

# Runx2 deficiency in chondrocytes causes adipogenic changes in vitro

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## Summary

**Runx2** (runt-related transcription factor 2) is an important transcription factor for chondrocyte differentiation as well as for osteoblast differentiation. To investigate the function of **Runx2** in chondrocytes, we isolated chondrocytes from the rib cartilage of *Runx2*-deficient (*Runx2*<sup>-/-</sup>) mice and examined the effect of *Runx2* deficiency on chondrocyte function and behavior in culture for up to 12 days. At the beginning of the culture, *Runx2*<sup>-/-</sup> chondrocytes actively proliferated, had a polygonal shape and expressed type II collagen; these are all characteristics of chondrocytes. However, they gradually accumulated lipid droplets that stained with oil red O and resembled adipocytes. Northern blot analysis revealed that the expression of adipocyte-related differentiation marker genes including *PPAR*γ (*peroxisome proliferator-activated receptor* γ), *aP2* and *Glut4* increased over time in culture, whereas expression of type II collagen decreased. Furthermore, the expression of *Pref-1*, an important inhibitory gene of adipogenesis, was

remarkably decreased. Adenoviral introduction of *Runx2* or treatment with transforming growth factor-β, retinoic acid, interleukin-1β, basic fibroblast growth factor, platelet-derived growth factor or parathyroid hormone inhibited the adipogenic changes in *Runx2*<sup>-/-</sup> chondrocytes. *Runx2* and transforming growth factor-β synergistically upregulated *interleukin-11* expression, and the addition of interleukin-11 to the culture medium reduced adipogenesis in *Runx2*<sup>-/-</sup> chondrocytes. These findings indicate that depletion of *Runx2* resulted in the loss of the differentiated phenotype in chondrocytes and induced adipogenic differentiation in vitro, and show that *Runx2* plays important roles in maintaining the chondrocyte phenotype and in inhibiting adipogenesis. Our findings suggest that these *Runx2*-dependent functions are mediated, at least in part, by interleukin-11.

Key words: *Runx2*, *Cbfa1*, Chondrocyte, Adipocyte, TGF-β, IL-11

## Introduction

Connective tissues are composed of several highly specified cell types, including skeletal myoblasts, adipocytes, chondrocytes, osteoblasts and fibroblasts. These cells are believed to be derived from a common precursor – that is, multipotential mesenchymal stem cells (Taylor and Jones, 1979; Cornelius et al., 1994). Generally, multipotent precursor cells undergo two steps to acquire tissue-specific phenotype (Sager and Kovac, 1982; Weintraub et al., 1991). The first step is commitment or determination that the multipotential cells are conducted to a particular lineage. The next step is cell maturation or terminal differentiation where the cells proceed through a specific program and show a functionally specialized phenotype. Recent studies have revealed that several transcriptional factors regulate the determination of cellular lineage. They include *MyoD* and *myf-5* in myogenesis (Weintraub, 1993), *PPAR*γ (*peroxisome proliferator-activated receptor* γ) and the *C/EBP* (*CCAAT/enhancer-binding protein*) family in adipogenesis (Tontonoz et al., 1994; Freytag et al., 1994), the *Sox* family in chondrogenesis (de Crombrughe et al., 2001), and *Runx2* (runt-related transcription factor 2) and

osterix in osteogenesis (Komori, 2002; Nakashima et al., 2002).

The *Runx2/Cbfa1* (*core binding factor α1*) is a master regulatory gene for osteoblast differentiation. *Runx2* belongs to the runt-domain gene family, the members of which have a unique DNA-binding domain that is homologous to the *Drosophila* pair-rule gene *runt* (Kania et al., 1990; Ogawa et al., 1993). Targeted disruption of *Runx2* resulted in a complete lack of bone formation due to maturational arrest of osteoblasts, showing that *Runx2* is an essential transcription factor for osteoblast differentiation (Komori et al., 1997; Otto et al., 1997). In addition, several recent studies indicate that *Runx2* is involved in regulating the reciprocal differentiation pathways of osteoblast and adipocyte lineages (Chen et al., 1998; Lecka-Czernik et al., 1999; Gori et al., 1999; Kobayashi et al., 2000).

The endochondral ossification process consists of a series of cellular events: mesenchymal cell condensation, differentiation of mesenchymal cells into chondrocytes, chondrocyte proliferation, matrix synthesis by chondrocytes, chondrocyte maturation and mineralization of the matrix. Previous studies

indicate that Runx2 plays an important role in chondrocyte maturation in the process of endochondral ossification. Overexpression of *Runx2* in chondrocytes accelerates chondrocyte maturation and matrix mineralization in vitro and endochondral bone formation in vivo (Enomoto et al., 2000; Takeda et al., 2001; Ueta et al., 2001). Chondrocyte maturation is disturbed in *Runx2*<sup>-/-</sup> mice (Inada et al., 1999; Kim et al., 1999), and overexpression of the dominant-negative form of *Runx2* in chondrocytes induces severe delay of endochondral ossification and suppresses chondrocyte maturation (Ueta et al., 2001; Stricker et al., 2002). *Runx2* expression initially occurs in mesenchymal cells just before their differentiation into chondrocytes, and then its expression is maintained in chondrocytes until the cells complete the terminal differentiation step, although its expression level varies according to the stage of differentiation. This suggests that Runx2 may play other roles in the regulation of chondrocyte differentiation in addition to promoting chondrocyte maturation. In fact, *Runx2*<sup>-/-</sup> mice have not only suppressed endochondral ossification but also retarded cartilage growth and poor organization of growth plate structures. Transgenic mice expressing a dominant-negative form of *Runx2* had cartilage elements of reduced size. Also, the expression of a native truncated form of *Runx2* inhibited chondrogenic differentiation of a prechondrogenic cell line, ATDC5, suggesting that Runx2 may be involved in the early phase of chondrocyte differentiation before the cells enter the terminal differentiation step (Akiyama et al., 1999). These findings indicate that Runx2 may be involved in multiple aspects of the differentiation and function of chondrocytes.

To elucidate further the function of Runx2 in chondrocytes, we isolated chondrocytes from cartilage tissues of *Runx2*<sup>-/-</sup> mice and examined how the deficiency of *Runx2* affected chondrocyte function. We found that *Runx2*<sup>-/-</sup> chondrocytes spontaneously underwent adipocyte differentiation, which was accompanied by loss of the chondrocyte phenotype, indicating that Runx2 is involved in maintenance of the chondrocyte phenotype and inhibition of adipogenesis.

