

# Cell Growth Regulatory Role of Runx2 during Proliferative Expansion of Preosteoblasts<sup>1</sup>

Jitesh Pratap,<sup>2</sup> Mario Galindo,<sup>2</sup> S. Kaleem Zaidi, Diana Vradii, Bheem M. Bhat, John A. Robinson, Je-Yong Choi, Toshisha Komori, Janet L. Stein, Jane B. Lian, Gary S. Stein, and Andre J. van Wijnen<sup>3</sup>

Department of Cell Biology and Cancer Center, University of Massachusetts Medical School, Worcester, Massachusetts 01655 [J. P., M. G., S. K. Z., D. V., J. L. S., J. B. L., G. S. S., A. J. v. W.]; Bone Metabolism/Osteoporosis, Women's Health Research Institute, Wyeth Research, Collegeville, Pennsylvania 19426 [B. M. B., J. A. R.]; Department of Biochemistry, Kyungpook National University, Daegu, Republic of Korea 700-422 [J.-Y. C.]; and Department of Molecular Medicine, Osaka University Medical School, Osaka, Japan 565-0871 [T. K.]

## ABSTRACT

The Runx2 (CBFA1/AML3/PEBP2 $\alpha$ A) transcription factor promotes lineage commitment and differentiation by activating bone phenotypic genes in postproliferative osteoblasts. However, the presence of Runx2 in actively dividing osteoprogenitor cells suggests that the protein may also participate in control of osteoblast growth. Here, we show that Runx2 is stringently regulated with respect to cell cycle entry and exit in osteoblasts. We addressed directly the contribution of Runx2 to bone cell proliferation using calvarial osteoblasts from wild-type and Runx2-deficient mice (*i.e.*, Runx2<sup>-/-</sup> and Runx2 <sup>$\Delta$ C/ $\Delta$ C</sup>). Runx2 <sup>$\Delta$ C/ $\Delta$ C</sup> mice express a protein lacking the Runx2 COOH terminus, which integrates several cell proliferation-related signaling pathways (*e.g.*, Smad, Yes/Src, mitogen-activated protein kinase, and retinoblastoma protein). Calvarial cells but not embryonic fibroblasts from Runx2<sup>-/-</sup> or Runx2 <sup>$\Delta$ C/ $\Delta$ C</sup> mutant mice exhibit increased cell growth rates as reflected by elevations of DNA synthesis and G<sub>1</sub>-S phase markers (*e.g.*, cyclin E). Reintroduction of Runx2 into Runx2<sup>-/-</sup> calvarial cells by adenoviral delivery restores stringent cell growth control. Thus, Runx2 regulates normal osteoblast proliferation, and the COOH-terminal region is required for this biological function. We propose that Runx2 promotes osteoblast maturation at a key developmental transition by supporting exit from the cell cycle and activating genes that facilitate bone cell phenotype development.

## INTRODUCTION

Stringent positive and negative control of the proliferative expansion of mesenchymal cells, osteoprogenitor cells, and immature osteoblasts is critical for normal skeletal development and bone formation. Osteoprogenitors represent mesenchymal cells that are committed to the bone lineage and can differentiate into osteoblasts, which are the principal cells that contribute to skeletogenesis by mediating extracellular matrix mineralization. Osteoprogenitors proliferate in response to mitotic growth factors and must expand into the appropriate number of osteoblasts to support normal formation of distinct skeletal elements. Osteoprogenitor expansion reflects the balance of cell growth and survival. This balance is controlled by both circulating factors (*e.g.*, growth factors, cytokines, and steroid hormones) and tissue architecture-related signals (*e.g.*, cell-cell contact and cell adhesion) that have either growth-stimulatory or inhibitory effects.

Cell growth control is mediated in part at the transcriptional level, and there are cell cycle stage-specific demands for *de novo* synthesis of proteins (*e.g.*, histones and cyclins; Ref. 1). Yet, there is a paucity of data on transcription factors known to control cell growth of osteoblasts. The Runt-related transcription factor Runx2 has a well-defined role in mediating the final stages of osteoblast maturation and

is required for normal osteogenesis. Runx2 deficiency or mutations affecting the function of Runx2 protein cause severe bone abnormalities in mouse and human (2–5). Deletion of the COOH terminus of Runx2, which interacts with a series of cell signaling responsive cofactors, generates bone defects that are comparable with the Runx2 null mouse (5). Runx2 is up-regulated during osteoblast differentiation to support the activation of bone-specific genes. However, Runx2 is already expressed at early stages of chondrogenesis (6–9), in actively proliferating immature osteoblasts (10, 11), and in C2C12 myoblast cells before BMP-2-dependent osteogenic differentiation (10–12). The expression of Runx2 in distinct proliferating mesenchymal cell types does not necessarily result in activation of mature bone phenotypic markers. These observations raise the question of whether Runx2 has a regulatory function in proliferating osteoblasts before osteoblast maturation.

In this study, we provide evidence that Runx2 is tightly regulated during entry into and exit from the cell cycle, and that Runx2 supports stringent control of osteoblast cell growth. Hence, our results indicate that Runx2 has a dual biological role in the osteogenic lineage by attenuating osteoblast growth and promoting bone phenotype maturation.

## MATERIALS AND METHODS

**Cell Growth Analysis.** The osteoblastic cell line MC3T3-E1 was maintained in  $\alpha$ -MEM supplemented with 10% FBS.<sup>4</sup> Cells were seeded in either six-well or 100-mm plates at  $0.08 \times 10^6$  cells/well or  $0.4 \times 10^6$  cells/plate, respectively. The growth medium was changed every 2 days and cultured until confluent (at 8 days). For serum deprivation experiments, cells were grown for 3 days, then washed three times in PBS, and refed with  $\alpha$  MEM plus 10, 5, 2.5, 1, or 0% FBS. Cells were maintained in culture for 2 days before harvesting. Growth rates were assessed by cell counting and FACS analysis.

MC3T3 cells were synchronized in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle by serum starvation. Briefly, exponentially growing cells in  $\alpha$ -MEM plus 10% FBS were washed three times in PBS on day 3 and cultured in serum-free medium for 48 h. Then, the cells were stimulated to progress through the cell cycle by removing medium and adding  $\alpha$ -MEM plus 10% FBS. After serum stimulation, cells were harvested at selected time points for Western blot analysis and FACS analysis.

The distribution of cells at specific cell cycle stages was evaluated by flow cytometry. Cells were trypsinized, washed with PBS, and fixed in 70% ethanol at  $-20^\circ\text{C}$  overnight. Cells were stained with propidium iodide and subjected to FACS analysis based on DNA content (13). The samples ( $1 \times 10^6$  cells) were analyzed for cell cycle distribution using the FACStar cell sorter and Consort 30 software (Becton Dickinson, Mountain View, CA).

