Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling

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Runx2 and phosphatidylinositol 3-kinase (PI3K)–Akt signaling play important roles in osteoblast and chondrocyte differentiation. We investigated the relationship between Runx2 and PI3K-Akt signaling. Forced expression of Runx2 enhanced osteoblastic differentiation of C3H10T1/2 and MC3T3-E1 cells and enhanced chondrogenic differentiation of ATDC5 cells, whereas these effects were blocked by treatment with IGF-I antibody or LY294002 or adenoviral introduction of dominant-negative (dn)–Akt. Forced expression of Runx2 or dn-Runx2 enhanced or inhibited cell migration, respectively, whereas the enhancement by Runx2 was abolished by treatment with LY294002 or adenoviral introduction of dn-Akt. Runx2 up-regulated PI3K subunits (p85 and p110β) and Akt, and their expression patterns were similar to that of Runx2 in growth plates. Treatment with LY294002 or introduction of dn-Akt severely diminished DNA binding of Runx2 and Runx2-dependent transcription, whereas forced expression of myrAkt enhanced them. These findings demonstrate that Runx2 and PI3K-Akt signaling are mutually dependent on each other in the regulation of osteoblast and chondrocyte differentiation and their migration.

Introduction

The formation of bone structures in vertebrates involves osteoblast and chondrocyte differentiation. Osteoblast and chondrocyte differentiation are regulated by many secreted differentiation factors including TGFβs, bone morphogenetic proteins, insulin-like growth factors (IGFs), FGFs, parathyroid hormone (PTH), PTH-related peptide, thyroid hormone, Indian hedgehog, and retinoic acid (Karaplis, 2002). Furthermore, transcription factors play fundamental roles in osteoblast and chondrocyte differentiation. Runx2 (runt-related transcription factor 2)/Cbfa1 (core binding factor 1)/Pebp2αA (polyoma enhancer binding protein 2αA) and Osterix are essential for osteoblast differentiation (Komori et al., 1997, Otto et al., 1997, Nakashima et al., 2002); Sox5, Sox6, and Sox9 are essential for chondrocyte differentiation (Bi et al., 1999, Smits et al., 2001); and Runx2 plays an important role in terminal chondrocyte differentiation (Enomoto et al., 2000, Ueta et al., 2001).

Runx2 is a transcription factor that belongs to the Runx family (Komori 2002). Runx2-deficient (Runx2−/−) mice completely lack bone formation owing to the absence of osteoblasts (Komori et al., 1997, Otto et al., 1997). Sox5, Sox6, and Sox9 are essential for chondrocyte differentiation (Bi et al., 1999, Smits et al., 2001); and Runx2 plays an important role in terminal chondrocyte differentiation (Enomoto et al., 2000, Ueta et al., 2001).

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dominant-negative (dn) form of Runx2 (dn-Runx2) in chondrocytes accelerates or decelerates chondrocyte maturation, respectively, indicating that Runx2 is a positive regulatory factor in chondrocyte maturation (Ueta et al., 2001). Further, introduction of dn-Runx2 inhibited cell condensation in insulin-induced chondrogenesis of ATDC5 cells (Akiyama et al., 1999). Thus, Runx2 plays crucial roles in osteoblast and chondrocyte differentiation (Komori 2002).

Mutant mice that lack both Igf1 and Igf2 and mice that lack the Igf1r show severe retardation of bone development, and both Irs1-deficient mice and Irs2-deficient mice show an osteopenic phenotype (Liu et al., 1993, Ogata et al., 2000, Akune et al., 2002). Therefore, IGFs and their signaling molecules play important roles in skeletal development by regulating osteoblast and chondrocyte differentiation. Akt is a serine-threonine kinase whose amino terminus contains a pleckstrin homology, and is activated by various extracellular stimuli including insulin and IGF through the phosphatidylinositol 3-kinase (PI3K) pathway (Scheid and Woodgett, 2001). In various cell culture systems, PI3K-Akt signaling has been implicated as a critical pathway for the differentiation of skeletal component cells including chondrocytes, osteoblasts, myoblasts, and adipocytes (Kaliman et al., 1996, Sakae et al., 1998, Hidaka et al., 2001, Ghosh-Choudhury et al., 2002). Further, bone development is severely delayed in mice lacking both Akt1 and Akt2 (Peng et al., 2003). However, the mechanism of the differentiation of skeletal component cells mediated by PI3K-Akt signaling remains to be clarified.

