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# The Cell Cycle Regulator p27$^{kip1}$ Contributes to Growth and Differentiation of Osteoblasts[1]

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## ABSTRACT

**The cyclin-dependent kinase (cdk) inhibitors are key regulators of cell cycle progression. p27 and p21 are members of the Cip/Kip family of cdk inhibitors and regulate cell growth by inactivating cell cycle stage-specific CDK-cyclin complexes. Because down-regulation of osteoprogenitor proliferation is a critical step for osteoblast differentiation, we investigated expression of p27 and p21 during development of the osteoblast phenotype in rat calvarial osteoblasts and in proliferating and growth-inhibited osteosarcoma ROS 17/2.8 cells. Expression of these proteins indicates that p21, which predominates in the growth period, is related to proliferation control. p27 levels are maximal postproliferatively, suggesting a role in the transition from cell proliferation to osteoblast differentiation. We directly examined the role of p27 during differentiation of osteoprogenitor cells derived from the bone marrow (BM) of p27$^{-/-}$ mice. BM cells from p27 null mice exhibited increased proliferative activity compared with BM cells from wild-type mice and formed an increased number and larger size of osteoblastic colonies, which further differentiated to the mineralization stage. Although p27$^{-/-}$ adherent marrow cells proliferate faster, they retain competency for differentiation, which may result, in part, from observed higher p21 levels compared with wild type. Histological studies of p27$^{-/-}$ bones also showed an increased cellularity in the marrow cavity compared with the p27$^{+/+}$. The increased proliferation in bone does not lead to tumorigenesis, in contrast to observed adenomas in the null mice. Taken together, these findings indicate that p27 plays a key role in regulating osteoblast differentiation by controlling proliferation-related events in bone cells.**

## INTRODUCTION

In eukaryotic cells, progression through the cell cycle is regulated by cdks[3] and their activating subunits, the cyclins (1, 2). cdks mediate phosphorylation of regulatory factors that contribute to control of transitions between sequential phases of the cell cycle (3). Different combinations of cyclins and cdks are required at appropriate times for orderly progression through the cell cycle. However, their activation is regulated by feedback mechanisms that prevent premature entry of cells into the next stage of the cell cycle, before completion of necessary macromolecular events (4).

Two families of protein inhibitors that negatively regulate activity of cyclin-cdk complexes have been identified in mammalian cells. The Cip/Kip family includes p21$^{Cip1}$ (also designated WAF1; Refs. 5–9), p27$^{Kip1}$ (10, 11), and p57$^{Kip2}$ (12, 13). The second family includes p18, p19, p15, and p16 (14–17). Cip/Kip family members inhibit the kinase activities of cdk2 in complex with cyclin A or E and cdk4/cdk6 in complex with cyclins $D_1$, $D_2$, or $D_3$. The inhibition of kinase activity by the Cip/Kip proteins provides a mechanism for negative regulation of cell proliferation that is associated with cell cycle arrest, differentiation, and/or apoptosis. In many different cell types, p21$^{Cip1}$ expression is induced in response to DNA damage to promote cell cycle arrest (5, 18), whereas p27$^{Kip1}$ induction is observed in several tissues after growth inhibition, mediated either by the growth factor TGF-$\beta$ or by serum deprivation (10, 11, 19).

Several recent studies suggest that Cip/Kip CDIs may support differentiation of a variety of cell types (reviewed in Ref. 20). MyoD, a skeletal muscle-specific bHLH transcription factor that induces terminal cell cycle arrest associated with skeletal muscle differentiation, has been shown to induce the levels of p21$^{Cip}$ (21). *In situ* hybridization of developing mouse embryos demonstrates that p21 mRNA is localized to tissues that primarily contain postmitotic differentiated cells (22). p27$^{Kip1}$ may be required for restriction point control to exit the cell cycle; the levels of p27$^{Kip1}$ are increased in fibroblasts deprived of serum mitogens and consequently arrested in $G_1$ (23). Mice nullizygous for p27 demonstrate a number of phenotypes related to defects in differentiation, including a body size one-third larger than the WT controls, female sterility associated with a defect in luteal cell differentiation, and pituitary adenomas (24–26). p57$^{Kip2}$ expression is also associated with terminally differentiated cells (12, 13), including rat calvarial osteoblasts (27), suggesting linkage to exit from the cell cycle and/or expression of phenotypic properties. Indeed, mice lacking p27$^{KIP2}$ display altered proliferation and differentiation of several mesenchymal cell types, indicating muscle, cartilage, and ossification defects (28).

