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Reconstitution of Runx2/Cbfa1-Null Cells Identifies a Requirement for BMP2 Signaling Through a Runx2 Functional Domain During Osteoblast Differentiation

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Abstract The Runx2/Cbfa1 transcription factor is a scaffolding protein that promotes osteoblast differentiation; however, the specific Runx2-functional domains required for induction of the osteogenic lineage remain to be identified. We approached this question using a TERT-immortalized cell line derived from calvaria of Runx2-null mice by reconstituting the osteogenic activity with wild-type and deletion mutants of Runx2. The presence or absence of osteogenic media (β-glycerol phosphate and ascorbic acid) and/or with BMP2 did not stimulate osteoblastic gene expression in the Runx2-null cells. However, cells infected with wild-type Runx2 adenovirus showed a robust temporal increase in the expression of osteoblast marker genes and were competent to respond to BMP2. Early markers (i.e., collagen type-1, alkaline phosphatase) were induced (four- to eightfold) at Days 4 and 8 of culture. Genes representing mature osteoblasts (e.g., Runx2, osteopontin, bone sialoprotein, osteocalcin) were temporally expressed and induced from 18- to 36-fold at Days 8 and 12. Interestingly, TGFβ and Vitamin D-mediated transcription of osteoblast genes (except for osteopontin) required the presence of Runx2. Runx2 lacking the C-terminal 96 amino acids (Runx2 Δ432) showed a pattern of gene expression similar to wild-type protein, demonstrating the Groucho interaction and part of the activation domain are dispensable for Runx2 osteogenic activity. Upon further deletion of the Runx2 C-terminus containing the nuclear matrix targeting signal and Smad-interacting domain (Δ391), we find none of the osteoblast markers are expressed. Therefore, the Runx2 391-432 domain is essential for execution of the BMP2 osteogenic signal. J. Cell. Biochem. 100: 434–449, 2007.

Key words: Runx2; transcriptional regulation; BMP2/TGFβ osteogenic signaling; $1,25(OH)_2D_3$

Musculoskeletal tissues are composed of a variety of specialized cell types, such as fibroblasts, adipocytes, chondrocytes, osteoblasts, and skeletal myoblasts. In vivo, a common multipotential mesenchymal stem cell (MSC) is thought to give rise to these cell types [Baksh et al., 2004; Kassem, 2004]. One of the defining characteristics of MSC is their self-renewal potential over extended time periods [Baksh et al., 2004]. The multilineage differentiation of MSCs involves commitment to a specific lineage followed by terminal differentiation. Recent genetic studies have identified several essential regulators that control bi- or uni-potential differentiation of MSC to specific pathways. They include PPARγ (peroxisome proliferator-activated receptor γ) and the C/EBP (CCAAT/enhancer-binding protein) family for adipogenesis [Wang et al., 1995; Barak et al., 1999], MyoD and myf-5 in myogenesis [Rudnicki et al., 1993; Braun and Arnold, 1995, 1996], Sox 9 and family members in chondrogenesis [Bi et al., 1999; Smits et al., 2001], and Runx2 (runt-related transcription factor 2) and the zinc

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finger-containing factor osterix in skeletogenesis [Komori et al., 1997; Choi et al., 2001; Nakashima et al., 2002].

Runx2 belongs to the runt-domain gene family that also includes Runx1 and Runx3. All three members share a highly conserved DNA-binding domain that is homologous to the runt gene in Drosophila [Kania et al., 1990; Ogawa et al., 1993a,b]. All Runx family members can bind the same DNA sequences and can heterodimerize with Cbfβ [Javed et al., 2000, 2001; Kundu et al., 2002]. Deletion of the *Runx2* gene results in embryonic or perinatal lethality with a complete absence of mineralized skeleton [Komori et al., 1997; Choi et al., 2001]. Mutations in Runx2 are associated with cleidocranial dysplasia, a dominant skeletal disorder in humans characterized by supernumerary teeth and delay in suture closure of the fontanels [Lee et al., 1997; Mundlos et al., 1997].

Differential transcriptional regulation of a large number of bone-tissue specific genes by Runx2 is well documented [Lian et al., 2004]. Selective interaction of Runx2 with various co-regulatory factors contributes to enhancement (ATF4, C/EBP, AP1, TAZ) [D'Alonzo et al., 2002; Gutierrez et al., 2002; Hong et al., 2005; Xiao et al., 2005] or suppression (Groucho, Hes-1, YAP, HDAC) of Runx2-transcriptional activity during osteoblast maturation [Javed et al., 2000; McLarren et al., 2000; Schroeder et al., 2004; Zaidi et al., 2004]. Furthermore, both direct and indirect mechanisms have been proposed for Runx2 involvement in chromatin remodeling, promoter organization, and hormonal responsive transcription of target genes [Javed et al., 1999; McCarthy et al., 2003; Sierra et al., 2003; Gutierrez et al., 2004; Young et al., 2005]. Runx2 is responsive to a number of key signaling pathways to control cell type-specific gene expression (Notch, BMP/TGFβ, Wnt, MAPK, PKC) [Lee et al., 2002; Kim et al., 2003; Zaidi et al., 2003; Selvamurugan et al., 2004a; Gaur et al., 2005; Papachristou et al., 2005; Shen and Christakos, 2005]. Thus, Runx2 acts at multiple levels to achieve cell fate determination.

To elucidate further the function of Runx2 in driving cell fate, we used mouse telomerase reverse transcriptase (TERT) expression to establish normal diploid, immortalized cell lines from the homozygous pups of Runx2-null [Komori et al., 1997] or Runx2-C-terminal-deleted knock-in mutation animals [Choi et al., 2001]. It has been shown that expression of the catalytic subunit of telomerase, TERT, in cultured human primary cells reconstitutes telomerase activity and allows immortal growth [Bodnar et al., 1998; Kiyono et al., 1998; Jiang et al., 1999]. Unlike embryonic stem cells, adult MSCs which lack telomerase activity [Zimmermann et al., 2003] show defined ex vivo proliferation capability, reaching senescence and losing multi-lineage differentiation potential after 30–50 population doublings in culture. Thus, it is necessary and critical to develop new strategies to prolong the replicative capacity of MSCs without impairing their multi-potentiality. Several studies have shown that ectopic expression of TERT in postnatal MSCs can extend their life span to more than 260 population doublings, while maintaining their multilineage differentiation potential [Simonsen et al., 2002; Shi et al., 2002]. Importantly, these TERT-transduced immortalized MSCs have normal karyotype and do not cause tumor formation in xenogenic transplants; these characteristics make them an attractive candidate source of cells for tissue repair and regeneration. For these reasons, we selected TERT-immortalization of calvarial-derived cells from Runx2-null mice representing putative osteoprogenitor cells to address mechanisms of osteoblastogenesis.

We characterized calvarial tissue-derived cells from Runx2 knock-out and knock-in mice to determine their ability to differentiate along the osteoblast lineage in response to osteogenic signals (BMP2, TGFβ, or 1,25-dihydroxyvitamin D3) in the presence or absence of Runx2 wild-type and mutant proteins. In long-term culture, neither osteogenic media nor BMP2 were permissive for Runx2-deficient cells to acquire the osteoblastic phenotype. Reintroduction of wild-type Runx2 by adenovirus infection rescued the osteoblast phenotype, which was enhanced in the presence of BMP2. TGFβ and Vitamin D-responsive transcription of the osteoblast marker genes, with the exception of osteopontin, required the presence of Runx2. Using a series of Runx2-deletion mutants, we established that a 41 amino acid domain in the carboxyl terminus of Runx2 that contains the nuclear matrix targeting signal and the Smad-interacting domain is necessary for programming these mesenchymal cells to the osteogenic lineage. Taken together, these data indicate that our immortalized Runx2-deficient

calvarial cells represent an immature mesenchymal cell pool that can be differentiated to osteoblasts in a Runx2-dependent manner. BMP2 signaling alone is insufficient to promote osteoblastogenesis in the absence of Runx2.

MATERIAL AND METHODS

Cell Culture and Immortalized Cell Lines

Cells were isolated from calvarial tissues of 17.5 dpc mouse embryos of the Runx2-null mouse [Komori et al., 1997] and Runx2 knock-in animals [Choi et al., 2001]. Diploid cells from the homozygous pups ($Runx2^{-/-}$ and $Runx2^{\Delta C/\Delta C}$) were obtained essentially as described previously [Pratap et al., 2003]. Briefly, calvaria were minced and subjected to three sequential digestions (8, 10, and 26 min) with collagenase P (Roche Molecular Biochemicals, Indiana, IN) at 37°C. Cells released from the third digest were collected and resuspended in α-MEM supplemented with 10% FBS and plated at a density of 0.5×10^6 cells/6-well plate. Cells were transfected with mouse telomerase (mTERT) expression vector, a kind gift from Dr. Robert Weinberg, Massachusetts Institute of Technology [Lundberg et al., 2002]. Plasmid DNA (2 μg) was mixed with Fugene 6 reagent at a ratio of 2:6 and the mixture was overlayed on cells for 4 h. Cells were then washed and fed with α-MEM supplemented with 10% FBS. After 4 days, cells were passed and selected with 10 μg/ml of G418. Neomycin resistant cells were cultured to passage 17 in selection media, at which time cells were frozen and maintained in media without G418. Cells were grown to passage 40 for assessment of the phenotypic potential and reproducibility of the established cell lines. Most of the experimental data were confirmed with at least two independent passage numbers. For differentiation assays, cells were cultured in osteogenic media (α-MEM supplemented with 10% FBS, 10 mM of β-glycerol phosphate, and 50 μg/ml of ascorbic acid).

