Journal of Cellular Biochemistry 93:374–383 (2004)

Dexamethasone Inhibits Insulin-Induced Chondrogenesis of ATDC5 Cells by Preventing PI3K-Akt Signaling and DNA Binding of Runx2

Takashi Fujita,[1] Ryo Fukuyama,[2,3] Hirayuki Enomoto,[2] and Toshihisa Komori[2]*

[1]Department of Pharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, 573-0101, Japan
[2]Department of Developmental and Reconstructive Medicine, Division of Oral Cytology and Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan
[3]Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Hiroshima International University, Kure, 737-0112, Japan

Abstract Glucocorticoids play important roles in cell growth and differentiation. In this study, we investigated the effect of application of dexamethasone (DEX) at the early stage of chondrogenesis using the prechondrogenic cell line, ATDC5, which differentiates into chondrocytes in the presence of insulin. When ATDC5 cells were cultured in the presence of DEX and insulin, DEX inhibited insulin-induced cellular condensation and subsequent cartilaginous nodule formation, and reduced proteoglycan synthesis and type II collagen expression dose-dependently. Pretreatment with 10^{-8} M DEX for 1 day inhibited insulin-induced Akt phosphorylation, but not ERK1/2 phosphorylation, in ATDC5 cells. Treatment of ATDC5 cells with insulin for more than 2 days upregulated the levels of phosphatidylinositol 3-kinase (PI3K) subunit proteins, p85 and p110, and Akt, whereas the upregulation was inhibited in the presence of 10^{-8} M DEX. In electrophoresis mobility shift assays (EMSAs), treatment with 10^{-8} M DEX inhibited DNA binding of Runx2 during culture of ATDC5 cells with insulin. Reporter assays using osteocalcin promoter showed that DEX inhibited Runx2-dependent transcription dose-dependently. Adenoviral introduction of dominant-negative (dn)-*Akt* or dn-*Runx2* into ATDC5 cells inhibited cellular condensation and reduced proteoglycan synthesis upon incubation with insulin, whereas adenoviral introduction of Akt or Runx2 prevented the inhibition of chondrogenesis by DEX. These findings indicate that DEX inhibits chondrogenesis of ATDC5 cells at the early stage by downregulating Akt phosphorylation as well as the protein levels of PI3K subunits and Akt, thereby suppressing PI3K-Akt signaling, and by inhibiting DNA binding of Runx2 and Runx2-dependent transcription. J. Cell. Biochem. 93: 374–383, 2004.

Key words: chondrogenesis; dexamethasone; PI3K; Akt; ATDC5; Runx2

Glucocorticoids are known to play important roles in cell growth and differentiation [Beato, 1989]. Various glucocorticoids have been shown to maintain the chondrocyte phenotype and enhance proteoglycan synthesis in chondrocytes [Calcagno et al., 1970; Jones and Addison, 1975; Kato and Gospodarowicz, 1985; Takigawa et al., 1988; Grigoriadis et al., 1989, 1996; Itagane et al., 1991; Quarto et al., 1992]. Further, glucocorticoids may stimulate or suppress cell growth depending on the culture conditions or the chondrocyte phenotype [Jones and Addison, 1975; Silbermann and Maor, 1985; Takigawa et al., 1988; Itagane et al., 1991]. Although the effects of glucocorticoids on chondrocytes at the late stage of chondrogenesis have been extensively examined, their effects on prechondrogenic cells at the early stage of chondrogenesis remain to be clarified.

During embryogenesis, skeletal formation begins with condensation of mesenchymal cells

Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology; Grant number: 12137204; Grant sponsor: Japan Science Society (Sasakawa Scientific Research Grant) (to TF).

*Correspondence to: Toshihisa Komori, Department of Developmental and Reconstructive Medicine, Division of Oral Cytology and Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan.
E-mail: komorit@net.nagasaki-u.ac.jp

Received 8 February 2004; Accepted 4 May 2004
DOI 10.1002/jcb.20192

and their differentiation into chondrocytes. These chondrocytes then undergo a program of proliferation and maturation [Thorogood and Hinchliffe, 1975]. The importance of cellular condensation and cell–cell interactions in chondrogenesis and in the maintenance of the chondrocyte phenotype has been shown, and studies on chondrocyte differentiation in vitro have been performed under high-density culture conditions [Hickok et al., 1998]. However, the requirement of high cellular density makes it difficult to investigate the onset of cellular condensation, which is one of the key events in chondrogenesis. ATDC5 cells have been shown to be a useful in vitro model for examining chondrocyte differentiation. ATDC5 cells show the characteristics of undifferentiated prechondrogenic cells in the growing phase [Atsumi et al., 1990], and maintain the potential for chondrogenesis. When cultured with insulin, ATDC5 cells undergo cellular condensation in the postconfluent phase and acquire the phenotype of chondrocytes, characterized by proteoglycan synthesis and type II collagen expression [Atsumi et al., 1990; Akiyama et al., 1996; Shukunami et al., 1996].

The insulin signaling process involves a cascade of events initiated by the binding of insulin to its receptor and activation of the intrinsic tyrosine kinase which elicits tyrosine phosphorylation of insulin receptor substrates (IRSs). The binding of the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (p85) to the tyrosine-phosphorylated IRSs results in activation of the catalytic subunit of PI3K (p110). Akt (also called PKB), a serine/threonine protein kinase, is activated through the PI3K pathway [Cantley, 2002]. Glucocorticoids have been shown to affect insulin signaling in adipocytes [Turnbow et al., 1994; Burén et al., 2002]. Dexamethasone (DEX) downregulated the expression of IRS-1, PI3K, and Akt proteins and Akt phosphorylation in adipocytes. In myoblasts, DEX upregulated p85 expression, which, in turn, competed with the PI3K heterodimer for binding at IRS-1, thereby inhibiting PI3K activation; DEX also reduced the level of IGF-1-induced phosphorylation of Akt [Singleton et al., 2000]. Thus, the effects of DEX on insulin signaling are still controversial and remains to be clarified.

Runx2 is a transcription factor that belongs to the Runx family [Komori, 2002]. Runx2 acquires the capacity for DNA binding and transcriptional activation by forming a heterodimer with Cbfb [Kundu et al., 2002; Miller et al., 2002; Yoshida et al., 2002]. Targeted disruption of Runx2 results in a complete lack of bone formation due to the absence of osteoblasts, demonstrating that Runx2 is essential for osteoblast differentiation [Komori et al., 1997; Otto et al., 1997]. Runx2 also plays important roles in chondrocyte maturation and in maintenance of the chondrocyte phenotype [Inada et al., 1999; Kim et al., 1999; Enomoto et al., 2000, 2004; Takeda et al., 2001; Ueta et al., 2001; Stricker et al., 2002]. In Runx2-deficient mice, mesenchymal condensation for intramembranous ossification is poor, while mesenchymal condensation and subsequent cartilaginous skeletal formation are not grossly impaired [Komori et al., 1997; Otto et al., 1997; Inada et al., 1999; Kim et al., 1999]. However, when the dominant-negative form of Runx2 (dn-Runx2) was stably transfected in ATDC5 cells, insulin did not induce cellular condensation of these ATDC5 cells, indicating that Runx2 is required for insulin-induced cellular condensation of ATDC5 cells [Akiyama et al., 1999].