## Materials and Methods

### Cell culture

*Runx2*<sup>-/-</sup> mice were generated as previously described (Komori et al., 1997). Chondrocytes were isolated from the ribs (excluding sternum) of wild-type or *Runx2*<sup>-/-</sup> E18.5 embryos and cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (1:1) hybrid medium (GIBCO BRL, New York, NY) containing 10% fetal bovine serum (FBS) (GIBCO BRL) according to the method previously described (Shimomura et al., 1975). Chondrocytes were plated at a density of  $1 \times 10^6$  cells/dish in 60 mm plates ( $5 \times 10^4$  cells/cm<sup>2</sup>) (Corning, New York, NY) and cultured for 12 days. Cells were maintained at 37°C in a humidified 5% CO<sub>2</sub>/95% air atmosphere. The medium was changed every other day. RNA was extracted from cells before plating (day 0) and from cultured cells when the cells became confluent (3 days after plating) (day 3). RNA was also extracted from cells every 3 days after confluence (on days 6, 9 and 12).

We investigated the inhibitory effect of various factors on adipogenesis of *Runx2*<sup>-/-</sup> chondrocytes. *Runx2*<sup>-/-</sup> primary chondrocytes were plated at a density of  $1 \times 10^5$  cells/well ( $5 \times 10^4$  cells/cm<sup>2</sup>) in 24 multi-well plates. Two days after inoculation, cultures were stimulated for 10 days with transforming growth factor- $\beta$  (TGF- $\beta$ ) (1 ng/ml), retinoic acid ( $10^{-8}$  M), interleukin-1 $\beta$  (IL-1 $\beta$ ) (10 ng/ml), basic fibroblast growth factor (bFGF) (10 ng/ml), platelet-derived

growth factor (PDGF) (200 ng/ml), parathyroid hormone (PTH) ( $10^{-7}$  M), bone morphogenetic protein 2 (BMP-2) (50 ng/ml), insulin-like growth factor 1 (IGF-1) (100 ng/ml), T3 ( $10^{-7}$  M) or interleukin-11 (IL-11) (100 ng/ml). The medium was changed with fresh medium containing the respective factor every other day. Cells were stained with oil red O or Alcian blue, or reacted with an antibody against type II collagen after replating.

### Cytochemical and immunocytochemical procedures

Cells were plated at a density of  $1 \times 10^5$  cells/well in 24 multi-well plates and subjected to cytochemical and immunocytochemical procedures. One day after plating (day 1), the expression of type II collagen was examined by immunocytochemistry. In the case of cell cultures with various factors, the cells were replated at a density of  $1 \times 10^5$  cells/well in 24 multi-well plates after the stimulation with each factor for 10 days, and subjected to the examination of type II collagen expression. Cultures were washed twice with PBS, fixed with 100% ethanol and permeabilized by incubation with PBS containing 0.5% Triton X-100 for 15 minutes. Cultures were then incubated with a polyclonal antibody against type II collagen (Cosmo Bio, Tokyo, Japan). Localization of the antibody was visualized by the ABC method using a Vectorstain ABC kit (Vector Laboratories, Burlingame, CA). Cells were fixed with formalin and stained with Alcian blue and/or oil red O (Kobayashi et al., 2000).

### RNA extraction and northern blot analysis

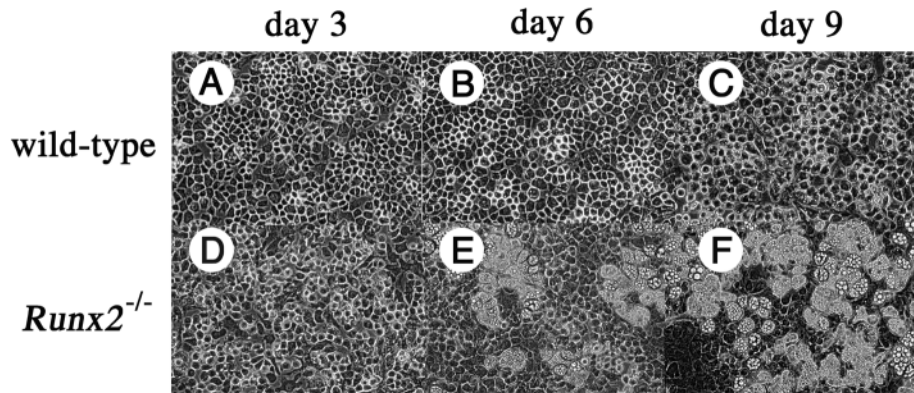
Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA was denatured with 6% formamide, subjected to electrophoresis on 1.0% agarose gels and blotted onto Hybond N membranes (Amersham Life Science, Tokyo, Japan). The membranes were hybridized with <sup>32</sup>P-labeled probes, including a 0.4 kb fragment of mouse type II collagen cDNA (Metsäranta et al., 1991), a 0.5 kb fragment of mouse type X collagen cDNA (Apte and Olsen, 1993), a 2.0 kb fragment of mouse *PPAR $\gamma$*  cDNA, a 0.5 kb fragment of mouse *aP2* cDNA, a 1.5 kb fragment of mouse *Glut4* cDNA and a 1.6 kb fragment of rat *Pref-1* cDNA (Okamoto et al., 1998). Hybridization was performed as described previously (Inada et al., 1999).

### Adenoviral introduction of *Runx2* into *Runx2*<sup>-/-</sup> chondrocytes

As the two Runx2 isoforms, type I and type II Runx2, showed similar functions in chondrocyte maturation in vivo (Ueta et al., 2001), we used type II *Runx2* cDNA in the gene transfer experiment. Bicistronic adenovirus vectors expressing type II *Runx2* and enhanced green fluorescent protein (EGFP) or EGFP alone were generated as previously described (Yoshida et al., 2002). *Runx2*<sup>-/-</sup> chondrocytes were plated at a density of  $1 \times 10^5$  cells/dish in collagen-coated 24-well plates or at a density of  $1 \times 10^6$  cells/dish in collagen-coated 60 mm plates ( $5 \times 10^4$  cells/cm<sup>2</sup>). When the cells reached confluence, they were infected with either EGFP-expressing or *Runx2*-and-EGFP-expressing virus for 2 hours. The infected cells were cultured for 12 days and stained with oil red O or cultured for 6 days and harvested for northern blot analysis. For real-time reverse transcription-polymerase chain reaction (RT-PCR), the infected cells were harvested at the indicated times after the onset of viral infection. In the analysis of synergistic effect of Runx2 and TGF- $\beta$ , TGF- $\beta$  (1 ng/ml) was added at the time of adenoviral infection.