Adenoviral Infections

Adenovirus expressing wild-type and C-terminal deletion mutants of Runx2 were generated via Adenovator system (Q BIOgene, Carlsbad, CA) and have been reported previously [Afzal et al., 2005]. $Runx2^{-/-}$ cells were plated at a density of 1×10^5 cells per well of a 6-well plate or 1×10^6 cells per 100-mm plate. Cultures at 70% confluence were infected with the Runx2 adenovirus in α-MEM without serum. After 90 min, cells were washed with serum free α-MEM and fed with complete α-MEM till harvest.

Western Blot Analysis

Whole cell lysates from $Runx2^{-/-}$, $Runx2^{-/-}$ cells infected with various adenoviruses and $Runx2^{\Delta C/\Delta C}$ cell lines were resolved by 10% polyacrylamide gel electrophoresis and protein transferred to Immobilon (Millipore) membrane. The blots were blocked in PBS-T (0.1% Tween 20) containing 2.5% nonfat dry milk for at least 1 h. Mouse monoclonal primary antibodies used were anti-Runx2 [Afzal et al., 2005], anti-tubulin (Sigma-Aldrich, MO), and anti-laminB (Santa Cruz, CA). Blots were incubated for 1 h at room temperature with 1:5,000 dilution of the primary antibody in PBS-T solution containing 1% milk. The blots were washed three times with the PBS-T solution and incubated for 1 h at room temperature with 1:5,000 dilution of the horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz, CA). After three washes with PBS-T solution, the immunoreactive bands were detected with ECL (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) by exposing blots to XAR-5 film (Kodak, Inc., Rochester, NY).

Alkaline Phosphatase Cytochemistry

$Runx2^{-/-}$ cells infected with various adenoviruses or uninfected $Runx2^{\Delta C/\Delta C}$ were cultured for 12 days in the absence or presence of BMP2. Cells were washed twice in 0.1 M cacodylic buffer and subsequently fixed in 2% formaldehyde in cacodylic buffer for 10 min at room temperature. After washing with 0.1 M cacodylic buffer, cells were treated with ALP histological stain solution prepared freshly. Following reagents purchased from Sigma-Aldrich were added in distilled water for a 50 ml total volume: 25 mg of Napthol AS-Mx phosphate disodium salt, 1.4 ml of NN dimethyl formamide, 25 ml of tris maleate buffer (0.2 M), and 50 mg of fast red salt. The solution was filter sterilized and immediately added to the wells. The plates were incubated at 37°C for 30 min or until color development was observed. The wells were then rinsed with distilled water and stained cells were visualized using an inverted microscope.

In Situ Immunofluorescence

$Runx2^{-/-}$ and $Runx2^{\Delta C/\Delta C}$ cells were plated at a density of 0.6×10^5cells/well on gelatin-coated

coverslips in 6-well plates. Cells were processed in situ for whole-cell (WC) and digital microscopic analyses were carried out as described previously [Javed et al., 2000]. Runx2 was detected by a rabbit polyclonal antibody at a dilution of 1:400 (Santa Cruz Biotechnology) and tubulin with a mouse monoclonal antibody against α-tubulin (Sigma-Aldrich, MO) at a dilution of 1:400. Secondary antibodies used were Alexa 488 anti-rabbit or Alexa 568 anti-mouse (Molecular Probes) at a dilution of 1:800. Slides were examined on a Zeiss Axioplan 2 microscope fitted with epifluorescence (Carl Zeiss Jena, Germany) attached to a CCD camera. Images were saved and processed using Metamorph imaging software, version 6.1 (Universal Imaging, Downingtown, PA).

Northern Blot Analysis

Total RNA was isolated from Runx2-null cells cultured in various conditions by using TRIzol reagents (Life Technology, Inc., Gaithersburg, MD) according to the manufacturer's specifications. For each treatment and time point, 20 μg of total RNA was separated in a 1% agarose-formaldehyde gel. Integrity of the RNA and equal loading of samples were assessed by ethidium bromide staining. RNA was transferred onto Hybond Plus membranes (Amersham Pharmacia Biotech, Buckinghamshire, England) in 20× standard saline citrate solution (SSC) overnight and immobilized by UV cross linking for 1 min. Blots were hybridized with cDNA probes specific for histone H4, alkaline phosphates, collagen type I, osteonectin, osteocalcin, Runx2, and GAPDH essentially as described previously [Gutierrez et al., 2002]. All hybridization was performed overnight at 42°C in hybridization buffer [5× SSC, 5× Denhart's solution, 1% sodium dodecyl sulphate (SDS), 10% dextran sulphate, 50% formamide] and the blots were washed extensively in buffer containing 0.5× SSC and 0.1% SDS at 55°C. Data were analyzed after overnight exposure using a Storm 840 PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

RT-PCR and Quantitative Real Time PCR

Total RNA (1 μg) was reversed transcribed using advantage RT-for-PCR kit (Clontech, Palo Alto, CA) following the manufacturer's protocol. Briefly, RNA was denatured at 65°C for 5 min and RT reaction was carried out with 200 U of MMLV reverse transcriptase enzyme in a final reaction of 20 μl. Freshly reverse transcribed cDNA (1 μl) and the fluorescent SYBR Green I dye was used to monitor DNA synthesis (SYBR Green PCR master mix, Applied Biosystems, Foster City, CA) in a real-time PCR assay. Specific primers designed to amplify mouse ALP, type I collagen, OP, BSP, OC, and Runx2 transcripts are as shown in Table I.

The PCR was carried out following a cycling protocol: an initial denaturation step at 95°C for 10 min followed by 40 repeat cycles each of a denaturation at 95°C (10 min), annealing 60°C (15 sec), and extension at 60°C (60 sec). Detection of the fluorescent product was carried out at the end of the 60°C extension period. Gene expression was normalized to the GAPDH as housekeeping gene. PCR products were subjected to a melting curve analysis and the data was analyzed and quantified with the ABI Prism 7000 SDS analysis software (Applied Biosystems).

RESULTS

Establishment of Runx2-Null Cell Model and Requirement of Runx2 for Osteogenic Differentiation

For understanding molecular mechanisms that are operative in Runx2-dependent cell differentiation, we have established calvarial-tissue-derived cell lines from two mouse models. The Runx2-null ($Runx2^{-/-}$) mouse dies shortly after birth and the Runx2 knock-in ($Runx2^{\Delta C/\Delta C}$) mouse that expresses a C-terminally truncated Runx2 protein from the P1 and P2 promoters (Fig. 1A) dies in utero after 17.5 dpc. Therefore,

TABLE I. Nucleotide Sequence of Primers Used for Quantitative RT-PCR Detection

Gene	Primer sequence
Runx2	For 5′-CGG TTC AGA GTG GAC TC-3′
	Rev 5′-GAC TCT GTA AGC GGG TCT GC-3′
Collagen I	For 5′-GTA TCT GCC ACA ATG GCA CG-3′
	Rev 5′-CTT CAT TGC ATT GCA CGT CAT-3′
BSP	For 5′-GCA CTC CAA CTG CCC AAG A-3′
	Rev 5′-TTT TGG AGC CCT GCT TTC TG-3′
Osteocalcin	For 5′-CTG ACA AAG CCT TCA TGT CCA A-3′
	Rev 5′-GCG CCG GAG TCT GTT CAC TA-3′
Alkaline phosphatase	For 5′-TTG TGC GAG AGA AAG GAG A-3′
	Rev 5′-GTT TCA GGG CAT TTT TCA AGG T-3′
Osteopontin	For 5′-TTT GCT TTT GCC TGT TTG C-3′
	Rev 5′-CAG TCA CTT TCA CCG GGA GG-5′

for comparison of age-matched development of calvarial tissue from the Runx2 mouse models, cells were isolated by a limited collagenase digestion from homozygous pups at E17.5 dpc and transfected with mouse telomerase (mTERT) expression vector. Cells were selected for neomycin resistance and considered immortalized after passage 17. Both established cell lines exhibited similar morphologic features as revealed by immunofluorescence and bright field microscopy (Fig. 1B,C). The absence ($Runx2^{-/-}$) and presence ($Runx2^{\Delta C/\Delta C}$) of the Runx2 protein in these cell lines was confirmed using in situ immunofluorescence and biochemical approaches (Fig. 1D). Thus, we have successfully generated calvarial-tissue-derived cell lines that are either deficient or express a truncated version of Runx2 protein.

For assessment of their phenotypic potential, both cell lines were cultured for 12 days in growth medium (α-MEM supplemented with 10% FBS) or in osteo-inductive medium (growth media supplemented with 10 mM of β-glycerol phosphate and 50 μg/ml of ascorbic-acid). Although cells reach full confluency by Day 7, nodule formation (multilayered osteoblast colonies) was not observed during 20 days of culture (data not shown). Even treatment of these cultures with BMP2 for 12 days failed to initiate formation of osteoblast nodules.