In this study, we examined the effects of application of a glucocorticoid on chondrogenesis of ATDC5 cells and its effects on insulin signaling and Runx2 functioning. Here, we show that DEX inhibits insulin-induced chondrogenesis of ATDC5 cells at the stage of cellular condensation by downregulating the levels of PI3K subunit proteins, p85 and p110, the level of Akt protein, Akt phosphorylation, DNA binding of Runx2, and Runx2-dependent transcription.

MATERIALS AND METHODS

Cell Culture, Alcian Blue Staining, and Measurement of Proteoglycan Content

The ATDC5 chondrogenic cell line, purchased from RIKEN Cell Bank, was cultured as previously described [Enomoto et al., 2000]. Briefly, ATDC5 cells were cultured in DMEM/Ham's F12 (1:1) hybrid medium (GIBCO BRL, New York, NY) supplemented with 5% fetal bovine serum (FBS) (GIBCO BRL), 10 μg/ml human transferrin (Roche Diagnostics, Mannheim, Germany), and 3×10^{-8} M sodium selenite (Sigma Chemical Co.). ATDC5 cells were plated at an initial density of 1×10^5 cells/well in 6-well plates or at 2×10^4 cells/well in 24-well plates (Corning, NY). To induce chondrogenesis, cells were cultured in the above medium supple-

mented with 10 μg/ml of human recombinant insulin (Wako Pure Chemical, Osaka, Japan). The medium was changed every other day. For Alcian blue staining, the wells were rinsed twice with PBS and fixed with 95% methanol at −20°C for 2 min. They were then stained overnight with 0.1% Alcian blue in 0.1 M HCl. After the wells were rinsed three times with distilled water, the dye was extracted with 6 M guanidine–HCl [Akiyama et al., 1996]. Total optical density of extracted dye was measured at 620 nm and designated as A_{620} as previously described [Atsumi et al., 1990; Akiyama et al., 1996].

RNA Extraction and Northern Blot Analysis

Total RNA was extracted using ISOGEN (Nippon Gene) according to the manufacturer's instructions. A 0.4-kb fragment of mouse *Col2a1* cDNA [Inada et al., 1999] was labeled with [α-^{32}P]dCTP, and hybridization was performed as previously described [Enomoto et al., 2000].

Western Blot Analysis

Proteins were extracted from ATDC5 cells, equal amounts of proteins (10 μg) were separated on a 10% gel by SDS–PAGE, and Western blotting was performed as previously described [Yoshida et al., 2002], using anti-phospho-ERK1/2 (New England Biolabs, Beverly, MA), anti-ERK1/2 (New England Biolabs), anti-phospho-Akt (New England Biolabs), anti-Akt (New England Biolabs), anti-p85 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-p110β (Santa Cruz Biotechnology, Inc.), anti-Runx2 [Yoshida et al., 2002], anti-Cbfb [Yoshida et al., 2002], and horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (New England Biolabs). Although p110α was strongly detected in C3H10T1/2 cells on Western blot analysis using anti-p110α antibody (Santa Cruz Biotechnology, Inc.), it was barely detectable in ATDC5 cells (data not shown). For semiquantification of the blots, the enhanced chemiluminescence exposures were scanned into a PPC Macintosh computer utilizing an EPSON GT7600 with Adobe Photoshop 5.0 software. The images were quantified utilizing NIH Image 1.61 software. The levels of phospho-ERK1/2 or phospho-Akt protein were then divided by the level of ERK1/2 or Akt protein, respectively, to generate normalized values for activated Akt in each sample. In the time-course studies of the levels of p85, p110, Akt, Runx2, and Cbfb in ATDC5 cells after insulin stimulation, the mean level of each protein in ATDC5 cells that were maintained in the absence of DEX and insulin on day 0 was defined as 1 and the levels of each protein on subsequent days were expressed as relative values.

Electrophoresis Mobility Shift Assay (EMSA)

Cells were homogenized using a Dounce homogenizer (B-type pestle) in four volumes of buffer A which contained 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 μg/ml of each protease inhibitor (*p*-amidinophenyl-methanesulfonyl fluoride, leupeptin, pepstatin A, and aprotinin), and NP-40 was added at 0.6%. After standing on ice for 5 min, the homogenates were centrifuged at 20,000*g* for 5 min. Pellets were resuspended in four volumes of buffer B (400 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, and 1 mg/ml each of the above protease inhibitors in 50 mM Tris-HCl buffer, pH 7.5) and after standing on ice for 30 min, the suspensions were centrifuged at 20,000*g* for 20 min. Double-stranded OSE2 oligonucleotides, which contain a Runx2 binding element [Thirunavukkarasu et al., 1998], were labeled with [α-^{32}P]dATP and Klenow fragment, and purified by Nick column. 2.5 μg nuclear extracts were incubated with 10 fmol of the labeled probe with a specific activity of 3–5×10^6 cpm/pmol DNA in binding buffer consisting of 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 1 mg poly(dI-dC). Protein–DNA complexes were resolved on 6% nondenaturing polyacrylamide gels. We carried out competition with either a 100-fold molar excess of unlabeled OSE2 oligonucleotides or a 100-fold molar excess of mutated OSE2 oligonucleotides [Thirunavukkarasu et al., 1998]. For supershift experiments, monoclonal antibody against Runx2 [Yoshida et al., 2002] was added to the entire mixture.

Reporter Assay

The day before transfection, ATDC5 cells were plated on 48-well multiplates at a density of 2.5×10^4 cells/ml. They were transfected with 0.2 μg of p147mOG2/luciferase reporter construct (p147-luc) and 0.002 μg of pRL-CMV vector, which was used to normalize the transfection efficiencies, using FuGENE 6 (Roche) according to the manufacturer's protocol. After

transfection, DEX-containing medium was added to the plates. After culture for 24 h, the cells were treated with insulin for 24 h, and then the cells were harvested. Luciferase activities were assayed using the Luciferase Reporter Assay System (Promega, Madison, WI) and a model TD20/20 luminometer (Turner BioSystems, Sunnyvale, CA).