### Real-time RT-PCR

Total RNA that had been extracted from *Runx2*<sup>-/-</sup> chondrocytes or skeletons of wild-type and *Runx2*<sup>-/-</sup> mice was reverse transcribed using M-MLV (Moloney murine leukemia virus) reverse transcriptase.



**Fig. 1.** Morphological changes of chondrocytes in culture. Chondrocytes isolated from the ribs of wild-type (A-C) and *Runx2*<sup>-/-</sup> (D-F) embryos at E18.5 were inoculated in DMEM/F12 hybrid medium containing 10% FBS at a density of  $1 \times 10^5$ /plate in 24-well plates, and cultured for up to 9 days. Phase-contrast images at days 3, 6 and 9 (with day 0 as the day of plating) are shown. Magnification,  $\times 50$ .

The cDNA (7.5 ng total RNA equivalent) was mixed with SYBR Green PCR Master Mix (Applied Biosystems) and analyzed by real-time PCR using the ABI7700 (Applied Biosystems). Primers used for amplification were as follows: type X collagen, 5'-AGAACGG-CACGCTACGAT-3' and 5'-AGGTAGCCTTTGCTGTACTCATC-AT-3'; *IL-11*, 5'-GCATGTACAATGGCTGCGC-3' and 5'-CAAGA-GCTGTAAACGGCGG-3'; *Runx2*, 5'-CCGCACGACAACCGCAC-CAT-3' and 5'-CGCTCCGGCCCCACAAATCTC-3'. The obtained CT (cycle number at which amplification threshold of detection was reached) values for *IL-11* and *Runx2* were normalized to those of rodent *GADPH* (Applied Biosystems) expression by the  $\Delta\Delta CT$  method. The mean  $\Delta\Delta CT$  was converted to relative expression value by the equation,  $2^{-\Delta\Delta CT}$ , and the range was calculated by the equation,  $2^{-(\Delta\Delta CT + \text{Stdev} \Delta\Delta CT)}$ .

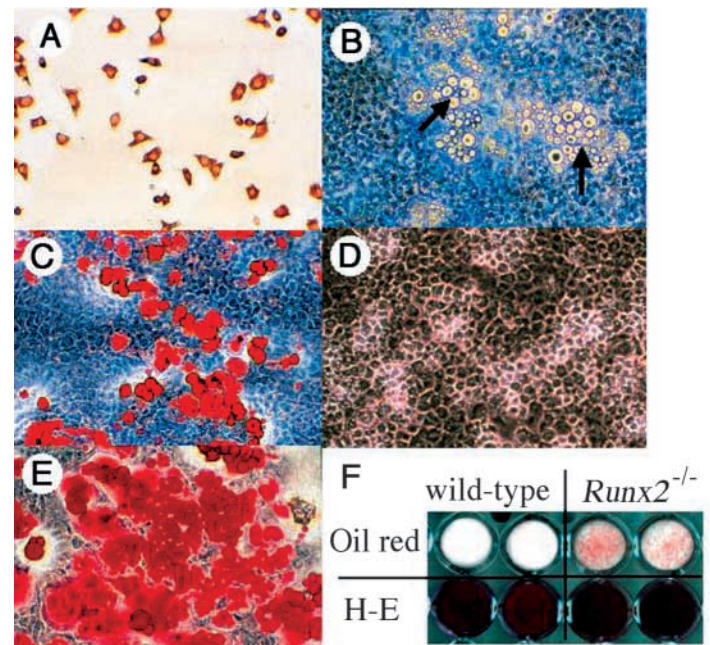
## Results

*Runx2*<sup>-/-</sup> chondrocytes displayed the morphology of adipocytes

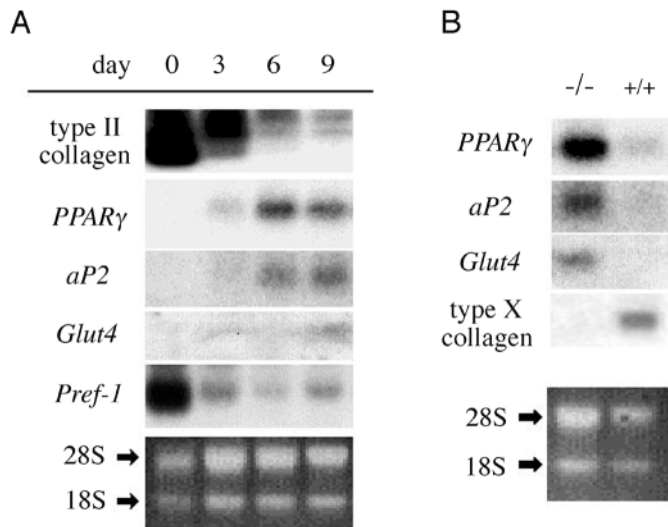
We isolated chondrocytes from the ribs of E18.5 *Runx2*<sup>-/-</sup> embryos and cultured them in monolayer. The *Runx2*<sup>-/-</sup> chondrocytes actively proliferated and showed a polygonal shape until the cells reached confluence (3 days after inoculation) (Fig. 1D). The morphology of *Runx2*<sup>-/-</sup> chondrocytes was similar to that of wild-type chondrocytes isolated from the ribs of E18.5 wild-type mice (Fig. 1A). On day 6 of culture, some of the *Runx2*<sup>-/-</sup> cells were enlarged and had accumulated many vacuoles (Fig. 1E), whereas the wild-type cells remained small and polygonal and had no vacuoles (Fig. 1B). On day 9 of culture, over 50% of the cells contained vacuoles in the *Runx2*<sup>-/-</sup> cultures and these cells looked very similar to adipocytes (Fig. 1F), whereas the wild-type cells retained the morphology of chondrocytes (Fig. 1C).