Calvarial osteoblasts were isolated from wild-type and homozygous mouse embryos at 17.5 dpc. Runx2-deficient mice were identified by soft X-ray analysis and genotyped by using PCR analysis as described previously (5). Normal diploid osteoblasts are obtained from the central bone area (*i.e.*

Received 3/21/03; revised 5/29/03; accepted 6/9/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported by NIH Grants AR39588 and AR48818.

<sup>2</sup> These authors contributed equally to this study.

<sup>3</sup> To whom requests for reprints should be addressed, at Department of Cell Biology and Cancer Center, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655. Phone: (508) 856-5625; Fax: (508) 856-6800; E-mail: andre.vanwijnen@umassmed.edu.

<sup>4</sup> The abbreviations used are: FBS, fetal bovine serum; FACS, fluorescence-activated cell sorter; TCA, trichloroacetic acid; RT-PCR, reverse transcription-PCR; dpc, days postcoitum; GFP, green fluorescent protein; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; TGF, transforming growth factor; CDK, cyclin-dependent kinase; CBFA, core binding factor alpha; PEBP, polyoma enhancer binding protein; AML, acute myelogenous leukemia; BMP, bone morphogenetic protein.

removing suture tissue) of calvaria from 17.5 dpc embryos. Cells were isolated and maintained as described previously (14). 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. Osteoblasts in the third digest were collected and resuspended in  $\alpha$ -MEM supplemented with 10% FBS. Cells were plated at a density of  $1 \times 10^6$  cells/six-well plate.

Measurement of DNA synthesis in calvarial cells was performed by [<sup>3</sup>H]thymidine incorporation (15). Briefly, calvarial cells isolated from wild-type or homozygotes were plated in 12-well plates at  $5 \times 10^4$  cell/well. After 24 h, [<sup>3</sup>H]thymidine was added to culture medium to a final concentration of 5  $\mu$ Ci/ml and incubated at 37°C for 30 min. Medium was removed by aspiration, and cells were washed twice with ice-cold serum-free  $\alpha$ -MEM. Cells were extracted twice with 10% TCA on ice for 5 min. TCA precipitates were solubilized by adding 10% SDS for 2 min at room temperature. Cells were harvested, and the amount of radioactivity was measured by liquid scintillation counting (Beckman Instruments, Inc., Fullerton, CA).

**Western Blot Analysis.** Cell cycle and cell growth markers of MC3T3-E1 cells and primary calvarial cells were analyzed by western blot analysis as described previously (16, 17). Briefly, total cellular protein or nuclear extracts were resolved in 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA). Blots were incubated with a 1:2000 dilution of each primary antibody for 1 h. Rabbit polyclonal antibodies (CDK2, cyclin A, cyclin B1, E2F-1, and cyclin E), mouse monoclonal antibody (cyclin D1), and goat polyclonal antibodies (actin) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibodies specific for lamin B (Zymed Laboratories, Inc., San Francisco, CA) and Runx2 (18) were also used in these studies. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) for 1 h. Immunoreactive protein bands were visualized by chemiluminescent detection (ECL kit; Amersham Pharmacia Biotech Inc., Piscataway, NJ), and signal intensities were quantitated by densitometry. Each experiment was repeated at least three times.

**Adenoviral Infection of Calvarial Osteoblasts.** Calvarial cells were transduced with an adenovirus vectors expressing human RUNX2 under control of the CMV promoter or the corresponding empty vector (pQBI-Ad-CMV5 GFP; Q.BIOgene, Carlsbad, CA). Virus particles were administered at 50 plaque-forming unit/cell in  $\alpha$ -MEM with 1% FBS and incubated for 1 h at 37°C. Dishes were rotated every 5 min for the first 15 min to ensure that all of the cells were exposed to the virus. After 1 h, medium was aspirated, and cultures were rinsed twice with serum-free medium, and then fresh medium supplemented with 10% FBS was added to the dishes. Growth rates were monitored by counting cells at regular intervals.

**Northern Blot Analyses and RT-PCR.** Total RNA was isolated separately from the head and lower body of eviscerated embryos at 17.5 dpc or from MC3T3 cells by using TRIzol reagents (Life Technologies, Inc., Rockville, MD). Standard procedures were used for northern blot analyses and for hybridization with a probe specific for alkaline phosphatase, collagen type 1, osteopontin, and glyceraldehyde-3-phosphate dehydrogenase, and RT-PCR was carried out by using primers specific for mouse osteocalcin sequence: forward primer 5'-TCT GAC AAA CCT TCA TGT CC-3' and reverse primer: 5'-AAA TAG TGA TAC CGT AGA TGC G-3'.

**RESULTS**

**Runx2 Expression Is Stringently Regulated with respect to Cell Growth.** To understand the relationship between osteoblast proliferation and Runx2 expression, we analyzed Runx2 levels during the proliferative expansion of mouse MC3T3 cells and subsequent cessation of growth. The cell growth was monitored by cell counting at each day of the culturing period. The data show that cells reach confluence after 6–7 days and that proliferation has ceased because of contact inhibition (Fig. 1A). This growth inhibition is reflected by a decrease in the levels of the cell cycle markers cyclin E (G<sub>1</sub>-S related) and cyclin A (S/G<sub>2</sub>-related) and the proliferation-related kinase CDK2, as determined by western blot analysis (Fig. 1B). Strikingly, whereas Runx2 protein is maintained at low but detectable levels in actively proliferating MC3T3 cells, the levels are dramatically in-

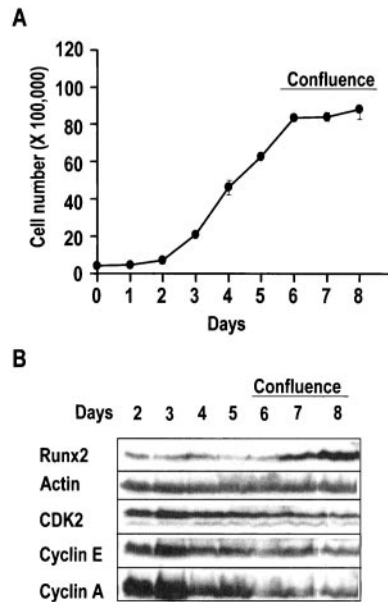


Fig. 1. Runx2 expression is inversely correlated with proliferation in mouse osteoblastic MC3T3 cells. A, the graph shows the growth profile of mouse osteoblastic MC3T3 cells, which were cultured until confluence. Proliferation was monitored by determining cell number (X axis) at daily intervals (Y axis). B, western blot analysis shows the levels of Runx2 and cell cycle regulatory proteins during proliferation and cessation of cell growth of MC3T3 cells. Protein levels of actin were measured as an internal standard.

creased as cell division ceases (Fig. 1B). Hence, these data indicate that the level of Runx2 is inversely correlated with the rate of osteoblast proliferation.