During bone formation, differentiating osteoblasts progress through well defined stages of maturation, including cell proliferation, matrix synthesis, and a final stage of differentiation concomitant with extracellular matrix mineralization. This developmental sequence is supported by a temporal expression of cell growth and phenotype-related genes (29). Previous studies from our laboratory have demonstrated selective expression of several different cyclins and cdks during osteoblast maturation (30, 31). For example, cyclins B and E are selectively up-regulated after cessation of proliferation in mature osteoblasts undergoing matrix mineralization. Therefore, there is a requirement to control the activities of cell cycle regulatory factors (such as the cyclins) during the growth and postproliferative periods of osteoblasts.

To understand the mechanisms by which osteoblast growth is down-regulated at key transition points from proliferation and growth arrest to differentiation, we have evaluated the roles of p21 and p27 in regulating development of the osteoblast phenotype. We show that during osteoblast differentiation, p21 is proliferation-related, whereas p27 is more abundant in mature osteoblasts. We established that ablation of p27 results in precocious proliferation of BM-derived osteoprogenitor cells and apparent acceleration of osteoblast differentiation. Our studies of the p27 null mice suggest a key role for p27 in regulating the proliferation to differentiation transition in osteoblasts.

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[3] The abbreviations used are: cdk, cyclin-dependent kinase; TGF, transforming growth factor; CDI, cdk inhibitor; ROB, rat osteoblast; ROS, rat osteosarcoma; WT, wild-type; KO, knockout; MM, matrix maturation; AP, alkaline phosphatase; BM, bone marrow; BMC, BM cell; GI, growth inhibited.

## MATERIALS AND METHODS

**Cell Cultures.** Primary cell cultures were established from animals maintained in accordance with NIH guidelines for the care and use of laboratory animals. Normal osteoblasts were isolated from fetal rat calvariae (21 days of gestation) and maintained in MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Atlanta Biological, Norcross, GA), 200 mM L-glutamine, and antibiotics (500 mM penicillin and 500 mM streptomycin; Sigma Chemical Co., St. Louis, MO). Media were changed every 2 days until confluent. For mineralization, cells were fed with the same media supplemented with ascorbic acid (50 $\mu$g/$\mu$l) and $\beta$-glycerophosphate (10 mM; Sigma Chemical Co.; Ref. 32). These ROBs were treated with 100 pM TGF-$\beta$ or $10^{-8}$ M $1,25(OH)_2D_3$ (Sigma Chemical Co.) for 24 h before collection for protein assays. Both factors were added on day 7 (proliferation period), day 11 (GI, MM stage), or day 21 (differentiation, mineralization period).

Primary stromal cells were cultured after flushing the BM from femurs and tibias of $p27^{+/+}$ (WT) or $p27^{-/-}$ (KO) male mice, 6 weeks of age, by previously described procedures for osteoprogenitor enrichment in the plastic adherent population of mouse marrow (33, 34). Cells were rinsed extensively and passed through a 0.45-$\mu$m filter before seeding at $3 \times 10^6$ cells/well into 6-well plates. Cells were maintained in culture with $\alpha$ MEM supplemented with 20% heat-inactivated FCS. After 4 days, nonadherent cells were removed, and media were changed every other day to complete media containing 50 $\mu$M ascorbic acid and 10 mM $\beta$-glycerophosphate to induce mineralization. The adherent marrow stromal cells were cultured for 7 or 20 days.

ROS 17/2.8 Cells (35) were maintained in F12 (Life Technologies, Inc.) supplemented with 5% FCS. Cells were plated at a density of $7 \times 10^5$/100-mm dish and collected during the proliferation and density-dependent growth inhibition periods. Growth inhibition was achieved by plating the cells at a higher density, $1 \times 10^6$/100 mm-dish and harvesting at confluency.

**AP.** AP activity was assessed cytochemically in BM-cultured cells from WT and $p27^{-/-}$ mice maintained in mineralization media for 7 or 20 days. The cell layers were washed twice with ice-cold PBS and fixed with 2% paraformaldehyde before staining. Cells were incubated 15–30 min at 37°C in Tris buffer containing naphthol AS-BI phosphate and Fast Red Violet LB-salt using Sigma reagents.

**Calcium Measurement.** Mineral deposition in the BMC cultures was identified histochemically by staining 2% paraformaldehyde-fixed cells with von Kossa solution (32). Total calcium content in the extracellular matrix was determined at day 7 and day 20 of culture. After dissolving the matrix in 0.5 N HCl (1 ml/well), cells were sonicated and an aliquot was used for calcium determination calorimetrically using a calcium detection kit (Sigma Chemical Co.).

**DNA Content Analysis.** Cells were scraped off in 0.5 N HCl and sonicated. DNA was precipitated using 10% perchloroacetic acid and resuspended in 10 mM Tris-1 mM EDTA buffer (pH 7.5) before measurement. Total DNA content was quantified fluorimetrically using a DyNA quart 200 fluorimeter (Pharmacia Biotechnology, San Francisco, CA). An aliquot of DNA solution was incubated with Hoechst 33258 reagent (36), and fluorescence was measured at excitation and emission wavelengths of 350 nm and 455 nm, respectively. Bovine thymus DNA was used as standard.