To investigate the changes in protein expression in *Runx2*<sup>-/-</sup> chondrocytes, we carried out immunocytochemical and cytochemical analyses. Most of the *Runx2*<sup>-/-</sup> cells expressed type II collagen at the time of plating, indicating that freshly isolated chondrocytes from *Runx2*<sup>-/-</sup> cartilage still express the chondrocyte phenotype (Fig. 2A). On day 6, wild-type cultures accumulated cartilage-specific proteoglycan, as determined by Alcian blue staining (data not shown). The *Runx2*<sup>-/-</sup> cultures were also positive on Alcian blue staining and even the cells that had vacuoles were surrounded by proteoglycan (Fig. 2B, arrows). To determine whether the vacuoles contained neutral fat, the

cells were stained with oil red O. The vacuoles in the cells of *Runx2*<sup>-/-</sup> cultures were strongly stained with oil red O (Fig. 2C). On day 9 of culture, the *Runx2*<sup>-/-</sup> cells contained more lipid droplets (Fig. 2E), whereas the wild-type cultures were completely negative on oil red O staining (Fig. 2D). The low-magnitude views clearly showed that the *Runx2*<sup>-/-</sup> cultures contained many cells with neutral fat that was stained by oil red O, and that the wild-type cultures did not (Fig. 2F).



**Fig. 2.** Adipocyte differentiation of *Runx2*<sup>-/-</sup> chondrocytes during culture. Chondrocytes isolated from the ribs of wild-type (D, F) and *Runx2*<sup>-/-</sup> (A-C, E, F) embryos at E18.5 were inoculated at a density of  $1 \times 10^5$ /plate in 24-well plates, and cultured for up to 9 days. (A) Immunocytochemistry using anti-type II collagen antibody of *Runx2*<sup>-/-</sup> chondrocytes 1 day after plating. (B, C) *Runx2*<sup>-/-</sup> chondrocytes were stained with Alcian blue (B) or with Alcian blue and oil red O (C) at 6 days after plating. Cells with vacuoles are surrounded by proteoglycan, as shown by staining with Alcian blue (B, arrows). (D-F) Wild-type (D, F) and *Runx2*<sup>-/-</sup> (E, F) chondrocytes were stained with oil red O 9 days after plating. Low-magnification views are shown in (F). The plates were also stained with hematoxylin and eosin (H-E) to confirm the presence of the cells (F). Magnification A-E,  $\times 50$ .

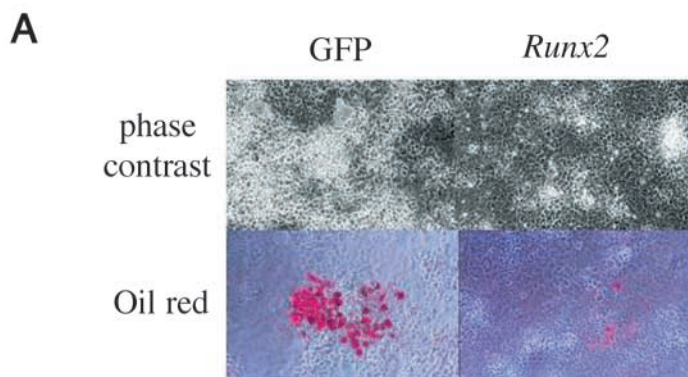


#### Expression of adipocyte-related genes in *Runx2*<sup>-/-</sup> chondrocytes

To characterize the phenotype of adipocyte-like *Runx2*<sup>-/-</sup> chondrocytes in detail, we next examined the expression of differentiation marker genes of chondrocytes and adipocytes by northern blot analysis. In the *Runx2*<sup>-/-</sup> cultures, the level of type II collagen mRNA was high at the beginning of the culture

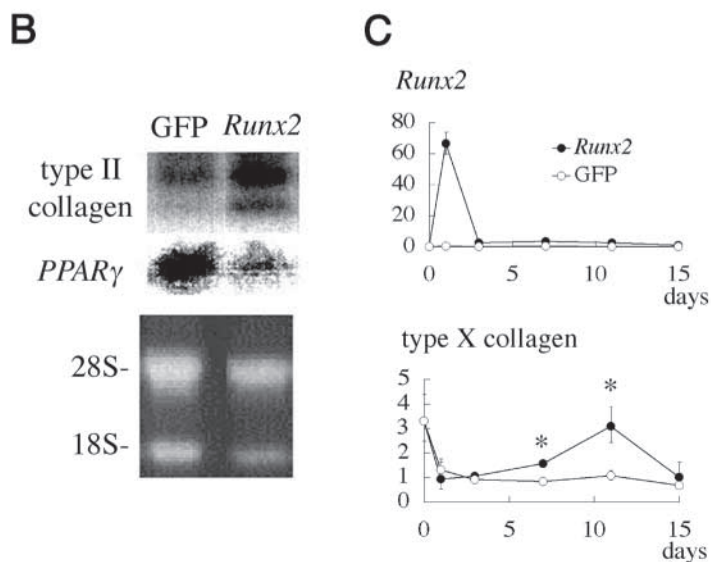
**Fig. 3.** Northern blot analysis for chondrocyte and adipocyte differentiation markers during cell culture of *Runx2*<sup>-/-</sup> and wild-type chondrocytes. Expression of chondrocyte and adipocyte differentiation markers in *Runx2*<sup>-/-</sup> chondrocytes on the indicated days (with day 0 as the day of plating) (A), and in *Runx2*<sup>-/-</sup> (-/-) and wild-type (+/+) chondrocytes on day 12 (B). Chondrocytes isolated from the ribs of *Runx2*<sup>-/-</sup> and wild-type embryos at E18.5 were plated at a density of  $1 \times 10^6$ /dish in 6 cm dishes and cultured in DMEM/F12 hybrid medium containing 10% FBS. Twenty  $\mu$ g of total RNA were loaded in each lane and hybridized with mouse cDNA of type II collagen, type X collagen, *PPARγ*, *aP2*, and *Glut4*, and rat cDNA of *Pref-1*. Gels stained with ethidium bromide are shown in the lowest panels as an internal control.

but decreased over time (Fig. 3A). Conversely, *PPARγ* mRNA and *aP2* mRNA, both of which are upregulated during adipogenesis, were first detected on day 3 and day 6, respectively, and their levels increased thereafter (Fig. 3A). Expression of *Glut4* mRNA, which is also highly expressed in adipocytes, was detected on day 9. Further, expression of *Pref-1* – which is abundantly expressed in pre-adipocytes, and its protein is known to be a major inhibitory factor of adipogenesis – was markedly decreased, in contrast to the increases in the expression levels of adipocyte marker genes (Fig. 3A). Expression of these adipocyte-related genes was maintained up to day 12 (Fig. 3B). By contrast, in the wild-type chondrocyte cultures, *PPARγ*, *aP2* and *Glut4* expression were barely detected, whereas type X collagen expression was upregulated on day 12 (Fig. 3B). These data indicate that *Runx2*<sup>-/-</sup> chondrocytes show the adipocyte phenotype over time in culture.



