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Identification of a potent combination of osteogenic genes for bone regeneration using embryonic stem (ES) cell-based sensor

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ABSTRACT **To identify potent bioactive factors for *in vivo* tissue regeneration by comprehensive screening remains a challenge for regenerative medicine. Here we report the development of an ES cell-based monitoring system for osteogenic differentiation, the identification of a potent combination of osteogenic genes using such a system, and an evaluation of its therapeutic potentials. ES cells were isolated from mice carrying a transgene expressing GFP driven by the 2.3 kb fragment of rat type I collagen α(1) promoter. Using these cells engineered to fluoresce on osteogenic differentiation, we screened cDNA libraries and combinations of major osteogenesis-related genes. Among them, the combination of constitutively active activin receptor-like kinase 6 (caALK6) and runt-related transcription factor 2 (Runx2) was the minimal unit that induced fluorescence. The combination efficiently induced osteogenic differentiation in various cell types, including terminally differentiated nonosteogenic cells. The cooperative action of the combination occurred through protein stabilization of core binding factor beta (Cbfb), induction of Runx2-Cbfb complex formation, and its DNA binding. Furthermore, transplantation of a monolayer sheet of fibroblasts transduced with the combination achieved bone regeneration within 4 wk in mouse calvarial bone defects. Thus, we successfully identified the potent combination of genes for bone regeneration, which helped broaden cell sources.—Ohba, S., Ikeda, T., Kugimiya, F., Yano, F., Lichtler, A. C., Nakamura, K., Takato, T., Kawaguchi, H., Chung, U. I. Identification of a potent combination of osteogenic genes for bone regeneration using embryonic stem (ES) cell-based sensor. *FASEB J.* 21, 1777–1787 (2007)**

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TRAUMA, DISEASE, AND DEVELOPMENTAL abnormalities resulting in skeletal defects often incur considerable morbidity (1). Bone grafts and prosthetic implant devices are the current strategies to repair irreversible skeletal damages. However, the bone grafts have shortcomings concerning both quantity (availability of bone graft material) and quality (donor site troubles, graft rejection, and disease transmission), and the prosthetic implants have shortcomings concerning quality (biocompatibility, function, and longevity). Regenerative medicine using the technique of tissue engineering attempts to provide solutions to such problems (2).

There are three components important for tissue regeneration: scaffolds, cells, and signaling pathways. Among them, autologous cell transplantation of mesenchymal stromal cells (MSCs) derived from bone marrow and adipose tissue using biodegradable scaffolds have been widely used in bone regeneration (3–6). However, MSCs are limited both in quantity and differentiation capacity. Their definition is vague; it is controversial whether they are real stem cells that require the ability for self-renewal and multipotency. From 10 ml of bone marrow fluid or adipose tissue, only 10^3 to 10^6 cells can be isolated (3, 7). To regenerate clinical bone defects, ~10^9 cells may be required (3), but it is difficult to expand MSCs by several rounds of passages without affecting their differentiation capacity (8). On the other hand, ES cells and multipotent adult progenitor cells are virtually unlimited in quantity; however, it is difficult and costly to isolate these cells, and their differentiation efficiency seems restricted (9–11). In short, the use of stem cells has not yet overcome these crucial hurdles and a new strategy needs to be developed. One solution for this conundrum is to establish a method to control osteoblast differentiation independent of cell sources and to apply this method to abundant autologous adult cells such as dermal fibroblasts for *in vivo* bone regeneration. For this purpose, we need to identify potent

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signals that can induce osteogenic differentiation even in nonosteogenic, nonstem cells.

Substantial progress has been made in the basic understanding of major osteogenic signaling molecules and genes such as bone morphogenetic proteins (BMPs), Hedgehogs, Runx2, Wnts, and insulin-like growth factors (IGFs) (12–16). Each factor, however, was effective on specific cell types, including stem cells and osteoblast progenitors (12, 17). In addition, although most of these individual molecules are endogenously expressed in various tissues, the region where osteogenesis occurs is restricted. These data suggest that these individual factors are not potent enough and that ideal signaling may be achieved by a new factor or combinations of factors.

In this study, we developed an ES cell-based monitoring system for osteogenic differentiation that enabled us to identify a potent combination of osteogenic genes through a convenient, reliable screening method. We then investigated the molecular mechanisms underlying the cooperative action of components of the combination. Finally, we tested its *in vivo* relevance through transplantation of skin fibroblasts transduced with it into the mouse bone defect model.

MATERIALS AND METHODS

Construction of retroviral cDNA library and functional cloning

Mouse cDNA libraries were constructed with mRNA isolated from mouse embryos at embryonic day 13.5 or 15.5. cDNAs were cloned unidirectionally into the pMX-Puro vectors. These vectors were transfected to platinum E cells using Fugene6 (Roche, Penzberg, Germany) according to the manufacturer's instructions and the cells were cultured for 48 h. The retroviral supernatant was infected into Col1a1GFP ES cells and cultured for 10 days. GFP fluorescence was observed using a fluorescence microscope.

Preparation of adenoviruses and plasmids

Adenoviral expression vectors encoding rat caSmoothened (caSmo) (18), *myc*-tagged human GLI3C'Δ*ClaI* (19), hemagglutinin (HA) -tagged mouse ca lymphoid enhancer factor 1 (LEF-1), HA-tagged mouse dominant negative (dn) LEF-1 (20), and mouse core binding factor beta (Cbfb; a generous gift from T. Komori, Nagasaki University, Nagasaki, Japan) were constructed using the AdenoX Expression System (Clontech, Palo Alto, CA, USA), according to the manufacturer's instructions. Adenoviruses expressing mouse Runx2 and Flag-tagged mouse dnRunx2 were generous gifts from R. Nishimura (Osaka University, Osaka, Japan); adenoviruses expressing HA-tagged mouse caALK6 and Flag-tagged mouse Smad6 from K. Miyazono (the University of Tokyo, Tokyo, Japan); adenoviruses expressing HA-tagged human insulin receptor substrate 1 (IRS-1) and HA-tagged human dnIRS-1 from W. Ogawa (Kobe University, Kobe, Japan).

Cell culture

NIH3T3 and HeLa were obtained from the Riken Cell Bank (Tsukuba, Japan) and the JCRB Cell Bank (Osaka, Japan), respectively; mesenchymal stromal cells (hMSCs) and human dermal fibroblasts (hDFs) were from Cambrex (East Rutherford, NJ, USA). Wild-type (WT) calvaria cells were isolated from C57BL/6N mice as described (16). Cbfb−/− calvaria cells were generous gifts from T. Fujita and T. Komori (Nagasaki University, Nagasaki, Japan). These cells were maintained in high glucose Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (Sigma-Aldrich), 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma-Aldrich). Col1a1GFP-ES cells were isolated as described (21) from blastocysts carrying the Col1a1GFP transgene obtained by mating Col1a1GFP transgenic mice with WT mice. Maintenance of isolated ES cells, formation of embryoid bodies (EBs), and induction of their subsequent differentiation were performed as described (9, 22).

For functional cloning using retroviral cDNA libraries, the retroviral supernatant was used to infect Col1a1GFP ES cells and was cultured for 10 days. For screening of adenoviral vectors, each adenovirus was infected at 50 multiplicities of infection. After infection, the cells were cultured in DMEM supplemented with 1× insulin-transferin-selenium + 1 (Sigma-Aldrich) and 1% penicillin/streptomycin (serum-free DMEM) or serum-free osteogenic medium, which is serum-free DMEM supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 50 mM β-glycerophosphate (Sigma-Aldrich), and 50 μg/ml ascorbic acid phosphate (Wako Pure Chemicals Industry, Ltd., Osaka, Japan). For an analysis of calcification, von Kossa staining was performed as described (23).

Real-time RT-polymerase chain reaction (real-time RT-PCR)

Total RNA was extracted using an ISOGEN Kit (Wako Pure Chemicals Industry, Ltd., Tokyo, Japan) and treated with DNase I (Qiagen, Hilden, Germany). After reverse transcription using a Takara RNA PCR Kit, AMV version 2.1 (Takara Shuzo Co., Shiga, Japan), PCR was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and QuantiTect SYBR Green PCR Master Mix (Qiagen), according to the manufacturer's instructions. The mRNA copy number of a specific gene in the total RNA was calculated as described (23). All reactions were run in triplicate. The primer sequences are available upon request.

Immunoblot and coimmunoprecipitation

Preparation of whole cell lysates was performed using a radio-immunoprecipitation assay buffer as described (24). Separated extraction of cytoplasmic and nuclear proteins was performed using an NE-PER Kit (Pierce Chemical Co., Rockford, IL, USA). Immunoblotting was performed as described (22) using anti-HA mouse monoclonal antibody (mAb) (1:1000; Santa Cruz Biolaboratories, Santa Cruz, CA, USA), anti-Flag rabbit antibody (1:1000; Sigma-Aldrich), anti-Myc antibody (1:1000; Upstate, Lake Placid, NY, USA), anti-Smo rabbit polyclonal antibody (pAb) (H-300, 1:200; Santa Cruz Biolaboratories), anti-Runx2 mouse mAb (1:1000; MBL, Nagoya, Japan), anti-PEBP2β mouse mAb (1:1000; MBL), or anti-actin rabbit antibody (1:1000; Sigma-Aldrich). Secondary antibodies (HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG; Promega, Madison, WI, USA) were used at a dilution of 1:10,000.

Coimmunoprecipitation assays were performed using a Catch and Release kit (Upstate) according to the manufacturer's instructions. After being treated with 100 μg of dithiobis (succinimidyl propionate, a reducible chemical cross-linker) (DSP, Pierce Chemical Co.) per milliliter for 20

min, the cell lysates were incubated with 5 μg of anti-Runx2 antibodies at 4°C for 4 h. Immune complexes were eluted and subjected to SDS-PAGE.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using a Chromatin Immunoprecipitation (ChIP) Assay Kit (Upstate), 5 μg of anti-Runx2 antibodies, and 5 μg of anti-PEBP2β antibodies according to the manufacturer's instructions. PCR was performed to amplify the promoter region of the osteocalcin gene containing OSE2 site. The primer sequences are available upon request.

Generation of mouse dermal fibroblast (mDF) cell sheets

Skin tissues were obtained from the backs of 8-wk-old male transgenic mice expressing GFP ubiquitously (C57BL/6-Tg-N(act-enhanced GFP (EGFP))OsbC14-Y01-FM131, GFP transgenic mice, Riken Bioresource Center, Tsukuba, Japan). Isolation of mDFs was performed as described (25). Briefly, after trypsinization with 0.25% trypsin (Gibco BRL, Rockville, MD, USA) in Hank's balanced salt solution (Gibco BRL), the dermis was manually separated from the epidermis and digested with 3.5% collagenase (Wako Pure Chemicals Industry, Ltd.) in DMEM. Isolated mDFs were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin. To generate cell sheets, the original approach (26) was modified by using collagen films to support the cell sheets because dermal fibroblasts exhibited weak cell-cell adhesion. The mDFs were plated onto collagen films (CELLGEN; Koken, Tokyo, Japan) after adenoviral infection and cultured in serum-free osteogenic medium for 1 wk to induce calcification.