PDGF, IGF, and VEGF work as chemotactic factors through PI3K, and PI3K-Akt signaling is a major pathway for chemotaxis through G-protein–coupled receptors in Dicystostelium discoideum and in neutrophils and through tyrosine kinase receptors in fibroblasts. After activation of PI3K at the leading edge, Akt rapidly accumulates by binding to PtdIns(3,4,5)P3 via its pleckstrin homology domain, leading to activation of Akt by phosphorylation. Akt likely mediates cell migration at least partly by activating Rac and p21-activated protein kinase (Ridley et al., 2003).

Although Runx2 is an important transcription factor for osteoblast and chondrocyte differentiation, and PI3K-Akt signaling is deeply involved in the differentiation of skeletal component cells, the relationship between Runx2 and PI3K-Akt signaling is not known. In this study, we investigated the involvement of PI3K-Akt signaling in the function of Runx2 using immature mesenchymal C3H10T1/2 cells, immature osteoblastic MC3T3-E1 cells, and prechondrogenic ATDC5 cells, and found that Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling.

Results

PI3K-Akt signaling is involved in Runx2-dependent osteoblast differentiation

To elucidate if PI3K-Akt signaling is involved in Runx2-dependent osteoblast and chondrocyte differentiation, we established Runx2 or dn-Runx2 stable transfectants from an immature osteoblastic cell line, MC3T3-E1, an immature mesenchymal cell line, C3H10T1/2, and an insulin-dependent prechondrogenic cell line, ATDC5 (Fig. 1, A and B). The differentiation of MC3T3-E1 cells was enhanced in Runx2 stable transfectants but inhibited in dn-Runx2 stable transfectants in comparison with that in the wild-type MC3T3-E1 cells, as indicated by the levels of ALP activity and calcium deposition (Fig. 2, A and B). MC3T3-E1 cells secrete IGF-I (Huang et al., 2000). The Runx2-induced enhancement of ALP activity and mineralization was blocked by treatment with either a neutralizing antibody against IGF-I or a PI3K inhibitor, LY294002, in a dose-dependent manner (Fig. 2 C), indicating that IGF-I receptor-PI3K signaling is required for Runx2 activity in MC3T3-E1 cells. Further, adenoviral introduction of dn-Akt blocked Runx2-induced ALP activity and mineralization (Fig. 2 D). ALP activity was also induced in Runx2 stable transfectants from C3H10T1/2 cells, whereas it was abrogated by adenoviral introduction of dn-Akt (Fig. 2 E). These findings indicate that PI3K-Akt signaling is involved in Runx2-dependent osteoblast differentiation.
PI3K-Akt signaling is involved in Runx2-dependent chondrocyte differentiation

We investigated whether or not PI3K-Akt signaling is involved in Runx2-dependent chondrocyte differentiation using ATDC5 cells. ATDC5 cells express Col1a1 until the cells reach confluence. After confluence, ATDC5 cells condensate and show chondrogenic characteristics including proteoglycan synthesis and Col2a1 expression in the presence of insulin (Shukunami et al., 1996). Further, ATDC5 cells mature to hypertrophic chondrocytes, which express Col10a1 (Enomoto et al., 2000). Adenoviral introduction of Runx2 promoted proteoglycan synthesis and Col2a1 expression and then Col10a1 expression, whereas treatment with LY294002 completely prevented proteoglycan production and Col2a1 and Col10a1 expression in Runx2-overexpressing cells (Fig. 3). Further, adenoviral introduction of dn-Akt attenuated the Runx2-enhanced proteoglycan production and Col2a1 and Col10a1 expression (Fig. 3). These findings indicate that PI3K-Akt signaling is involved in Runx2-induced chondrocyte differentiation of ATDC5 cells.