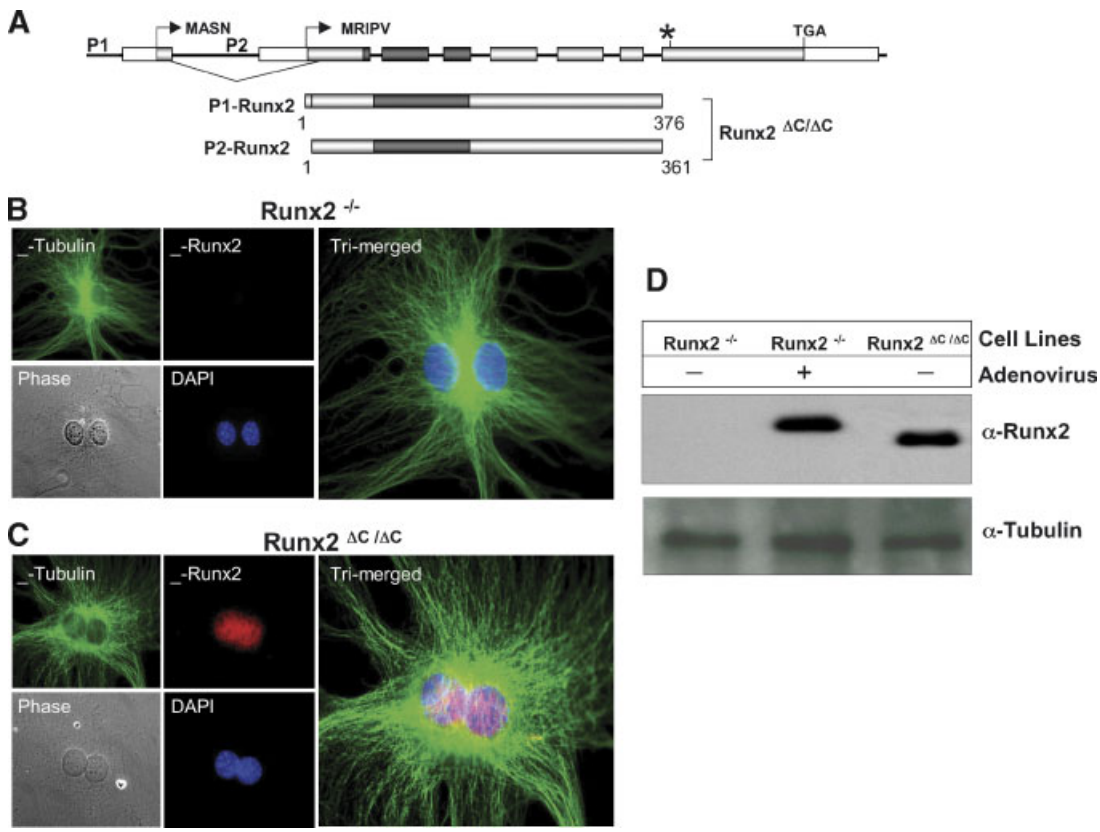

Fig. 1. Cell and nuclear morphology of the established Runx2-null cell line. **A**: Schematic illustration of the Runx2 genomic locus showing the origin of the two protein isoforms (**top panel**). Boxes denote various exons (clear-untranslated regions, gray-translated regions, dark-exon encoding DNA-binding domain). Star represent introduced premature stop codon in the $Runx2^{\Delta C/\Delta C}$ knock-in mice. Amino acid numbers of the Runx2 proteins derived from the promoter 1 and 2 usage in the $Runx2^{\Delta C/\Delta C}$ cells are indicated. **B**: Fetal calvarial tissue (E 17.5) from $Runx2^{-/-}$ and $Runx2^{\Delta C/\Delta C}$ animals were isolated and digested with collagenase as described in Method section. Cells were cultured in αMEM supplemented with 10% FBS and immortalized by transfection with 5 μg of mouse telomerase expression vector. Cells at passage 20 from (B) $Runx2^{-/-}$ and (**C**) $Runx2^{\Delta C/\Delta C}$ were plated on gelatin coated glass cover slips and processed for in situ immunofluorescence. Cells were fixed in 4% formaldehyde, permeabilized and stained for Tubulin and Runx2. Nuclei are revealed by DAPI staining. Tri-merged represent montage of Runx2, Tubulin, and DAPI signals. Magnifications 63×. Runx2 nuclear signal is observed only in $Runx2^{\Delta C/\Delta C}$ preparations. **D**: Whole cell lysates from control null and adeno-virus infected $Runx2^{-/-}$ cells as well as $Runx2^{\Delta C/\Delta C}$ cells were resolved on SDS–PAGE for western blot analysis. Blot was probed with mouse monoclonal antibody for Runx2, strip, and re-probed for tubulin antigen as a loading control.

Thus, in the complete absence of Runx2 or in the presence of the Runx2 ΔC mutant, the E17.5 cell lines cannot be induced to the osteoblast lineage under appropriate culture conditions.

For a mechanistic understanding of a failure of these cells to differentiate in these conditions, we isolated RNA from the Runx2-null cells at different days from parallel cultures. Northern blot analysis indicates that in all conditions, untreated and in the presence of BMP2 in either growth or osteoinductive media, cells withdrew from the cell cycle as reflected by termination of histone H4 expression, a cell cycle marker (Fig. 2). However, we did not observe expression of any of the key osteogenic differentiation marker genes in control or BMP2-treated cells. Thus, osteo-inductive cocktail or BMP2 is not sufficient to initiate expression of osteoblast-related genes in Runx2$^{-/-}$ cells.

To establish the osteogenic potential of Runx2$^{-/-}$ cells, we infected the cells with a Runx2-expressing adenovirus and isolated RNA from control and Runx2 reconstituted cells at Days 4, 8, and 12 for analysis of differentiation towards the osteoblast lineage. Reintroduction of Runx2 in these cells resulted in a robust temporal induction of genes considered hallmarks of mature osteoblast phenotype (Fig. 2). Peak expression of early markers Alkaline phosphatase (ALP) and collagen type I (Col I) was noted at Day 8 followed by late markers osteopontin (OP) and osteocalcin (OC) at Day 12. Taken together, these findings demonstrate that we have established a cell model system in which Runx2 activity is required to initiate a program of gene expression for osteoblast differentiation.

Requirement of Runx2 for Vitamin D and TGFβ-Responsive Transcription of Osteoblast Marker Genes

We and others have reported that integrity of Runx2 DNA-binding sequences in target gene promoters is critical for their response to various physiologic regulators such as 1,25-dihydroxyvitamin D_3 (Vitamin D_3), BMP2, and TGFβ [Javed et al., 1999; Chikazu et al., 2002; Selvamurugan et al., 2004b]. We took advantage of the absence of Runx2 in these cells to address the physiologic responses of endogenous genes during osteogenic differentiation. Control and Runx2 virus-infected cells were cultured in osteo-inductive media for 12 days and treated with 20 ng of TGFβ or 10^{-8} M Vitamin D_3 for 24 h. Consistent with the previous observation, control cells (not infected

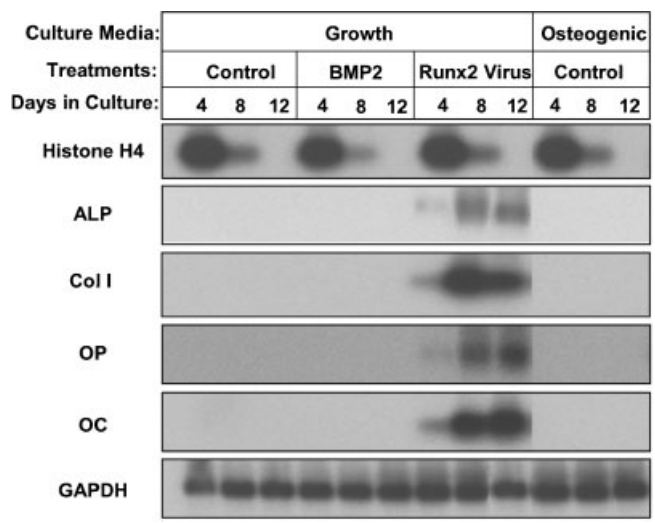

Fig. 2. Runx2 is required for osteoblastic differentiation of the null cell line. Commitment of Runx2 null cell to osteoblast lineages was tested in either control media (αMEM supplemented with 10% FBS) or osteogenic media (αMEM supplemented with 10% FBS, 10 mM of β-glycerol phosphate, and 50 μg/ml of ascorbic-acid). Runx2-null cells were plated in 100-mm dishes, treated for 24 h with 100 ng/ml of BMP2, or infected with 50 MOI of Runx2-expressing adenovirus and cultured for 12 days. Total RNA was isolated from all treatment groups at indicated days and 10 μg was separated on 1% formaldehyde-agarose gel. Northern blot analysis for cell cycle and osteogenic markers was performed as described in Methods. Runx2-null cell cultured in osteogenic cocktail do not differentiate and induction of osteoblastic differentiation was noted only in Runx2-reconstituted cells.