Transplantation of mDF cell sheets

Mice were anesthetized with ketamine/xylazine (80 and 5 mg/kg) solution through intraperitoneally injection and a round craniotomy defect (4 mm in diameter) was manually created as described (27). mDF cell sheets cut into a round shape (5 mm in diameter) were placed to cover the defects. At 2, 4, and 8 wk after the operation for analyses, mice were euthanized by asphyxiation with carbon dioxide. Because substantial spontaneous bone regeneration occurred at 8 wk but not at 4 wk, we chose to evaluate the induction of bone formation within 4 wk after surgery (28). To assess bone regeneration, radiological analysis, tissue preparation, H&E staining, and immunohistological analysis using a rabbit pAb against GFP (Molecular Probes, Inc., Eugene, OR, USA) were performed as described (23). The area of the regenerated bone detected by X-ray for each animal was measured using the NIH Image. The ratio of the regenerated bone area to the original defect area (RBA/ODA) was calculated and used as the index of bone regeneration. Animal experiments were performed according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

Statistical analysis

The means of groups were compared by ANOVA and the significance of differences was determined by *post hoc* testing using Bonferroni's method.

RESULTS

ES cell-based screening for potent osteogenic genes

To optimize the osteogenic condition through screening a large number of genes and signaling pathways, a convenient, reliable, and low-background monitoring system for osteogenic differentiation was required. To establish such a system, we isolated ES cells from mice carrying a transgene expressing the GFP driven by the 2.3 kb fragment of rat type I collagen α (1) promoter (Col1a1GFP) (29); we call them Col1a1GFP ES cells hereafter. We previously reported restrictive expression of GFP in bone tissues of these transgenic mice (29) (Supplemental Fig. 1*A*), suggesting that GFP fluorescence in Col1a1GFP ES cells is produced only when they differentiate into osteoblastic cells (Supplemental Fig. 1*B*). Indeed, expression of GFP fluorescence and osteocalcin mRNA showed the same temporal pattern in osteogenic cultures of Col1a1GFP ES cells (Supplemental Fig. 1*C, D*). These findings indicated that the site of insertion of Col1a1GFP did not interfere with its expression and that GFP fluorescence controlled by Col1a1 promoter reliably reflected osteogenic differentiation, suggesting that this system might allow us to monitor osteogenic differentiation easily, precisely, and noninvasively without analyzing differentiation markers or staining cells. Using this system, we planned to identify much more potent genes for bone regeneration than conventional ones. For identification, we set three criteria: such genes have to induce osteogenic differentiation (**Fig. 1*A***) within a week in serum-free medium (Fig. 1*B*) and in various cell types (Fig. 1*C*), including nonosteogenic cells. The first two criteria were set in order to find osteogenic stimuli that were rapid and potent enough for clinical applications and to avoid the confounding influences of cytokines and the potential contamination of pathogens contained in the serum (30). We set the third in order to broaden the range of the cell types that respond to the osteogenic stimuli.

We first tried to clone a single potent osteogenic gene through functional screening of the retroviral cDNA libraries. We chose cDNA libraries isolated from mouse embryos at embryonic day 13.5 or 15.5, because bone formation started around embryonic day 14.0 (31). After construction of two retroviral cDNA libraries, we infected Col1a1GFP ES cells with either one under the condition that a single copy of the retroviral genome would be integrated into a host chromosome and screened for a single gene that induced osteogenic differentiation in serum-free medium using GFP fluorescence as an indicator. Although we observed the cells for as long as 10 days, no GFP-positive cells appeared (data not shown).

We next chose five signaling pathways (BMPs, Hedgehogs, Runx2, Wnts, and insulin-like growth factor 1, or IGF-1) based on the *in vivo* phenotypes of their mutant animals (12–16) and attempted to screen their random combinations. After preparation of adenoviral vectors encoding genes activating or inhibiting these signaling pathways (**Table 1**), specific expression of each protein was confirmed by immunoblot analysis (Fig. 1*A*). We infected Col1a1GFP ES cells with each combination of genes, including the neutral one encoding LacZ

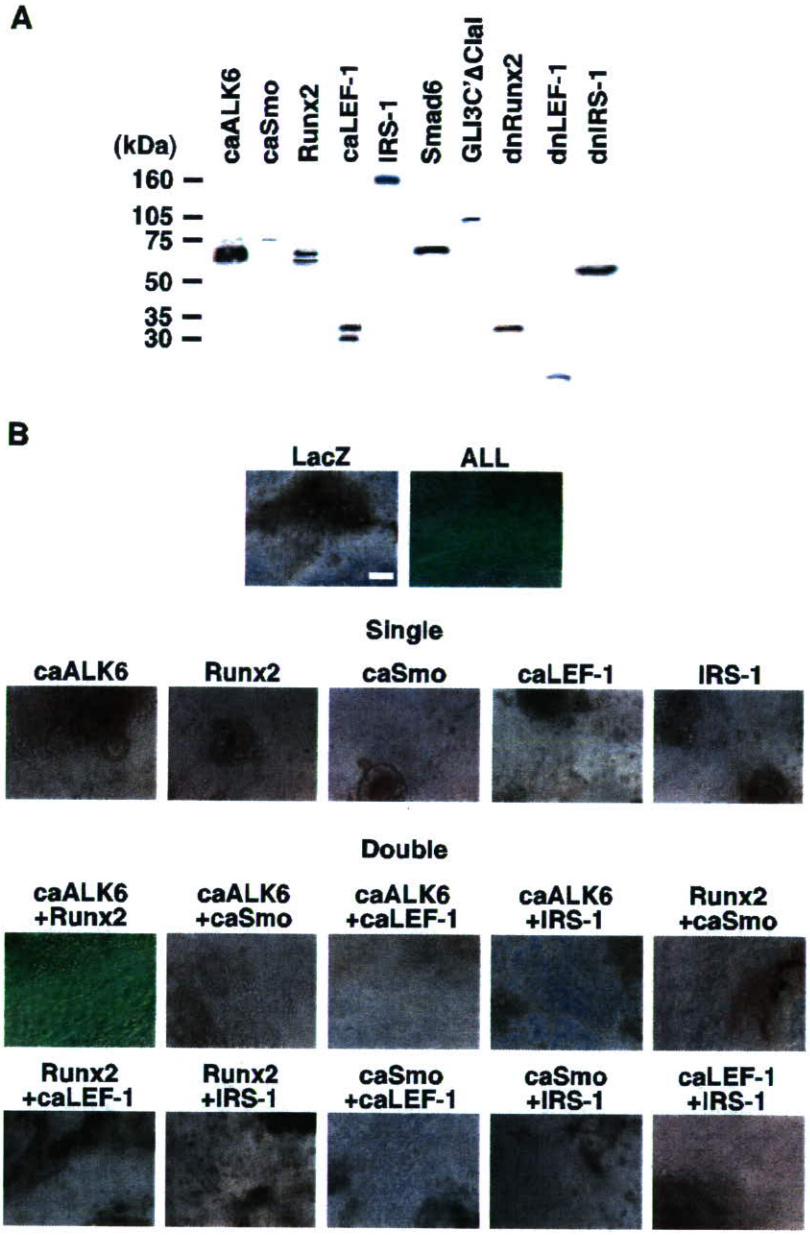

Figure 1. Screening for potent osteogenic genes using Col1a1GFP ES cells. *A)* Protein expression of adenovirally introduced genes in ES cells. Five days after infection, expression of each protein was analyzed by immunoblot analysis. *B)* GFP fluorescence of Col1a1GFP ES cells 7 days after infection with adenoviruses expressing osteogenic genes in various combinations. Single, stimulation of one signaling pathway; Double, stimulation of two signaling pathways; ALL, stimulation of all signaling pathways. Bar, 500 μm.

(3^5=243 combinations), then screened for the combination that induced osteogenic differentiation within a week and in serum-free medium. Although the stimulation of all signaling pathways (ALL) strongly induced GFP fluorescence within a week, stimulation of each signaling pathway failed to do so, as expected. Among all combinations, caALK6+Runx2 was found to be the minimal unit that induced GFP fluorescence as potently as ALL (Fig. 1*B*). Neither the number of GFP-positive cells nor the strength of their GFP fluorescence was significantly enhanced by adding the other genes (data not shown). These data suggest that caALK6+Runx2 may be the combination meeting our criteria.

TABLE 1. *Osteogenesis-related genes used in screening for potent combinations*

Signaling pathway	Gene for activation	Gene for inhibition	Gene for control
BMP	Mouse caALK6	Mouse Smad6	LacZ
Hedgehog	Rat caSmo	Human GLI3C'ΔClaI	LacZ
Runx2	Mouse Runx2	Mouse dnRunx2	LacZ
Wnt	Mouse caLEF-1	Mouse dnLEF-1	LacZ
IGF-I	Human IRS-1	Human dnIRS-1	LacZ

Osteogenic induction in stem cells and terminally differentiated nonosteogenic cells by caALK6+Runx2

To confirm that caALK6+Runx2 actually induced the osteoblast phenotype in mouse ES cells (mESs), we analyzed the expression of osteoblast marker genes and matrix calcification. caALK6+Runx2 strongly induced mRNA expression of the osteoblast marker genes within a week in serum-free medium (**Fig. 2*A***). As shown by von Kossa staining, caALK6+Runx2 induced matrix calcification within 10 days (Fig. 2*B*), but other individual factors did not (data not shown). The level of osteocalcin expression induced by caALK6+Runx2 was comparable to that induced by ALL and that of mouse primary osteoblasts, whereas other individual factors did not induce its expression (Fig. 2*C*).

In serum-free medium, caALK6+Runx2 exerted similar effects on human hMSCs (supplemental Fig. 2), hDFs (**Fig. 3**), and terminally differentiated nonosteogenic cell lines, including NIH3T3 cells (32) (**Fig. 4*A*, *B***) and HeLa cells (33) (Fig. 4*C*). It is worth noting that vascular endothelial growth factor A (VEGF-A) and matrix metalloproteinase-13 (MMP-13), which played important roles in angiogenesis and tissue remodeling, respectively, were strongly induced by caALK6+Runx2 in hDFs (Fig. 3*A*). The results from nonosteogenic cell lines (Fig. 4) enabled us to exclude the possibility that a small number of stem cells mingled in primary DFs might selectively expand and differentiate into osteoblasts. In addition, caALK6+Runx2 failed to induce expression of the type X collagen, a differentiation marker of hypertrophic chondrocytes (data not