#### Inhibition of adipogenic changes of *Runx2*<sup>-/-</sup> chondrocytes by introduction of *Runx2* or by treatment with TGF- $\beta$ , retinoic acid, IL-1 $\beta$ , bFGF, PDGF, PTH or IL-11

To confirm that the induction of adipogenic changes in *Runx2*<sup>-/-</sup> chondrocyte cultures depended on loss of the *Runx2* gene, we infected *Runx2*-containing adenovirus into

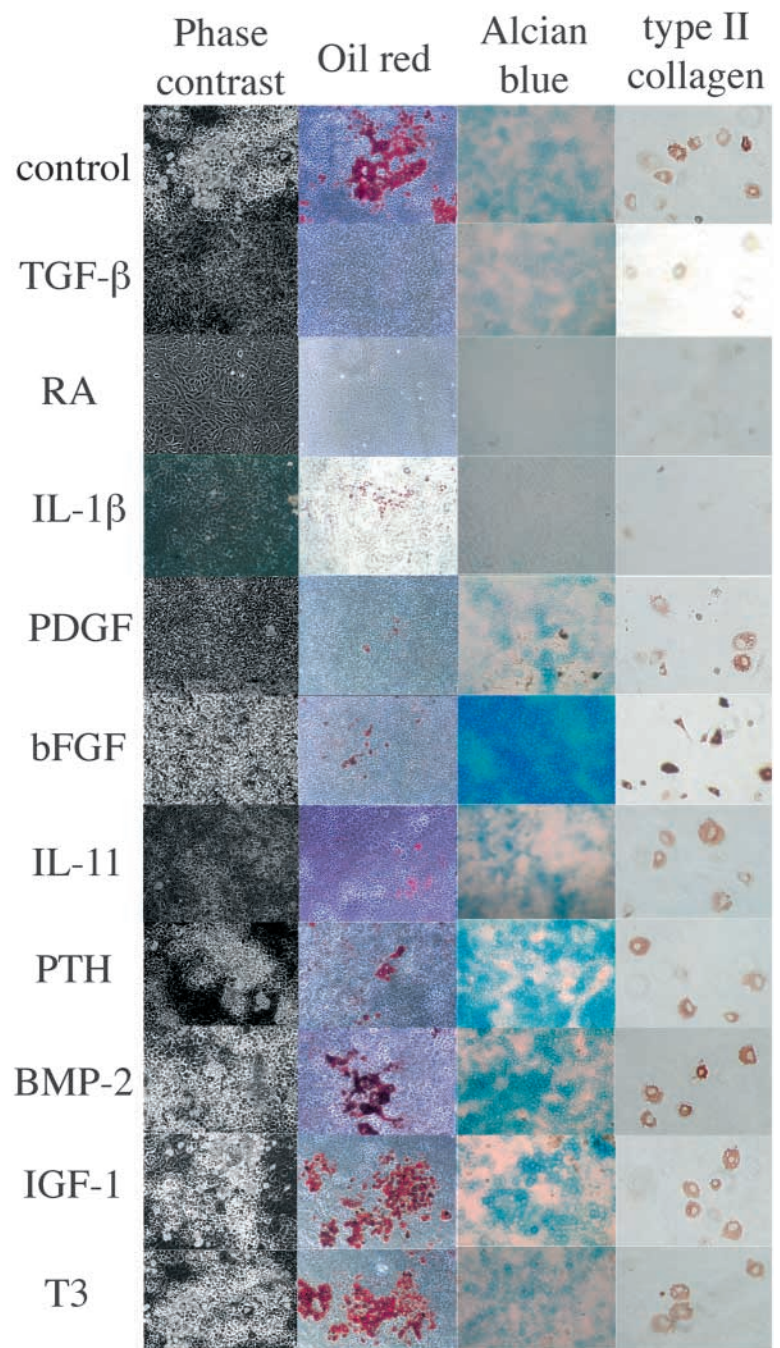


**Fig. 4.** Inhibition of adipogenesis by the adenoviral introduction of *Runx2* in *Runx2*<sup>-/-</sup> chondrocytes. *Runx2*<sup>-/-</sup> chondrocytes infected with either EGFP-expressing (GFP) or *Runx2*-and-EGFP-expressing (*Runx2*) virus were cultured for 12 days and stained with oil red O (A), cultured for 6 days and harvested for northern blot analysis (B), or cultured for 15 days and harvested for real-time RT-PCR analysis at indicated days (C). Phase-contrast images are shown in (A) ( $\times 50$ ). In northern blot analysis, 20  $\mu$ g of total RNA were loaded in each lane and hybridized with mouse type II collagen or *PPARγ* cDNA (B). The gel stained with ethidium bromide is shown as an internal control. In real-time RT-PCR analysis, *Runx2* and type X collagen expression were examined (C). EGFP-expressing and *Runx2*-and-EGFP-expressing adenovirus infection are shown by open circles and closed circles, respectively. The value of *Runx2* or type X collagen in *Runx2*-and-EGFP-expressing adenovirus infection on 15-day culture was defined as 1, and relative values are shown. Data represent the mean of three wells. \* $P < 0.05$  as determined by one-way ANOVA. Similar results were obtained in three independent experiments and representative data are shown.

these chondrocytes (Fig. 4). Although the infection of EGFP-containing adenovirus mildly reduced the adipogenic changes in *Runx2*<sup>-/-</sup> chondrocytes, the introduction of *Runx2* severely inhibited the adipogenic changes and the cells retained the morphology of chondrocytes (Fig. 4A). Six days after infection, we compared the expression of type II collagen and *PPAR* $\gamma$  in the *Runx2*-and-EGFP-infected and EGFP-infected cultures (Fig. 4B). Introduction of *Runx2* in *Runx2*<sup>-/-</sup> chondrocytes inhibited the downregulation of type II collagen expression as well as the upregulation of *PPAR* $\gamma$  expression. Furthermore, the introduction of *Runx2* slightly upregulated type X collagen expression on 7–11-day culture after the infection, although the level of the induction was extremely low (Fig. 4C). These findings indicate that loss of the chondrocyte phenotype and acquirement of the adipogenic phenotype are closely linked to *Runx2* deficiency.

