of E18.5 tibias were fixed with 4% formaldehyde solution in PBS for 15 minutes followed by digestion in Proteinase K (10 μg/ml) for 10 minutes. TUNEL staining was performed using Fluorometric TUNEL System (Promega, WI). After mounting with DAPI reagent to stain nuclei, the samples were analyzed under a fluorescence microscope using a standard fluorescein filter set to view the green fluorescence of fluorescein at 520±20 nm; and view blue DAPI fluorescence at 460 nm.

In situ hybridization

Radiolabeled probes for *Col2a1*, *Col10a1* and *Mmp13* were created by transcribing linearized antisense complementary deoxyribonucleic acid (DNA) in the presence of [^{35}S]UTP using T7 polymerases at 37℃ for 2 hours. DNA was removed with RNAse-free DNase. The labeled RNA was purified using a mini Quick Spin RNA Column. All probes have been previously characterized (Dong et al., 2010). Sections (5 μm thick) of E18.5 tibias were prepared. After dewaxing and rehydration, sections were pretreated with 10 μg/ml Proteinase K, 0.2N hydrogen chloride and 0.1 M triethanolamine at room temperature. Hybridization was performed at 55℃ for 18 hours. Non-specific binding was reduced by adding 10 μg/ml RNase A and several washes in SSC. After dipping in nuclear-type emulsion emulsion, the slides were exposed for 3–7 days at 4℃ followed by developing and fixation with Kodak developer and fixer. The slides were counterstained with Toluidine Blue, dehydrated and coverslipped.

Primary chondrocyte isolation

Three-day-old neonatal mice were euthanized and genotyped using tail tissues obtained at the time of death. The anterior rib cage and sternum were harvested, washed with phosphate buffered saline (PBS), and then digested with 2 mg/ml Pronase (Roche) in PBS in a 37℃ water bath with continuous shaking for 60 minutes. This was followed by incubation in a solution of 3 mg/ml collagenase D (Roche)/Dulbecco's modified Eagle's medium (DMEM) for 90 minutes at 37℃. The soft tissue debris was thoroughly removed. The remaining sterna and costosternal junctions were further digested in a fresh collagenase D solution in Petri dishes in a 37℃ incubator for 5 hours with intermittent shaking. The digestion solution was filtered to remove all residual bone fragments, and centrifuged. The cells were washed and collected for RNA analysis.

Cell culture and transfection

Rat chondrosarcoma (RCS) cells were cultured in DMEM supplemented with 10% fetal calf serum at 37℃ under 5% CO_2. DNA plasmids were transiently transfected into RCS cells in 6 cm culture dishes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Empty vector was used to keep the total amount of transfected DNA constant in each group in all experiments. FLAG–EGFP plasmid was co-transfected as an internal control for transfection efficiency. Western blot and immunoprecipitation (IP) assays were performed 24 hours after transfection.

Western blotting and ubiquitylation assay

Western blotting and in vivo ubiquitylation assay were performed as described previously (Shen et al., 2006; Zhang et al., 2010). For the Runx2 ubiquitylation

assay, the proteasome inhibitor MG132 (10 μM) was added to the cell culture 4 hours before cells were harvested. The rat anti-Runx2 monoclonal antibody was purchased from Marine Biological Laboratory (MBL, town, MA). The rabbit anti-CDK4 (C-22) polyclonal antibody and the rabbit anti-ubiquitin (FL-76) polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from RCS cells and primary mouse chondrocytes using RNAzol B solution (Tel-Test, town, TX). DNAse-I-treated total RNA was reverse transcribed-using oligo-(dT) and cDNA was amplified by PCR in a total volume of 20 μl solution containing 10 μl SYBR Green Master Mix (Thermo Scientific), 1 μl of the diluted (1:5) cDNA, and 10 pM of forward and reverse primers specific for the genes listed in supplementary material Table S1.

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