**Western Blot Analysis.** Cells were harvested in ice-cold PBS and centrifuged 10 min at $2500 \times g$. Cell pellets were then resuspended in EBC lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% NP40, 1 mM EDTA, 10 $\mu$g/ml trypsin inhibitor, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml pepstatin, 20 $\mu$g/ml TPCK, 1 mM DTT, and 1 mM PMSF), in which they were incubated for 15 min on ice, then centrifuged for 10 min at $2000 \times g$. The supernatants were collected for Western analysis. After total protein concentrations were quantified by Bradford assay, 25 or 30 $\mu$g whole cell extract protein were subjected to SDS-PAGE (12%), followed by electroblotting onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Rockville Center, NY) or Immobilon P membranes (Millipore Corp., Bedford, MA). Immunodetection was performed with the enhanced chemiluminescence ECL-Plus, following the manufacturer's instructions (Amersham Corp., Arlington Heights, IL). Proteins were detected using specific polyclonal antibodies to p15, p16, p21, and p27 generated to the COOH terminus of each protein (Santa Cruz Biotechnology, Santa Cruz, CA).

**Histological Analysis.** Tibia and femur of WT and p27 null mice, 4–5 weeks of age, were fixed in 2.5% paraformaldehyde, demineralized in 10% EDTA (pH 7.5), and prepared for paraffin embedding. Longitudinal sections were deparaffinized by standard procedures and stained with either H&E or with Toluidine blue. Multiple serial sections of bones were examined from WT and $p27^{-/-}$ mice ($n = 2$), with each $p27^{+/+}$ and $p27^{-/-}$ pair from the same litter. Comparisons of marrow volume, growth plates, trabeculae density, and diaphyseal cortical bone width were made on similar bone planes.

## RESULTS

**Stage-specific Expression of CDIs during Development of the Osteoblast Phenotype.** We assessed the levels of p21 and p27 in primary cultured rat calvarial osteoblasts during the principal developmental stages of growth and differentiation: the proliferation period (3 days of culture), MM period (11 days), and the terminally differentiated mineralization stage (22 days). To evaluate cellular protein levels in relation to growth and differentiation of osteoblasts, we monitored the consequential effects of physiological regulators of osteoblast maturation. Fig. 1 shows that during osteoblast differentiation, basal levels of p21 are high in proliferating cells and down-regulated in the immediate postproliferative MM period, and that, subsequently, p21 exhibits increased expression. Levels of p27 are low in proliferating normal osteoblasts and substantially increased in postproliferative osteoblasts during differentiation.

TGF-$\beta$ treatment of osteoblasts during the proliferation period results in loss of competency for differentiation at later postconfluent stages. TGF-$\beta$ initially arrests growth and then exerts a mitogenic effect on osteoblasts (37–39). To examine the mechanisms associated with TGF-$\beta$ blockade of osteoblast differentiation, we assessed the effect of TGF-$\beta$ on expression of p21 and p27 CDIs. TGF-$\beta$ does not alter p21 expression in the proliferative period, whereas it slightly reduces p21 levels at the differentiation stage in osteoblasts. p27 levels have been reported to be enhanced in several *in vitro* models that are GI by TGF-$\beta$ treatment (40). Although low levels of p27 are found in proliferating cells, TGF-$\beta$ has a slight inhibitory effect on p27 levels in proliferating osteoblasts. In contrast, in postproliferative mature osteoblasts, TGF-$\beta$ slightly increased p27 expression.

Vitamin D promotes osteoblast differentiation (41); thus, for 24 h, we treated primary osteoblasts with $10^{-8}$ M $1,25(OH)_2D_3$ to observe the effects on p21 and p27 expression. Vitamin D3 significantly increases p21 cellular levels in proliferating cells, but inhibits p21 expression in differentiated osteoblasts. Vitamin D treatment induced a significant increase in p27 levels in proliferating cells and, to a lesser extent, in differentiated cells. These observations are consistent with the known antiproliferative effect of $1,25(OH)_2D_3$ on these osteoblasts (42). Taken together, our findings show that in ROB cells, p21 and p27 have an opposite pattern of expression during osteoblast differentiation. p21 seems to be a proliferation-related CDI, reflected by reduced expression during osteoblast differentiation. In contrast, p27 is more abundant after exit from the cell cycle, during postproliferative osteoblast differentiation. These results support the hypothesis that p27 may regulate late stages of osteoblast maturation.