To examine directly the correlation between Runx2 and cell growth, we assessed Runx2 levels during inhibition of MC3T3 proliferation by serum deprivation (Fig. 2). Cells were cultured in complete medium for 3 days until the onset of exponential growth (see Fig. 1A) and were then maintained for 2 additional days in either normal or reduced serum concentrations ranging from 10% to 0%. Reduced serum concentrations diminished MC3T3 cell growth as established by cell counting (Fig. 2A). Decreased growth is reflected by a strongly decreased number of S phase cells and increased representation of cells arrested in the G<sub>0</sub>/G<sub>1</sub> phase as revealed by FACS analysis (Fig. 2B). Western blot analysis demonstrates that growth arrest by serum deprivation increases the levels of Runx2 5-fold (Fig. 2, D and E). We note that cells exit the cell cycle because of serum deprivation and, thus, remain in a subconfluent state (Fig. 2A). In addition, bone phenotypic markers, which are normally up-regulated in differentiated osteoblasts, are not elevated in serum-deprived cells (Fig. 2C). Taken together, these data establish that Runx2 is induced as a consequence of cell proliferation arrest rather than contact inhibition or onset of differentiation.

**Runx2 Levels Are Down-Regulated on Cell Cycle Entry.** Because Runx2 levels are increased when cells are arrested in G<sub>0</sub>/G<sub>1</sub>, we experimentally addressed whether this elevation is reversible and whether Runx2 levels are regulated when cells are allowed to re-enter cell cycle. Cells arrested in G<sub>0</sub>/G<sub>1</sub> for 48 h were stimulated by the addition of serum, and cell cycle progression was monitored by flow cytometric analysis (Fig. 3A). The percentage of cells in S phase is dramatically increased within the first 24 h of cell cycle traverse from quiescence (0 h, 3.6% cells in S phase; 24 h, 17.6% cells in S phase). The resumption of cell proliferation is reflected by increased levels of the cell cycle regulatory proteins, cyclins A and E, as well as the growth factor-dependent increase in CDK2. Our key finding is that Runx2 levels decline after cell cycle entry (4–8 h) and are lowest when cells exhibit active proliferation at 24 h (Fig. 3B). Thus, the

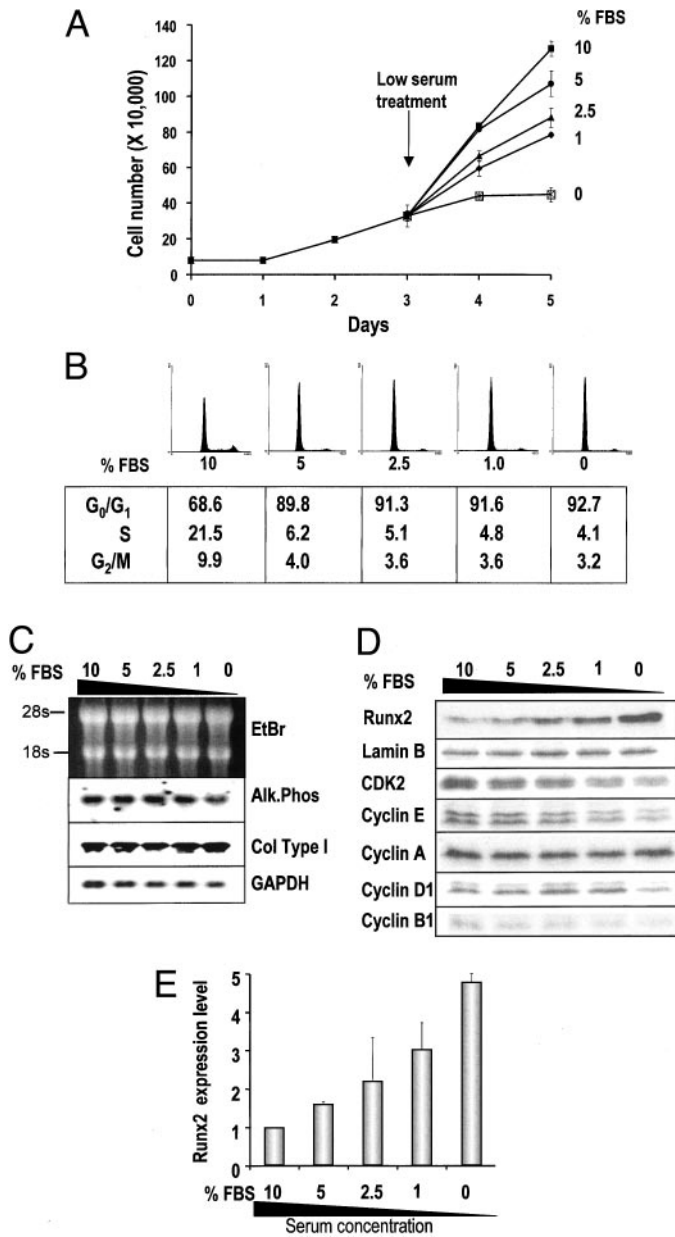


Fig. 2. Runx2 protein is increased in MC3T3 cells arrested in G<sub>0</sub>/G<sub>1</sub> by serum deprivation. *A* and *B*, cell growth rates (*A*) and cell cycle distribution (*B*) were determined for MC3T3 cells, which were grown in the presence of reduced serum concentrations (from 10% to 1%) or in the absence of serum (0%) for 48 h after modulation. *C*, northern blot analysis of cells harvested 48 h after serum reduction or deprivation were analyzed for expression of bone phenotypic markers. *D*, western blot analysis was performed to assess Runx2 protein levels relative to a panel of cell cycle markers and lamin B. *E*, graphic presentation of Runx2 levels during serum deprivation shows a gradual increase up to 5-fold on decreasing serum concentrations.

elevation of Runx2 in quiescent cells is reversed when cells progress from G<sub>0</sub>/G<sub>1</sub> to S phase in MC3T3 cells. We conclude that the increase in Runx2 levels that is evident when cells become contact inhibited (Fig. 1*B*) or serum deprived (Fig. 2*D*) and the decrease in Runx2 levels when cells re-enter the cell cycle (Fig. 3*B*) all clearly indicate that Runx2 is regulated stringently with respect to the G<sub>0</sub>/G<sub>1</sub> transition.