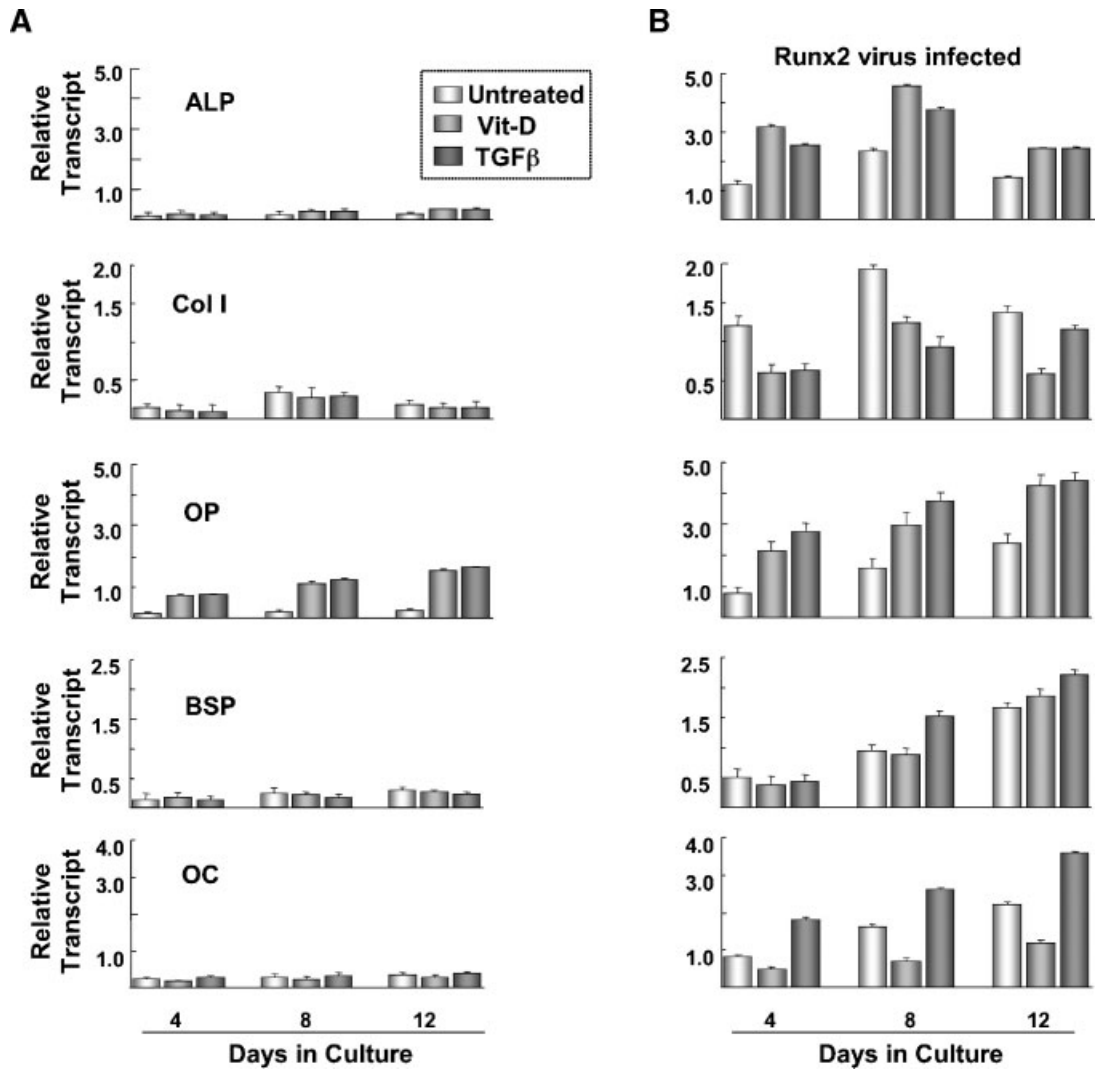

Fig. 3. Vitamin D responsive transcription of osteopontin is independent of Runx2. Runx2-null cells were cultured in osteogenic media in 100-mm dishes for 12 days and treated with 20 ng of TGFβ or 10^{-8} M Vitamin D for 24 h prior to harvesting. **A**: Control Runx2 null cells or (**B**) cells infected with 50 MOI of Runx2 adenovirus were harvested at indicated days and total RNA isolated. RT-PCR analyses were carried out for early and late differentiation markers as described in methods. Primers used in QPCR to analyze gene expression are shown in Table I. Data were normalized with GAPDH values and are representative of two independent determinations of duplicate samples. In control cells, only osteopontin gene is responsive to Vitamin D treatment where as cells reconstituted with Runx2 infection a differential and temporal response of all target genes was seen by TGFβ and Vitamin D.

with Runx2) showed no expression of osteoblast marker genes (Fig. 3A). Addition of TGFβ or Vitamin D_3 failed to activate expression of all markers at any time point during differentiation, except for osteopontin. When cells were infected with Runx2 adenovirus and then treated with TGFβ or Vitamin D_3, a robust and differential response was seen (Fig. 3B). While both treatments resulted in a 2.8- to 3-fold-enhanced expression of ALP, a 50% reduction in expression of Col I occurred at Days 4 and 8 in response to Vitamin D, consistent with known regulation of Col I by Vitamin D_3 [Bedalov et al., 1998]. No significant changes in levels of BSP expression could be seen throughout the differentiation time course with either treatment. Vitamin D_3 caused a 50% reduction and TGFβ enhanced expression of the OC at later time points. These findings are consistent with responses of the endogenous genes in osteoblasts that express significant levels of Runx2 [Van Leeuwen et al., 2001].

Surprisingly, we observed a 3–5 fold-enhanced expression of OP gene expression in control

cultures upon treatment with both TGFβ and Vitamin D (Fig. 3A). Reintroduction of Runx2 also resulted in a 3–5 fold increased basal expression which was further enhanced 2–3 fold in the presence of both TGFβ and Vitamin D. Taken together, these findings demonstrate a critical role of Runx2 in mediating TGFβ or Vitamin D-responsive transcription of osteoblast target genes.

A Specific 41 Amino Acid Fragment in the Carboxyl Terminus of Runx2 Controls Osteogenic Differentiation of Reconstituted Runx2-Null Cells

Our cell model system that requires Runx2 activity for osteoblast differentiation provided us a viable opportunity to identify region(s) of Runx2 that mediate its osteogenic function in a biological assay. We generated a series of adenoviruses expressing deletion mutants of Runx2 protein (Fig. 4A). All of the mutant Runx2 proteins enter the nucleus and bind DNA (data not shown). Runx2-null cells were infected with pre-determined multiplicity of infection (MOI) of wild-type and mutant Runx2 viruses. Cells were cultured in 6-well dishes for 12 days in media containing osteo-inductive cocktail and harvested for protein. We find a sustained and comparable expression of Runx2 proteins throughout the differentiation time course (Fig. 4B). Parallel cultures of Runx2-infected cells, in the presence or absence of BMP2, were stained for ALP activity (Fig. 5A). A temporal increase in ALP activity was noted only in cells infected with WT and Δ432-mutant Runx2 viruses. Addition of BMP2 resulted in progressively enhanced ALP staining in Runx2 WT and Δ432-expressing cells. Strikingly, no ALP activity could be detected at any time point in cells infected with control or Runx2 Δ391 viruses (Fig. 5A). Thus, Runx2 sequences between 391 and 432 are essential for induction of alkaline phosphatase, an early marker of osteoblast differentiation.

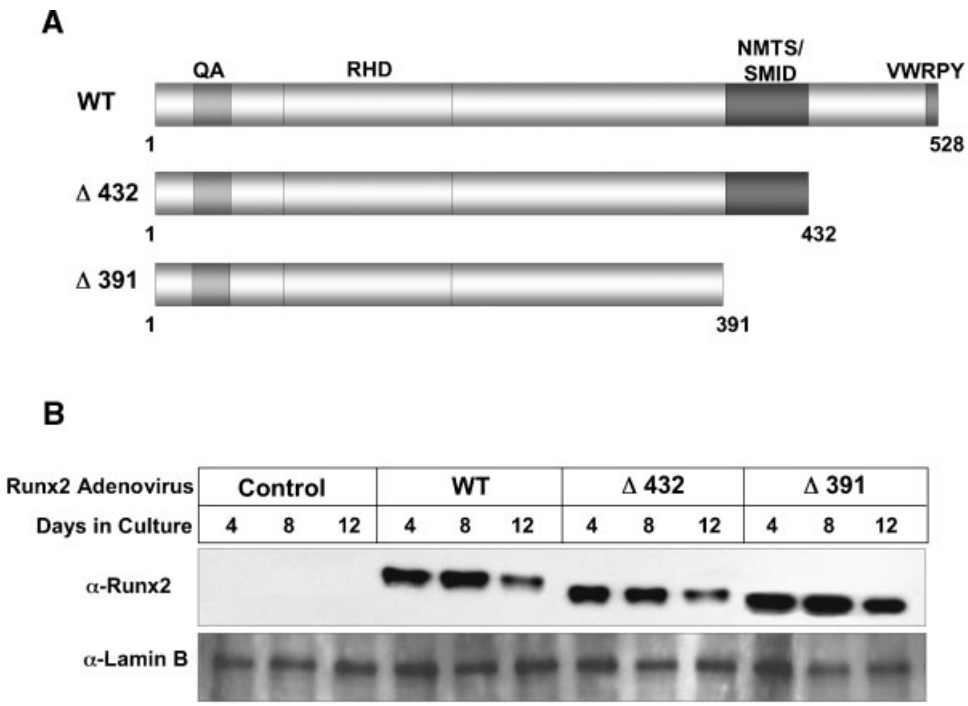

Fig. 4. Runx2 expression in reconstituted null cells is sustained throughout differentiation time course. For reconstitution assay, Runx2-null cells were infected with adenovirus expressing wild-type or mutant Runx2 protein. **A**: Schematic illustration of the wild-type and mutant Runx2 proteins. Runx2 protein domains include the QA (poly glutamic-acid and alanine stretch, the conserved domains for all Runx factors: the RHD, runt homology DNA binding domain; NMTS/SMID, nuclear matrix targeting signal/Smad-interacting domain; and VWRPY, TLE-interacting conserved penta-peptide) are indicated. Total number of amino acids for each Runx2 protein is indicated underneath. **B**: Runx2$^{-/-}$ cells at passage 19 were infected with wild-type (50 MOI), Δ432 (50 MOI), and Δ391 (50 MOI), and cultured for 12 days in 6-well dishes. Cells at indicated days were directly lysed in plate as described in methods. For comparison of Runx2 expression levels, equal amount of cell lysates were resolved on 10% SDS–PAGE for western blot analysis. Blots were probed with monoclonal Runx2 antibody, stripped and reprobed with LaminB antigen, used as loading control.