We also assessed expression of p21 and p27 in ROS 17/2.8 cells, which exhibit abrogated growth-differentiation relationships when compared with normal diploid osteoblasts. ROS 17/2.8 cells express differentiation genes constitutively, but the bone phenotype mineralized matrix is lacking in these cultures. However, some phenotypic markers are up-regulated at confluency (43). Fig. 2 shows that basal levels of p21 are ~2-fold greater in proliferative cells compared with confluent GI cells. The expression of p27 is significantly higher in GI cultures than in the proliferating cells. Thus, similar to normal osteoblasts (ROBs), p21 seems to be a proliferation-related CDI, whereas p27 expression is coupled to the

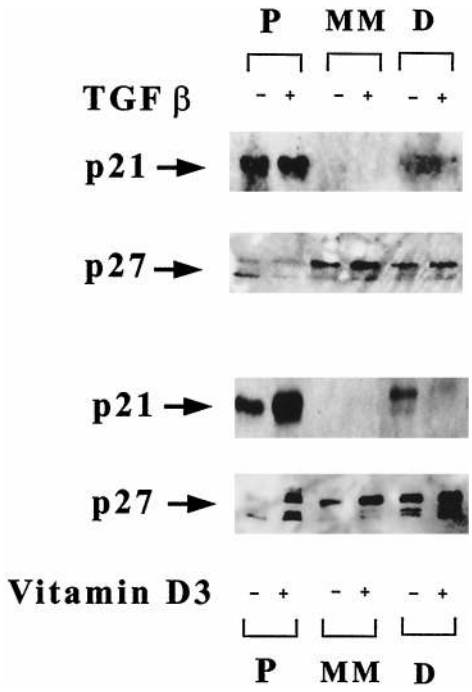


Fig. 1. Reciprocal expression of p21 and p27 during osteoblast differentiation. Western blot analysis of cell extracts from rat calvarial osteoblasts at three stages of differentiation: proliferating (*P*), *MM*, and differentiation (*D*). Proteins (25 $\mu$g) were separated by SDS-PAGE, and specific bands for p21 (*top*) and p27 (*bottom*) were detected using polyclonal antibodies. Cells were treated (+ *lane*) for 24 h with either TGF-$\beta$ (100 pM) or vitamin D3 (10 nM).

mature osteoblast phenotype in the GI ROS 17/2.8 cells. However, only subtle differences in response to TGF-$\beta$ and vitamin D are observed when compared with ROB cells. The effect of TGF-$\beta$ on p21 in proliferating ROS 17/2.8 cells is similar to that observed in Mv1LU cells (44). In contrast, vitamin D decreased p21 in GI cells. Consistent with vitamin D3 inhibition of cell growth concomitant with induction of bone phenotypic differentiation-related genes in osteosarcoma cells (43), p27 increased significantly in proliferating cells after treatment with Vitamin D3.

**Bones of p27 Null Mice Are Larger than Those of WT Littermates.** Although p27 null mice are from 20–40% larger in size than WT littermates, X-rays of the skeleton revealed normal anatomy (24). Overall tibia and femoral bone lengths of $p27^{-/-}$ mice are greater than that of WT mice (24). In our studies, we examined cell and tissue organization of bone. Fig. 3*A* reveals the increased size of the bone and increased width of diaphyseal cortical bone shafts of KO mice compared with WT mice (Fig. 3*B*). Measurements along the bone shaft ranged from 31–40 $\mu$m in WT bone and 60.5–70 $\mu$m in $p27^{-/-}$ mice. Both osteoblasts and osteoclasts on trabecular bone surfaces were of normal appearance in bones of $p27^{-/-}$ mice (data not shown). The overall width of the growth plate is very similar between WT and p27 null mice, with no noted disorganization of the maturation zones (Fig. 3, *C* and *D*), although in some sections the proliferating zone of chondrocytes appears slightly larger. A striking difference between WT and null mice in long bone appearance was observed in cellularity of the marrow cavity (Fig. 3, *A* and *B*). Cellular density of the marrow was greater in $p27^{-/-}$ mice in all sections examined. Notably, the marrow of WT mice shows a typical morphological appearance (Fig. 3*B*), whereas densely packed cells are observed throughout the marrow and particularly along the endosteal surfaces of $p27^{-/-}$ mice (Fig. 3*A*).

**$p27^{Kip1}$ Regulates the Number of Osteoprogenitor Cells in BM.** To address an involvement of p27 in the development of the preosteoblast phenotype, we examined the competency of osteoprogenitor cells from $p27^{-/-}$ mice compared with $p27^{+/+}$ mice to differentiate using a BMC culture model. The adherent marrow cell population forms cell colonies that can be directed to different mesenchymal cell lineages dependent on the culture conditions (45). When ascorbic acid and $\beta$-glycerophosphate are included in the medium, osteogenic differentiation occurs. Fig. 4 shows staining of the cells for AP, an early marker of osteoblasts, at 7 and 20 days of culture. In 7-day cultures derived from BM of $p27^{-/-}$ mice, there is an increased representation of AP-stained colonies compared with cultures from WT mice (Fig. 4*A*). These differences between the two populations of cells persist after 20 days of culture (Fig. 4*B*) and reflect both an increased number and size of the osteogenic colonies. The results suggest an accelerated maturation of osteoprogenitors from the p27 null mice relative to those from the p27 WT mice.