**Runx2 Deficiency Causes Loss of Stringent Cell Growth Control.** The increased levels of Runx2 in growth-arrested cells suggest that this protein may actively support the nondividing state and participate in cell growth-inhibitory mechanisms. To study the regulatory role of Runx2 in proliferation *in vivo*, we examined the growth

properties of primary calvarial cells isolated from the Runx2<sup>-/-</sup> (knockout) and Runx2<sup>ΔC/ΔC</sup> (knock-in) mouse models. Runx2<sup>ΔC/ΔC</sup> mice exhibit a skeletal phenotype comparable with the Runx2<sup>-/-</sup> mice and express a COOH-terminally truncated Runx2 protein that is incapable of supporting osteoblast maturation (5). During *ex vivo* growth, we observed that osteogenic cells from the calvarial regions of Runx2<sup>ΔC/ΔC</sup> mice exhibit increased cell density as compared with wild-type cells (Fig. 4*A*). As expected, the mutant cells express lower levels of bone phenotypic markers (Fig. 4*B*). We directly compared the rate of proliferation of calvarial cells isolated from wild-type, Runx2<sup>ΔC/ΔC</sup>, and Runx2<sup>-/-</sup> embryos at 17.5 dpc (Fig. 4, *C* and *D*). Equal numbers of cells were plated in MEM supplemented with 10% FBS, and growth profiles were monitored for up to 4 weeks by cell counting at regular intervals. Cells from Runx2<sup>ΔC/ΔC</sup> and Runx2<sup>-/-</sup> mutant mice show significantly enhanced proliferation compared with wild-type cells (Fig. 4, *C* and *D*). The differences in cell growth appear to be restricted to the osteogenic lineage, because no differences in proliferation were observed for wild-type and ΔC/ΔC mutant embryonic fibroblasts (Fig. 4*E*); fibroblasts do not express endogenous Runx2 (data not shown; Ref. 19). The increased proliferative potential of calvarial cells from Runx2<sup>ΔC/ΔC</sup> and null mice compared with wild-type cells is reflected by increased DNA synthesis rates measured by [<sup>3</sup>H]thymidine incorporation (Fig. 4*F*) and elevated levels of cell cycle markers (*e.g.*, cyclin E) determined by western blot analysis (Fig. 4*G*). Taken together, our data indicate that Runx2-deficient calvarial cells exhibit increased growth potential and suggest that wild-type Runx2 may normally function as a cell growth inhibitor in immature osteoblasts.

**Reintroduction of Runx2 into Runx2-deficient Cells Restores Cell Growth Control.** Because Runx2 deficiency increases the rate of cell proliferation in osteoblasts, we examined whether reintroduction of Runx2 can restore stringent cell growth control. Runx2 null calvarial cells were transduced with an adenoviral vector expressing wild-type Runx2 (Fig. 5). Under our experimental conditions, >90% of the cells express exogenous proteins as established by microscopic

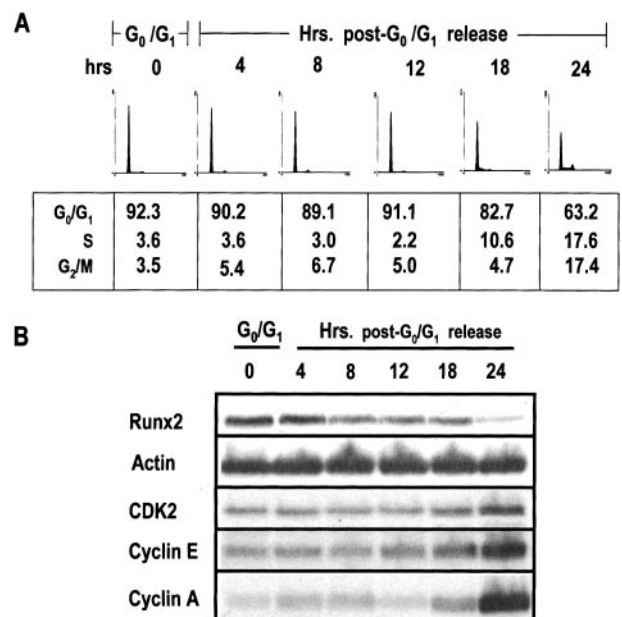


Fig. 3. Release from G<sub>0</sub>/G<sub>1</sub> to S phase decreases Runx2 protein levels in mouse osteoblastic MC3T3 cells. *A*, cell cycle stages were monitored by flow cytometric analysis in cells arrested in G<sub>0</sub>/G<sub>1</sub> phase by serum deprivation and then allowed to proceed to S phase after serum stimulation. Cells were harvested at multiple time points (*i.e.*, 0, 4, 8, 12, 18, and 24 h) after serum stimulation. *B*, levels of Runx2, cell cycle regulatory proteins, and actin were examined at the same time points by western blot analysis.