Runx2 controls cell migration through PI3K-Akt signaling

When PI3K-Akt signaling was inhibited, Runx2-dependent osteoblast and chondrocyte differentiation were abol-
ished. Therefore, we examined cell migration, which is controlled by PI3K-Akt signaling, in chemotaxis assays using Runx2 or dn-Runx2 stable transfectants from MC3T3-E1 cells. Random migration was enhanced in the Runx2 stable transfectants both in the absence or presence of a chemotactic factor, PDGF, compared with that in wild-type MC3T3-E1 cells, whereas it was suppressed in the dn-Runx2 stable transfectants, indicating that Runx2 enhances cell motility (Fig. 4, A and B). Further, PDGF-induced chemotaxis was enhanced in the Runx2 stable transfectants compared with that in the wild-type cells, whereas it was suppressed in the dn-Runx2 stable transfectants. Similar results were obtained using Runx2 or dn-Runx2 stable transfectants from C3T10T1/2 cells and ATDC5 cells.
cells (unpublished data). Treatment with LY294002 completely abolished the PDGF-induced chemotaxis of Runx2 stable transfectants (Fig. 4 C). Indeed, PDGF-induced phosphorylation of Akt in wild-type MC3T3-E1, C3T10T1/2, and ATDC5 cells, which were inhibited by treatment with LY294002 (Fig. 4 D and not depicted). Adenoviral introduction of dn-Akt into Runx2 stable transfectants abolished the enhancement of chemotaxis induced by Runx2 in the cell migration assay (Fig. 4 E). Similar results were also obtained using MC3T3-E1 cells infected with Runx2-expressing adenovirus (unpublished data). These findings indicate that Runx2 enhances cell migration through PI3K-Akt signaling.

**Runx2 up-regulates PI3K and Akt protein levels**

To investigate whether or not Runx2 affects PI3K-Akt signaling, we examined the expression levels of the PI3K subunits, p85 and p110, and Akt in Runx2 or dn-Runx2 stably transfected cells by Western blot analysis (Fig. 5). Overexpression of Runx2 up-regulated the levels of p85, p110β, and Akt in C3H10T1/2, MC3T3-E1, and ATDC5 cells. Further, phosphorylated Akt was increased in parallel with the level of total Akt protein. In contrast, overexpression of dn-Runx2 down-regulated the levels of p85, p110β, and Akt proteins in MC3T3-E1 cells and ATDC5 cells. There were no differences in the levels of p110α and p110β proteins among the wild-type cells, Runx2 stable transfectants and dn-Runx2 stable transfectants in the C3H10T1/2 cells, MC3T3-E1 cells, and ATDC5 cells. p110γ was up-regulated only in the Runx2 stably transfected MC3T3-E1 cells compared with the respective wild-type cells. These findings indicate that Runx2 positively regulates PI3K-Akt signaling by up-regulating the p85, p110β, and Akt protein levels in immature mesenchymal cells, immature osteoblastic cells, and prechondrogenic cells.

We next examined if up-regulation of p85, p110β, and Akt protein levels was due to the transcriptional regulation by RT-PCR analysis. Overexpression of Runx2 up-regulated mRNA for p110β in C3H10T1/2, MC3T3-E1, and ATDC5 cells, whereas overexpression of dn-Runx2 down-regulated mRNA for p110β in MC3T3-E1 and ATDC5 cells. mRNAs for p85β, Akt1, Akt2, and Akt3 were also mildly up-regulated by overexpression of Runx2, but mRNAs for p85β, Akt1, Akt2 were subtly down-regulated by overexpression of dn-Runx2. In p85α, there was no transcriptional regulation by Runx2. Therefore, these findings indicate that p110β is mainly regulated by Runx2 at transcriptional level, whereas p85 and Akt are regulated by Runx2 at both transcriptional and protein levels.

As the levels of p85, p110β, and Akt proteins were up-regulated by Runx2, their expression patterns in tibial growth plates were examined in wild-type mouse embryos at E16.5 by immunohistochemistry. The p85, p110β, and Akt protein levels were up-regulated at the stage of prehypertrophic chondrocytes, which express Pthr1, and the up-regulation was maintained in hypertrophic chondrocytes, which express Col10a1 (Fig. 6). The Runx2 protein level was also up-regulated at the stage of prehypertrophic chondrocytes (Fig. 6: Inada et al., 1999; Kim et al., 1999; Enomoto et al., 2000). Therefore, we also examined p85, p110β, and Akt expression in the tibial growth plates of dn-Runx2 transgenic embryos under the control of the Col2a1 promoter (Ueta et al., 2001) at E18.5. Up-regulation of p85, p110β, and Akt protein levels was not ob-
PI3K-Akt signaling enhances DNA binding of Runx2 and Runx2-dependent transcription

