Cell Growth Regulatory Role of Runx2 during Proliferative Expansion of Preosteoblasts

Jitesh Pratap, Mario Galindo, S. Kaleem Zaidi, Diana Vrdii, 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

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 (CBAF1/AML3/PEBP2αA) 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+/− and Runx2−/−/H9004). Runx2−/−/H9004 mutant 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+/− or Runx2−/−/H9004 mutant mice exhibit increased cell growth rates as reflected by elevations of DNA synthesis and G1/S phase markers (e.g., cyclin E). Reintroduction of Runx2 into Runx2−/−/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 mice 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 α-MEM supplemented with 10% FBS.4 Cells were seeded in either six-well or 100-mm plates at 0.08 × 106 cells/well or 0.4 × 106 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 α 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 G1/G0 phase of the cell cycle by serum starvation. Briefly, exponentially growing cells in α 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 α-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°C overnight. Cells were stained with propidium iodide and subjected to FACS analysis based on DNA content (13). The samples (1 × 106 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).

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.

1 Supported by NIH Grants AR39588 and AR48818.
2 These authors contributed equally to this study.
3 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.

4 The abbreviations used are: FBS, fetal bovine serum; FACS, fluorescence-activated cell sorter; TCA, trichloroacetic acid; RT-PCR, reverse transcription-PCR; dpc, days postconitum; GFP, green fluorescent protein; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; TGF, transforming growth factor; CDR, cyclin-dependent kinase; CBFA, core binding factor alpha; PEBP, polyoma enhancer binding protein; AML, acute myelogenous leukemia; BMP, bone morphogenetic protein.
RUNX2 CONTROL OF OSTEOBLAST PROLIFERATION

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 daily intervals (Y axis) at daily intervals (X axis). A, 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.

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. Increased 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 G2/G1 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 differentiatation.

Runx2 Levels Are Down-Regulated on Cell Cycle Entry. Because Runx2 levels are increased when cells are arrested in G2/G1, 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 G2/G1 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
RUNX2 DEFICIENCY CAUSES LOSS OF STRINGENT 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

properties of primary calvarial cells isolated from the Runx2-/- (knockout) and Runx2^Ac^Ac (knock-in) mouse models. Runx2^Ac^Ac mice exhibit a skeletal phenotype comparable with the Runx2-/- 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^Ac^Ac mice exhibit increased cell density as compared with wild-type cells (Fig. 4A). As expected, the mutant cells express lower levels of bone phenotypic markers (Fig. 4B). We directly compared the rate of proliferation of calvarial cells isolated from wild-type, Runx2^Ac^Ac, and Runx2-/- 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^Ac^Ac and Runx2-/- 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 DCA/CAC mutant embryonic fibroblasts (Fig. 4E); fibroblasts do not express endogenous Runx2 (data not shown; Ref. 19). The increased proliferative potential of calvarial cells from Runx2^Ac^Ac and null mice compared with wild-type cells is reflected by increased DNA synthesis rates measured by [3H]thymidine incorporation (Fig. 4F) and elevated levels of cell cycle markers (e.g., cyclin E) determined by western blot analysis (Fig. 4G). 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

elevation of Runx2 in quiescent cells is reversed when cells progress from G_0/G_1 to S phase in MC3T3 cells. We conclude that the increase in Runx2 levels that is evident when cells become contact inhibited (Fig. 1B) or serum deprived (Fig. 2D) and the decrease in Runx2 levels when cells re-enter the cell cycle (Fig. 3B) all clearly indicate that Runx2 is regulated stringently with respect to the G_0/G_1 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

Fig. 3. Release from G_0/G_1 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_0/G_1 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.

Downloaded from cancerres.aacrjournals.org on October 20, 2017. © 2003 American Association for Cancer Research.
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 changes 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 related (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>2ΔC/ΔC</sup> mice have
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 $G_0/G_1$ 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 D3, ascorbic acid, BMP-2, dexamethasone, TGF-$\beta$, 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 definitive 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 hemorgeric regulator of a DNA tumor virus (PEBP2aC; Ref. 19), a nuclear matrix protein from osteosarcoma cells (34, 35), as well as 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 (PEBP2aC), 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 $G_1$ 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 Centrocell 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 phenotype 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


Cell Growth Regulatory Role of Runx2 during Proliferative Expansion of Preosteoblasts

Jitesh Pratap, Mario Galindo, S. Kaleem Zaidi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/17/5357

Cited articles
This article cites 45 articles, 20 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/17/5357.full#ref-list-1

Citing articles
This article has been cited by 29 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/17/5357.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.