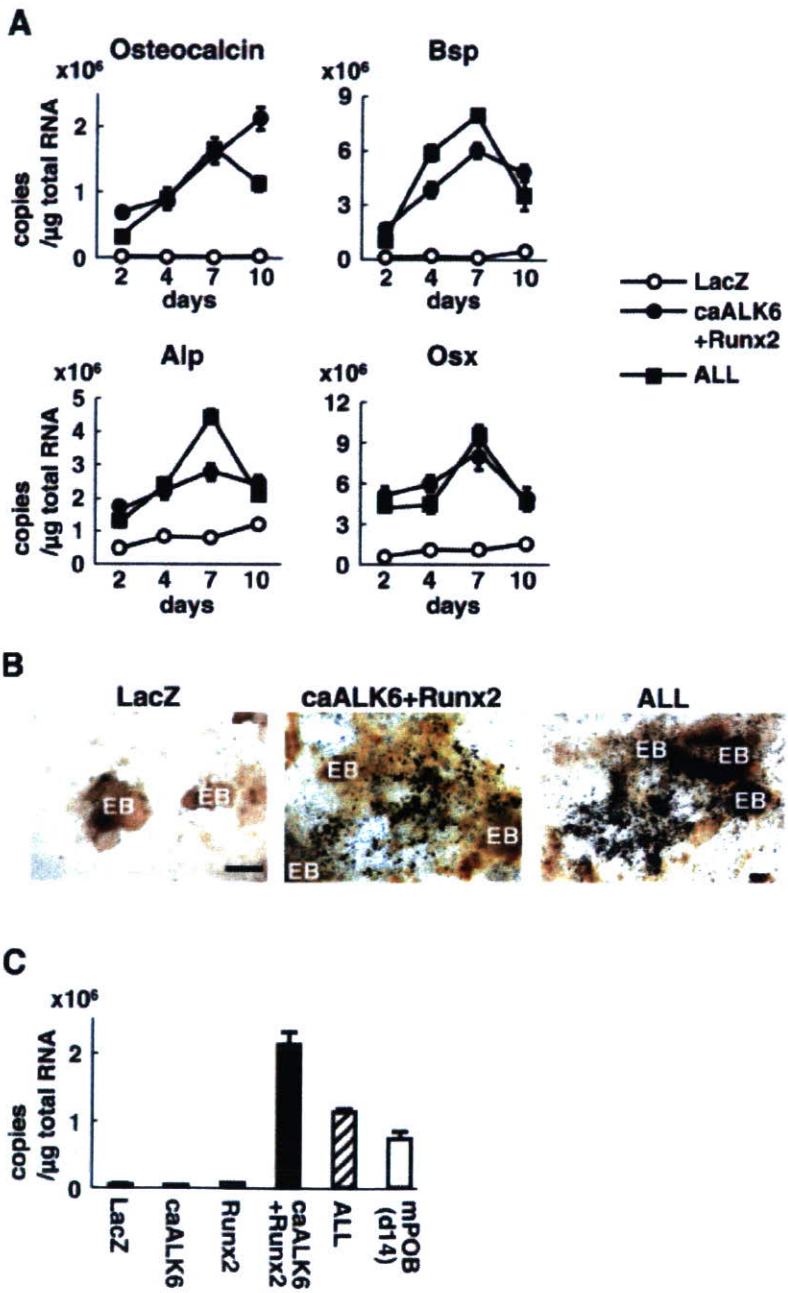

Figure 2. Osteogenic induction of mESs by caALK6+Runx2. *A)* Induction of mRNA expression of osteoblast marker genes by caALK6+Runx2. mESs were cultured in serum-free osteogenic medium for 10 days after the indicated adenoviral infections and real-time RT-PCR analysis was performed. Bsp, bone sialoprotein; Alp, alkaline phosphatase; Osx, osterix. Data are means ± SDs of 3 wells per group. *B)* Induction of matrix calcification by caALK6+Runx2. Ten days after infection, calcification was assessed using von Kossa staining. Calcification was stained black. EB, embryoid body. Bar, 500 μm. *C)* Comparison of individual factors with caALK6+Runx2 regarding induction of osteocalcin mRNA expression 10 days after infection. Mouse primary osteoblasts (mPOB) were cultured in osteogenic medium for 2 wk after isolation from WT mice. Data are means ± SDs of 3 wells per group.

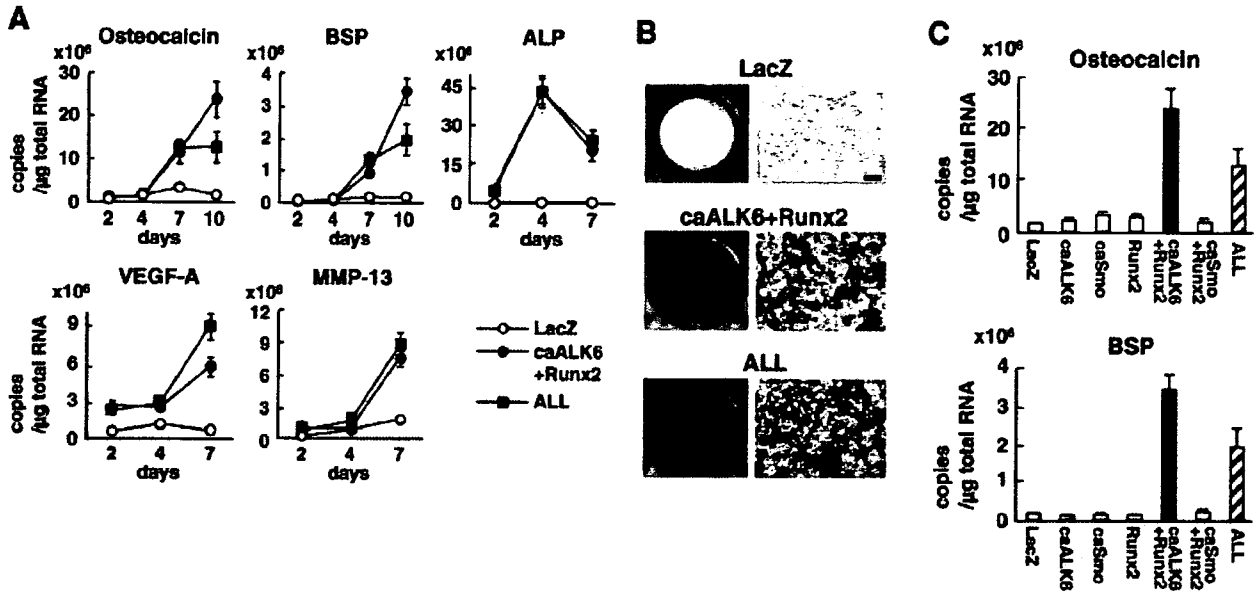

Figure 3. Osteogenic induction of hDFs by caALK6+Runx2. *A)* Induction of mRNA expression of osteoblast marker genes by caALK6+Runx2. hDFs were cultured in serum-free osteogenic medium for 10 days after adenoviral infection and real-time RT-PCR analysis was performed. Data are means ± SDs of 3 wells per group. *B)* Induction of matrix calcification by caALK6+Runx2. 10 days after infection, calcification was assessed using von Kossa staining. Left panels show culture wells; right panels show part of culture wells at higher magnification. Bar, 500 μm. *C)* Comparison of individual factors with caALK6+Runx2 regarding induction of osteocalcin and BSP mRNA expression 10 days after infection. Data are means ± SDs of 3 wells per group.

shown), ruling out the possibility that mineralization was induced by hypertrophic chondrocytes rather than osteoblasts.

Taken together, caALK6+Runx2 is the minimal unit that meets the optimization criteria (Fig. 3*A*) and in stem cells and terminally differentiated nonosteogenic cells (Fig. 3*B*), suggesting that caALK6+Runx2 is the potent osteogenic unit that meets all three criteria.

The molecular mechanisms underlying the cooperative action of caALK6+Runx2

The data so far strongly suggest the presence of a synergistic action between caALK6, which transduces BMP signaling, and Runx2 for the induction of osteogenic differentiation. Because Cbfb forms a complex with Runx2 and enhances its DNA binding and transcriptional activation (34, 35), we hypothesized that Cbfb might be involved in the synergistic action. To clarify the role of Cbfb, the effects of the loss or overexpression of Cbfb on caALK6+Runx2-induced differentiation were investigated. Cbfb-KO (−/−) cells failed to induce mRNA expression of osteocalcin in response to caALK6+Runx2, which was restored by adenoviral reintroduction of Cbfb to the level of that of WT cells (**Fig. 5*A***). This regulation was also observed in mRNA expression of ALP and osteopontin (data not shown). In NIH3T3 stimulated by caALK6+Runx2, additional treatment with Cbfb accelerated the speed of the osteocalcin expression without enhancing its maximum expression level (Fig. 5*B*). These data suggested that Cbfb was necessary for the synergistic action of BMP signaling and Runx2 and might be regulated by the two signaling pathways, leading us to focus on Cbfb in investigating the molecular mechanisms underlying the synergistic action.

When Runx2 was overexpressed in NIH3T3, its mRNA and protein expressions were up-regulated, with the protein being accumulated in the nucleus, which was not altered by coinfection with caALK6 (**Fig. 6*A, B***). At the basal level, NIH3T3 cells expressed a moderate amount of endogenous Cbfb mRNA, which was not altered by infection with caALK6, Runx2, or both (Fig. 6*A*). On the other hand, the basal expression of Cbfb protein was weak, which was markedly increased in the nucleus by Runx2 or caALK6+Runx2 (Fig. 6*B*). Although Runx2 and Cbfb were colocalized in the nucleus upon infection with Runx2 or caALK6+Runx2, coimmunoprecipitation analysis revealed that these two proteins bound to each other only upon infection with the latter (Fig. 6*B*). Upon treatment with the ubiquitin-proteasome inhibitors such as lactacystin and MG132, the Cbfb protein level was increased without being affected by infection with Runx2 or caALK6+Runx2 (Fig. 6*C*). In addition, chromatin immunoprecipitation analysis revealed that caALK6+Runx2, but not Runx2 alone, recruited Cbfb to the osteocalcin promoter whereas Runx2 was constantly recruited (Fig. 6*D*).

Taken together, these data suggest the following molecular mechanisms. *1)* Overexpressed Runx2 protects Cbfb from degradation by the ubiquitin proteasome, and both proteins accumulate in the nucleus. *2)* Upon activation of BMP signaling by caALK6, Runx2 associates with Cbfb, and the complex of Runx2 and Cbfb binds to the promoters of the osteoblast-specific genes to activate their expression. This is a sequential

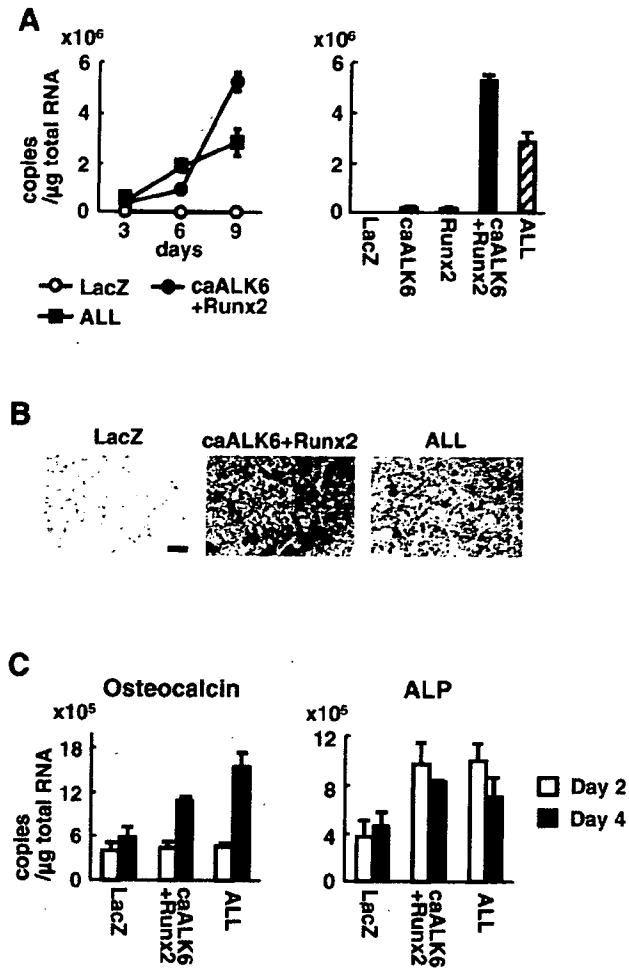

Figure 4. Osteogenic induction of nonosteogenic cells by caALK6+Runx2. *A)* Induction of osteocalcin mRNA expression by caALK6+Runx2 in NIH3T3 cells. Cells were cultured in serum-free osteogenic medium for 9 days after adenoviral infection and real-time RT-PCR analysis was performed. Data are means ± SDs of 3 wells per group. *B)* Induction of matrix calcification by caALK6+Runx2 in NIH3T3 cells. 9 days after infection, calcification was assessed using von Kossa staining. Bar, 500 μm. *C)* Induction of mRNA expression of osteoblast marker genes by caALK6+Runx2 in HeLa cells. Cells were cultured in serum-free DMEM for 4 days after adenoviral infection. Data are means ± SDs of 3 wells per group.

and progressive process, a cascade of molecular interactions *via* regulation of Cbfb, which leads to transcription of the target gene. Thus, caALK6+Runx2 synergistically elicits a potent osteogenic signaling that is distinct from its component.