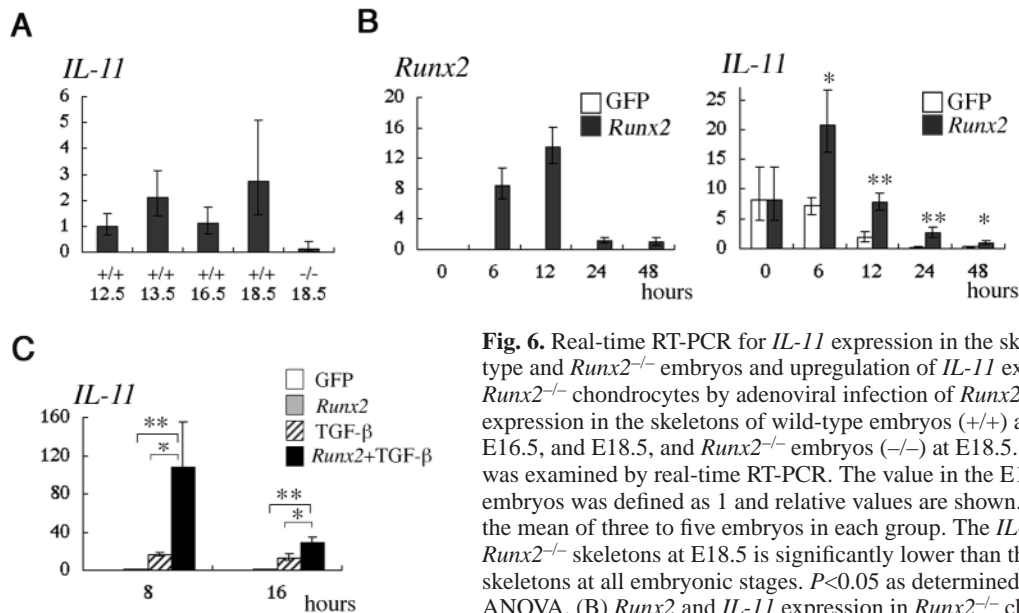
As adipogenic changes were not observed in *Runx2*<sup>-/-</sup> cartilage in vivo, we treated *Runx2*<sup>-/-</sup> chondrocytes with secreted factors including TGF- $\beta$ , retinoic acid, IL-1 $\beta$ , PDGF, bFGF, PTH, BMP-2, IGF-1 and T3, which have been reported to affect the differentiation, proliferation or function of chondrocytes (Centrella et al., 1988; Goldring et al., 1988; Kato and Iwamoto, 1990; Koike et al., 1990; Bohme et al., 1992; Chen et al., 1992; Zou et al., 1997), in order to identify factors that prevent the adipogenic change of *Runx2*<sup>-/-</sup> chondrocytes. Treatment of *Runx2*<sup>-/-</sup> chondrocytes with TGF- $\beta$  or retinoic acid completely inhibited the adipogenic changes, and treatment with IL-1 $\beta$ , PDGF, bFGF or PTH also inhibited the adipogenic changes (Fig. 5). By contrast, treatment with BMP-2, IGF-1 or T3 did not inhibit the adipogenic changes of *Runx2*<sup>-/-</sup> chondrocytes (Fig. 5). We treated *Runx2*<sup>-/-</sup> chondrocytes with IL-11, which has been reported to inhibit adipocyte differentiation of bone marrow stromal cells (Du and Williams, 1994). Treatment with IL-11 inhibited the adipogenic changes of *Runx2*<sup>-/-</sup> chondrocytes (Fig. 5). The cells, which showed no adipogenic change after the treatment with each factor, retained the abilities to produce proteoglycan and type II collagen except the cells treated with retinoic acid or IL-1 $\beta$ , which lost the characteristics of chondrocytes as shown by the absence of proteoglycan and type II collagen production (Fig. 5). Further, the treatment with bFGF greatly enhanced the production of proteoglycan and type II collagen and the treatment with PTH or BMP-2 mildly enhanced proteoglycan production, whereas the treatment with TGF- $\beta$  mildly reduced the production of proteoglycan and type II collagen (Fig. 5).

Maier et al. (Maier et al., 1993) reported that treatment with IL-1 or TGF- $\beta$  induced *IL-11* expression in articular chondrocytes (Maier et al., 1993). We investigated the steady-state level of *IL-11* expression in the skeletal tissues of wild-type embryos and *Runx2*<sup>-/-</sup> embryos (Fig. 6A). *IL-11* was expressed in chondrocytes as well as in bone marrow cells, because the levels of *IL-11* expression in cartilaginous skeleton at E12.5



**Fig. 5.** Inhibition of adipogenesis of *Runx2*<sup>-/-</sup> chondrocytes by soluble differentiation factors. *Runx2*<sup>-/-</sup> primary chondrocytes were plated at a density of  $1 \times 10^5$  cells/well ( $5 \times 10^4$  cells/cm<sup>2</sup>) in 24 multi-well plates. Two days after inoculation, the cells were further cultured for 10 days without (control) or with a factor including TGF- $\beta$  (1 ng/ml), retinoic acid (RA) ( $10^{-8}$  M), IL-1 $\beta$  (10 ng/ml), PDGF (200 ng/ml), bFGF (10 ng/ml), IL-11 (100 ng/ml), PTH ( $10^{-7}$  M), BMP-2 (50 ng/ml), IGF-1 (100 ng/ml) or T3 ( $10^{-7}$  M), and stained with oil red O or Alcian blue. After culture, the cells were replated for the examination of type II collagen expression by immunocytochemistry. Magnification: phase contrast, oil red and Alcian blue,  $\times 50$ ; type II collagen,  $\times 100$ .

and E13.5 were similar to those in skeletons at E16.5 and E18.5, when the cartilaginous skeleton was partly or largely



**Fig. 6.** Real-time RT-PCR for *IL-11* expression in the skeletons of wild-type and *Runx2*<sup>-/-</sup> embryos and upregulation of *IL-11* expression in *Runx2*<sup>-/-</sup> chondrocytes by adenoviral infection of *Runx2*. (A) *IL-11* expression in the skeletons of wild-type embryos (+/+) at E12.5, E13.5, E16.5, and E18.5, and *Runx2*<sup>-/-</sup> embryos (-/-) at E18.5. *IL-11* expression was examined by real-time RT-PCR. The value in the E12.5 wild-type embryos was defined as 1 and relative values are shown. Data represent the mean of three to five embryos in each group. The *IL-11* expression in *Runx2*<sup>-/-</sup> skeletons at E18.5 is significantly lower than those in wild-type skeletons at all embryonic stages. *P*<0.05 as determined by one-way ANOVA. (B) *Runx2* and *IL-11* expression in *Runx2*<sup>-/-</sup> chondrocytes infected with either EGFP-expressing (open columns) or *Runx2*-and-