Adenoviral and Retroviral Transfers

Bicistronic adenovirus vectors expressing type II *Runx2* and enhanced green fluorescent protein (EGFP) or EGFP alone were generated as previously described [Enomoto et al., 2003]. Dn-*Runx2*- and -EGFP-expressing adenovirus vector was generated by inserting the 421 bp DNA fragment containing the runt domain of *Runx2* into the *Eco*RI-*Bam*HI sites of the pACCMV-IRES-EGFP vector [Enomoto et al., 2003]. *Akt*-expressing and dn-*Akt*-expressing adenoviruses were generated as described previously [Fujio and Walsh, 1999]. ATDC5 cells were plated at a density of 2×10^4 cells/well in 24-well plates. For EGFP-expressing, *Akt*-expressing, or *Runx2*- and -EGFP-expressing adenovirus infection, after ATDC5 cells were cultured in the absence or presence of 10^{-8} M DEX for 2 days, the cells were infected with adenovirus at a multiplicity of infection (MOI) of 10 for 12 h. For dn-*Akt*- or dn-*Runx2*- and -EGFP-expressing adenovirus infection, at 2 days before confluence, we infected ATDC5 cells at an MOI of 10 for 12 h. *Cbfb*-expressing retrovirus was prepared as previously described [Yoshida et al., 2002]. ATDC5 cells were plated at a density of 2×10^4 cells/well in 24-well plates, and 24 h later, the cells were incubated with retroviral supernatants in the presence of 4 μg/ml polybrene for 12 h.

RESULTS

DEX Inhibited Cellular Condensation and Subsequent Cartilaginous Nodule Formation in ATDC5 Cells

When ATDC5 cells were incubated with insulin, the ATDC5 cells began to aggregate and formed cartilaginous nodules. On day 9 (day 0 was set at confluence), many cartilaginous nodules were observed and the plate was strongly stained with Alcian blue, indicating that ATDC5 cells had differentiated to chondrocytes and produced a large amount of proteoglycan (Fig. 1A,D). However, upon incu-

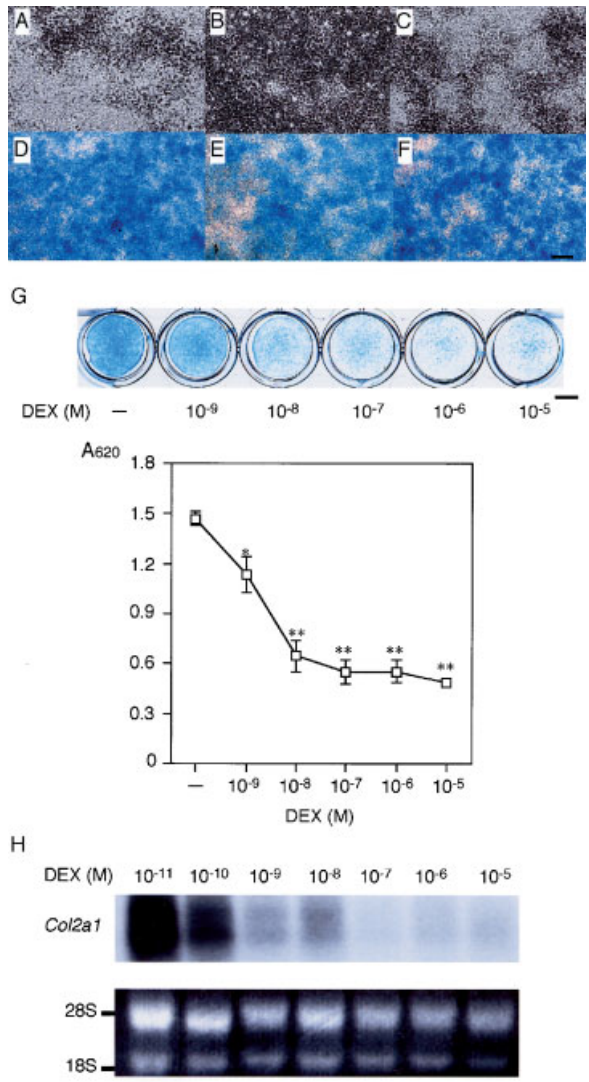


Fig. 1. Effect of dexamethasone (DEX) on insulin-induced chondrogenesis of ATDC5 cells. **A–F**: Microphotographs of phase contrast (A–C) and Alcian blue staining (D–F) of ATDC5 cells treated with insulin (A, D), with insulin plus 10^{-8} M DEX (B, E), or with insulin plus 10^{-8} M DEX plus 10^{-6} M RU486 (C, F) for 12 days. Bar (A–F), 10 μm. **G**: Proteoglycan production. ATDC5 cells were cultured with insulin and the indicated concentration of DEX. After a 12-day culture, the cells were stained with Alcian blue. To obtain quantitative data, the dye was extracted with 6 M guanidine–HCl, and the concentration of Alcian blue was measured at 620 nm. Total optical density of extracted dye was measured at 620 nm and designated as A_{620}. Values are expressed as the mean ± SD of three wells. $^*P < 0.05$ and $^{**}P < 0.01$ versus control as determined by one-way ANOVA. Two independent experiments were performed and gave similar results. Representative pictures of cells stained with Alcian blue are shown in the **upper panel**. Bar, 500 μm. **H**: Northern blot analysis of *Col2a1*. ATDC5 cells were cultured in the presence of insulin and the indicated concentration of DEX for 12 days. Twenty micrograms of total RNA was loaded and hybridized with ^{32}P-labeled *Col2a1* probe. 28S and 18S ribosomal bands stained with ethidium bromide are shown in the **lower panel** as internal controls. Two independent experiments were performed and gave similar results.

bation of ATDC5 cells with insulin and 10^{-8} M DEX, DEX strongly inhibited cellular condensation, and subsequent nodule formation and the ATDC5 cells produced less proteoglycan (Fig. 1B,E). When ATDC5 cells were cultured with insulin, DEX, and RU486, which is an inhibitor of the glucocorticoid receptor, the antichondrogenic effects of DEX were abolished and chondrogenesis was restored, as shown by their morphology and staining with Alcian blue (Fig. 1C,F). Extraction and quantitation of Alcian blue confirmed the dose-dependent, inhibitory effect of DEX on proteoglycan synthesis (Fig. 1G). Northern blot analysis showed that DEX also suppressed type II collagen expression in a dose-dependent manner (Fig. 1H). Insulin-induced cellular condensation was also inhibited by DEX in a concentration-dependent manner (data not shown). These findings indicate that DEX inhibited insulin-induced chondrogenesis of ATDC5 cells by preventing cellular condensation through the glucocorticoid receptor.