In vivo osteogenic effects of dermal fibroblast cell sheets stimulated by caALK6+Runx2

To investigate the *in vivo* relevance of caALK6+Runx2, we tested whether DFs stimulated by caALK6+Runx2 would have osteogenic effects on bone defects. For transplantation of DFs, we modified the cell sheet technology (26) to create sheets of cells by using collagen films to support the cell sheets. Autologous mouse DFs (mDFs) were infected with adenoviruses expressing LacZ, caALK6, Runx2, or caALK6+Runx2, cultured on collagen membranes, then transplanted as monolayer cell sheets onto bone defects created in the mouse calvarias. To estimate the contribution of donor cells, transgenic mice expressing GFP ubiquitously (36)

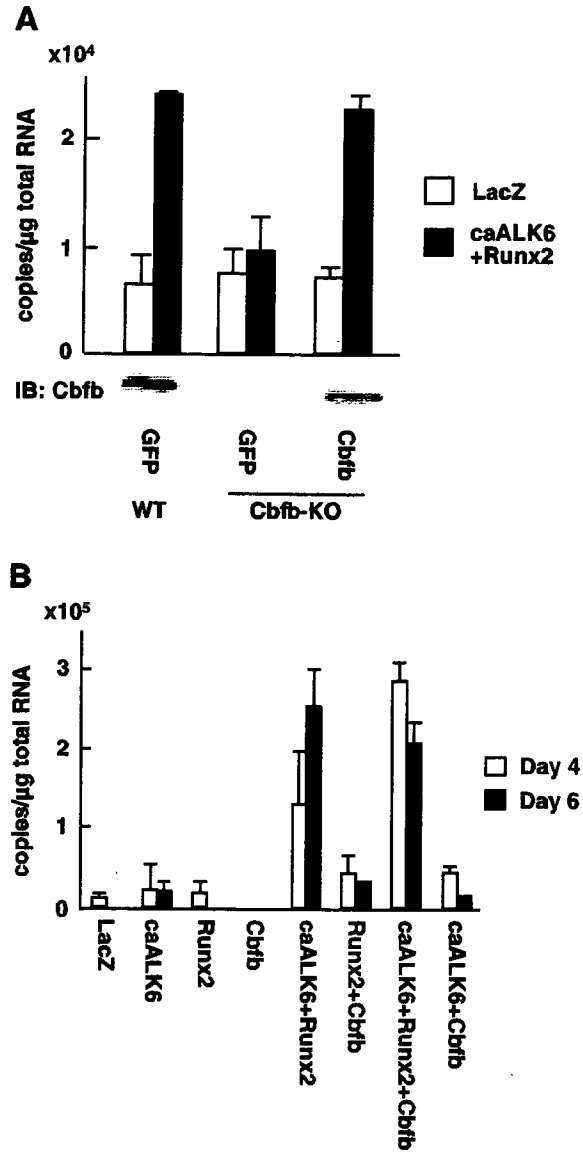

Figure 5. Involvement of Cbfb in the osteogenic induction by caALK6+Runx2. *A)* Requirement of Cbfb for osteogenic induction by caALK6+Runx2. WT and Cbfb−/− (KO) calvaria cells were cultured in serum-free osteogenic medium for 3 days after the indicated adenoviral infection. For rescue, an adenovirus expressing Cbfb was used to infect Cbfb-KO cells. Osteocalcin mRNA expression and Cbfb protein expression were determined by real-time RT-PCR analysis and by immunoblot analysis, respectively. Data are means ± SDs of 3 wells per group. *B)* Effects of Cbfb overexpression on osteogenic induction by caALK6+Runx2. NIH3T3 cells were cultured in serum-free osteogenic medium for 4 and 6 days after adenoviral infection. Osteocalcin mRNA expression was determined by real-time RT-PCR analysis. Data are means ± SDs of 3 wells per group.

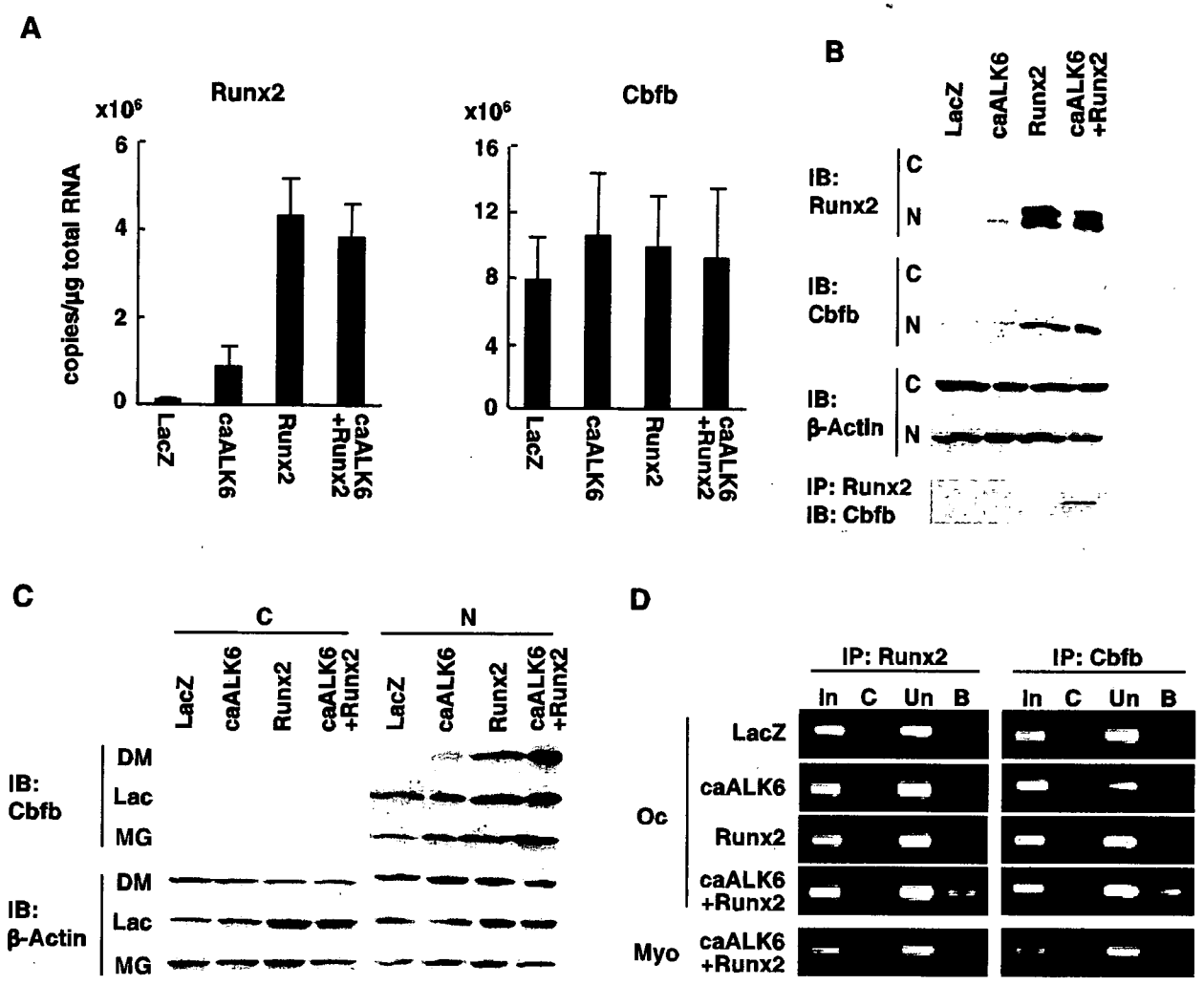

Figure 6. The molecular mechanisms underlying the synergistic action of caALK6+Runx2. *A)* Induction of mRNA of Runx2 and Cbfb by caALK6+Runx2. After the indicated adenoviral infection, NIH3T3 cells were cultured in serum-free osteogenic medium for 5 days and real-time RT-PCR analysis was performed. Data are means ± SDs of 3 wells per group. *B)* Protein expressions and physical association of Runx2 and Cbfb by caALK6+Runx2. Coimmunoprecipitation or immunoblotting were performed on NIH3T3 cells cultured for 5 days in serum-free osteogenic medium after adenoviral infection. IB, immunoblot; inositol phase (IP), immunoprecipitation; C, cytoplasmic fraction; N, nuclear fraction. *C)* Stabilization of Cbfb by the ubiquitin-proteasome inhibitors. Four days after adenoviral infection, NIH3T3 cells were treated with lactacystin (Lac, 20 μM) or MG132 (MG, 2 μM) overnight, then immunoblot analysis was performed. DM, DMSO. *D)* Recruitment of Runx2 and Cbfb to the osteocalcin promoter. Chromatin immunoprecipitation was performed on NIH3T3 cells cultured for 5 days in serum-free osteogenic medium after adenoviral infection. Oc, amplification of osteocalcin promoter sequence; Myo, amplification of myogenin promoter sequence as a negative control; In, PCR from total DNA input. *C)* PCR from the immunoprecipitation product with control serum; Un, PCR from the supernatant after immunoprecipitation; *B)* PCR from the immunoprecipitation product with specific antibodies.

were used as donors. Soft X-ray analysis revealed that while no bone formation occurred 2 wk after transplantation in either group, bone formation was strongly induced at 4 wk in the caALK6+Runx2-infected group (**Fig. 7*A***), which was confirmed by quantitative analysis of the regenerated bone area (Fig. 7*B*). Histological analysis revealed that woven bones with marrow cavities, thicker than the original calvaria bones, appeared 4 wk after transplantation (Fig. 7*C*). Immunohistochemistry for GFP revealed that both GFP-positive donor cells and GFP-negative host cells were observed in regenerated bone tissues (Fig. 7*D*), suggesting that the transplants induced osteogenic differentiation of recipient cells. In contrast, cell sheets of mDFs infected with either caALK6 or Runx2 did not mineralize the matrix in culture or induce bone formation when implanted (data not shown).