We found that treatment of Runx2 stable transfectants from C3H10T1/2 cells with U0126, a MEK inhibitor, or LY294002 inhibited ALP activity in a dose-dependent manner (Fig. 7 A). To investigate how PI3K-Akt signaling is involved in the activity of Runx2, we examined the effects of LY294002 on the DNA binding of Runx2 and Runx2-dependent transcription, and compared them with the effects of U0126. On electrophoretic mobility shift assay (EMSA) using OSE2 oligonucleotides that contain a Runx binding site, nuclear extracts from Runx2 stable transfectants of C3H10T1/2 cells formed specific Runx2-Cbfb-DNA complexes (Fig. 7 B). However, treatment of the Runx2 stable transfectants with LY294002 for 30 min drastically reduced the DNA-binding capacity of Runx2 to less than one-tenth (Fig. 7, B and C). In contrast, treatment of the cells with U0126 for 30 min had no effect on the DNA-binding capacity of Runx2 (Fig. 7, B), although prolonged treatment for 24 h gradually reduced the DNA binding of Runx2 up to one-fourth (Fig. 7 C). Treatment with LY294002 or U0126 did not affect the level of Runx2 protein in the Runx2 stable transfectants (Fig. 7 B). In reporter assays using the osteocalcin promoter, treatment with either LY294002 or U0126 for 48 h severely reduced the level of Runx2-dependent transcription in Runx2 stable transfectants from C3H10T1/2 cells (Fig. 7 D). Further, chromatin immunoprecipitation assays showed that treatment with LY294002 prevented the binding of Runx2 to endogenous osteocalcin promoter in wild-type MC3T3-E1 cells (Fig. 7 E). These findings indicate that the DNA-binding capacity of Runx2 and transcriptional activation by Runx2 are dependent on the activities of both PI3K and MEK, but the mechanisms through which PI3K and MEK regulate Runx2 function are quite different.

As the treatment with anti–IGF-I antibody severely inhibited osteoblastic differentiation of MC3T3-E1 cells (Fig. 2 C), we further examined the effect of the treatment with anti–IGF-I antibody in the DNA binding of Runx2. The DNA binding of Runx2 was diminished by the treatment with anti–IGF-I antibody (Fig. 7 F). However, overexpression of Runx2 failed to induce Igf1 (Fig. 7 G). These findings indicate that Runx2 is not involved in Igf1 induction, but IGF-I signaling plays an important role in Runx2-dependent osteoblastic differentiation of MC3T3-E1 cells.

Next, we examined the DNA binding of Runx2 and Runx2-dependent transcription in myrAkt and dn-Akt stable transfectants. DNA binding of Runx2 was strongly enhanced in myrAkt stable transfectants of MC3T3-E1 cells compared with that in wild-type MC3T3-E1 cells (Fig. 8 A). In reporter assays using the osteocalcin promoter, Runx2-dependent transcription was strongly enhanced in the myrAkt stable transfectants, but was absent in dn-Akt stable transfectants of MC3T3-E1 cells (Fig. 8 B). However, overexpression of myrAkt or dn-Akt did not affect the protein levels of Runx2 or Cbfb (Fig. 8 C). Adenoviral introduction of dn-Akt in Runx2 stable transfectants of C3H10T1/2 cells severely reduced the DNA binding of Runx2 (Fig. 8 D). In reporter assays using the osteocalcin promoter, introduction of dn-Akt in Runx2 stable trans-
fectants of C3H10T1/2 cells dose dependently inhibited Runx2-dependent transcription, which was similar to the effect of dn-Runx2 (Fig. 8 E). These findings indicate that PI3K-Akt signaling plays an important role in the DNA binding of Runx2 and transcriptional activation by Runx2.