DEX Prevented PI3K-Akt Signaling in Insulin-Induced Chondrogenesis of ATDC5 Cells by Downregulating Akt Phosphorylation and the Levels of p85, p110, and Akt Proteins

To investigate the mechanism through which DEX inhibited insulin-induced chondrogenesis of ATDC5 cells, we first examined the effect of DEX on insulin signaling in ATDC5 cells, because PI3K-Akt signaling is involved in chondrogenesis of ATDC5 cells [Hidaka et al., 2001]. The addition of insulin to ATDC5 cell cultures immediately induced the phosphorylation of ERK1/2 and Akt (Fig. 2). Next, ATDC5 cells

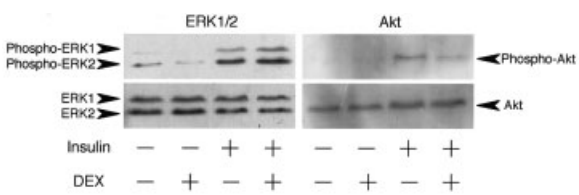


Fig. 2. Effect of DEX on the insulin-induced phosphorylation of ERK1/2 and Akt. After confluency, the medium was changed to FCS-free medium with or without 10^{-8} M DEX, and 24 h later, the cells were stimulated with 10 μg/ml insulin. Western blot analyses were performed. The maximal responses in the phosphorylation of Akt and ERK1/2 were obtained at 30 and at 10 min, respectively, after the insulin treatment in time-course analysis (data not shown). Insulin promoted Akt and ERK1/2 phosphorylation, and DEX reduced the level of insulin-induced Akt phosphorylation but not ERK1/2 phosphorylation. Data are representative of four independent experiments.

were incubated with DEX for 24 h, and then insulin was added to the cell cultures. Incubation with DEX for 24 h had no effect on the level of insulin-induced phosphorylation of ERK1/2, but it reduced the level of insulin-induced Akt phosphorylation (Fig. 2). Semiquantification of the blots showed that the level of Akt phosphorylation in the presence of DEX was one-third of that in the absence of DEX. When ATDC5 cells were treated with insulin, the levels of p85 and p110, which are the subunits of PI3K, increased on day 3 (day 0 was set as the day of insulin stimulation) (Fig. 3A,B). When DEX was added to ATDC5 cell cultures at 24 h before the insulin treatment, however, the levels of p85 and p110 did not increase up through 3 days after insulin treatment. In ATDC5 cell cultures in the absence of DEX, insulin treatment increased the Akt protein level on days 2 and 3. However, when DEX was added to ATDC5 cell cultures at 24 h before the insulin treatment, the Akt protein level did not increase up through 3 days after insulin treatment (Fig. 3C).

DEX Suppressed DNA Binding of Runx2 and Runx2-Dependent Transcription

We examined the involvement of Runx2 in the inhibition of insulin-induced chondrogenesis of ATDC5 cells at an early stage by DEX. When ATDC5 cells were cultured with insulin in the absence or presence of DEX for up to 3 days, there were no significant changes in the levels of Runx2 and Cbfb in the ATDC5 cells. (Fig. 3D,E). We next examined the DNA binding of Runx2 in insulin-treated ATDC5 cells by EMSA. When ATDC5 cells were cultured with insulin for 3 days, the DNA binding of Runx2 increased. However, when ATDC5 cells were first incubated with DEX for 24 h followed by the addition of insulin, the DNA binding of Runx2 gradually decreased after insulin treatment (Fig. 4). Reporter assays using the osteocalcin promoter showed that DEX reduced the level of Runx2-dependent transcriptional activation in a dose-dependent manner (Fig. 5). These findings indicate that DEX suppresses DNA binding of Runx2 and Runx2-dependent transcription during the chondrogenesis of ATDC5 cells.

Overexpression of Akt or Runx2 Abolished the Inhibitory Effect of DEX on the Chondrogenesis of ATDC5 Cells

To examine whether the suppression of either Akt or Runx2 leads to inhibition of

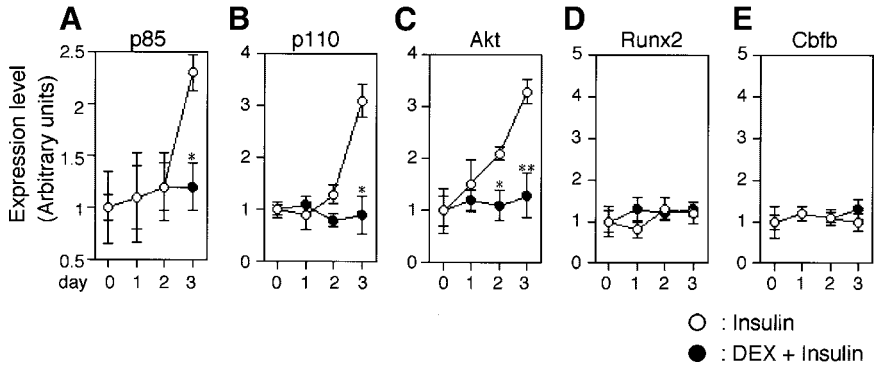


Fig. 3. Effect of DEX on the protein levels of p85, p110, Akt, Runx2, and Cbfb in ATDC5 cells after insulin stimulation. After confluency, ATDC5 cells were cultured in the absence or presence of 10^{-8} M DEX for 24 h, and the cells were stimulated with 10 μg/ml insulin. Samples were prepared on the indicated days of culture (day 0 was set as just before insulin stimulation). Ten micrograms of cell lysates was loaded, and the transferred membrane was incubated with anti-p85 (**A**), anti-p110 (**B**), anti-Akt (**C**), anti-Runx2 (**D**), or anti-Cbfb (**E**) antibodies. Semiquantitative Western blot analyses were performed as described in the ''Materials and Methods.'' The mean value in cells that were maintained in the absence of DEX and insulin on day 0 was defined as 1 and relative values are shown. ○, insulin; ●, insulin plus DEX. The values are mean ± SE of four independent experiments. *Versus no DEX treatment, $*P < 0.05$, $**P < 0.001$ as determined by Student's *t*-test.

chondrogenesis of ATDC5 cells, dn-*Akt* or dn-*Runx2* was adenovirally introduced into ATDC5 cells. Overexpression of dn-*Akt* or dn-*Runx2* in ATDC5 cells severely inhibited proteoglycan production (Fig. 6A). We next examined whether overexpression of Akt can prevent the inhibitory effect of DEX on the chondrogenesis of ATDC5 cells. Adenoviral introduction of *Akt* prevented the inhibition of proteoglycan production by DEX (Fig. 6B). Similarly, adenoviral introduction of *Runx2* prevented the inhibition of proteoglycan production by DEX, and the