DISCUSSION

We developed the Col1a1GFP system by combining osteoblast-specific expression of the Col1a1 promoter fragment with the easy and noninvasive detectability of GFP. Col1a1GFP cells served as a cell-based sensor, which allowed us to monitor the complex process of

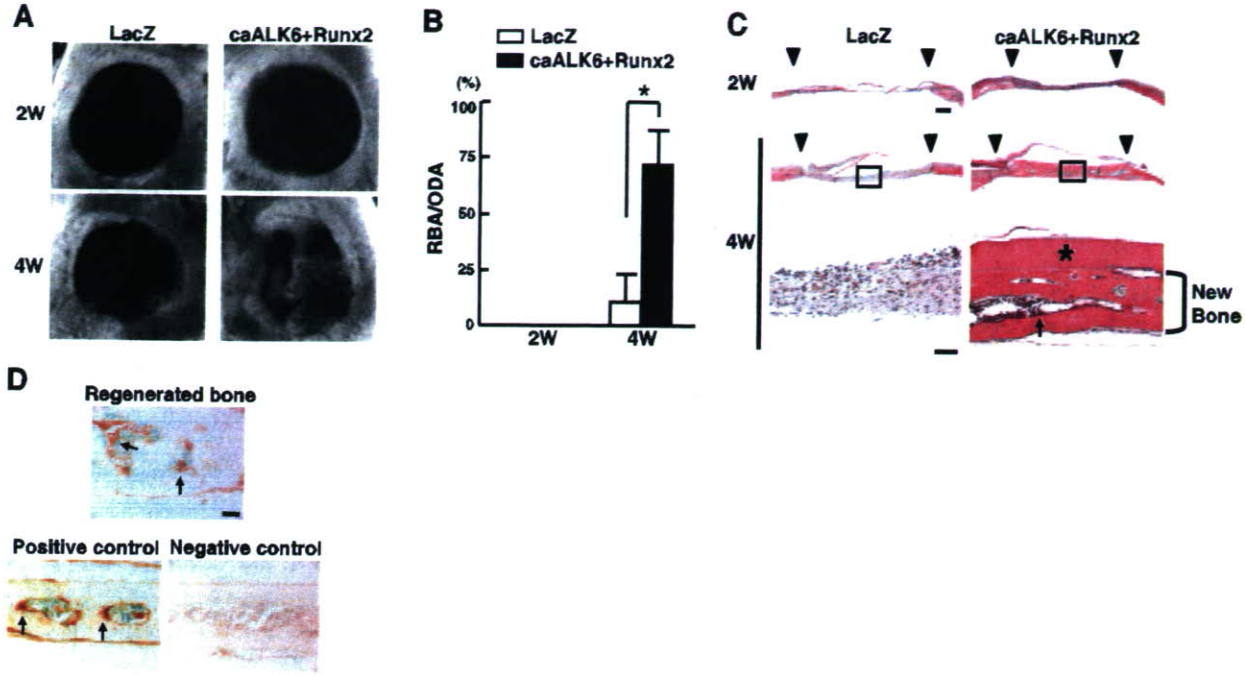

Figure 7. *In vivo* osteogenic effect of dermal fibroblast cell sheet stimulated by caALK6+Runx2. *A)* Soft X-ray images of calvarias transplanted with an mDF cell sheet infected with the indicated adenoviruses. Calvarias were isolated at 2 or 4 wk after transplantation. *B)* Quantitative analysis of bone regeneration. The ratio of the regenerated bone area to the original defect area (RBA/ODA) was measured radiologically by the NIH Image. Data are means ± SDs of 5 mice per group. $*P < 0.01$ *vs.* LacZ at 4 wk after transplantation. *C)* Histological analyses of calvarias transplanted with an mDF cell sheet infected with the indicated adenoviruses. Isolated calvarias were stained with H&E. Bottom panels show magnified views of boxed areas in the middle panels. Arrowheads, defect edges. Arrows, bone marrow cavities. Asterisk, a collagen film used to support the cell sheet. Bar: 500 μm in the upper panels, 100 μm in the bottom panels. *D)* Donor *vs.* host cell contribution to the regenerated bone. To detect donor cells from GFP transgenic mice, immunohistochemistry against GFP was performed. GFP protein was stained brown (arrows). Regenerated bone, regenerated bone in the caALK6+Runx2-infected group; positive control, a calvaria from a Col1a1GFP transgenic mouse in which all osteoblasts were positive for the staining; negative control, a calvaria from a WT mouse. Bar, 100 μm.

osteogenic differentiation of live cells in real time without analyzing differentiation markers or staining the cells. We chose to use ES cells isolated from Col1a1GFP mice over other cell types, because other primary cells need to be isolated for each experiment and immortalized cell lines often lose the characteristics of parental cells. ES cells are naturally immortalized, proliferating almost indefinitely without losing totipotency and have relatively low background noise, as demonstrated in the current study.

The screening of cDNA libraries using this system suggested that a single gene could not induce osteogenic differentiation under our experimental conditions. The integrity of the libraries seems high because we succeeded in isolating several chondrogenic genes in other experiments using them (data not shown). This result prompted us to screen combinations of genes rather than individual ones. Based on an educated guess, we chose five major osteogenic signaling pathways for the combination because random combinations of millions of genes or signaling pathways amount to an astronomical number, which is impossible to screen using this system or any technology available at this time. By setting the three criteria, we successfully identified caALK6+Runx2, which cooperatively induced osteogenic differentiation much more rapidly and potently than the conventional osteogenic genes. Two recent reports strongly support our results. Phimphilai *et al.* reported that the transcriptional activity of Runx2 required BMP signaling and that the sensitivity of cells to BMPs was enhanced by Runx2 (37). In addition, Runx2, especially its C terminus containing the nuclear matrix targeting signal and Smad interacting domain, was shown to be indispensable for execution of the BMP2 osteogenic signal (38). These reports indicate that BMP signaling and Runx2 require each other for their full osteogenic effects, suggesting they may act in synergism. We provided the potential molecular mechanism involving Cbfb for this synergism.

Regarding the interactions of BMP signaling, Runx2, and Cbfb, several points remain to be clarified. The first is the mechanism by which Cbfb is stabilized by Runx2. Although stabilization of Runx1 occurred by its dimerization with Cbfb (39), the stabilization of Cbfb was not related to the dimerization with Runx2, suggesting that Runx2 may stabilize Cbfb in an indirect manner. The second is the mechanism by which Cbfb enters the nucleus. Our data suggest that dimerization takes place in the nucleus, but Cbfb has no known nuclear local-

ization signal. Cbfb may enter the nucleus alone by an unknown mechanism (39); alternatively, there may be some other protein partner(s) helping Cbfb enter the nucleus. The third is the mechanism by which BMP signaling promotes dimerization of Runx2 and Cbfb. Because Runx2 is known to interact with Smad, Smads may promote dimerization; alternatively, other proteins interacting with Runx2 may mediate BMP signaling and dimerization (40, 41). These points are now under investigation. Worth noting is that Runx2+Cbfb did not up-regulate the expression of endogenous osteocalcin in our experiments, although Cbfb was shown to enhance Runx2-mediated transactivation of the osteocalcin promoter in the luciferase assay (34, 35). This difference likely reflects the difference in the chromatin structure of endogenous genes and exogenous plasmids (42), and likely accounts for the inability of either caALK6 or Runx2 alone to induce expression of the endogenous osteocalcin gene in our experiments.

Although applications of Runx2 or BMP signaling to bone regeneration have been reported, most depended on transplantation of stem cells, osteoblast lineage cells, or cell populations containing either one (17, 43–45). A few groups used primary fibroblasts transduced with BMP genes, but their *in vitro* calcification was never shown (27, 46). In addition, although the use of recombinant BMPs has been studied extensively as a clinically useful procedure in bone regeneration, a large amount of BMP is required and BMP-containing devices fail in a certain percentage of cases, raising concerns over costs and safety (47–49). As Franceschi *et al.* pointed out, the reasons may be related to a lack of controlled and sustained BMP delivery, its short biological half-life, and the inability of its presentation to mimic the biological condition (50). In the above studies, the possibility was not excluded that other signaling molecules, including their combinations with BMP, might exert a stronger effect on bone regeneration because neither BMP nor Runx2 was selected through comprehensive screening. Using the combination of caALK6+Runx2 identified through screening by the cell-based sensor, we succeeded in inducing both the expression of osteoblast marker genes and *in vitro* calcification in terminally differentiated fibroblasts and in inducing rapid bone regeneration by transplantation of a monolayer sheet of fibroblasts transduced with the combination.

In the present study, using the cell-based sensor system, we successfully identified a potent combination of genes for bone regeneration that helped broaden cell sources to terminally differentiated adult nonosteogenic cells. Our approach using cell-based sensors may provide tools to clarify mechanisms underlying regeneration of various tissues or, more generally, may help identify *in vivo* effective signaling factors, including their combinations for various biological processes including developmental and pathological ones. FJ

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A novel osteogenic helioxanthin-derivative acts in a BMP-dependent manner

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Abstract

To effectively treat serious bone defects using bone regenerative medicine, there is a need for the development of a small chemical compound that potently induces bone formation. We now report a novel osteogenic helioxanthin-derivative, TH. TH induced osteogenic differentiation in MC3T3-E1 cells, mouse primary osteoblasts, and mouse embryonic stem cells. The combination of TH and bone morphogenetic protein (BMP) 2 induced the mRNA expression of osteoblast marker genes and calcification in primary fibroblasts. The TH induced the mRNA of the inhibitor of DNA-binding 1 (Id-1), and its osteogenic effect was inhibited by Smad6 or Noggin. Furthermore, TH induced the mRNA expression of Bmp4 and Bmp6. These data suggest that TH exerts its potent osteogenic effect in a BMP-dependent manner by enhancing the effects of the existing BMPs and/or increasing the expression of Bmp4 and Bmp6. TH may help establish a more efficient bone regeneration system.

Keywords: Bone regeneration; Small compound; Osteogenic differentiation; Osteoblast; Fibroblast; ES cell; BMP

The treatment of serious bone defects remains a great challenge, and bone regenerative medicine has been drawing attention for the treatment of such defects [1]. The local transplantation of autologous multipotent cells has been widely used for bone regeneration [1,2]. For sufficient and timely bone formation, however, the current system requires the addition of exogenous signaling factors including BMPs [3], Hedgehogs [4], Runx2 [5], transforming growth factors (TGFs) [6], fibroblast growth factors (FGFs) [7], and vascular endothelial growth factors (VEGFs) [8]. These factors were locally applied using direct protein delivery or viral gene delivery.

Direct protein delivery, however, can suffer from protein instability and inadequate post-translational modifications of the recombinant proteins [9]. For example, although the use of BMPs has been extensively studied for bone regeneration, a large amount of BMP is required, and BMP-containing devices fail in a certain percentage of cases, raising concerns over costs and safety [10–12]. As Franceschi et al. pointed out, the reason may be due to its short biological half-life [13]. As for viral gene delivery, their clinical use is severely limited due to the potential risk of immunogenic responses and the difficulty in manipulation and mass production.

Thus, there is a clear need for the development of a small chemical compound that directly or indirectly induces bone formation. Despite recent successes with drugs inhibiting bone resorption, there is a limited number of reports on such anabolic agents that effectively increase bone formation. Statins [14], isoflavone derivatives [15,16], and TAK-778 [17] were reported to stimulate osteogenic differentiation, but their osteogenic activity was shown

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only in specific cell types including osteoblastic cells and stem cells. We hypothesize that, if we are able to identify a small compound that potently induces osteogenic differentiation, we may be able to broaden the cell sources for cell transplantation and thereby establish a more efficient bone regeneration system.

Through screening the small compound library of Takeda Chemical Industries, 4-(4-methoxyphenyl)pyrido[4′,3′:4,5]thieno[2,3-*b*]pyridine-2-carboxamide (TH) was found to induce alkaline phosphatase (ALP) activity in MC3T3-E1 cells. This study was aimed to investigate the osteogenic effect of TH and its molecular mechanism using an *in vitro* culture system.