To better evaluate the osteoblast phenotype, we performed quantitative expression analysis for mature osteoblast marker genes. Total RNA was isolated from control and Runx2-reconstituted cells and subjected to real-time RT-PCR with mouse specific primers (Table I). Consistent with the ALP enzyme activity, we observed a 3–4 fold-enhanced ALP transcript in cells infected with the WT and Δ432 viruses, but no change in Δ391 cultures when compared to control (Fig. 5B). A similar degree and pattern of induction of Col I mRNA was noted, with the maximum expression at Day 8. Osteopontin expression was induced 4–5 fold by the WT and 4–8 fold by the Δ432 Runx2 proteins. Again cells expressing the Δ391 mutant Runx2 protein did not show a change in the OP levels when compared to control cultures. When we analyzed late osteoblast markers BSP and OC, a 2–8 fold-enhanced expression was observed by the WT and Δ432-infected cells with the maximum transcript levels occurring in Day 12 samples. The failure of osteoblast marker gene activation in Δ391 reconstituted cells is not related to the expression of the mutant protein as comparable levels were sustained throughout the time course (Fig. 4B).

To further validate the failure of Δ391 mutant Runx2 protein to initiate the program of osteogenic differentiation as related to specific deletion of the C-terminus, we analyzed expression of the endogenous Runx2 gene (Fig. 5B). We designed primers in the 3′ un-translated region which is absent in our Runx2 viruses. Strikingly, a robust activation of the endogenous Runx2 gene was observed in cells reconstituted with WT (4- to 18-fold) and Δ432 (4- to 25-fold) Runx2 proteins. Cells infected with control or Δ391 mutant Runx2 adenovirus showed no expression of the Runx2 gene. This finding indicates that a 41 amino acid fragment in the carboxyl terminus of Runx2 supports Runx2 expression in these early osteoprogenitor-derived cells. As illustrated in Figure 4A, this fragment contains the unique Runx2 nuclear matrix targeting signal and the Smad-interacting domain. Taken together, our studies demonstrate that the 391–432 domain of Runx2 is essential for the function of this master regulator in controlling the developmental profile of gene expression throughout osteoblast differentiation.

Runx2 Is Required for Execution of BMP2 Signal and Osteogenic Differentiation

Bone morphogenetic proteins enhance osteogenic differentiation and mediate their signals through Smad transducer proteins. We and others have shown a direct physical and functional association between Runx2 and Smads

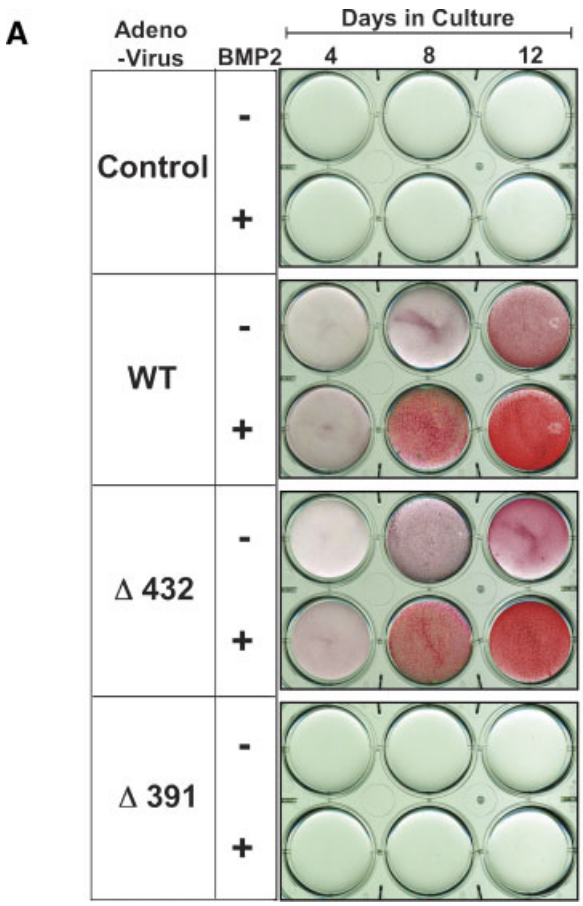

Fig. 5. A specific domain of 41 amino acid in the carboxyl terminus of Runx2 drives osteogenic differentiation of reconstituted null cells. The osteogenic capacity of the wild-type and deletion mutant Runx2 proteins were tested in reconstitution assay of Runx2$^{-/-}$ cell line. Cells were plated in 6-well dishes and upon confluency infected with Runx2 adenovirus for 4 h. Cells were then washed and cultured for indicated days in osteogenic media. **A**: Cells were fixed and immuno-cytochemistry for early marker alkaline phosphatase was carried as described in methods. Images of scanned ALP-stained plates are presented. **B**: Control and Runx2-infected cells were harvested for RNA isolation at indicated days. Real-time RT-PCR analyses were performed to assess expression of the Runx2 target genes during osteoblastic differentiation. Data are presented as relative transcript normalized to GAPDH levels in the same sample. All experiments were performed in duplicates and representative image are shown in (A) and mean normalized values with standard deviation in (B).

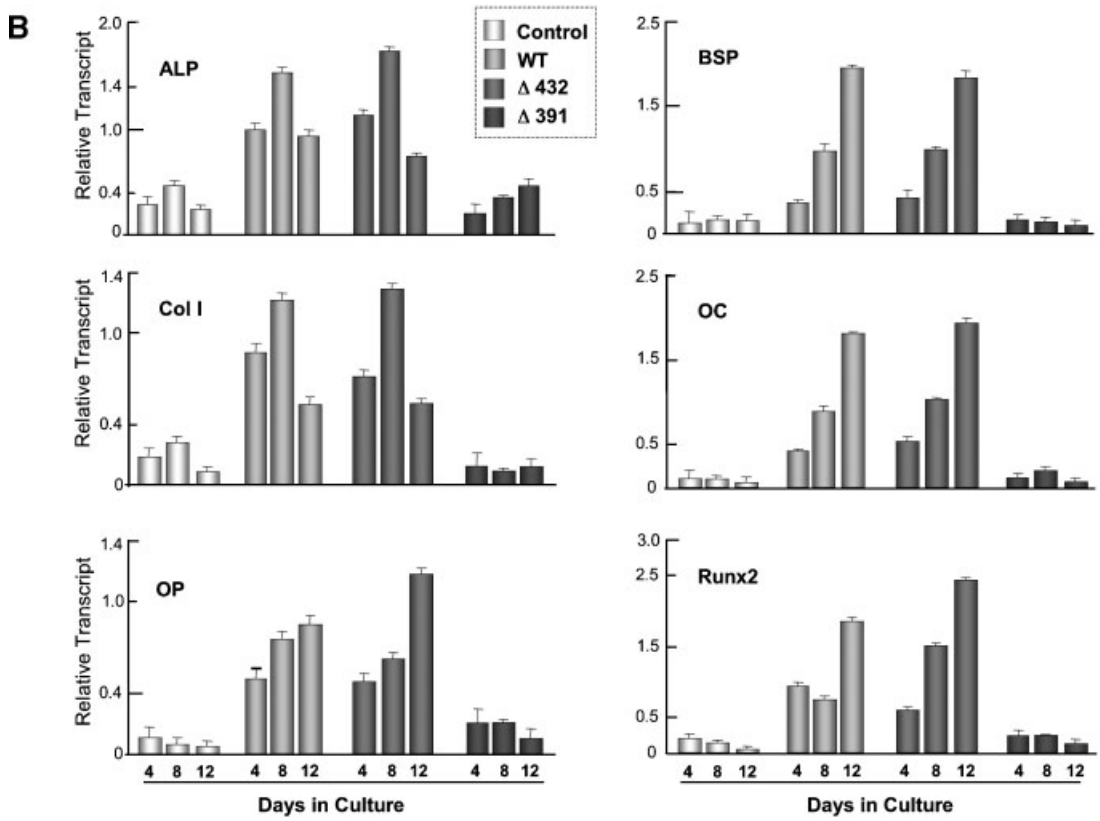

Fig. 5. (*Continued*)

[Lee et al., 2002; Afzal et al., 2005]. We found that Runx2$^{-/-}$ cells are unresponsive to the BMP2 osteogenic signal (see Figs. 2 and 5A). To test if this is related to defects in the BMP pathway or the absence of Runx2 activity, we reconstituted Runx2-null cells with adenoviruses expressing WT or mutant Runx2 proteins and cultured them for 12 days in the presence or absence of 100 ng/ml BMP2. Cells were harvested at Day 4, 8, and 12 and RNA isolated for real-time RT-PCR analysis to determine the extent of BMP2-mediated osteogenic differentiation (Fig. 6). Expression of early osteoblast marker genes (ALP and Col I) in cells infected with Runx2 WT and Δ432 virus was enhanced 3–7 fold by BMP2 treatment, while no change could be detected in Runx2 Δ391 reconstituted cells at any time point. In the presence of WT Runx2 protein, BMP2-induced expression of OP from 6–12-fold and 6–7 fold with Runx2 Δ432. BMP2 stimulated the transcription of the late marker BSP and OC genes from 5–7 fold by both WT and Δ432 Runx2 proteins. The integration of the BMP2 signal for maximal BSP and OC expression occurred at Day 4 and remained elevated for the 12-day culture period. OP expression is distinguished from other marker genes as maximal BMP2-mediated activation of OP requires Runx2 amino acids 432 to 528. Runx2 is an early target of the BMP2 signal [Lee et al., 2000; Balint et al., 2003] and we observed a 2–12-fold-enhanced expression of the endogenous Runx2 gene. It is important to note that BMP2 treatment at Day 4 resulted in only a small change of Runx2 mRNA (2-fold compare to 12-fold at later days). Thus, integration of the BMP signal by Runx2 for its positive autoregulation may involve additional secondary events.