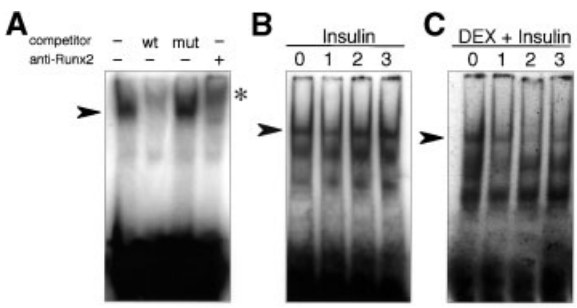


Fig. 4. Electrophoresis mobility shift assay (EMSA). After confluency, ATDC5 cells were cultured in the absence or presence of 10^{-8} M DEX for 24 h, and then the cells were stimulated with 10 μg/ml insulin. Nuclear extracts were prepared on the indicated days of culture (day 0 was set as just before insulin stimulation), and EMSA was performed using OSE2 oligonucleotides. **A**: EMSA using the nuclear extract from the cells on day 3 in the absence of DEX. The specific band (arrowhead) competed with unlabeled OSE oligonucleotides (wt, ×100), but not with mutated oligonucleotides (mut, ×100). Addition of antibody against Runx2 super-shifted the specific band (asterisk). **B**, **C**: EMSA using the nuclear extract from cells that were cultured in the absence of DEX (B) or in the presence of 10^{-8} M DEX (C). The intensity of the specific band (arrowhead) was elevated on day 2 after insulin treatment of cells that were cultured in the absence of DEX (B), while it was reduced on day 1 and lowest on day 2 in cells that were cultured in the presence of DEX (C). Data are representative of three independent experiments.

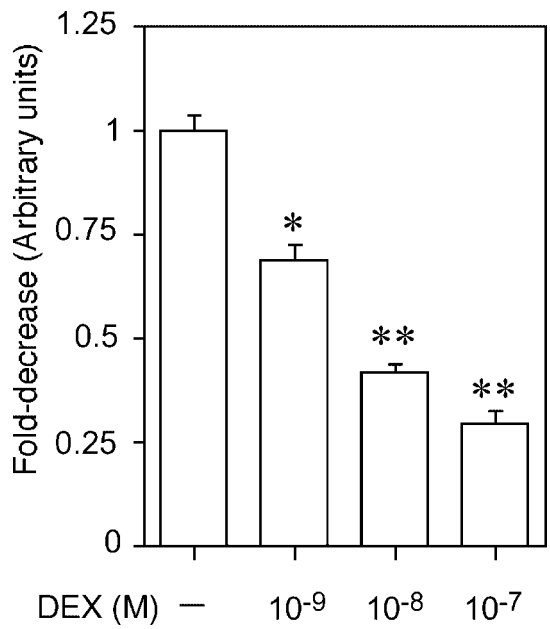
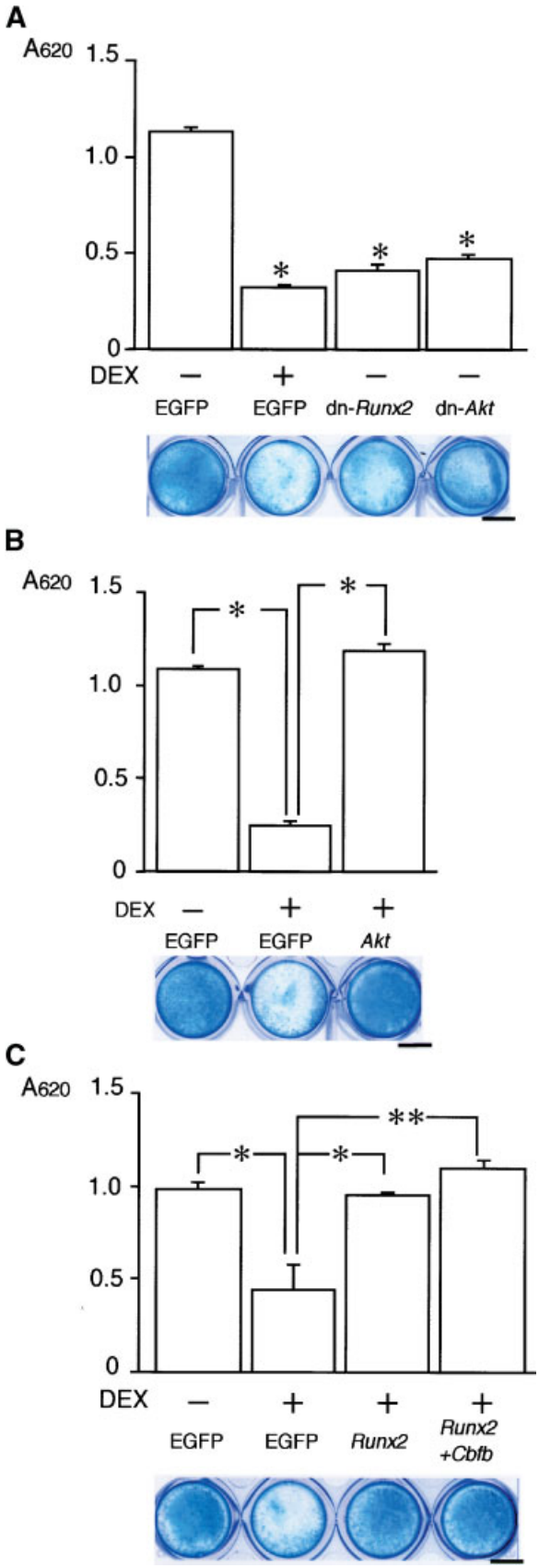


Fig. 5. Reporter assays. The reporter plasmid containing the osteocalcin promoter was co-transfected with *Renilla* luciferase vector into ATDC5 cells that were cultured in medium that did or did not contain the indicated concentration of DEX. One day after transfection, 10 μg/ml insulin was added to the medium, and luciferase activity was measured 24 h later. The luciferase activity was normalized to *Renilla* luciferase activity using the pRL-CMV vector, and calculated data are shown as fold-decrease ± SE. n = 4. *Versus no DEX treatment, $*P < 0.005$, $**P < 0.001$ as determined by Student's *t*-test. Similar results were obtained in three independent experiments and representative data are shown.



introduction of both *Runx2* and *Cbfb* completely restored the proteoglycan production (Fig. 6C). Upon dn-*Akt*- or dn-*Runx2*-expressing adenovirus infection, cellular condensation of the infected ATDC5 cells was severely inhibited. On the other hand, infection of *Akt*- or *Runx2*-expressing adenovirus enhanced cellular condensation (data not shown). These findings indicate that Akt and Runx2 are both involved in the DEX-induced inhibition of chondrogenesis of ATDC5 cells at the early stage.