Materials and methods

Reagents and vectors. TH (Fig. 1A) was synthesized at Takeda Chemical Industries (Osaka, Japan). The recombinant human (rh) BMP2 was provided by Astellas Pharma, Inc. (Tokyo, Japan). Noggin/Fc chimera was purchased from Sigma–Aldrich (St. Louis, MO); the anti-phospho-Smad1/5/8 antibody and anti-Smad1 antibody from Cell Signaling Technology (Beverly, MA); the anti-Runx2 antibody from MBL (Nagoya, Japan); the anti-actin antibody from Sigma–Aldrich; and the HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG from Promega (Madison, WI). The adenoviral vectors expressing Smad6 (AxSmad6), plasmids expressing mouse Runx2 and 12xGCCG-luc were generous gifts from K. Miyazono (the University of Tokyo); the plasmids expressing Cbfb were from T. Komori (Nagasaki University); and the 1050Oc-luc from G.S. Stein (University of Massachusetts Medical School).

Cell culture. The MC3T3-E1 cells and NIH3T3 cells were obtained from the Riken Cell Bank (Tsukuba, Japan); and the human dermal fibroblasts (hDFs) from Cambrex (East Rutherford, NJ). The heterozygous Runx2-null (Runx2+/−) mice were kindly provided by Dr. M.J. Owen (Wales College of Medicine). The homozygous Runx2-null (Runx2−/−) and wild-type (WT) mESs were isolated de novo as already described [18]. The primary osteoblasts (POBs) were isolated from the WT C57BL/6N mice as already described [19]. Isolation of the mouse DFs (mDFs) was performed as already described [20]. The NIH3T3 cells, mDFs, and hDFs were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich) containing 10% fetal bovine serum (FBS, Sigma–Aldrich) and 1% penicillin/streptomycin (Sigma–Aldrich) (10% FBS/DMEM); the MC3T3-E1 cells and POBs were maintained in αMEM containing 10% FBS and 1% penicillin/streptomycin (10% FBS/αMEM). For the osteogenic culture, these cells were cultured in 10% FBS/DMEM containing 50 μg/mL ascorbic acid phosphate (AsAP), 10 mM β-glycerophosphate (β-GP), and 0.1 μM dexamethasone (Dex) (osteogenic medium). For the serum-free osteogenic culture, the hDFs were cultured in DMEM containing ITS+1 (Sigma–Aldrich), 50 μg/mL AsAP, 10 mM β-GP, and 0.1 μM Dex (serum-free osteogenic medium). Alkaline phosphatase (ALP) and von Kossa stainings were performed as already described [21]. Maintenance of the isolated ES cells and induction of their subsequent differentiation were performed as already described [22].

Real-time RT-PCR analysis. The total RNA was extracted using an ISOGEN Kit (Wako Pure Chemicals Industry, Ltd., Tokyo, Japan) and treated with DNase I (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After reverse-transcription using Takara RNA PCR Kit (AMV) version 2.1 (Takara Shuzo Co., Shiga, Japan), PCR was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and QuantiTect SYBR Green PCR Master Mix (Qiagen). All reactions were run in triplicate and the mRNA copy number of a specific gene in the total RNA was calculated as already described [21]. All data were expressed as means ± SDs of triplicate wells. The primer sequences are available upon request.

Immunoblot analysis. For preparation of the whole cell lysates, the cells were lysed using the M-PER Kit (Pierce Chemical Co., Rockford, IL). The separated extraction of the cytoplasmic and nuclear proteins was performed using the NE-PER Kit (Pierce Chemical Co.). Immunoblotting

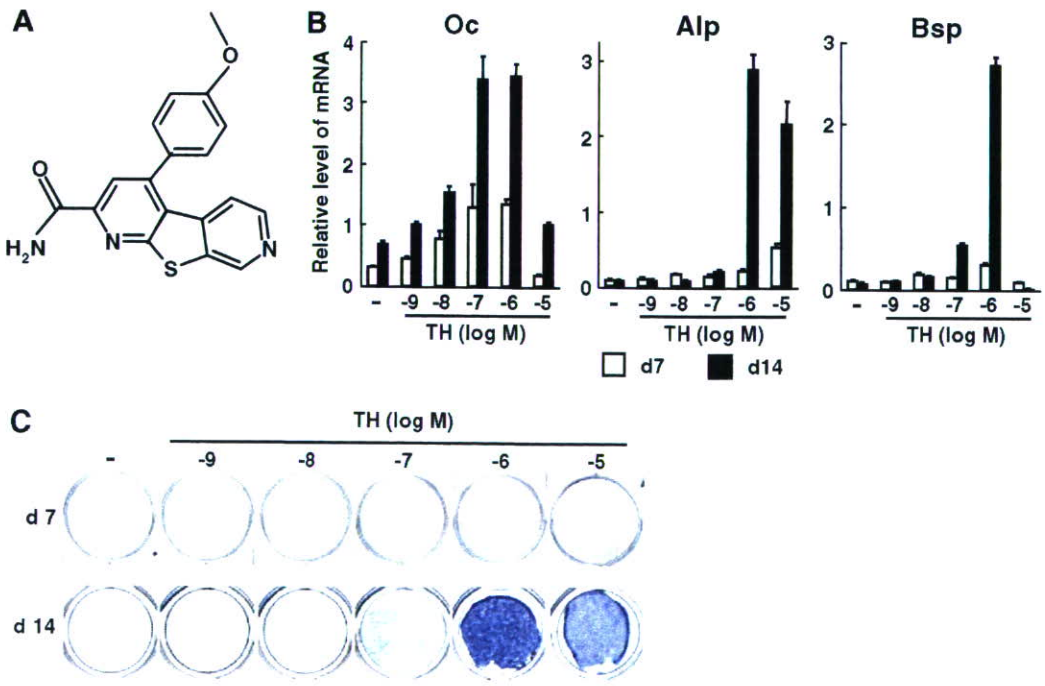

Fig. 1. Osteogenic induction of MC3T3-E1 cells by TH. (A) Chemical structure of TH. (B) mRNA expression of osteoblast marker genes in MC3T3-E1 cells treated with TH determined by real-time RT-PCR analysis. Oc, osteocalcin; Alp, alkaline phosphatase; Bsp, bone sialoprotein. (C) ALP staining of MC3T3-E1 cells treated with TH.

was performed as already described [22] using the anti-Runx2 antibody (1:1000), anti-phospho-Smad1/5/8 antibody (1:500), anti-Smad1 antibody (1:500) or anti-actin rabbit antibody (1:1000). The secondary antibodies were used at a dilution of 1:10,000.

Luciferase assay. The NIH3T3 cells were plated onto 24-well plates and they were transfected with 0.4 μg of a DNA mixture containing the test reporter plasmids, the control reporter plasmids encoding *Renilla* luciferase and the effecter plasmids using FuGENE6 (Roche, Penzberg, Germany). The dual luciferase assay was performed 48 h after transfection as already described [22]. All data were expressed as means ± SDs of triplicate wells.

Results and discussion

Osteogenic induction of MC3T3-E1 cells by TH in a dose-dependent manner

To examine the osteogenic effect of TH, we treated MC3T3-E1 cells, a pre-osteoblastic cell line, with TH at concentrations ranging from 10^{-9} to 10^{-5} M and then cultured them for 14 days. TH upregulated the osteocalcin expression in a dose-dependent manner and, at 10^{-6} and 10^{-7} M, induced the highest level of expression (Fig. 1B). TH at 10^{-6} M strongly induced the expression of Alp and bone sialoprotein (Bsp) (Fig. 1B). In all markers that we examined, TH at 10^{-5} M had a lower effect than TH at 10^{-6} M, probably due to its toxicity (Fig. 1B). ALP staining concurred with the osteocalcin expression (Fig. 1C). These data suggest that TH exerts a potent osteogenic effect on MC3T3-E1 cells in a dose-dependent manner, and its optimal concentration is around 10^{-6} M. On the other hand, TH did not induce osteogenic differentiation on C3H10T1/2 cells or bone marrow-derived mesenchymal stromal cells at 14 days (data not shown), suggesting that TH alone exerts its osteogenic effect on cells committed to the osteoblastic lineage under our experimental conditions.

Osteogenic induction of POBs by TH through activation of BMP signaling

To investigate the osteogenic effects of TH on primary cells, we treated the POBs with TH (10^{-6} M). TH strongly induced the mRNA expression of osteocalcin and Alp within 14 days (Fig. 2A). As shown by ALP staining and von Kossa staining, TH increased the ALP activity and calcified nodule formation (Fig. 2B).

Among the osteogenic factors that have been reported, the BMP family proteins are the most notable, because they have a strong ability to induce ectopic bone formation [23]. In addition, Runx2 has been shown to be indispensable for bone formation [24] and a potent inducer of osteogenic differentiation [25]. This knowledge led us to hypothesize that TH might exert its osteogenic effect by interacting with these factors. Indeed, at 14 days, TH strongly induced the mRNA expression of Id-1, which is

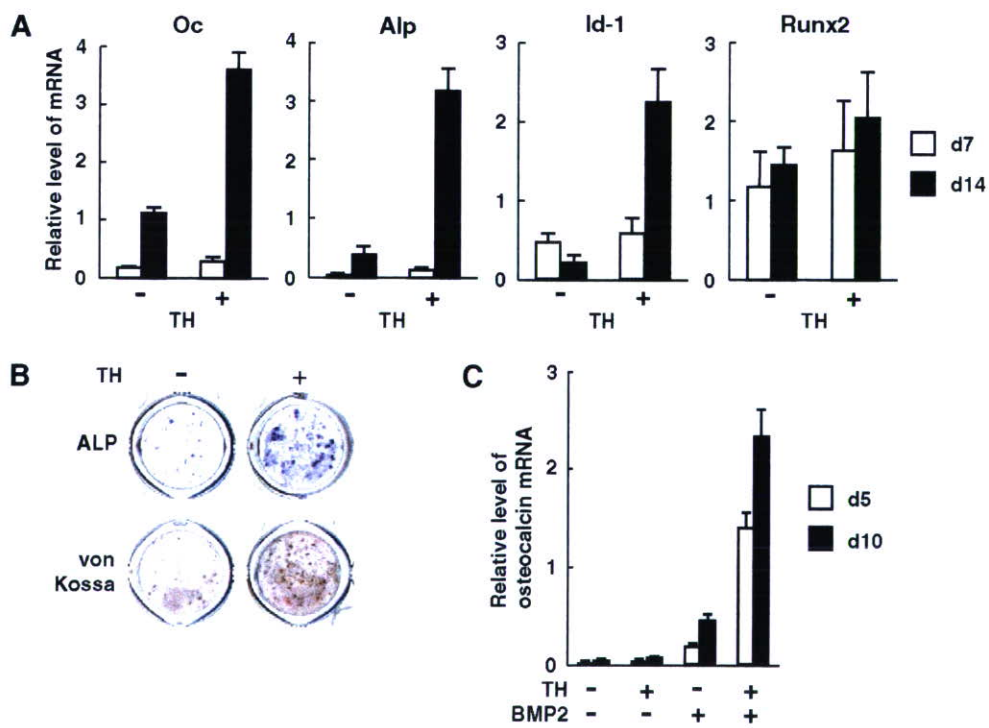

Fig. 2. Osteogenic induction of POBs (A,B) and mESs (C) by TH. (A) mRNA expression of osteoblast marker genes in POBs treated with TH (10^{-6} M), determined by real-time RT-PCR analysis. (B) ALP and von Kossa staining of POBs treated with TH (10^{-6} M) for 14 days. (C) Expression of osteocalcin mRNA in mESs treated with TH (10^{-6} M) and/or rhBMP2 (500 ng/mL), determined by real-time RT-PCR analysis. Cells were cultured in osteogenic medium.

one of the transcriptional targets of the BMP/Smad signaling activation (Fig. 2A). On the other hand, TH failed to upregulate the Runx2 mRNA expression and alter the expression or nuclear localization of the Runx2 protein (Figs. 2A and Supplementary Fig. 1). These results suggest that TH directly or indirectly activates the BMP/Smad signaling and that the osteogenic induction by TH may occur, at least partly, through this action.