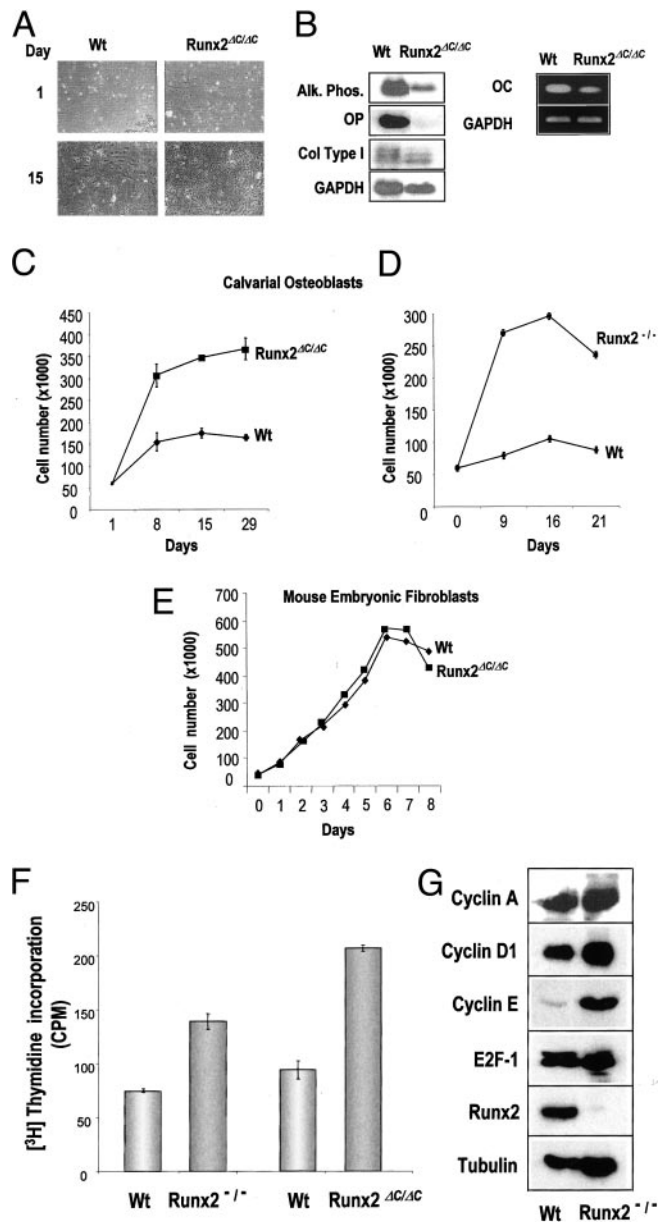


Fig. 4. Calvarial osteoblasts, but not fibroblasts from Runx2<sup>ΔC/ΔC</sup> knock-in embryos exhibit enhanced proliferation compared with wild-type cells. **A**, micrographs of calvarial cell cultures (days 1 and 15) from wild-type (Wt) and Runx2<sup>ΔC/ΔC</sup> embryos. Cells were initially plated at equal cell densities (i.e.,  $5 \times 10^4$  cells/well of a 24-well plate). **B**, expression levels of bone phenotypic markers [alkaline phosphatase (Alk.Phos.), osteopontin (OP), collagen type I (Col Type I), and osteocalcin (OC)] were examined by northern blotting or RT-PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase was used for normalization. **C** and **D**, growth profiles of calvarial cells from wild-type or Runx2<sup>ΔC/ΔC</sup> (C) and Runx2<sup>-/-</sup> mice (D) were established by counting cell number at regular intervals. **E**, growth profiles of embryonic fibroblasts from wild-type or Runx2<sup>ΔC/ΔC</sup> mice were determined by cell counts at indicated days. **F**, the graph shows thymidine incorporation of calvarial osteoblasts from wild-type, Runx2<sup>ΔC/ΔC</sup>, and Runx2<sup>-/-</sup>, which were plated at equal densities and incubated for 24 h with [<sup>3</sup>H]thymidine. Cells isolated from Runx2<sup>-/-</sup> or Runx2<sup>ΔC/ΔC</sup> mice exhibit increased [<sup>3</sup>H]thymidine incorporation compared with wild-type cells. **G**, western blot analysis shows increased levels of cyclin E but no appreciable changes in the levels of cyclin D1, cyclin A, and E2F. Tubulin levels were measured as an internal standard.

analysis of GFP, which is expressed from the same adenoviral vector that carries the recombinant Runx2 (Fig. 5A). Calvarial cells transduced with Runx2 adenovirus show significantly decreased proliferation throughout the 3-week period of *ex vivo* cell culture (Fig. 5B). In contrast, cells transduced by the control adenovirus vector do not exhibit appreciable alterations in growth relative to untreated cells

(Fig. 5B). These data establish that Runx2 is functionally linked to stringent cell growth control in osteogenic calvarial cells. On the basis of the results presented in this study, we propose that Runx2 protein levels and osteoblast proliferation are functionally interrelated (Fig. 6).

DISCUSSION

In this study, we provide evidence that the bone-related gene regulatory factor Runx2 contributes to cell growth control of osteogenic cells. Our results support the emerging concept that the biological activity of Runx2 is not restricted to activation of bone tissue-specific genes on differentiation into the osteoblast lineage. We find that calvarial cells isolated from Runx2<sup>-/-</sup> or Runx2<sup>ΔC/ΔC</sup> mice have

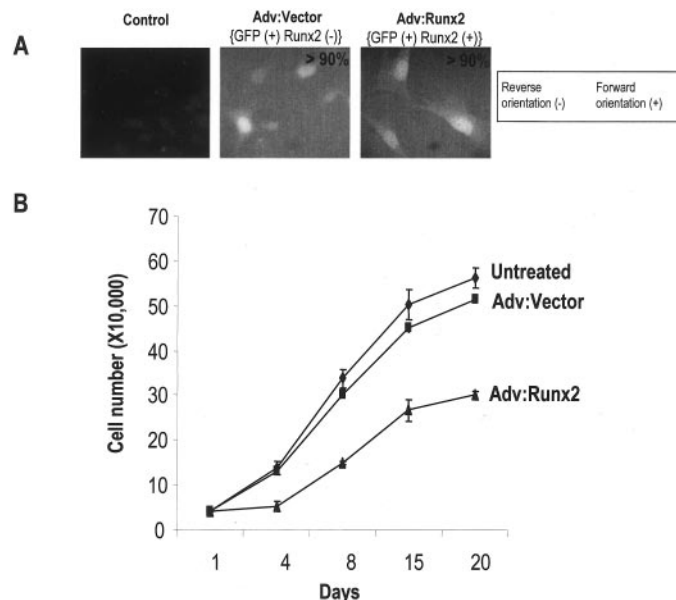


Fig. 5. Reintroduction of Runx2 decreases proliferation of Runx2-deficient calvarial osteoblasts. **A**, microscopic analysis of GFP expression shows efficient transduction (up to 90%) of primary mouse calvarial cells; the GFP protein is expressed from the same viral vector as the Runx2 protein. **B**, the graph shows cell growth profiles of calvarial osteoblasts from Runx2<sup>-/-</sup> that were transduced with an adenovirus vector expressing Runx2 or the corresponding control vector. Untreated cultures subject to mock transfection are shown for comparison. After transduction, cell growth was monitored at the indicated days. Cells transduced with the Runx2 containing adenovirus vector (Adv:Runx2) show a decreased proliferation rate compared with cells treated with the empty vector (Adv:Vector) or untreated cells. The Adv:Vector contains the GFP cassette in the forward orientation (+) and the Runx2 cDNA in reverse orientation (-). Adv:Runx2 contains both the GFP cassette and the Runx2 cDNA in forward orientation (+).

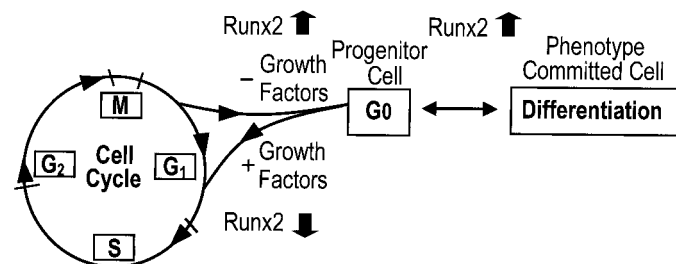


Fig. 6. Functional relationships between Runx2 protein level, and osteoblast cell growth and differentiation. The diagram shows the cell cycle stages in actively proliferating osteoblasts (G<sub>1</sub>, S, G<sub>2</sub>, and M), as well as nonproliferative quiescent (G<sub>0</sub>) and differentiated stages relative to changes in Runx2 levels (wide arrows). Runx2 levels are up-regulated (upward arrow) in response to growth factor deprivation (-Growth Factors) when cells exit the cell cycle and arrest in quiescence (G<sub>0</sub>/G<sub>1</sub>). Runx2 levels are additionally elevated during osteoblast differentiation, whereas Runx2 levels are down-regulated (downward arrow) on cell cycle entry during active proliferation in response to growth factors (+Growth Factors).

enhanced proliferative potential compared with their wild-type counterparts, based on enhanced cell growth rates, as well as elevated levels of DNA synthesis and cell cycle markers in Runx2 deficient cells. Most importantly, reintroduction of Runx2 into calvarial cells restores stringent growth control, establishing the functional requirement of Runx2 in normal cell growth and differentiation in osteoblasts. On the basis of our findings, we propose that Runx2 is a cell growth inhibitor that contributes to control of the G<sub>0</sub>/G<sub>1</sub> transition in osteogenic cells.