Marrow-derived osteoprogenitors from $p27^{-/-}$ mice also show earlier mineralization of the extracellular matrix, compared with WT animals. von Kossa silver staining indicates that after 20 days of culture, osteogenic colonies in the $p27^{-/-}$ cultures are mineralized, whereas those formed from WT cells have not yet advanced to this final stage of osteoblast maturation (data not shown). We confirmed these qualitative observations by determining the total calcium content of the mineralized extracellular matrix. Fig. 5 shows absence of calcium on day 7 when cells are proliferating and an increased calcium concentration (21-fold) on day 20 in the $p27^{-/-}$ cell layers, compared with the WT-derived cell cultures.

To determine whether the observed increase in the number of osteogenic colonies is due to a difference in cell proliferation and/or maturation, we assayed the DNA content in WT-derived cells compared with $p27^{-/-}$-derived cells after 7 days and 20 days of culture. Fig. 6 shows that at 7 days, $p27^{-/-}$-derived cell cultures contain significantly higher amounts of DNA compared with the WT, indi-

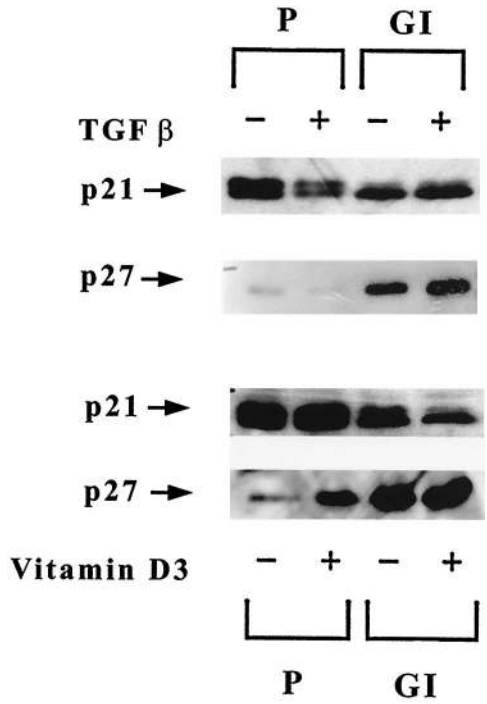


Fig. 2. p27 expression is regulated in osteosarcoma cells. Western blot analysis using 30 $\mu$g of whole cell protein extracts from proliferating (*P*) and GI ROS 17/2.8 cells. *Top*, specific bands for p21. *Bottom*, specific bands for p27. Cells were treated (+ *lane*) for 24 h with either TGF-$\beta$ (100 pM) or vitamin D3 (10 nM).