Osteogenic induction of mESs by TH and the combination of TH and rhBMP2

We next treated mESs with TH at concentrations ranging from 10^{-9} to 10^{-5} M and cultured them in osteogenic medium containing Dex, β-GP, and AsAp for 21 days. TH at 10^{-7} and 10^{-6} M strongly induced the mRNA expression of osteocalcin and Alp within 21 days, whereas TH at the other concentrations did not (Supplementary Fig. 2).

The Id-1 upregulation induced by TH (Fig. 2A) raised the possibility that the combination of TH and BMP2 (TH+rhBMP2) might exert a more potent osteogenic effect on mESs than TH alone. We treated mESs with TH and/or rhBMP2, and then cultured them in osteogenic medium. TH+rhBMP2 achieved a dramatic upregulation of the osteocalcin expression at 5 days of culture, whereas each individual factor did not (Fig. 2C). The upregulation was further enhanced at 10 days (Fig. 2C). When the ES cells were cultured in 10% FBS/DMEM, TH failed to induce osteogenic differentiation, even in the presence of rhBMP2 (data not shown), indicating the requirement of Dex, β-GP, and AsAp for the osteogenic effect of TH. Phillips et al. reported that compactin, a member of the Statin family, also required these three ingredients to induce expression of the osteoblast marker genes in the mESs [26].

Osteogenic induction of DFs by the combination of TH and rhBMP2

To examine the osteogenic effect of TH on terminally-differentiated non-osteoblastic cells, we treated dermal fibroblasts derived from the mDFs or hDFs cultured in serum-free osteogenic medium with TH or rhBMP2 or both. In mDFs, rhBMP2, but not TH, induced the mRNA expression of osteocalcin, Runx2 and Id-1; TH+rhBMP2 further increased the osteocalcin expression, with upregulation of Runx2 and Id-1 expression (Fig. 3A). Analysis of the calcification by von Kossa staining concurred with these results (data not shown). Furthermore, TH+rhBMP2 induced a considerable upregulation of the osteocalcin expression and calcification in the hDFs, whereas neither TH nor rhBMP2 alone induced them (Fig. 3B and C). This system using TH+rhBMP2, which induces osteogenic differentiation of DFs in serum-free osteogenic medium with-

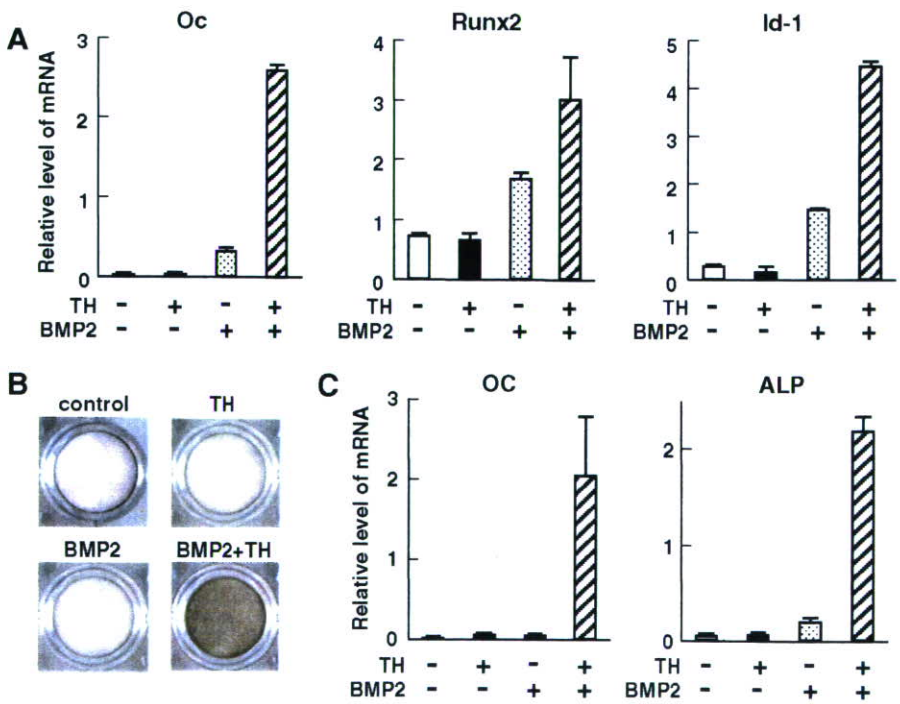

Fig. 3. Osteogenic induction of DFs by the combination of TH and rhBMP2. (A) mRNA expression of osteoblast marker genes in mDFs treated with TH (10^{-6} M) and/or rhBMP2 (500 ng/mL) determined by real-time RT-PCR analysis. Cells were cultured in osteogenic medium for 10 days. (B) von Kossa staining of hDFs treated with TH (10^{-6} M) and/or rhBMP2 (500 ng/mL). Cells were cultured in serum-free osteogenic medium for 14 days. (C) mRNA expression of osteoblast marker genes in hDFs treated with TH (10^{-6} M) and/or rhBMP2 (500 ng/mL). Cells were cultured in serum-free osteogenic medium for 14 days.

out exogenous gene transduction, may help broaden cell sources for bone regeneration, and help reduce concerns on disease transmission by animal or human serum, leading to the establishment of a safer and more efficient bone regeneration system than the conventional ones.

Involvement of BMP/Smad and Runx2 signaling in the osteogenic effect of TH

The present data suggest the involvement of the BMP/Smad signaling in the osteogenic effect of TH. To verify this possibility, we examined the effect of transduction with Smad6, which inhibited the BMP signaling by inactivating the receptors. Smad6 decreased the intensity of ALP staining and osteocalcin expression induced by TH in MC3T3-E1 cells (Fig. 4A), suggesting that the osteogenic effect of TH required activation of the BMP signaling.

There are two possible mechanisms underlying the involvement of BMP in the TH effect. First, TH may indirectly activate the BMP/Smad signaling through extracellular BMPs. Second, TH may directly activate the signaling by activating the BMP receptors or intracellularly modulating the function of the BMP-dependent Smads by an unknown mechanism, and induce transcription of the target genes. To differentiate between these two possibilities, we examined the treatment effects with Noggin, a potent BMP antagonist. The addition of Noggin into the culture media decreased the intensity of the ALP staining and the osteocalcin expression induced by TH in the MC3T3-E1 cells (Fig. 4B), suggesting that TH function requires extracellular BMP, supporting the first possibility. Furthermore, TH failed to induce the promoter activity of the 12xGCCG-luc reporter construct, which contained the DNA-binding site of Smad, within 48 h (Supplementary Fig. 3A), suggesting that TH is unlikely to directly activate the BMP/Smad signaling. In line with this result, TH failed to induce the phosphorylation of the BMP-dependent Smads within 180 min (Supplementary Fig. 3B).

TH may stimulate the synthesis of BMPs or amplify the activity of existing BMPs. To differentiate between these two possibilities, we investigated the mRNA expression of Bmp2, Bmp4, Bmp6, and Bmp7 in MC3T3-E1 cell treated with TH. These BMPs are known to be expressed in skeletal tissue and stimulate bone formation [21,27]. Interestingly, TH upregulated the mRNA expression of Bmp4 and Bmp6 (Fig. 4C), while there was no significant basal expression or TH-induced upregulation of Bmp2 and Bmp7 under our conditions (data not shown). Together

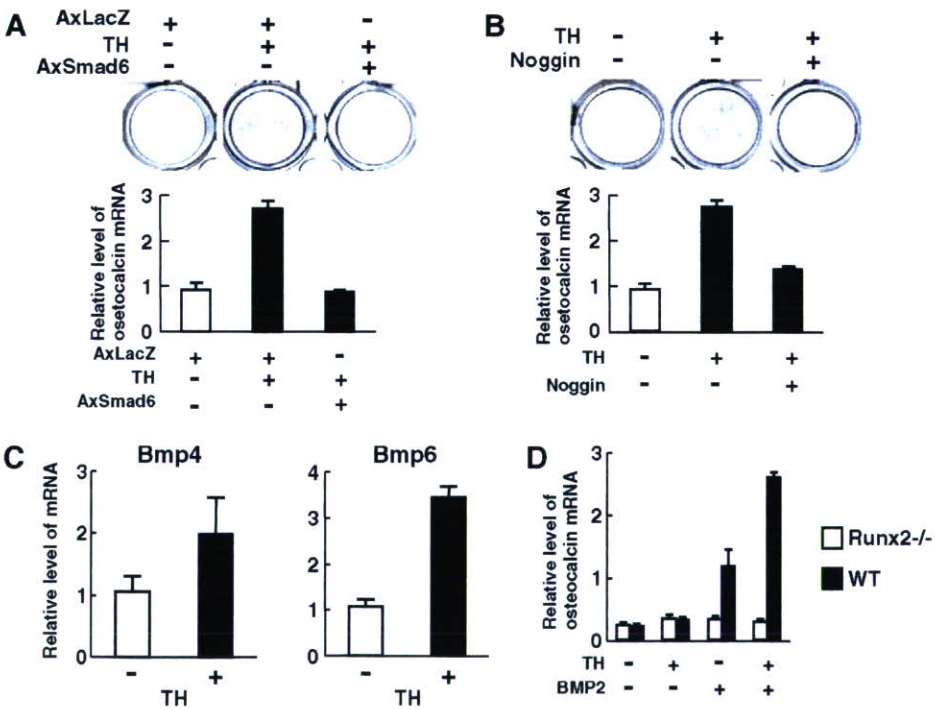

Fig. 4. Involvement of BMP/Smad and Runx2 signaling in the osteogenic effect of TH. (A) The effect of transduction with Smad6 on the osteogenic effect of TH, determined by ALP staining (upper panels) and real-time RT-PCR analysis of osteocalcin expression (lower panel). MC3T3-E1 cells were transduced with AxLacZ or AxSmad6, and cultured for 7 days with or without TH (10^{-6} M). (B) The effect of Noggin treatment on the osteogenic effect of TH, determined by ALP staining (upper panels) and real-time RT-PCR analysis of osteocalcin expression (lower panel). MC3T3-E1 cells were cultured for 7 days with TH (10^{-6} M) in the presence or absence of Noggin (1 μg/mL). (C) mRNA expression of Bmp4 and Bmp6 in MC3T3-E1 cells treated with TH (10^{-6} M) for 7 days. mRNA expression was determined by real-time RT-PCR analysis. (D) The effect of Runx2 deficiency on the osteogenic effect of TH. WT or Runx2−/− ES cells were cultured in osteogenic medium containing TH (10^{-6} M) and/or rhBMP2 (500 ng/mL). The mRNA expression of osteocalcin was analyzed by real-time RT-PCR.

with the results that TH synergistically enhances the osteogenic activity of rhBMP2 in mES (Fig. 2C) and DFs (Fig. 3), BMP4 and BMP6 induced by TH may enhance the activity of the extracellularly existing BMPs.

Finally, we examined the involvement of Runx2 in the BMP-dependent osteogenic effect of TH, because the osteogenic signaling of BMP was shown to require Runx2 [28]. In the Runx2-null ES cells, TH failed to upregulate the osteocalcin expression even in combination with rhBMP2 (Fig. 4D). In addition, TH did not activate the Runx2-dependent transcription in the reporter assay (Supplementary Fig. 3C). These data suggest that although Runx2 is indispensable for the osteogenic effect of TH, TH does not regulate the transcriptional activity of Runx2.