Taken together, our results support the conclusion that the 41 amino-acid fragment (391–432) of Runx2 possesses properties for both integration of the BMP2 signal and osteogenic differentiation. Thus, our cell model that permits Runx2 reconstitution, integration of osteogenic signals from other pathways, and phenotypic differentiation is a useful and unbiased system for examining Runx2-dependent and -independent regulatory mechanisms.

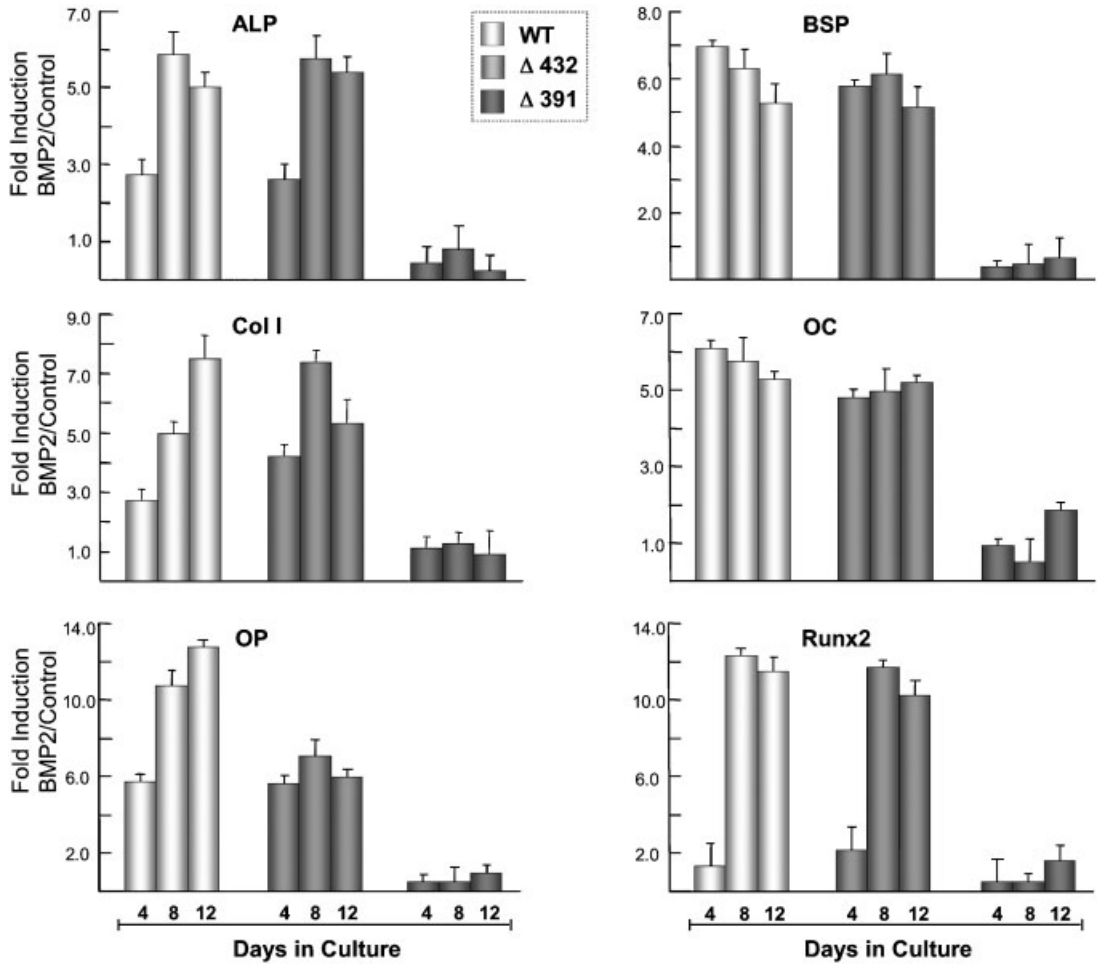

Fig. 6. Integration of BMP2 osteogenic signal by Runx2 during differentiation requires Smad-interacting domain. Runx2$^{-/-}$ cells were cultured in 6-well plates and 2 days later infected with the pre-determined MOI Runx2 adenoviruses. Cells were fed every other day with regular media in the presence or absence of 100 ng/ml of BMP2. Cells were harvested at indicated days and RNA isolated. Real time-PCR for selective marker genes were carried out as described earlier. All duplicate values were normalized to internal GAPDH levels and data are presented as fold induction (uninfected BMP2-treated/infected BMP2-treated).

DISCUSSION

One goal of the present work was to develop a model system with which to study the genetic events underlying the Runx2-controlled osteogenic differentiation of mesenchymal cells. Osteoblasts are derived from mesenchymal cells and are a crucial component of the skeleton. Investigations using cultured osteoblastic cells are instrumental for defining the biology of bone. However, primary cells in culture not only have a finite replicative lifespan [Hayflick and Moorhead, 1961], but their properties change with passage and therefore, limit the scope of their experimental manipulation and reproducibility. This practical limitation has fostered numerous efforts to develop skeletal-derived cell lines that possess unlimited replicative potential and maintain competency for differentiation. We have used expression of mTERT that allows cells to bypass senescence and become immortalized in a single step [Bodnar et al., 1998; Dickson et al., 2000] for establishing Runx2-null or -mutant cell lines. These cells represent a novel experimental model to identify molecular mechanisms critical for development of the osteoblast phenotype that either require Runx2 or are Runx2-independent.

Focusing on the Runx2-null cell line, we addressed the biological responses to osteogenic factors in the absence of Runx2. Runx2 is well established as an early requirement for commitment and differentiation of osteoblasts and bone formation. Recently, immediate early BMP2

response genes, for example homeodomain transcription factors, Runx2, and Osterix, have been shown to influence commitment to the osteoblast phenotype [Lee et al., 2003; Kim et al., 2004]. Gene ablation of Osterix, like Runx2, results in failure of bone formation; however, Osterix is downstream of Runx2 [Nakashima et al., 2002]. Thus, a compelling question is whether factors which have been characterized for promoting expression of genes that reflect the mature osteoblast phenotype are operative in the Runx2-null cell. It is also necessary to determine if Runx2-null cells can respond to physiologic regulators (BMP2, glucocorticoid, Vitamin D, TGFβ).

The immortalized primary calvarial-derived cell line we established from the Runx2-null as well as the $Runx2^{\Delta C/\Delta C}$ knock-in mouse model (data not shown), has in part features of an undifferentiated early mesenchymal cell. In contrast to MSCs, the cells cannot be induced to osteoblasts either with osteogenic media, BMP2, TGFβ, or $1,25(OH)_2D_3$ because they are completely lacking in all isoforms of functional Runx2. Thus, our immortalized cell line behaves analogously to Runx2-null primary cultured cells in their failure to differentiate to the osteoblast lineage [Pratap et al., 2003]. However, these and other Runx2-null cell lines have many of the pluripotent properties of mesenchymal progenitors, and under appropriate media conditions can be differentiated to the myogenic and adipocytic lineages ([Kobayashi et al., 2000; Enomoto et al., 2004] and our unpublished data). Here, we report that through subsequent introduction of Runx2, the TERT-immortalized Runx2-null cells can undergo osteoblast differentiation. Previous studies of Runx2-null cells isolated from perinatal mice showed weak alkaline phosphatase activity, a marker of the first stage of osteoblast differentiation in response to reintroduction of Runx2; but, these cells never proceeded to the final mineralization stage [Komori et al., 1997].

We also show for the first time that $1,25(OH)_2D_3$, a potent inducer of osteogenic genes, has a minimal effect in Runx2-null cells. Reconstitution by Runx2 restores Vitamin D_3 responsive transcription of early and late marker genes in the null cells. This finding is consistent with studies that have identified Runx2-VDR protein–protein interactions (e.g., in the rat osteocalcin gene) for Vitamin D enhanced transcription [Paredes et al., 2004]. Of particular note are the effects of Vitamin D on the mouse osteocalcin gene expression. Vitamin D is seen to downregulate osteocalcin, an established finding for the mouse gene in contrast to the human, rat, and chick osteocalcin genes which are highly upregulated [Lian et al., 1989; Gerstenfeld et al., 1990; Zhang et al., 1997; Gutierrez et al., 2004]. It is also interesting to note that the osteopontin gene is highly responsive to Vitamin D_3 even in the absence of Runx2, but its induction is not significantly different at Day 4 or 12.