DISCUSSION

Using the ATDC5 prechondrogenic cell line, which differentiates into chondrocytes when treated with insulin, we investigated the effects of application of a glucocorticoid at the early stage of chondrogenesis. DEX inhibited insulin-induced chondrogenesis of ATDC5 cells at the stage of cellular condensation. DEX inhibited insulin signaling by reducing Akt phosphorylation, and by downregulating the protein levels of the PI3K subunits, p85 and p110, and Akt. Further, DEX suppressed DNA binding of Runx2 and Runx2-dependent transcription without affecting the levels of the Runx2 and Cbfb proteins. Overexpression of dn-*Akt* or dn-*Runx2* severely inhibited insulin-induced chondrogenesis of ATDC5 cells, and the inhibitory effect of DEX on chondrogenesis was prevented by the overexpression of *Akt* or *Runx2*. These

Fig. 6. Adenoviral introduction of dn-*Runx2*, dn-*Akt*, *Akt*, or *Runx2* into ATDC5 cells. ATDC5 cells were plated at 2×10^4 cells/well in 24-well plates and cultured in medium supplemented with 10 μg/ml insulin. After confluence, the cells were cultured in the absence or presence of 10^{-8} M DEX for 2 days, and then infected with enhanced green fluorescent protein (EGFP)-, dn-*Runx2*- and -EGFP-, dn-*Akt*-, *Akt*-, or *Runx2*- and -EGFP-expressing adenovirus at an multiplicity of infection (MOI) of 10 for 12 h. The infected cells were cultured in the absence or presence of 10^{-8} M DEX for 8 days and stained with Alcian blue. After extraction, the concentration of Alcian blue was measured at 620 nm. Total optical density of extracted dye was measured at 620 nm and designated as A_{620}. Values are expressed as the mean ± SD of three wells. **A**: EGFP-, dn-*Runx2*- and -EGFP-, or dn-*Akt*-expressing adenovirus infection. $^*P < 0.001$ versus EGFP-expressing cells without DEX treatment as determined by one-way ANOVA. **B**: EGFP- or *Akt*-expressing adenovirus infection. $^*P < 0.001$. **C**: EGFP- or *Runx2*- and -EGFP-expressing adenovirus infection. For introduction of *Cbfb*, ATDC5 cells were infected with *Cbfb*-expressing retrovirus for 12 h before DEX treatment. $^*P < 0.005$ and $^{**}P < 0.001$. In each experiment, three independent experiments were performed and gave similar results. Representative pictures of cells stained with Alcian blue are also shown. Bars, 500 μm.

findings indicate that DEX inhibits insulin-induced chondrogenesis of ATDC5 cells by suppressing PI3K-Akt signaling and Runx2 activation.

The effect of DEX on the level of the PI3K subunit, p85, has been controversial. Treatment of myoblasts with a high dose of DEX (10^{-5} M) upregulated p85 expression [Singleton et al., 2000], while treatment of adipocytes with DEX (3×10^{-7} M) downregulated it [Burén et al., 2002]. In our analysis, treatment of chondrogenic ATDC5 cells with a physiologically relevant concentration of DEX (10^{-8} M) downregulated the levels of both p85 and p110 (Fig. 3A,B). Therefore, the dosage of DEX and the cell type seem to affect the action of DEX on the PI3K protein level. The inhibitory effects of DEX on the Akt protein level and Akt phosphorylation were consistent, although the time courses of the inhibitory effects of DEX on the PI3K and Akt protein levels differed. Treatment of adipocytes with DEX for 24 h reduced the p85 and Akt protein levels, leading to reduced Akt phosphorylation [Burén et al., 2002]. In the present study on ATDC5 cells, a prechondrogenic cell line, however, incubation with DEX for 24 h prior to the addition of insulin caused a reduction in the level of insulin-induced Akt phosphorylation at 30 min after the start of insulin stimulation, although the protein levels of p85, p110, and Akt were not affected by DEX at this time point (Figs. 2 and 3A–C). Therefore, our results indicate that DEX suppresses Akt phosphorylation without affecting the protein levels of p85, p110, and Akt, and that DEX inhibits PI3K-Akt signaling by reducing Akt phosphorylation as an early effect and by downregulating the p85, p110, and Akt protein levels as a late effect. In accordance with this response pattern, we found no glucocorticoid responsive elements in the promoter regions of the *p85α*, *Akt1*, *Akt2*, and *Akt3* genes by a database search. As DEX reduces the levels of IRS-1 mRNA and protein [Turnbow et al., 1994; Burén et al., 2002] and DEX exerted the anti-chondrogenic effects on ATDC5 cells through glucocorticoid receptor (Fig. 1C,F), the reduction of Akt phosphorylation by DEX may be caused by the transcriptional suppression of *IRS-1* by glucocorticoid–glucocorticoid receptor complexes. Indeed, many molecules interact with Akt and are involved in Akt phosphorylation [Brazil et al., 2002], and it is also possible that DEX modulates Akt phosphorylation by transcriptional regulation of the genes encoding Akt-interacting molecules. The mechanisms through which DEX regulates the p85, p110, and Akt protein levels and Akt phosphorylation need to be further investigated.

It has been shown that DEX reduces the level of Runx2 protein in primary osteoblasts [Chang et al., 1998]. When DEX was applied at an early stage of chondrogenesis to ATDC5 cells, however, it had no effect on the Runx2 and Cbfb protein levels, but it reduced DNA binding of Runx2 and Runx2-dependent transcription (Figs. 3D,E, 4, and 5). Our recent results showed that Runx2 and PI3K-Akt signaling are mutually dependent on each other in the regulation of osteoblast and chondrocyte differentiation [Fujita et al., 2004]. Runx2 upregulated PI3K subunits (p85 and p110β) and Akt, and PI3K-Akt signaling enhanced DNA binding of Runx2 and Runx2-dependent transcription [Fujita et al., 2004]. Therefore, the following cascade may be suggested for the anti-chondrogenic effects of DEX, DEX inhibits Akt phosphorylation; the inhibition of Akt phosphorylation leads to the reduction in Runx2 function; and the reduced Runx2 function decreases PI3K subunits and Akt. MAPK-dependent phosphorylation of Runx2 stimulates Runx2-dependent transcription [Xiao et al., 2000], and protein kinase A also phosphorylates the transactivation domain of Runx2 [Selvamurugan et al., 2000]. In contrast, Runx2 is negatively regulated by serine phosphorylation [Wee et al., 2002]. Therefore, DEX may regulate Runx2 function by phosphorylation or dephosphorylation of Runx2 through PI3K-Akt signaling. However, it is unlikely that PI3K-Akt signaling modulates Runx2 phosphorylation, because the level of Runx2 phosphorylation was not affected by Akt activation [Fujita et al., 2004]. Further, interactions with other transcription factors such as Ets, Smads, and C/EBP, with the transcription cofactor Rb, and with the transcriptional repressor TLE and YAP greatly influence Runx2 function [Sato et al., 1998; Javed et al., 2000; McCarthy et al., 2000; Zhang et al., 2000; Thomas et al., 2001; Gutierrez et al., 2002; Zaidi et al., 2004]. Therefore, it is also possible that DEX regulates Runx2 function by regulating one of these interacting proteins. Moreover, DEX may regulate the molecules that are related to the nuclear import and subnuclear localization of Runx2, because the fidelity of the subnuclear localization of Runx2 mediated by a

nuclear-matrix-targeting signal is required for Runx2 function [Choi et al., 2001]. These possibilities remain to be investigated.