In summary, we report a novel osteogenic drug, TH, which induces osteogenic differentiation in osteoblastic cells, ESs, and DFs. TH exerts its osteogenic effect in synergy with BMP2, probably through inducing the BMP4 and BMP6 expressions. The local use of TH in combination with the BMPs will help broaden the cell sources for bone regeneration and also help reduce concerns about cost and risks surrounding the use of BMPs. We are now studying *in vivo* bone regeneration by TH, and the results will be reported in the near future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.173.

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Identification and characterization of the human *SOX6* promoter

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Abstract

The present study attempted to identify and characterize the embryonic promoter of Sox6, a determinant regulator of chondrogenic differentiation. A common transcription start region for human and mouse Sox6 was initially identified, which contained a highly conserved sequence, A-box. Tandem repeats of A-box had a strong transcriptional activity both at the basal level and in response to Sox9. Cells carrying the 4xA-box-DsRed2 reporter fluoresced only upon chondrogenic differentiation. The 46-bp core enhancer region (CES6) was then identified in the 3′ half of A-box, within which a C/EBP-binding motif was identified. Overexpressed C/EBPβ activated the Sox6 promoter, and mutant 4xCES6 constructs lacking the C/EBP motif lost their basal activity. CES6 and nuclear extracts formed a specific complex, which was supershifted by anti-C/EBPβ antibody, and *in vitro* translated C/EBPβ specifically bound to CES6. Thus, we successfully identified the Sox6 promoter and its core enhancer and characterized the interactions with regulatory transcription factors.

Keywords: Sox6; Sox9; Cartilage; Promoter; CCAAT enhancer binding protein (C/EBP)

Cartilage regenerative medicine attempts to treat irreversible damage to permanent cartilage caused by injuries and diseases including osteoarthritis. Cartilage, however, has a limited regenerative capacity with a low remodeling speed and has unique physiological and mechanical properties [1], presenting a major hurdle in regenerative medicine. To overcome this difficulty, it is crucial to understand the mechanism of cartilage development.

Chondrogenesis, an essential process for limb development and growth, is preceded by the recruitment of undifferentiated mesenchymal cells into condensations, which then differentiate into chondrocytes that produce cartilage-specific matrix proteins like type II collagen and aggrecan [2,3]. To date, three members of the Sry-type high-mobility group (HMG) box protein (SOX) family, a transcription factor, Sox9 and its co-activators, Sox5 (L-Sox5) and Sox6, were shown to play pivotal roles in chondrogenesis [4]. Data obtained from mouse embryo chimeras containing homozygous *Sox9* mutant embryonic stem (ES) cells and from mouse limb buds with Sox9 inactivated by the Cre recombinase/*loxP* recombination system indicated that Sox9 is essential for the mesenchymal condensation and subsequent chondrogenesis [5,6]. On the other hand, patients with campomelic dysplasia caused by the *SOX9* heterozygous mutations and mice with *Sox9* heterozygous mutations present not only with hypoplastic development of the endochondral skeletal tissues, but also with non-skeletal defects such as sex-reversal and kidney, heart, and central nervous system anomalies [7–12], suggesting its additional roles in non-chondrogenic tissues. However, Sox9 activates endogenous chondrocyte-specific genes only in the chondrocyte lineage [13], suggesting that Sox9 is not the sole determinant of chondrogenic differentiation.

Sox5 and Sox6 are induced by Sox9, and are coexpressed with Sox9 in all the chondrogenic sites of the mouse

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embryos [4]. The deletion of *Sox9* after mesenchymal condensation causes a severe generalized chondrodysplasia of the mouse embryos [5], along with the missing expression of both Sox5 and Sox6 [5,14]. While single null mutant mice of *Sox5* or *Sox6* exhibit moderate chondrodysplastic phenotypes, double-null mutants of Sox5 and Sox6 die with a severe chondrodysplasia, in which most cells are arrested as condensed mesenchymal cells, suggesting the presence of a genetic redundancy between Sox5 and Sox6 [14]. We previously showed that the combination of SOX9, SOX5, and SOX6 (the SOX trio), but not SOX9 alone, potently stimulated the chondrogenic differentiation of the non-chondrogenic cells such as the human dermal fibroblasts [15]. Moreover, in the mouse undifferentiated mesenchymal cell line C3H10T1/2, Col2a1 mRNA induction was dependent on Sox6 [16]. These lines of evidence indicate that Sox6 and Sox5 in cooperation with SOX9 work as determinant switches for chondrogenic differentiation of the mesenchymal cells; however, the promoters of SOX5 and SOX6 have not been well characterized. Aimed at elucidating the crucial molecular signals for chondrogenesis, the present study attempted to identify and characterize the human and mouse embryonic SOX6 gene promoters.

Materials and methods

5′ RACE. 5′ RACE was performed using a Marathon-Ready cDNA kit derived from human fetus and mouse embryo (Clontech), according to the manufacturer's instructions. Both in humans and mice, two gene-specific primers were used for the first and nested PCRs. The primer sequences are available upon request. Products from the nested PCRs were cloned into pCR-2.1-TOPO or pCR-Blunt vectors (Invitrogen) and then sequenced.

Database analysis. The sequence spanning up to 3-kb upstream and 1-kb downstream of exon 1 of the human SOX6 gene was analyzed by a BLAST search [17]. The detected sequences were aligned by the Vector-NTI software (Invitrogen). Prediction of the transcription factor-binding sites in the SOX6 promoter was performed using the TFSEARCH web site (Computational Biology Research Center, AIST, Japan).

In situ hybridization. Anti-sense probes were prepared by *in vitro* transcription using the DIG RNA Labelling Mix (Roche) according to the manufacturer's instructions. The positions of the anti-sense probes in the mRNAs were as follows: Sox6_exon 1, nt 77–566 of mouse exon 1; Sox6_cds, nt 1584–1978 (GenBank, Accession No. AK030713); Sox9, nt 2848–3130 (GenBank Accession No. NM_011448). Paraffin embedded blocks and sections of mouse embryos (E17.5) for in situ hybridization (ISH) were obtained from Genostaff. Hybridization was performed with DIG-labeled anti-sense RNA probes at concentrations of 100 ng/mL in Probe Diluent (Genostaff) at 60 °C for 16 h. The sections were incubated with an anti-DIG AP conjugate (Roche) for 2 h. Coloring reactions were performed overnight with NBT/BCIP (Roche). The sections were counterstained with Kernechtrot (Mutoh), dehydrated, and then mounted with Malinol (Mutoh).

Plasmid construction. The long and short fragments of the human SOX6 promoter and exon 1 region (−2089 or −517 to IVS1+23, respectively) were PCR-amplified. The primer sequences are available upon request. The amplified promoter fragments were cloned into the *Mlu*I and *Xho*I sites of pGL3-Basic (Promega). The wild-type and mutant highly conserved short sequences in the human SOX6 promoter and its complement fragments were synthesized as oligonucleotides. After the annealing and kination of the sense and anti-sense oligonucleotides, double-strand DNA fragments were cloned into the *Eco*RI site of the modified pGL3-Basic containing additional cloning sites between the *Xho*I and *Hin*dIII sites of the original plasmid. The oligonucleotide sequences are available upon request. The 4xA-box fragment of SOX6 was excised from the pGL3-4xA-box at the *Bgl*II and *Hin*dIII site and cloned into the same site of pDsRed2-1 (Clontech). The SOX9-expression plasmid was prepared as described elsewhere [15]. The cDNA sequence of the human C/EBPα and C/EBPβ was PCR-amplified and cloned into the *Bgl*II and *Kpn*I sites of pCMV-HA (Clontech). The primer sequences are available upon request.

Cell cultures. The ATDC5 and HuH-7 cells were obtained from the RIKEN BioResource Center Cell Bank (Ibaraki, Japan). The HuH-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), supplemented with L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 mg/mL). The ATDC5 cells were maintained in DMEM/Ham's F-12 (1:1) containing 5% FBS, 10 μg/mL human transferrin, 30 nM sodium selenite and the above supplements.

Luciferase assays. The ATDC5 and HuH-7 cells were plated at 30,000 cells/well on 24-well plates 24 h before transfection. In each well, 200 ng of the pGL3-reporter plasmid, 100 ng of the expression plasmid and 4 ng of the pRL-TK (Promega) internal control plasmid were co-transfected using 1 μl of FuGENE6 transfection reagent (Roche), according to the manufacturer's instructions. After 48 h of transfection, cells were harvested and analyzed using a Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instruction. The relative luciferase activity was calculated by dividing the firefly luciferase activity of the constructs by the *Renilla* luciferase activity of the tyrosine kinase promoter (pRL-TK (Promega)) as the internal control.

Stable transfection. For generating stably transfected reporter cells, 3×10^5 of ATDC5 cells were plated onto a 60-mm culture dish. After 24 h, the cells were transfected by 3 μg of the 4xA-box-DsRed2 reporter plasmid. Two days after transfection, the cells were diluted and selected in the presence of 300 μg/mL G418. After 3 weeks of selection, 48 clones were picked and expanded. The cloned cells were observed under a fluorescent microscope after the induction of chondrogenesis by 10 μg/mL insulin.

Preparation of nuclear extracts and synthesis of proteins in vitro. The nuclear extracts from the HuH-7 cells were isolated as follows. Cell pellets of HuH-7 derived from a 100-mm cell culture dish were suspended in 400 mL of Buffer A (10 mM Hepes–KOH, 10 mM KCl, 0.1 mM EDTA, 0.1% NP-40, 1× complete proteinase inhibitor cocktail (Roche) [pH 7.8]). After vortexing and centrifugation, the pellets were resuspended in Buffer B (50 mM Hepes–KOH, 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 20% glycerol, 1× Complete [pH 7.8]) and incubated on a rotator apparatus at 4 °C for 30 min . After centrifugation at 15,000 rpm for 10 min, the supernatants were collected as nuclear extracts. The C/EBPβ protein was synthesized using the TNT T7 quick-coupled transcription/translation system (Promega) with the pCITE (Novagen) *in vitro* translation vector, in which the C/EBPβ cDNA was inserted at the *Bam*HI and *Not*I sites, according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA). The dsDNA fragments of the wild-type and mutant CES6 were prepared as described in the Plasmid Construction. The EMSA assays were performed using a DIG gel shift kit (Roche), according to the manufacturer's instructions. The nuclear extracts or synthesized proteins were incubated with 400 pg of the DIG-labeled CES6 probes in the presence of 1 μg poly(dI–dC) at room temperature for 20 min. For the competition experiments, 100-fold excess of unlabeled probes were added. For the super-shift experiments, 1 μg of the anti-C/EBPα (C-18) or anti-C/EBPβ (C-19) antibody (Santa Cruz Biotechnology) was added to the proteins and incubated at 4 °C for 1 h prior to the DNA binding reactions. The electrophoretic gel separations were run at 70 V in Novex 4–15% TBE gel for 110 min (Invitrogen).

Immunoblot analysis. Immunoblotting was performed as already described [15] using 10 μg of the nuclear extracts of HuH-7 and the anti-C/EBPα (C-18) or anti-C/EBPβ (C-19): (Santa Cruz Biotechnology) at a dilution of 1:100. The secondary antibodies were used at a dilution of 1:10,000.

GenBank Accession No. Genomic sequence of human SOX6, AC100865; genomic sequence of mouse Sox6, AC102696; genomic