We further examined whether or not Akt is involved in the phosphorylation of Runx2 by immunoprecipitation assays using anti-Runx2 antibody in myrAkt-transfected ATDC5 cells. The phosphorylation levels of tyrosine, serine, and threonine in Runx2 were similar between wild-type and myrAkt-transfected ATDC5 cells (Fig. 8 F). Further, a similar level of Runx2 was detected in wild-type and myrAkt-transfected ATDC5 cells in the immunoprecipitation assays using antiphosphotyrosine, antiphosphoserine, or antiphosphothreonine antibody (unpublished data). These findings suggest that phosphorylation of Runx2 is not a major mechanism for the activation of Runx2 by Akt.

**Discussion**

We showed that PI3K-Akt signaling is deeply involved in Runx2-dependent osteoblast and chondrocyte differentiation and their migration. Runx2 enhanced PI3K-Akt signaling by up-regulating the protein levels of PI3K subunits and...
Akt signaling regulates DNA binding of Runx2 and Runx2-dependent transcription. The activation of PI3K-Akt signaling through the activation of PI3K-Akt signaling regulates the DNA-binding activity of Runx2 by phosphorylation of some molecules that form a DNA-binding complex with Runx2 or their dephosphorylation through the activation of phosphatase, because interactions with other transcription factors such as Ets, Smads, and C/EBP, with the transcriptional repressor TLE greatly influence the activity of Runx2 (Komori, 2002). However, the mechanism of the phosphorylation or dephosphorylation of DNA-binding complex molecules containing Runx2 by PI3K-Akt signaling should be different from that by the MAPK pathway because the time courses of the inhibition of the DNA-binding activity of Runx2 were quite different between the treatments with LY294002 and U0126 (Fig. 7 C).

In accordance with our findings, bone development in Igf1/ Igf2 mutant mice, Igf1r mutant mice, and Akt1/Akt2 mutant mice is severely delayed (Liu et al., 1993, Peng et al., 2003). However, the delay in bone development in these mutant mice is still milder than that in Runx2−/− mice (Komori et al., 1997, Otto et al., 1997), suggesting that stimulation with other ligands such as VEGF in addition to IGFs may also be involved in the activation of PI3K-Akt signaling, and that Akt3 in addition to Akt1 and Akt2 may function in osteoblast and chondrocyte differentiation. However, in the case of MC3T3-E1 cells it is likely that IGF-I is a major ligand for the activation of PI3K-Akt signaling and Runx2 function and
that autocrine stimulation by IGF-I is important for differentiation because treatment with anti-IGF-I antibody severely inhibited the osteoblastic differentiation induced by Runx2 and DNA binding of Runx2 (Fig. 2 C and Fig. 7 F).

In growth plates, proliferating chondrocytes begin to mature to prehypertrophic chondrocytes and further mature to hypertrophic chondrocytes. In this maturation process, the Runx2, p85, p110β, and Akt protein levels were all up-regulated at the stage of prehypertrophic chondrocytes, and the up-regulation was maintained during the hypertrophic stage (Fig. 6). Further, Runx2 up-regulated the p85, p110β, and Akt protein levels (Fig. 5), and PI3K-Akt signaling activated Runx2 function (Figs. 7 and 8). Therefore, the positive feedback loop of Runx2 and PI3K-Akt signaling is likely to play important roles in the maturational process of chondrocytes at the growth plates.

We showed that Runx2 is involved in cell migration. Runx2 increased chemotaxis of C3H10T1/2, MC3T3-E1, and ATDC5 cells, and it was abrogated by treatment with LY294002 or the introduction of dn-Akt (Fig. 4 and not depicted), indicating that Runx2 induces cell migration through PI3K-Akt signaling. However, as IGF-I has no significant effect on the cell migration of MC3T3-E1 cells (Fukuyama et al., 2004), activation of another signaling pathway in addition to PI3K-Akt signaling pathway may be required for the cell migration, and Runx2 may be involved in the activation of both signaling pathways. The significance of chemotaxis in bone and cartilage formation has not been shown. However, as Runx2 is expressed in the precursors of osteoblasts and chondrocytes (Ducy et al., 1997, Otto et al., 1997), Runx2 may play a role in the migration of these precursors to the appropriate sites during skeletal development. Runx2 may also play a role in bone remodeling by inducing the migration of osteoblasts to the surface of bone that has undergone osteolysis by osteoclasts. As Runx2 expression is strongly induced in osteoblastic cells after bone fracture (Kawahata et al., 2003), chemotaxis enhanced by Runx2 should be important for the migration of osteoblastic cells to the healing area. Indeed, after a bone fracture, the positive feedback loop of Runx2 and PI3K-Akt signaling would play important roles in multiple healing processes including the migration of chondrogenic and osteoblastic cells and their precursors to the healing place, chondrocyte maturation that leads to the replacement of cartilage with bone, and osteoblast differentiation.