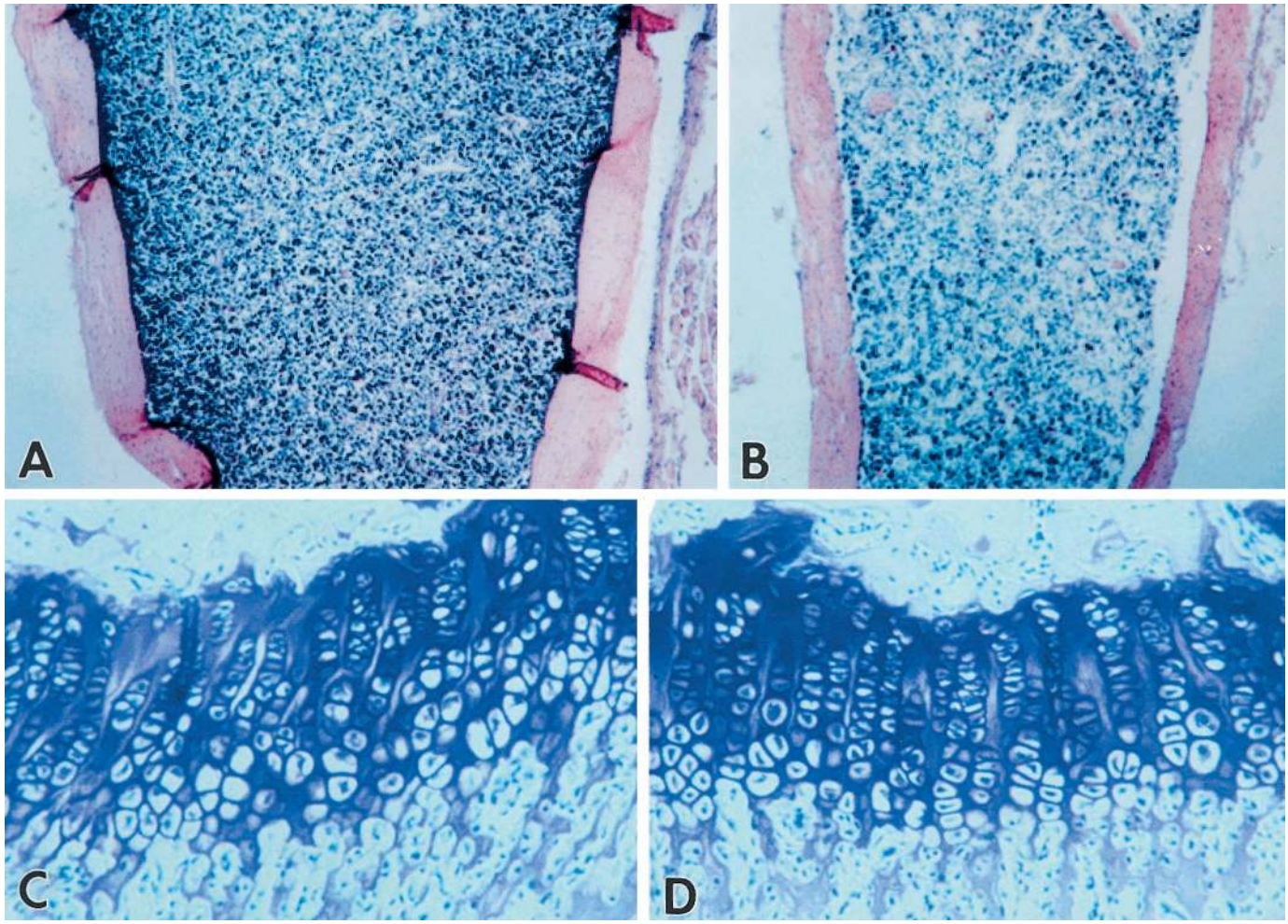


Fig. 3. Increased bone size and marrow cell density of $p27^{-/-}$ mice. Decalcified femur sections of $p27^{-/-}$ (*A* and *C*) and WT (*B* and *D*) mice, 5 weeks of age, from the same litter. *A* and *B*, the diaphysis and marrow stained with H&E (magnification, ×200). *C* and *D*, the chondrocyte zones of the growth plate revealed by Toluidine blue staining (magnification, ×250).

cating a faster proliferation of cells lacking p27. After 20 days, the levels of DNA were approximately the same in both KO- and WT-derived cultures. Together, these results indicate that the $p27^{-/-}$ mice exhibit increased growth of osteogenic cells, which cease proliferation and are competent to differentiate and develop the osteoblast phenotype earlier than cells from WT mice.

**Osteoblasts of p27 Null Mice Exhibit Elevated p21.** $p27^{-/-}$ animals show increased body size relative to their $p27^{+/+}$ WT littermates. Although no increase in systemic growth hormone or insulin-like growth factor-I has been observed in these animals (24), $p27^{-/-}$ mice have larger long bones than WT mice and show increased proliferation of marrow progenitor cells (see above). In several nonosseous tissues, the absence of p27 leads to the development of tumors; however, neither cartilage nor bone tissues show tumor abnormalities. To explore potential mechanisms by which cell growth is regulated in osteoblasts in the absence of p27, we extracted total proteins from cultured WT and p27 null BMCs at confluency and assessed the expression of other CDIs. Western blot analysis shows, as expected, that p27 is present only in extracts from WT cells (Fig. 7). We observed an increase in p21 levels in cells derived from $p27^{-/-}$ compared with WT animals. The Ink family member p16 is not expressed in mouse BMCs, and no differences in p15 expression were observed between WT and p27 null cells (data not shown). These results suggest a compensatory effect of p21 in the absence of p27, which may contribute to regulation of osteoblast proliferation and differentiation.

## DISCUSSION

During bone formation, osteoblasts undergo a series of events marked by phenotypic changes that contribute to tissue structure and function. Proliferating osteoprogenitors must exit the cell cycle to differentiate into mature osteoblasts, which will synthesize an extracellular matrix competent for mineral deposition, and end their differentiation as osteocytes in a mineralized matrix. In this study, we have demonstrated that the CDI $p27^{Kip1}$ is associated with the control of the osteoblast growth differentiation transition based on the following observations: (*a*) mice lacking p27 seem to have in their marrow an increased representation of osteoprogenitors, consistent with larger bones; (*b*) although p21 predominates in proliferating cells during development of the osteoblast phenotype *in vitro*, p27 is preferentially expressed in the immediate postproliferative stage of osteoblast maturation; (*c*) $1,25(OH)_2D_3$, a potent inducer of osteoblast differentiation, dramatically increases p27 expression during the growth period; and (*d*) p27 appears in GI osteosarcoma cells.