One key issue that remains to be addressed is how the up-regulation of Runx2, which is observed during the onset of quiescence in immature MC3T3 cells in response to withdrawal of serum growth factors, relates to the signaling pathways that control normal osteoblast growth and differentiation. Osteoblast-related expression or activity of Runx2 is tightly regulated by a broad spectrum of physiological agents including (1, 25)-dihydroxyvitamin D<sub>3</sub>, ascorbic acid, BMP-2, dexamethasone, TGF- $\beta$ , tumor necrosis factor- $\alpha$ , FGF-2, and parathyroid hormone (10, 20–29). Quiescence induced by serum deprivation of subconfluent MC3T3 cells is likely to result in intricate regulatory cross-talk between several principal signaling pathways that are either inactivated or derepressed after removal of ligands (*e.g.*, peptide growth factors or hormones, and steroids). Increased cell/cell contact that occurs during osteoblast differentiation may additionally influence the integration of the multiple signaling pathways that control progression of bone-phenotypic maturation. Furthermore, endogenous production of growth factors may increase the biological complexity of osteoblast-related signaling cascades.

FGF-2 is of particular interest because it stimulates bone cell proliferation and suppresses expression of bone-related markers [*e.g.*,  $\alpha$ 2(I) type I collagen]. FGF-2 promotes expression of TWIST and Egr-1 in undifferentiated MC3T3-E1 cells, and both transcription factors can repress collagen type I gene expression (22). FGF-2/FGFR signaling also stimulates the DNA binding activity, activation potential, and expression of Runx2 at both transcriptional and post-translational levels, and requires the activity of the protein kinase C pathway (*i.e.*, protein kinase C  $\delta$ ; Ref. 21). Consistent with these data, Yousfi *et al.* (23) have shown that TWIST is a positive physiological regulator of RUNX2 in osteoblasts. Thus, FGF-2/FGFR/TWIST-dependent modulation of Runx2 binding activity may preferentially affect expression of distinct genes (*e.g.*, collagen type I and osteocalcin) depending on the relative affinities of Runx2 binding sites. In proliferating cells, FGF-2/FGFR/TWIST signaling potentially generates a dual effect in which mature phenotypic markers are repressed, whereas Runx2 levels are elevated (21–23). In our study, we find that Runx2 is elevated in the absence of exogenous serum growth factors in quiescent MC3T3 cells. This elevation of Runx2 on serum withdrawal neither induces nor modulates expression of mature bone phenotypic target genes. Thus, compensatory gene regulatory events may negate the increased activity of Runx2 in a promoter-context-dependent manner. The proliferation suppression function of Runx2 indicates that Runx2 may remain active in control of cell growth regulatory genes.

Whereas Runx2 has a definite function in osteoblast maturation (10, 11, 30–33), the characterization of Runx2 as a regulator of osteoblast growth is consistent with data obtained in other biological contexts. For example, Runx2 protein has been characterized independently as the DNA binding component of a cellular heteromeric regulator of a DNA tumor virus (PEBP2 $\alpha$ A; Ref. 19), a nuclear matrix protein from osteosarcoma cells (34, 35), as well a factor encoded by a T-cell tumor integration locus (36). Runx2 cooperates with the *c-myc* gene in promoting formation of T-cell lymphomas (36, 37). Furthermore, Runx2 is highly expressed in Ha-ras transformed, but not normal NIH3T3 fibroblasts (19). When activated by retroviral integration in

T cells, Runx2 acts as an oncoprotein that stimulates cell growth (36, 37). For example, forced expression of Runx2 in transgenic mice under the CD2 promoter was found to interfere with early T-cell development and to predispose mice to lymphomas (38). The observation that Runx2 can act as an inhibitor or stimulator of cell growth indicates that the physiological microenvironment dictates the biological activity of Runx2 in osseous and nonosseous cells. Consistent with this concept, it has been shown that the tumor suppressor protein retinoblastoma and the TGF- $\beta$ /BMP-2 responsive Smads interact directly with the COOH terminus of Runx2 (39), which may modify the cell growth and transcriptional properties of Runx2.

The bone tissue-specific role of Runx2 in cell growth and differentiation is evident in Runx2-deficient mice (2–5) and from the data presented here. In addition, gene knockouts of the two other Runt-related transcription factors, Runx1 (AML1) and Runx3 (PEBP2 $\alpha$ C), result in severe tissue-specific defects (40–42). Strikingly, Runx3 deficiency causes hyperplasia in the gastric mucosa of null mice because of promotion of proliferation and suppression of apoptosis in stomach epithelial cells (42). Furthermore, mutations and deletions in the *Runx1* and *Runx3* genes have been linked to acute myelogenous leukemia (40) or gastric cancer (42), respectively. Our finding that Runx2 is required for stringent cell growth control in osteoblasts establishes that all three of the Runt domain-containing transcription factors have tissue-specific roles in control of cell proliferation.

The mechanism by which Runx factors control cell growth must ultimately be reflected by the activation and/or repression of Runx target genes capable of affecting kinetic components of the cell cycle. For example, Runx1 (AML1) is capable of affecting cell cycle progression through G<sub>1</sub> in part by directly or indirectly controlling the genes for cyclins D2 and D3 (43), the CDK inhibitor p21 in hematopoietic cells (44), the apoptosis-related gene *Bcl-2* (45), as well as hematopoietic growth factors and/or their receptors (46). It has been shown recently that Runx2 is capable of controlling transcription of the gene encoding the p21 protein in mesenchymal cells (47), and the Centrella laboratory demonstrated that Runx2 regulates the TGF- $\beta$  type I receptor promoter (48). In addition, we observed here elevated levels of cyclin E in Runx2-deficient cells. Runx-dependent control of cyclins, CDK inhibitors, growth factors, and growth factor receptors is particularly relevant, because together they function as components of cell signaling pathways that control cell cycle entry and/or the subsequent transitions between different cell cycle stages.

The key findings presented in our study that support a cell growth-suppressive function for Runx2 in mesenchymal bone cell progenitors and the known cell cycle-related target genes of Runx proteins together suggest that Runx2-dependent transcriptional control in immature osteoblasts may directly or indirectly regulate proliferation by (de)sensitizing cells to bone-related external stimuli. The bone-related cell growth function for Runx2 that has been revealed by our data provides a new conceptual dimension to understand the previously established role for Runx2 in osteoblast maturation (31, 33). We propose that Runx2 is biologically important, because the factor controls osteoblast maturation at a key developmental transition by functionally supporting exit from the cell cycle and by activating genes that support bone cell phenotypic development.