We showed the linkage of Runx2 and the PI3K-Akt signaling pathway in osteoblast and cartilage differentiation and their migration. However, PI3K-Akt signaling is involved in multiple cell functions including cell proliferation, apoptosis, cell growth, and glucose metabolism in addition to cell differentiation and migration. Therefore, the cell phenotypes resulting from the coupling of Runx2 and PI3K-Akt signaling may be more complex, and they need to be further investigated.

**Materials and methods**

**Cell cultures**

C3H10T1/2 and ATDC5 cells were purchased from RIKEN Cell Bank. MC3T3-E1 subclone 4 was a gift from R.T. Franceschi (University of Michigan School of Dentistry, Ann Arbor, MI; Xiao et al., 2000). C3H10T1/2 cells were cultured in BME and MC3T3-E1 cells were cultured in α-MEM containing 10% FBS (GIBCO BRL). ATDC5 cells were cultured as previously described (Enomoto et al., 2000). Cells were treated with anti–IGF-I antibody (Upstate Biotechnology), LY294002 (Calbiochem), or U0126 (Calbiochem). The percentages of dead cells, which were less than 3% by a trypan blue exclusion assay, were not significantly different among all of the experiments.

**Establishment of stably transfected cells**

To generate a dn-Runx2-expressing vector, a 421-bp DNA fragment containing the runt domain of Runx2 was subcloned into pSG5 (Stratagene). The vectors, myrAkt, which has the c-Src myristoylation sequence fused in frame to the NH₂ terminus of the Akt coding sequence, and dn-Akt (T308A, S473A), were gifts from K. Walsh (St. Elizabeth’s Medical Center, Boston, MA; Fujio et al., 1999). The day before transfection, cells were plated on 35-mm dishes at a density of 10⁴ cells per milliliter. Runx2-expressing vector (Harada et al., 1999), dn-Runx2-expressing vector, myr-Akt-expressing vector, and/or dn-Akt-expressing adenoviruses was transfected using FuGENE 6 (Roche). A vector containing the neomycin-resistant gene and the respective vector were cotransfected in the cells. Cells were grown to confluence, trypsinized, plated at low density, and selected in the presence of 400 μg/ml G418 for 3 wk. Colonies were isolated by digestion with trypsin/EDTA for 5 min at 37°C within stainless steel cloning rings. Four to five independent clones were established in each expression vector.

**Adenoviral transfer**

Biortic adenosine-activating expressing vector II Runx2 and EGFP or EGFP alone were generated as previously described (Yoshida et al., 2002). Dn-Akt-expressing adenovirus was a gift from K. Walsh (Fujio et al., 1999). Cells were plated at a density of 2 × 10⁴ cells per well in 24-well plates. They were infected with EGFP-expressing, Runx2-and-EGFP-expressing, and/or dn-Akt-expressing adenoviruses at a multiplicity of infection of 10 or 20 for 12 or 24 h.

**Cytochemical and immunohistochemical examinations and in situ hybridization**

Detection and quantification of ALP activity, von Kossa staining, Alizarin red staining, and calcium quantification were performed as described previously (Enomoto et al., 2000; Kobayashi et al., 2000). Dn-Runx2 transgenic mice were generated as previously described (Ieta et al., 2001). Immunohistochemical analysis was performed using rabbit anti-p85 (Upstate Biotechnology), rabbit anti-p110 (Santa Cruz Biotechnology, Inc.), rabbit anti-Akt (New England Biolabs, Inc.), or mouse monoclonal anti-Runx2 (Yoshida et al., 2002) antibody as described previously (Iliu et al., 2001). In situ hybridization was performed using a 0.8-kb fragment of mouse Pht1 cDNA and a 0.65-kb fragment of mouse Col10a1 cDNA for probes as previously described (Ieta et al., 2001). Sections were counterstained with methyl green. The images were acquired by Axioskop 2 Plus (Carl Zeiss Microimaging, Inc.) with an objective lens (PlanNeofluar, 40× 0.75) and AxioCam HRC (Carl Zeiss Microimaging, Inc.) using AxioVision 3.0 at 22°C. The images were processed in size and brightness using Adobe Photoshop 5.5. Before the study, all experiments were reviewed and approved by Osaka University Medical School Animal Care and Use Committee.