Both BMP2 and TGFβ are known to stimulate Runx2 expression but only BMP2 leads to commitment to the osteogenic phenotype in a trans-differentiation C2C12 model [Lee et al., 1999]. Interestingly, TGFβ responsiveness of most of the osteoblast marker genes was dependent on Runx2. The osteopontin gene also exhibited a similar Runx2-independent response to TGFβ treatment as observed with $1,25(OH)_2D_3$. Osteopontin is a broadly expressed gene, for example, bone, kidney, aorta, pancreas, and cancer cells. Although its expression in bone is upregulated with bone mineralization and by Runx2, our studies show that physiologic regulation of basal expression of osteopontin is not dependent on Runx2. It is striking that TGFβ upregulates osteocalcin 1.5- to 2-fold in the presence of Runx2 indicating its potent effects in promoting osteoblast differentiation in this cell line. TGFβ has been reported to repress OC expression and not lead to expression in non-osteogenic cell models [Kang et al., 2005]. Yet in vivo TGFβ can contribute to bone formation. Thus, this immortalized mesenchymal Runx2-null cell line is advantageous for identifying Runx2-dependent mechanisms that promote osteoblast differentiation.

A key finding of our study of the $Runx2^{\Delta C/\Delta C}$ knock-in cells, which express Runx2 Δ376, is that the C-terminus of Runx2 is the critical domain for mediating the BMP2 signal, commitment to the osteogenic lineage and subsequent differentiation to the mature osteoblast. This finding is consistent with the failure of Runx2 Δ391 and Δ376 deletion mutant proteins to rescue osteoblast differentiation of the Runx2-null cells. The molecular mechanism by which Runx2 mediates BMP2 commitment and differentiation to osteoblasts was first suggested by the in vivo phenotype of the Runx2 ΔC/ΔC mouse which resulted in complete absence of bone formation, a phenotype similar

to the null mouse. This knock-in mutation deleted the C-terminus which contains the unique nuclear matrix targeting signal characteristic of the Runx gene family. Our studies show that the Runx2 deletion mutant that still retains these interaction domains (Δ432) is equally competent to activate the endogenous Runx2 and Runx2 target genes as wild-type protein. Thus, the activation and repression domains between 432 and 528, identified by earlier studies, including the VWRPY domain that interacts with both repressor TLE/Groucho and activator isoform Grg5 [McLarren et al., 2001; Wang et al., 2004], do not appear to be necessary when compared to the 391–432 domain. Thus, while Runx2 is a scaffolding protein that interacts with many factors to attenuate gene expression for physiologic levels, the effects of the interacting proteins in the 432–528 domain appear to be dispensable for basal activation and physiologic responsiveness of the target genes reflecting osteoblast differentiation. However, the osteopontin gene is an exception and requires an interacting protein(s) between 432 and 528 for maximal induction by BMP2 on Day 4. BMP2 stimulation of osteopontin in the presence of Runx2 Δ432 is 50% less than that observed for the wild-type Runx2 at Days 8 and 12. This suggests a selective requirement of the 432–528 region for coregulatory protein interactions in the mature osteoblast, but not in the proliferating cell, for BMP2 induction of osteopontin. This finding is further supported by the complete failure of osteogenic differentiation and BMP2 response in the $Runx2^{\Delta C/\Delta C}$ immortalized cell line (data not shown). These cells express physiological levels of the C-terminal-truncated Runx2 protein from the endogenous Runx2 locus. Although expressed proteins lack the 391–432 domain, they do retain normal DNA binding and are competent to interact with CBFβ heteromeric partner [Choi et al., 2001; Javed et al., 2000, 2001].

The C-terminus contains one of the most important protein–protein interactions with the coregulatory Smad proteins [Afzal et al., 2005]. Previous studies have shown that BMP2 induction of osteoblast differentiation in C2C12 cells is dependent on Runx2 [Lee et al., 2000]. Our earlier study established that the Smad-interacting domain and NMTS overlap and the domain from 391 to 432 was required for robust expression of the alkaline phosphatase marker of early differentiation [Afzal et al., 2005]. Because BMP2 will induce endogenous Runx2 in C2C12 cells, the present study using Runx2-null cells provides the definitive evidence for the requirement of Runx2 to transduce the BMP2-Smad osteogenic signal into expression of appropriate genes for osteoblast differentiation. Taken together, our findings strongly suggest that although Runx2 binding to DNA is important, formation of SMID/NMTS dependent multi-regulatory protein complexes are also essential for commitment and progression of the osteoblast phenotype.

REFERENCES

Afzal F, Pratap J, Ito K, Ito Y, Stein JL, van Wijnen AJ, Stein GS, Lian JB, Javed A. 2005. Smad function and intranuclear targeting share a Runx2 motif required for osteogenic lineage induction and BMP2 responsive transcription. J Cell Physiol 204:63–72.

Baksh D, Song L, Tuan RS. 2004. Adult mesenchymal stem cells: Characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med 8:301–316.

Balint E, Lapointe D, Drissi H, van der Meijden C, Young DW, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2003. Phenotype discovery by gene expression profiling: Mapping of biological processes linked to BMP-2-mediated osteoblast differentiation. J Cell Biochem 89:401–426.

Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, Evans RM. 1999. PPAR gamma is required for placental, cardiac, and adipose tissue development. Mol Cell 4:585–595.

Bedalov A, Salvatori R, Dodig M, Kapural B, Pavlin D, Kream BE, Clark SH, Woody CO, Rowe DW, Lichtler AC. 1998. 1,25-Dihydroxyvitamin D3 inhibition of col1a1 promoter expression in calvariae from neonatal transgenic mice. Biochim Biophys Acta 1398:285–293.

Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. 1999. Sox9 is required for cartilage formation. Nat Genet 22:85–89.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science 279:349–352.

Braun T, Arnold HH. 1995. Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. EMBO J 14:1176–1186.

Braun T, Arnold HH. 1996. Myf-5 and myoD genes are activated in distinct mesenchymal stem cells and determine different skeletal muscle cell lineages. EMBO J 15:310–318.

McCarthy TL, Chang WZ, Liu Y, Centrella M. 2003. Runx2 integrates estrogen activity in osteoblasts. J Biol Chem 278:43121–43129.

Chikazu D, Li X, Kawaguchi H, Sakuma Y, Voznesensky OS, Adams DJ, Xu M, Hoshio K, Katavic V, Herschman HR, Raisz LG, Pilbeam CC. 2002. Bone morphogenetic protein 2 induces cyclo-oxygenase 2 in osteoblasts via a Cbfal binding site: Role in effects of bone morphogenetic

protein 2 in vitro and in vivo. J Bone Miner Res 17:1430–1440.

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

D'Alonzo RC, Selvamurugan N, Karsenty G, Partridge NC. 2002. Physical interaction of the activator protein-1 factors c-Fos and c-Jun with Cbfa1 for collagenase-3 promoter activation. J Biol Chem 277:816–822.

Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, Weinberg RA, Louis DN, Li FP, Rheinwald JG. 2000. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol 20:1436–1447.

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

Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PVN, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2005. Canonical WNT signaling promotes osteogenesis by directly stimulating RUNX2 gene expression. J Biol Chem 280:33132–33140.

Gerstenfeld LC, Kelly CM, Von Deck M, Lian JB. 1990. Effect of 1,25-dihydroxyvitamin D3 on induction of chondrocyte maturation in culture: Extracellular matrix gene expression and morphology. Endocrinology 126: 1599–1609.

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

Gutierrez S, Liu J, Javed A, Montecino M, Stein GS, Lian JB, Stein JL. 2004. The vitamin D response element in the distal osteocalcin promoter contributes to chromatin organization of the proximal regulatory domain. J Biol Chem 279:43581–43588.

Hayflick M, Moorhead PS. 1961. The serial cultivation of human diploid cell strains. Exp Cell Res 25:585–621.

Hong JH, Hwang ES, McManus MT, Amsterdam A, Tian Y, Kalmukova R, Mueller E, Benjamin T, Spiegelman BM, Sharp PA, Hopkins N, Yaffe MB. 2005. TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. Science 309:1074–1078.

Javed A, Gutierrez S, Montecino M, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 1999. Multiple Cbfa/AML sites in the rat osteocalcin promoter are required for basal and vitamin D responsive transcription and contribute to chromatin organization. Mol Cell Biol 19:7491–7500.

Javed A, Guo B, Hiebert S, Choi J-Y, Green J, Zhao S-C, Osborne MA, Stifani S, Stein JL, Lian JB, van Wijnen AJ, Stein GS. 2000. Groucho/TLE/R-Esp proteins associate with the nuclear matrix and repress RUNX (CBFa/AML/PEBP2a) dependent activation of tissue-specific gene transcription. J Cell Sci 113:2221–2231.

Javed A, Barnes GL, Jassanya BO, Stein JL, Gerstenfeld L, Lian JB, Stein GS. 2001. runt homology domain transcription factors (Runx, Cbfa, and AML) mediate repression of the bone sialoprotein promoter: Evidence for promoter context-dependent activity of Cbfa proteins. Mol Cell Biol 21:2891–2905.