In contrast to our results, 10^{-8} M DEX enhanced cartilaginous nodule formation in another chondrogenic cell line, RCJ 3.1C5 [Grigoriadis et al., 1989]. Recently, however, similar to our results, it was reported that a high dose of DEX (10^{-6} M) inhibited insulin-induced chondrogenesis of ATDC5 cells, although the mechanism for the inhibition was not resolved [Siebler et al., 2002]. Our findings suggest that the effect of glucocorticoids applied at the early stage of chondrogenesis varies in different types of cells depending on the signaling pathway for cellular condensation, and that glucocorticoids effectively inhibit cellular condensation in cells that are dependent on insulin/IGF signaling and Runx2 for their cellular condensation.

ACKNOWLEDGMENTS

We thank K. Walsh for the *Akt*-expressing and dn-*Akt*-expressing adenovirus vectors, Y. Ito for the Runx2 antibody, S. Sumitani for helpful discussion, and M. Yanagita for secretarial assistance.

REFERENCES

Akiyama H, Hiraki Y, Sigeno C, Kohno H, Shukunami C, Tuboyama T, Kasai R, Suzuki F, Konishi J, Nakamura T. 1996. 1,25-Dihydroxyvitamin D3 inhibits cell growth and chondrogenesis of a clonal mouse EC cell line, ATDC5. J Bone Miner Res 11:22–27.

Akiyama H, Kanno T, Ito H, Terry A, Neil J, Ito Y, Nakamura T. 1999. Positive and negative regulation of chondrogenesis by splice variants of PEBP2αA/CBFα1 in clonal mouse EC cells, ATDC5. J Cell Physiol 181:169–178.

Atsumi T, Miwa Y, Kimata K, Ikawa Y. 1990. A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells. Cell Diff Dev 30:109–116.

Beato M. 1989. Gene regulation by steroid hormones. Cell 56:335–344.

Brazil DP, Park J, Hemmings BA. 2002. PKB binding proteins: Getting in on the Akt. Cell 111:293–303.

Burén J, Liu HX, Jensen J, Eriksson JW. 2002. Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase, and protein kinase B in primary cultured rat adipocytes. Eur J Endocrinol 146:419–429.

Calcagno M, Goyena H, Arrambide E, Arruti de Urse C. 1970. Action of cortisone and cortisol upon biosynthesis of chondroitin sulfate in femur in vitro cultures of chick embryo. Exp Cell Res 63:131–137.

Cantley LC. 2002. The phosphoinositide 3-kinase pathway. Science 296:1655–1657.

Chang DJ, Ji C, Kim KK, Casinghino S, McCarthy TL, Centrella M. 1998. Reduction in transforming growth factor β receptor I expression and transcription factor CBFa1 on bone cells by glucocorticoid. J Biol Chem 273: 4892–4896.

Choi JY, Pratap J, Javed A, Zaidi SK, Xing L, Balint E, Dalamangas S, Boyce B, van Wijnen AJ, Lian JB, Stein JL, Jones SN, Stein GS. 2001. Subnuclear targeting of Runx/Cbfa/AML factors is essential for tissue-specific differentiation during embryonic development. Proc Natl Acad Sci USA 98:8650–8655.

Enomoto H, Enomoto-Iwamoto M, Iwamoto M, Nomura S, Himeno M, Kitamura Y, Kishimoto T, Komori T. 2000. Cbfa1 is a positive regulatory factor in chondrocyte maturation. J Biol Chem 275:86695–86702.

Enomoto H, Shiojiri S, Hoshi K, Furuichi T, Fukuyama R, Yoshida C, Kanatani N, Nakamura R, Mizuno A, Zanma A, et al. 2003. Induction of osteoclast differentiation by Runx2 through RANKL and OPG regulation and partial rescue of osteoclastogenesis in Runx2−/−mice by RANKL transgene. J Biol Chem 278:23971–23977.

Enomoto H, Furuichi T, Zanma A, Yamana K, Yoshida C, Sumitani S, Yamamoto H, Enomoto-Iwamoto M, Iwamoto M, Komori T. 2004. Runx2 deficiency in chondrocytes causes adipogenic changes in vitro. J Cell Sci 117:417–425.

Fujio Y, Walsh K. 1999. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. J Biol Chem 274: 16349–16354.

Fujita T, Azuma Y, Fukuyama R, Hattori Y, Yoshida CA, Koida M, Ogita K, Komori T. 2004. Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. J Cell Biol (in press).

Grigoriadis AE, Aubin JE, Heersche JN. 1989. Effects of dexamethasone and vitamin D3 on cartilage differentiation in a clonal chondrogenic cell population. Endocrinology 125:2103–2110.

Grigoriadis AE, Heersche JN, Aubin JE. 1996. Analysis of chondroprogenitor frequency and cartilage differentiation in a novel family of clonal chondrogenic rat cell lines. Differentiation 60:299–307.

Gutierrez S, Javed A, Tennant DK, van Rees M, Montecino M, Stein GS, Stein JL, Lian JB. 2002. CCAAT/enhancer-binding proteins (C/EBP) β and δ activate osteocalcin gene transcription and synergize with Runx2 at the C/EBP element to regulate bone-specific expression. J Biol Chem 277:1316–1323.

Hickok NJ, Haas AR, Tuan RS. 1998. Regulation of chondrocyte differentiation and maturation. Microsc Res Tech 43:174–190.

Hidaka K, Kanematsu T, Takeuchi H, Nakata M, Kikkawa U, Hirata M. 2001. Involvement of the phosphoinositide 3-kinase/protein kinase B signaling pathway in insulin/IGF-I-induced chondrogenesis of the mouse embryonal carcinoma-derived cell line ATDC5. Int J Biochem Cell Biol 33:1094–1103.

Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, Himeno M, Sato M, Yamagiwa H, Kimura T, Yasui N, et al. 1999. Maturational disturbance of chondrocytes in Cbfa1-deficient mice. Dev Dyn 214:279–290.

Itagane Y, Inada H, Fujita K, Isshiki G. 1991. Interactions between steroid hormones and insulin-like growth factor-I in rabbit chondrocytes. Endocrinology 128:1419–1424.

Javed A, Guo B, Hiebert S, Choi JY, Green J, Zhao SC, Osborne MA, Stifani S, Stein JL, Lian JB, et al. 2000. Groucho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (CBFα/AML/PEBP2α) dependent activation of tissue-specific gene transcription. J Cell Sci 113:2221–2231.

Jones KL, Addison J. 1975. Pituitary fibroblast growth factor as a stimulator of growth in cultured rabbit articular chondrocytes. Endocrinology 97:359–365.

Kato Y, Gospodarowicz D. 1985. Stimulation by glucocorticoid of the synthesis of cartilage-matrix proteoglycans produced by rabbit costal chondrocytes in vitro. J Biol Chem 260:2364–2373.

Kim IS, Otto F, Zabel B, Mundlos S. 1999. Regulation of chondrocyte differentiation by Cbfa1. Mech Dev 80:159–170.

Komori T. 2002. Runx2, a multifunctional transcription factor in skeletal development. J Cell Biochem 87:1–8.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao Y, Inada M, et al. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Kundu M, Javed A, Jeon JP, Horner A, Shum L, Eckhaus M, Muenk M, Lian JB, Yang Y, Nuckolls GH, et al. 2002. Cbfβ interacts with Runx2 and has a critical role in bone development. Nat Genet 32:639–644.

McCarthy TL, Ji C, Chen Y, Kim KK, Imagawa M, Ito Y, Centrella M. 2000. Runt domain factor (Runx)-dependent effects on CCAAT/enhancer-binding protein δ expression and activity in osteoblasts. J Biol Chem 275:21746–21753.

Miller J, Horner A, Stacy T, Lowrey C, Lian JB, Stein G, Nuckolls GH, Speck N. 2002. The core-binding factor b subunit is required for bone formation and hematopoietic maturation. Nat Genet 32:645–649.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GWH, Beddington RSP, Mundlos S, Olsen BR, et al. 1997. *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771.

Quarto R, Campanile G, Cancedda R, Dozin B. 1992. Thyroid hormone, insulin, and glucocorticoids are sufficient to support chondrocyte differentiation to hypertrophy: A serum-free analysis. J Cell Biol 119:989–995.

Sato M, Morii E, Komori T, Kawahata H, Sugimoto M, Terai K, Shimizu H, Yasui T, Ogihara H, Yasui N, et al. 1998. Transcriptional regulation of osteopontin gene in vivo by PEBP2A/CBFA1 and ETS1 in the skeletal tissues. Oncogene 17:1517–1525.

Selvamurugan N, Pulumati MR, Tyson DR, Partridge NC. 2000. Parathyroid hormone regulation of the rat collagenase-3 promoter by protein kinase A-dependent transactivation of core binding factor a1. J Biol Chem 275:5037–5042.

Shukunami C, Sigeno C, Atsumi T, Ishizeki K, Suzuki F, Hiraki Y. 1996. Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: Differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor. J Cell Biol 133: 457–468.

Siebler T, Robson H, Shalet SM, Williams GR. 2002. Dexamethasone inhibits and thyroid hormone promotes differentiation of mouse chondrogenic ATDC5 cells. Bone 31:457–464.

Silbermann M, Maor G. 1985. Receptor-mediated glucocorticoid inhibition of cell proliferation in mouse growth cartilage in vitro. Acta Endocrinol (Copenh) 108:343–350.

Singleton JR, Baker BL, Thorburn A. 2000. Dexamethasone inhibits insulin-like growth factor signaling and potentiates myoblast apoptosis. Endocrinology 141: 2945–2950.

Stricker S, Fundele R, Vortkamp A, Mundlos S. 2002. Role of *Runx* genes in chondrocyte differentiation. Dev Biol 245:95–108.

Takeda S, Bonnamy JP, Owen MJ, Ducy P, Karsenty G. 2001. Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. Genes Dev 15:467–481.

Takigawa M, Takano T, Nakagawa K, Sakuda M, Suzuki F. 1988. Hydrocortisone stimulation of proliferation and glycosaminoglycan synthesis in rabbit craniofacial chondrocytes in vitro. Arch Oral Biol 33:893–899.

Thirunavukkarasu K, Mahajan M, Mclarren KW, Stifani S, Karsenty G. 1998. Two domains unique to osteoblast-specific transcription factor Osf2/Cbfa1 contribute to its transactivation function and its inability to heterodimerize with Cbfβ. Mol Cell Biol 18:4197–4208.

Thomas DM, Carty SA, Piscopo DM, Lee JS, Wang WF, Forrester WC, Hinds PW. 2001. The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell 8:303–316.

Thorogood PV, Hinchliffe JR. 1975. An analysis of the condensation process during chondrogenesis in the embryonic chick hind limb. J Embryol Exp Morphol 33:581–606.

Turnbow MA, Keller SR, Rice KM, Garner CW. 1994. Dexamethasone down-regulation of insulin receptor substrate-1 in 3T3-L1 adipocytes. J Biol Chem 269:2516–2520.

Ueta C, Iwamoto M, Kanatani N, Yoshida C, Liu Y, Enomoto-Iwamoto M, Ohmori T, Enomoto H, Nakata K, Takada K, et al. 2001. Skeletal malformations caused by overexpression of Cbfa1 or its dominant negative form in chondrocytes. J Cell Biol 153:87–99.

Wee HJ, Huang G, Shigesada K, Ito Y. 2002. Serine phosphorylation of RUNX2 with novel potential functions as negative regulatory mechanisms. EMBO Rep 3: 967–974.

Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, Franceschi RT. 2000. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem 275:4453–4459.

Yoshida CA, Furuichi T, Fujita T, Fukuyama R, Kanatani N, Kobayashi S, Satake M, Takada K, Komori T. 2002. Core-binding factor β interacts with Runx2 and is required for skeletal development. Nat Genet 32:633–638.

Zaidi SK, Sullivan AJ, Medina R, Ito Y, Van Wijnen AJ, Stein JL, Lian JB, Stein GS. 2004. Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. EMBO J 23:790–799.

Zhang YW, Yasui N, Ito K, Huang G, Fujii M, Hanai J, Nogami H, Ochi T, Miyazono K, Ito Y. 2000. A RUNX2/PEBP2αA/CBFA1 mutation displaying impaired transactivation and Smad interaction in cleidocranial dysplasia. Proc Natl Acad Sci USA 97:10549–10554.