**Northern blot and RT-PCR**

Northern blot was performed using a 0.4-kb fragment of mouse Col2a1 cDNA, a 0.65-kb fragment of mouse Col10a1 cDNA, and a 0.85-kb fragment of mouse GAPDH cDNA, as described previously (Inada et al., 1999). For RT-PCR, cDNA (10 ng total RNA equivalent) was amplified by Amp Taq DNA polymerase (PerkinElmer) using the following primers: p85β, 5'-ATTCACCCCTCTCCCAAA-3' and 5'-GGCTGTCCTCATTCCATCA-3'; p85β, 5'-CGCAACACGGAGACACTGTT-3' and 5'-TACGCGACAGACAGGAGAAT-3'; p110β, 5'-CTGTGACCCCCCAGGAAAAAT-3' and 5'-CATACTCACCCTCCTCCAC-3'; Akt1, 5'-CGTACGCTGGTCTTTGTCGAGAAGAGATTT-3' and 5'-CCTCTGGCCAGCAGTTTAGTGTACG-3'; Akt2, 5'-GTCGCACACAGTCTGAAGC-3' and 5'-GAGGAGGGTGGGAACCCGAC-3'; Akt3, 5'-AAGGGTGGTGTCAGACAGGAG-3' and 5'-CTCTGGGCACTGGTGCTTCTT-3'; hypoxanthin phosphoribosyl transferase (Hprt), 5'-GCTGGTAAGACGGACCTTCT-3' and 5'-CCAAGGACTAGAACAACG-3'; 18S rRNA, 5'-GCATGTTCTTCCCTCAGG-3' and 5'-CTTTCTGGGCACTGGTGCTTCTT-3'. Amplified products were verified by subcloning and sequence analysis. PCR products were transferred to nylon membranes and hybridized with the respective 32P-labeled probes.
Western blot and immunoprecipitation analyses

Western blot analyses were performed using nuclear extracts or whole cell lysates as described previously (Yoshida et al., 2002). The blots were first incubated with rabbit anti-p85α antibody, which recognizes both p85α and p85β (Upstate Biotechnology); rabbit anti-p110α antibody; rabbit anti-p110β antibody; rabbit anti-p110α antibody; rabbit anti-p110β antibody (Santa Cruz Biotechnology, Inc.); rabbit anti-Akt antibody, which recognizes Akt1, Akt2, and Akt3 (New England Biolabs, Inc.); rabbit anti-phospho-Akt antibody (New England Biolabs, Inc.); goat anti-Runx2 antibody (Santa Cruz Biotechnology, Inc.); or monoclonal Chibb antibody (a gift from Y. Ito, Institute of Molecular and Cell Biology, Singapore; Yoshida et al., 2002); and then with HRP-conjugated anti-rabbit or anti-mouse IgG (New England Biolabs, Inc.) or anti-goat IgG (Santa Cruz Biotechnology, Inc.). Immunoprecipitation was performed using Seize™X Protein G Immunoprecipitation kit (Pierce Chemical Co.) to avoid contamination of IgG band according to the manufacturer’s protocol using nuclear extract samples, anti-Runx2 antibody (Santa Cruz Biotechnology, Inc.), and antiphosphotyrosine, antiphosphoserine, and antiphosphothreonine mAbs (Seikagaku Corp.).

Cell migration assay

Cell migration assays were performed using PDGF-BB (PeproTech) as described previously (Fukuyama et al., 2004). Cells were placed in the upper wells at 10^5 cells per well. After culture for 12 h, the filters were removed and stained with Diff-Quick (International Re-Enforcement and Culture of Japan (T. Fujita, Y. Azuma, and T. Komori) and by the colonies, and M. Yanagita for secretarial assistance.

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