It is well known that entry into and progression through the cell cycle are promoted by cyclins and cdk complexes, which facilitate the transition between different phases of the cell cycle. In earlier studies,

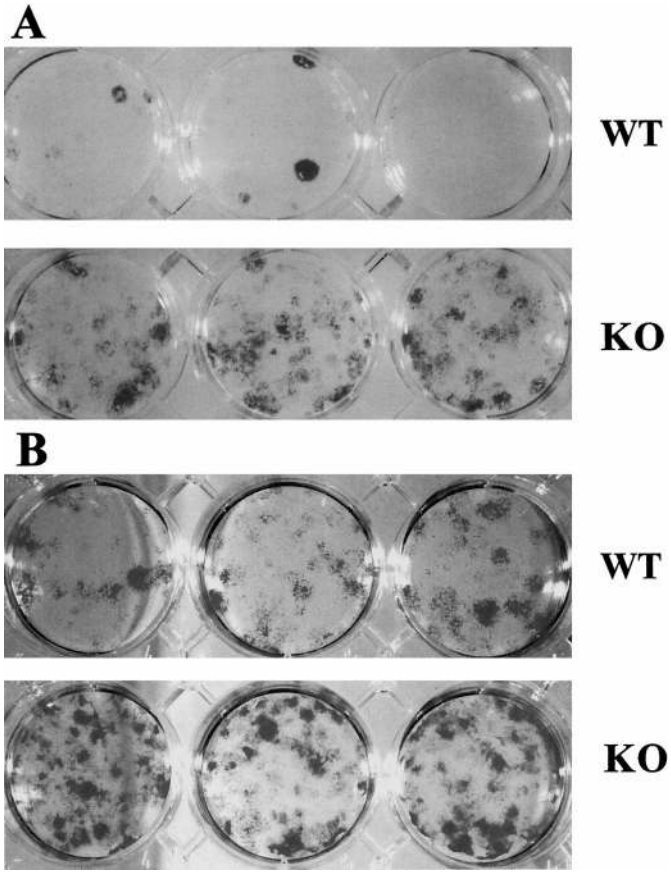


Fig. 4. Formation of osteogenic colonies in BMCs from WT and p27 null mice. BMCs were isolated from long bones of *WT* and p27 null mice (*KO*), 6 weeks of age. Cells were seeded at a density of $3 \times 10^6$ cells/well in 6-well plates. Nonadherent hematopoietic cells were removed after 4 days of culture in complete media, and mesenchymal cells were then cultured for 7 days (*A*) or 20 days (*B*) in mineralization media containing ascorbic acid (50 $\mu$M) and $\beta$-glycerophosphate (10 mM). AP staining reveals colonies of osteoblastic cells.

we reported a significant up-regulation of cyclin E in postproliferative osteoblasts (30). Because cyclin E-Cdk complexes are targeted by the Cip/Kip family of CDIs, which include p21 and p27, we investigated these CDIs during growth and maturation of the osteoblast phenotype. The observed protein levels at three stages of osteoblast differentiation suggest that p21 expression is developmentally biphasic with high levels in proliferating osteoblasts, a sharp decline in postproliferative cells, and induction in more differentiated osteoblasts in the mineralization period. The enhancement of p21 expression during osteoblast proliferation after treatment with Vitamin D3 could be explained by its effect on the p21 promoter, as described previously (46). Notably, p21 is associated with protection of osteoblastic cells against apoptosis (47); the appearance of p21 at the early mineralization stage is consistent with this role during this period of osteoblast maturation (48, 49).

The expression profile of p27 during development of the osteoblast phenotype reflects a function in promoting the switch from proliferation to differentiation because it is abundantly expressed through the MM and mineralization periods. A functional role for p27 in regulating initiation of differentiation as cells exit the cell cycle is further supported by the enhancer effect of $1,25(OH)_2D_3$, a differentiation-promoting hormone and the absence of an effect on p27 levels by TGF-$\beta$, which delays differentiation of osteoblasts. In epithelial cells, TGF-$\beta$ arrests the cell cycle and induces expression of p27 (10). However, p27 declines on mitogenic stimulation of resting cells (50, 51), similar to the known effects of TGF-$\beta$ in osteoblasts (38). Thus, expression of p27 is tightly coupled to modifications in growth and postproliferative differentiation. Consistent with these findings is a related observation in a ROS cell line (UMR-106) treated with parathyroid hormone. Parathyroid hormone increases p27 expression in these cells and blocks entry into S phase, inhibiting cell proliferation