EGFP-expressing (closed columns) adenovirus. Infected cells were harvested at the indicated times after the onset of viral infection, and *Runx2* and *IL-11* expression were examined by real-time RT-PCR. The value of *Runx2* or *IL-11* in *Runx2*-and-EGFP-expressing adenovirus infection at 48 hours was defined as 1, and relative values are shown. Data represent the mean of three wells. \**P*<0.05 and \*\**P*<0.01 as determined by one-way ANOVA. Similar results were obtained in three independent experiments, and representative data are shown. (C) Synergistic induction of *IL-11* mRNA by *Runx2* and TGF-β. *Runx2*<sup>-/-</sup> chondrocytes were infected with EGFP-expressing (GFP) or *Runx2*-and-EGFP-expressing (*Runx2*) adenovirus. *Runx2*<sup>-/-</sup> chondrocytes and *Runx2* overexpressing *Runx2*<sup>-/-</sup> chondrocytes were treated with TGF-β (1 ng/ml) for the indicated times. The value of *IL-11* in *Runx2*-and-EGFP-expressing adenovirus infection at 8 hours was defined as 1, and relative values are shown. The induction of *IL-11* mRNA by *Runx2* was much weaker than that by TGF-β but *Runx2* hugely induced *IL-11* mRNA in the presence of TGF-β. Data represent mean of four wells. \**P*<0.01 and \*\**P*<0.001 as determined by one-way ANOVA. Similar results were obtained in three independent experiments and representative data are shown.

replaced by bone and bone marrow cells (Fig. 6A). *IL-11* expression was reduced in the *Runx2*<sup>-/-</sup> cartilaginous skeleton. Interestingly, adenoviral introduction of *Runx2* into *Runx2*<sup>-/-</sup> chondrocytes upregulated the level of *IL-11* mRNA expression, although the expression level was still low (Fig. 6B,C). We next examined whether *Runx2* and TGF-β synergistically induce *IL-11* expression. The treatment with TGF-β induced *IL-11* expression, and adenoviral introduction of *Runx2* synergistically upregulated the *IL-11* expression (Fig. 6C). We also examined the effect of *Runx2* on *IL-1β* expression. However, *IL-1β* expression was not reduced in the *Runx2*<sup>-/-</sup> cartilaginous skeleton, adenoviral introduction of *Runx2* into *Runx2*<sup>-/-</sup> chondrocytes failed to induce *IL-1β* expression, and TGF-β had no effect on *IL-1β* expression (data not shown). These findings indicate that soluble factors also play important roles in the maintenance of the chondrocyte phenotype and inhibition of adipogenesis of chondrocytes, and suggest that the *Runx2*-dependent functions of maintenance of chondrocyte phenotype and inhibition of adipogenesis are mediated, at least in part, by *IL-11*.

## Discussion

We found that *Runx2*<sup>-/-</sup> chondrocytes gradually undergo adipogenic changes in vitro, as determined by analyzing the cell morphology, lipid accumulation and expression of adipocyte marker genes. These adipogenic changes of *Runx2*<sup>-/-</sup> chondrocytes were prevented by adenoviral introduction of

*Runx2* or treatment with TGF-β, retinoic acid, *IL-1β*, PDGF, bFGF, PTH or *IL-11*. Our findings indicate that *Runx2* inhibits adipocyte differentiation and has potent activity for maintaining the phenotype of chondrocytes in addition to promoting chondrocyte maturation.

## Expression of adipogenic phenotype in *Runx2*<sup>-/-</sup> chondrocytes

There have been many reports of multipotent mesenchymal cell lines that have the ability to differentiate into several types of cells including adipocytes, osteoblasts, myoblasts and chondrocytes under specific conditions. This diversity of mesenchymal cell differentiation is also observed in bone marrow cells. Also, mesenchymal cells have the capacity to shift their cell lineage to a different cell lineage even after they display one specific phenotype (Nathanson and Hay, 1980; Bennett et al., 1991; Cancedda et al., 1992; Hu et al., 1995). In our study, we observed that a large percentage of *Runx2*<sup>-/-</sup> chondrocytes in vitro expressed the adipocyte phenotype over time. The immature chondrocytes of xiphoids in newborn mice have been shown to differ from other chondrocytes and become adipocytes under a defined in vitro condition (Heermeier et al., 1994). However, normal immature chondrocytes do not undergo adipogenesis, especially under the chondrocyte culture condition that we used in this study (Leboy et al., 1989; Iwamoto et al., 1993; Enomoto et al., 2000). The simultaneous induction of adipocyte differentiation and chondrocyte

differentiation in bone marrow cell cultures and mesenchymal cell line cultures is rare. Thus, the conversion of chondrocytes to adipocytes is a unique phenomenon that occurs on depletion of Runx2. Indeed, adenoviral introduction of *Runx2* into *Runx2*<sup>-/-</sup> chondrocytes inhibited adipogenic differentiation.

### Role of Runx2 in inhibition of adipogenesis

Recent studies have suggested that Runx2 is involved in regulating both adipocyte differentiation and osteoblast differentiation (Lecka-Czernik et al., 1999; Gori et al., 1999; Kobayashi et al., 2000). *Runx2*<sup>-/-</sup> calvarial cells differentiated into adipocytes at high frequency (Kobayashi et al., 2000). When adipogenesis was induced in a bone marrow-derived cell line with characteristics of both osteoblasts and adipocytes by forced expression of *PPAR*γ, osteogenesis was disturbed and the level of *Runx2* expression decreased (Lecka-Czernik et al., 1999). BMP-2 promotes osteoblast differentiation and inhibits adipocyte differentiation in human bone marrow stromal cells; the effect of BMP-2 was associated with an increase in *Runx2* expression, and downregulation of *Runx2* by antisense treatment reduced the level of BMP-2-induced alkaline phosphatase expression (Gori et al., 1999). These findings suggest that Runx2 is involved in the determination of osteoblast and adipocyte lineages from the same precursors. Taken together with our finding that *Runx2* deficiency stimulates expression of the adipogenic phenotype, we suggest that Runx2 is likely to be an inhibitor that prevents mesenchymal cells from entering an adipogenic cell lineage.