Jiang XR, Jimenez G, Chang E, Frolkis M, Kusler B, Sage M, Beeche M, Bodnar AG, Wahl GM, Tlsty TD, Chiu CP. 1999. Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. Nat Genet 21:111–114.

Kang JS, Alliston T, Delston R, Derynck R. 2005. Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. EMBO J 24:2543–2555.

Kania MA, Bonner AS, Duffy JB, Gergen JP. 1990. The Drosophila segmentation gene runt encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. Genes Dev 4:1701–1713.

Kassem M. 2004. Mesenchymal stem cells: Biological characteristics and potential clinical applications. Cloning Stem Cells 6:369–374.

Kim HJ, Kim JH, Bae SC, Choi JY, Kim HJ, Ryoo HM. 2003. The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2. J Biol Chem 278:319–326.

Kim YJ, Lee MH, Wozney JM, Cho JY, Ryoo HM. 2004. Bone morphogenetic protein-2-induced alkaline phosphatase expression is stimulated by Dlx5 and repressed by Msx2. J Biol Chem 279:50773–50780.

Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ. 1998. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396:84–88.

Kobayashi H, Gao Y, Ueta C, Yamaguchi A, Komori T. 2000. Multilineage differentiation of Cbfa1-deficient calvarial cells in vitro. Biochem Biophys Res Commun 273:630–636.

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

Kundu M, Javed A, Jeon JP, Horner A, Shum L, Eckhaus M, Muenke M, Lian JB, Yang Y, Nuckolls GH, Stein GS, Liu PP. 2002. Cbfbeta interacts with Runx2 and has a critical role in bone development. Nat Genet 32:639–644.

Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P, Karsenty G. 1997. Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. Nat Genet 16:307–310.

Lee MH, Javed A, Kim HJ, Shin HI, Gutierrez S, Choi JY, Rosen V, Stein JL, van Wijnen AJ, Stein GS, Lian JB, Ryoo HM. 1999. Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor b1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. J Cell Biochem 73:114–125.

Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC. 2000. Runx2 is

a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between runx2 and smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20:8783–8792.

Lee KS, Hong SH, Bae SC. 2002. Both the Smad and p38 MAPK pathways play a crucial role in Runx2 expression following induction by transforming growth factor-beta and bone morphogenetic protein. Oncogene 21:7156–7163.

Lee MH, Kwon TG, Park HS, Wozney JM, Ryoo HM. 2003. BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. Biochem Biophys Res Commun 309:689–694.

Lian J, Stewart C, Puchacz E, Mackowiak S, Shalhoub V, Collart D, Zambetti G, Stein G. 1989. Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. Proc Natl Acad Sci USA 86:1143–1147.

Lian JB, Javed A, Zaidi SK, Lengner C, Montecino M, van Wijnen AJ, Stein JL, Stein GS. 2004. Regulatory controls for osteoblast growth and differentiation: Role of Runx/Cbfa/AML factors. Crit Rev Eukaryot Gene Expr 14:1–41.

Lundberg AS, Randell SH, Stewart SA, Elenbaas B, Hartwell KA, Brooks MW, Fleming MD, Olsen JC, Miller SW, Weinberg RA, Hahn WC. 2002. Immortalization and transformation of primary human airway epithelial cells by gene transfer. Oncogene 21:4577–4586.

McLarren KW, Lo R, Grbavec D, Thirunavukkarasu K, Karsenty G, Stifani S. 2000. The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the runt-related factor cbfa1. J Biol Chem 275:530–538.

McLarren KW, Theriault FM, Stifani S. 2001. Association with the nuclear matrix and interaction with groucho and RUNX proteins regulate the transcription repression activity of the basic helix loop helix factor Hes1. J Biol Chem 276:1578–1584.

Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JHM, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR. 1997. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell 89:773–779.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29.

Ogawa E, Inuzuka M, Maruyama M, Satake M, Naito-Fujimoto M, Ito Y, Shigesada K. 1993a. Molecular cloning and characterization of PEBP2b, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2a. Virology 194:314–331.

Ogawa E, Maruyama M, Kagoshima H, Inuzuka M, Lu J, Satake M, Shigesada K, Ito Y. 1993b. PEBP2/PEA2 represents a family of transcription factors homologous to the products of the Drosophila runt gene and the human AML1 gene. Proc Natl Acad Sci USA 90:6859–6863.

Papachristou DJ, Papachristou GI, Papaefthimiou OA, Agnantis NJ, Basdra EK, Papavassiliou AG. 2005. The MAPK-AP-1/-Runx2 signalling axes are implicated in chondrosarcoma pathobiology either independently or via up-regulation of VEGF. Histopathology 47:565–574.

Paredes R, Arriagada G, Cruzat F, Olate J, van Wijnen A, Lian J, Stein G, Stein J, Montecino M. 2004. The Runx2 transcription factor plays a key role in the 1a,25-dihydroxy vitamin D3-dependent upregulation of the rat osteocalcin (OC) gene expression in osteoblastic cells. J Steroid Biochem Mol Biol 89-90:269–271.

Pratap J, Galindo M, Zaidi SK, Vradii D, Bhat BM, Robinson JA, Choi J-Y, Komori T, Stein JL, Lian JB, Stein GS, van Wijnen AJ. 2003. Cell growth regulatory role of Runx2 during proliferative expansion of pre-osteoblasts. Cancer Res 63:5357–5362.

Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH, Jaenisch R. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75:1351–1359.

Schroeder TM, Kahler RA, Li X, Westendorf JJ. 2004. Histone deacetylase 3 interacts with runx2 to repress the osteocalcin promoter and regulate osteoblast differentiation. J Biol Chem 279:41998–42007.

Selvamurugan N, Kwok S, Alliston T, Reiss M, Partridge NC. 2004a. Transforming growth factor-beta 1 regulation of collagenase-3 expression in osteoblastic cells by cross-talk between the Smad and MAPK signaling pathways and their components, Smad2 and Runx2. J Biol Chem 279:19327–19334.

Selvamurugan N, Kwok S, Partridge NC. 2004b. Smad3 interacts with JunB and Cbfa1/Runx2 for transforming growth factor-beta1-stimulated collagenase-3 expression in human breast cancer cells. J Biol Chem 279:27764–27773.

Shen Q, Christakos S. 2005. The vitamin D receptor, Runx2, and the Notch signaling pathway cooperate in the transcriptional regulation of osteopontin. J Biol Chem 280:40589–40598.

Shi S, Gronthos S, Chen S, Reddi A, Counter CM, Robey PG, Wang CY. 2002. Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression. Nat Biotechnol 20:587–591.

Sierra J, Villagra A, Paredes R, Cruzat F, Gutierrez S, Javed A, Arriagada G, Olate J, Imschenetzky M, van Wijnen AJ, Lian JB, Stein GS, Stein JL. 2003. Regulation of the bone-specific osteocalcin gene by p300 requires Runx2/Cbfa1 and the vitamin D3 receptor but not p300 intrinsic histone acetyltransferase activity. Mol Cell Biol 23:3339–3351.

Simonsen JL, Rosada C, Serakinci N, Justesen J, Stenderup K, Rattan SI, Jensen TG, Kassem M. 2002. Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. Nat Biotechnol 20:592–596.

Smits P, Li P, Mandel J, Zhang Z, Deng JM, Behringer RR, de CB, Lefebvre V. 2001. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. Dev Cell 1:277–290.

Van Leeuwen JP, van Driel M, van den Bemd GJ, Pols HA. 2001. Vitamin D control of osteoblast function and bone extracellular matrix mineralization. Crit Rev Eukaryot Gene Expr 11:199–226.

Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR, Darlington GJ. 1995. Impaired energy homeostasis in C/EBP alpha knockout mice. Science 269:1108–1112.

Wang W, Wang YG, Reginato AM, Glotzer DJ, Fukai N, Plotkina S, Karsenty G, Olsen BR. 2004. Groucho homologue Grg5 interacts with the transcription factor

Runx2-Cbfa1 and modulates its activity during postnatal growth in mice. Dev Biol 270:364–381.

Xiao G, Jiang D, Ge C, Zhao Z, Lai Y, Boules H, Phimphilai M, Yang X, Karsenty G, Franceschi RT. 2005. Cooperative interactions between activating transcription factor 4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression. J Biol Chem 280:30689–30696.

Young DW, Pratap J, Javed A, Weiner B, Ohkawa Y, van Wijnen A, Montecino M, Stein GS, Stein JL, Imbalzano AN, Lian JB. 2005. SWI/SNF chromatin remodeling complex is obligatory for BMP2-induced, Runx2-dependent skeletal gene expression that controls osteoblast differentiation. J Cell Biochem 94:720–730.

Zaidi SK, Young DW, Pockwinse SH, Javed A, Lian JB, Stein JL, van Wijnen AJ, Stein GS. 2003. Mitotic partitioning and selective reorganization of tissue specific transcription factors in progeny cells. Proc Natl Acad Sci USA 100:14852–14857.

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

Zhang R, Ducy P, Karsenty G. 1997. 1,25-dihydroxyvitamin D3 inhibits osteocalcin expression in mouse though an indirect mechanism. J Biol Chem 272:110–116.

Zimmermann S, Voss M, Kaiser S, Kapp U, Waller CF, Martens UM. 2003. Lack of telomerase activity in human mesenchymal stem cells. Leukemia 17:1146–1149.