## ACKNOWLEDGMENTS

We thank Judy Rask for expert assistance in preparation of the manuscript and Dr. Yoshiaki Ito and Kosei Ito (National University of Singapore, Singapore) for the generous gift of monoclonal antibody against Runx2. We also thank Kelly Almeida for excellent laboratory assistance and the members of our research group, and specifically Amjad Javed, for stimulating discussion throughout the course of these studies.

## REFERENCES

- van Wijnen, A. J., Stein, G. S., Stein, J. L., and Lian, J. B. Cell cycle control of transcription at the G<sub>1</sub>/S phase transition. In: A. Giordano and K. J. Soprano (eds.), *Cell Cycle Inhibitors in Cancer Therapy: Current Strategies*, pp. 31–48. Totowa, NJ: Humana Press, Inc., 2003.
- Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y.-H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., and Kishimoto, T. Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*, 89: 755–764, 1997.
- Mundlos, S., Otto, F., Mundlos, C., Mulliken, J. B., Aylsworth, A. S., Albright, S., Lindhout, D., Cole, W. G., Henn, W., Knoll, J. H. M., Owen, M. J., Mertelsmann, R., Zabel, B. U., and Olsen, B. R. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell*, 89: 773–779, 1997.
- Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W. H., Bedington, R. S. P., Mundlos, S., Olsen, B. R., Selby, P. B., and Owen, M. J. *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*, 89: 765–771, 1997.
- Choi, J.-Y., Pratap, J., Javed, A., Zaidi, S. K., Xing, L., Balint, E., Dalamangas, S., Boyce, B., van Wijnen, A. J., Lian, J. B., Stein, J. L., Jones, S. N., and Stein, G. S. Subnuclear targeting of Runx/Cbfa/AML factors is essential for tissue-specific differentiation during embryonic development. *Proc. Natl. Acad. Sci., USA*, 98: 8650–8655, 2001.
- Inada, M., Yasui, T., Nomura, S., Miyake, S., Deguchi, K., Himeno, M., Sato, M., Yamagiwa, H., Kimura, T., Yasui, N., Ochi, T., Endo, N., Kitamura, Y., Kishimoto, T., and Komori, T. Maturational disturbance of chondrocytes in *Cbfa1*-deficient mice. *Dev. Dyn.*, 214: 279–290, 1999.
- Lengner, C. J., Drissi, H., Choi, J.-Y., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. Activation of the bone related *Runx2/Cbfa1* promoter in mesenchymal condensations and developing chondrocytes of the axial skeleton. *Mech. Dev.*, 114: 167–170, 2002.
- Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A., and de Crombrugge, B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.*, 16: 2813–2828, 2002.
- Stricker, S., Fundele, R., Vortkamp, A., and Mundlos, S. Role of runx genes in chondrocyte differentiation. *Dev. Biol.*, 245: 95–108, 2002.
- Banerjee, C., Javed, A., Choi, J.-Y., Green, J., Rosen, V., van Wijnen, A. J., Stein, J. L., Lian, J. B., and Stein, G. S. Differential regulation of the two principal Runx2/Cbfa1 N-terminal isoforms in response to bone morphogenetic protein-2 during development of the osteoblast phenotype. *Endocrinology*, 142: 4026–4039, 2001.
- Lee, K. S., Kim, H. J., Li, Q. L., Chi, X. Z., Ueta, C., Komori, T., Wozney, J. M., Kim, E. G., Choi, J. Y., Ryoo, H. M., and Bae, S. C. Runx2 is a common target of transforming growth factor  $\beta$ 1 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, 2000.
- Ryoo, H.-M., van Wijnen, A. J., Stein, J. L., Lian, J. B., and Stein, G. S. Detection of a proliferation specific gene during development of the osteoblast phenotype by mRNA differential display. *J. Cell. Biochem.*, 64: 106–116, 1997.
- van der Meijden, C. M., Lapointe, D. S., Luong, M. X., Peric-Hupkes, D., Cho, B., Stein, J. L., van Wijnen, A. J., and Stein, G. S. Gene profiling of cell cycle progression through S-phase reveals sequential expression of genes required for DNA replication and nucleosome assembly. *Cancer Res.*, 62: 3233–3243, 2002.
- Owen, T. A., Aronow, M., Shalhoub, V., Barone, L. M., Wilming, L., Tassinari, M. S., Kennedy, M. B., Pockwinse, S., Lian, J. B., and Stein, G. S. Progressive development of the rat osteoblast phenotype *in vitro*: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J. Cell. Physiol.*, 143: 420–430, 1990.
- Stein, G. S., Stein, J. L., Lian, J. B., Last, T. J., Owen, T., and McCabe, L. Synchronization of normal diploid and transformed mammalian cells. In: J. E. Celis (ed.), *Cell Biology: A Laboratory Handbook*, vol. 1, pp. 282–287. San Diego, CA: Academic Press, Inc., 1994.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. *Current Protocols in Molecular Biology*, pp. 10.8.1–10.8.16. New York: John Wiley and Sons, Inc., 1987.
- Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*, 76: 4350–4354, 1979.
- Zhang, Y. W., Yasui, N., Ito, K., Huang, G., Fujii, M., Hanai, J., Nogami, H., Ochi, T., Miyazono, K., and Ito, Y. A RUNX2/PEBP2 $\alpha$ A/CBFA1 mutation displaying impaired transactivation and Smad interaction in cleidocranial dysplasia. *Proc. Natl. Acad. Sci. USA*, 97: 10549–10554, 2000.
- Ogawa, E., Maruyama, M., Kagoshima, H., Inuzuka, M., Lu, J., Satake, M., Shigesada, K., and Ito, Y. 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, 1993.
- Viereck, V., Siggelkow, H., Tauber, S., Raddatz, D., Schutze, N., and Hufner, M. Differential regulation of *Cbfa1/Runx2* and osteocalcin gene expression by vitamin-D<sub>3</sub>, dexamethasone, and local growth factors in primary human osteoblasts. *J. Cell Biochem.*, 86: 348–356, 2002.
- Kim, H. J., Kim, J. H., Bae, S. C., Choi, J. Y., Kim, H. J., and Ryoo, H. M. 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, 2003.
- Fang, M. A., Glackin, C. A., Sadhu, A., and McDougall, S. Transcriptional regulation of  $\alpha$ 2(I) collagen gene expression by fibroblast growth factor-2 in MC3T3-E1 osteoblast-like cells. *J. Cell Biochem.*, 80: 550–559, 2001.