*PPAR*γ is expressed even in normal cartilage, although its expression level is lower than that in adipose tissues. Chondrocytes expressing *PPAR*γ do not display other characteristics of adipocytes and *PPAR*γ has been predicted to modulate the production and effects of inflammatory cytokines such as IL-1 and tumor necrosis factor α (TNFα) in these cells (Bordji et al., 2000). Although *PPAR*γ expression was suppressed in freshly isolated *Runx2*<sup>-/-</sup> chondrocytes, *PPAR*γ expression gradually increased during culture (Fig. 3), indicating that Runx2 is necessary for the suppression of *PPAR*γ expression in vitro. However, this also indicates that *PPAR*γ expression can be suppressed in vivo by Runx2-independent pathways, probably through signaling by extracellular matrix proteins and secreted differentiation factors.

Another possible mechanism through which Runx2 inhibits adipogenesis is regulation of *Pref-1* gene expression. *Pref-1*, an EGF-like protein, is regarded as a tissue-specific, important inhibitory factor against adipogenesis. Continuous expression of *Pref-1* in 3T3L1 pre-adipocyte cells abolished adipocyte differentiation (Smas and Sul, 1993), and mice lacking *Pref-1* showed accelerated adiposity (Moon et al., 2002). Further, it has been reported that downregulation of *Pref-1* expression is an important step for adipocyte differentiation induced by dexamethasone (Smas et al., 1999). Although freshly isolated *Runx2*<sup>-/-</sup> chondrocytes expressed a high level of *Pref-1* mRNA (Fig. 3A), they rapidly lost *Pref-1* expression in culture. Thus, *Runx2* expression is required to maintain *Pref-1* expression in vitro but not in vivo.

### Maintenance of chondrogenic phenotype by Runx2

On depletion of Runx2, chondrocytes lost their established

phenotype and took an unexpected pathway to adipocytes. In other words, Runx2 may play an important role in expression of the chondrogenic phenotype. Misexpression of a dominant-negative form of *Runx2* suppresses the expression of chondrocyte marker genes such as *aggrecan*, type IX collagen and *Sox9* in chick primary chondrocytes (Iwamoto et al., 2003). However, in primary chondrocyte cultures, a dominant-negative form of *Runx2* does not induce adipogenesis (Ueta et al., 2001; Iwamoto et al., 2003). There might be differences in the characteristics of chondrocytes that develop in the complete absence of Runx2 from the beginning and chondrocytes that normally develop in the presence of Runx2 and then suddenly lose Runx2 function. It may indicate that *Runx2*<sup>-/-</sup> chondrocytes was triggered for differentiation into adipocytes at an early stage of the differentiation but it was not sufficient for adipogenesis in vivo.

Our present result is contradictory to the phenotype of *Runx2*<sup>-/-</sup> mice in which chondrocytes organize cartilage tissues, but not adipose tissues. Therefore, it is suggested that some signals are transduced from extracellular matrix proteins or soluble differentiation factors in vivo, but these signals are insufficient for maintaining the chondrocyte phenotype in vitro. The soluble differentiation factors may include TGF-β and retinoic acid, which completely inhibited adipogenic differentiation of *Runx2*<sup>-/-</sup> chondrocytes (Fig. 5). Furthermore, IL-1β, PDGF, bFGF, PTH and IL-11, which reduced the extent of adipogenic changes of *Runx2*<sup>-/-</sup> chondrocytes, may also be involved in the inhibition of adipogenic differentiation in vivo. In agreement with our findings, TGF-β, retinoic acid, IL-1β, PDGF, FGF and IL-11 have been shown to inhibit the differentiation of adipogenic cell lines or to inhibit adipocyte formation in human long-term bone marrow culture (Kuri-Harcuch, 1982; Ignatz and Massague, 1985; Navre and Ringold, 1989; Kawashima et al., 1991; Delikat et al., 1993; Hauner et al., 1995). Although retinoic acid and IL-1β strongly inhibited adipogenic changes in *Runx2*<sup>-/-</sup> chondrocytes, they also abolished the production of proteoglycan and type II collagen as previously reported (Benya and Padilla, 1986; Horton et al., 1987; Goldring et al., 1988; Lefebvre et al., 1990; Lum et al., 1996; Bolton et al., 1996).

Adenoviral introduction of *Runx2* into *Runx2*<sup>-/-</sup> chondrocytes induced *IL-11* expression, and Runx2 and TGF-β synergistically upregulated the *IL-11* expression (Fig. 6). Although the sequence of the promoter region of mouse *IL-11* gene is not available, three putative Runx2 binding sites were found near the transcription start site of human *IL-11* gene on database search. Thus, the *IL-11* gene is likely to be one of the downstream genes responsible for Runx2-dependent inhibition of adipogenic differentiation. Although IL-11 has been shown to inhibit adipogenesis in bone marrow stromal cells by enhancing BMP action through STAT3 (signal transducer and activator of transcription 3) (Takeuchi et al., 2002), another signal pathway from IL-11 should play an important role in the inhibition of adipogenic change of *Runx2*<sup>-/-</sup> chondrocytes, because BMP-2 treatment failed to inhibit it (Fig. 5). It was reported that TNFα and IL-11 secreted by malignant breast epithelial cells inhibited adipocyte differentiation by downregulation of *C/EBPα* and *PPAR*γ expression (Meng et al., 2001). Therefore, IL-11 is likely to inhibit adipogenesis by downregulating the essential adipogenic transcription factors, *C/EBPα* and *PPAR*γ, although the signal pathway remains to be clarified.

Our present result is also contradictory to the finding that there were no adipogenic changes in the chondrocytes of which differentiation had been induced by BMP-2 in *Runx2*<sup>-/-</sup> calvarial cells in vitro (Kobayashi et al., 2000). It may indicate that calvarial cells were more potent in secreting differentiation factors for maintaining chondrocyte phenotypes than *Runx2*<sup>-/-</sup> chondrocytes. Interestingly, BMP-2 inhibited adipogenic change of *Runx2*<sup>-/-</sup> calvarial cells but not *Runx2*<sup>-/-</sup> chondrocytes (Kobayashi et al., 2000) (Fig. 5). Therefore, it is also possible that the chondrocytes of which differentiation was induced by BMP-2 in vitro may be different from the chondrocytes that differentiated in vivo in the gene expression profiles related to adipogenesis.

In conclusion, our study indicates that *Runx2* plays multiple roles in the regulation of chondrocyte differentiation. These roles include maintenance of the chondrocyte phenotype or inhibition of adipogenic differentiation, in addition to induction of chondrocyte maturation.

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