- Yousfi, M., Lasmoles, F., Kern, B., and Marie, P. J. TWIST inactivation reduces CBFA1/RUNX2 expression and DNA binding to the osteocalcin promoter in osteoblasts. *Biochem. Biophys. Res. Commun.*, 297: 641–644, 2002.
- Drissi, H., Pouliot, A., Kooloos, C., Stein, J. L., Lian, J. B., Stein, G. S., and van Wijnen, A. J. 1,25(OH)<sub>2</sub>Vitamin D<sub>3</sub> suppresses the bone-related Runx2/Cbfa1 gene promoter. *Exp. Cell Res.*, 274: 323–333, 2002.
- Gilbert, L., He, X., Farmer, P., Rubin, J., Drissi, H., van Wijnen, A. J., Lian, J. B., Stein, G. S., and Nanes, M. S. Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/PEBP2 $\alpha$ A) is inhibited by tumor necrosis factor- $\alpha$ . *J. Biol. Chem.*, 277: 2695–2701, 2002.
- Swarthout, J. T., D'Alonzo, R. C., Selvamurugan, N., and Partridge, N. C. Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. *Gene*, 282: 1–17, 2002.
- Komori, T. Runx2, a multifunctional transcription factor in skeletal development. *J. Cell Biochem.*, 87: 1–8, 2002.
- Marie, P. J. Fibroblast growth factor signaling controlling osteoblast differentiation. *Gene*, in press, 2003.
- Franceschi, R. T., and Xiao, G. Regulation of the osteoblast-specific transcription factor, Runx2: Responsiveness to multiple signal transduction pathways. *J. Cell Biochem.*, 88: 446–454, 2003.
- Banerjee, C., Hiebert, S. W., Stein, J. L., Lian, J. B., and Stein, G. S. An AML-1 consensus sequence binds an osteoblast-specific complex and transcriptionally activates the osteocalcin gene. *Proc. Natl. Acad. Sci. USA*, 93: 4968–4973, 1996.
- Banerjee, C., McCabe, L. R., Choi, J.-Y., Hiebert, S. W., Stein, J. L., Stein, G. S., and Lian, J. B. Runt homology domain proteins in osteoblast differentiation: AML-3/CBFA1 is a major component of a bone specific complex. *J. Cell. Biochem.*, 66: 1–8, 1997.
- Ducy, P., and Karsenty, G. Two distinct osteoblast-specific *cis*-acting elements control expression of a mouse osteocalcin gene. *Mol. Cell Biol.*, 15: 1858–1869, 1995.
- Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*, 89: 747–754, 1997.
- Bidwell, J. P., Fey, E. G., van Wijnen, A. J., Penman, S., Stein, J. L., Lian, J. B., and Stein, G. S. Nuclear matrix proteins distinguish normal diploid osteoblasts from osteosarcoma cells. *Cancer Res.*, 54: 28–32, 1994.
- Merriman, H. L., van Wijnen, A. J., Hiebert, S., Bidwell, J. P., Fey, E., Lian, J., Stein, J., and Stein, G. S. The tissue-specific nuclear matrix protein, NMP-2, is a member of the AML/CBF/PEBP2/runt domain transcription factor family: interactions with the osteocalcin gene promoter. *Biochemistry*, 34: 13125–13132, 1995.
- Stewart, M., Terry, A., Hu, M., O'Hara, M., Blyth, K., Baxter, E., Cameron, E., Onions, D. E., and Neil, J. C. Proximal insertions induce the expression of bone-specific isoforms of PEBP2 $\alpha$ A (CBFA1): evidence for a new myc collaborating oncogene. *Proc. Natl. Acad. Sci. USA*, 94: 8646–8651, 1997.
- Vaillant, F., Blyth, K., Terry, A., Bell, M., Cameron, E. R., Neil, J., and Stewart, M. A full-length *Cbfa1* gene product perturbs T-cell development and promotes lymphomagenesis in synergy with *myc*. *Oncogene*, 18: 7124–7134, 1999.
- Vaillant, F., Blyth, K., Andrew, L., Neil, J. C., and Cameron, E. R. Enforced expression of Runx2 perturbs T cell development at a stage coincident with  $\beta$ -selection. *J. Immunol.*, 169: 2866–2874, 2002.
- Thomas, D. M., Carty, S. A., Piscopo, D. M., Lee, J. S., Wang, W. F., Forrester, W. C., and Hinds, P. W. The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol. Cell*, 8: 303–316, 2001.
- Okuda, T., van Deursen, J., Hiebert, S. W., Grosfeld, G., and Downing, J. R. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*, 84: 321–330, 1996.
- Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A. H., and Speck, N. A. Disruption of the *Cbfa2* gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc. Natl. Acad. Sci. USA*, 93: 3444–3449, 1996.
- Li, Q. L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X. Z., Lee, K. Y., Nomura, S., Lee, C. W., Han, S. B., Kim, H. M., Kim, W. J., Yamamoto, H., Yamashita, N., Yano, T., Ikeda, T., Ito, H., Inazawa, J., Abe, T., Hagiwara, A., Yamagishi, H., Ooe, A., Kaneda, A., Sugimura, T., Ushijima, T., Bae, S. C., and Ito, Y. Causal relationship between the loss of RUNX3 expression and gastric cancer. *Cell*, 109: 113–124, 2002.
- Strom, D. K., Nip, J., Westendorf, J. J., Linggi, B., Lutterbach, B., Downing, J. R., Lenny, N., and Hiebert, S. W. Expression of the AML-1 oncogene shortens the G(1) phase of the cell cycle. *J. Biol. Chem.*, 275: 3438–3445, 2000.
- Lutterbach, B., Westendorf, J. J., Linggi, B., Isaac, S., Seto, E., and Hiebert, S. W. A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia. *J. Biol. Chem.*, 275: 651–656, 2000.
- Klampfer, L., Zhang, J., Zelenetz, A. O., Uchida, H., and Nimer, S. D. The AML1/ETO fusion protein activates transcription of BCL-2. *Proc. Natl. Acad. Sci. USA*, 93: 14059–14064, 1996.
- Rhoades, K. L., Hetherington, C. J., Rowley, J. D., Hiebert, S. W., Nucifora, G., Tenen, D. G., and Zhang, D. E. Synergistic up-regulation of the myeloid-specific promoter for the macrophage colony-stimulating factor receptor by AML1 and the t(8;21) fusion protein may contribute to leukemogenesis. *Proc. Natl. Acad. Sci. USA*, 93: 11895–11900, 1996.
- Westendorf, J. J., Zaidi, S. K., Cascino, J. E., Kahler, R., van Wijnen, A. J., Lian, J. B., Yoshida, M., Stein, G. S., and Li, X. Runx2 (Cbfa1, AML-3) interacts with histone deacetylase 6 and represses the p21(CIP1/WAF1) promoter. *Mol. Cell Biol.*, 22: 7982–7992, 2002.
- Ji, C., Eickelberg, O., McCarthy, T. L., and Centrella, M. Control and counter-control of TGF- $\beta$  activity through FAST and Runx (CBFA) transcriptional elements in osteoblasts. *Endocrinology*, 142: 3873–3879, 2001.