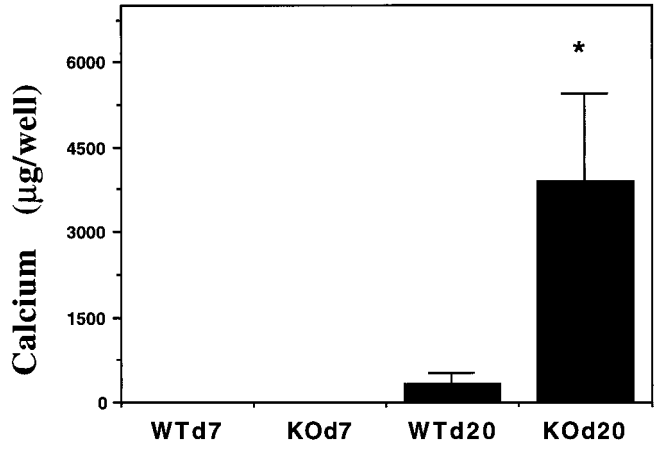


Fig. 5. Mineralization of the osteogenic colonies in BM cultures from WT and p27 nullizygous mice. Cell layers of BM cultures derived from *WT* and p27 null mice (*KO*), 6 weeks of age, were harvested on day 20 for analysis of calcium content of the extracellular matrix (as detailed in "Materials and Methods"). Values are means ± SD of duplicate determinations from wells ($n = 3$) for different mice ($n = 3$) from each group. *, $P < 0.01$ significance by Student's $t$ test.

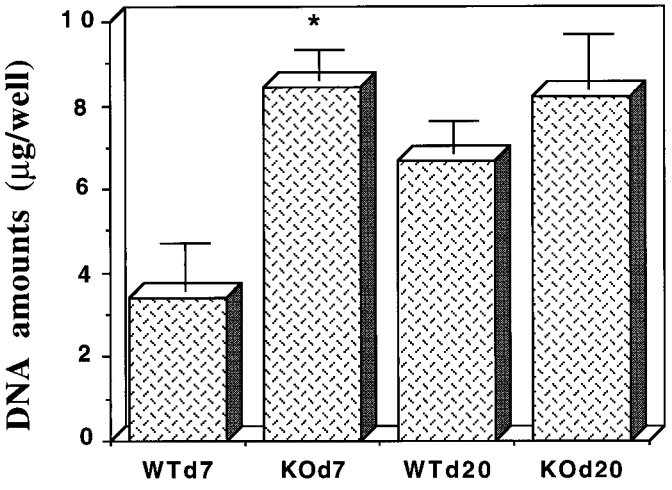


Fig. 6. Growth rate of marrow cells from WT and p27 null mice. Proliferation of p27 null mice (*KO*) marrow-cultured cells is compared with *WT* by quantitating DNA levels in both populations after 7 and 20 days of culture. DNA contents are ± SD of samples ($n = 6$) from three different WT or p27 null mice. *, $P < 0.01$ significance by Student's $t$ test.

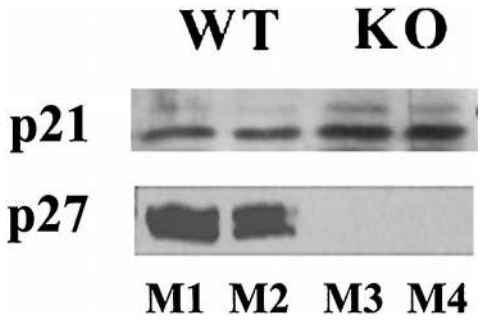


Fig. 7. p21 expression in p27 null mice compared with their WT littermates. Western blot analysis of cell-extract proteins from confluent marrow cells cultured from two WT (*M1* and *M2*) and two p27 null (*M3* and *M4*) mice. Proteins (30 $\mu$g) were loaded, and p21 (*top*) and p27 (*bottom*) were detected using specific antibodies (see "Materials and Methods").

(52). The up-regulation of p27, in concert with antiproliferative effects that correlate to expression of differentiation, is also observed in our studies in GI ROS 17/2.8 cells. Our observations of increased p27 expression during maturation of the osteoblast phenotype strengthens the growing concept that up-regulation of p27 is associated with control of the proliferation to differentiation transition (53, 54).

We used a p27-nullizygous mouse model to explore the mechanisms by which p27 regulates osteoblast differentiation. Mice lacking p27 exhibit several abnormalities, among which are multiple adenomas, female sterility, and enhanced growth. This increase in body weight is not accompanied by an increase in serum levels of growth hormone or insulin-like growth factor (24). Histological analysis shows that $p27^{-/-}$ bones have essentially normal growth plates and normal bone tissue morphology (Fig. 3). However, BM cavities appear proportionately larger, but with an increased marrow cell density, observed particularly on the endosteal surface. This hypercellularity, coupled with a significant 4–5-fold increase in formation of osteogenic colonies from marrow-derived adherent stromal cell cultures of the $p27^{-/-}$ mice compared with $p27^{+/+}$ mice, may explain the enhanced growth of the bones in the absence of p27.

We explored the mechanisms by which both the growth and differentiation of adherent mesenchymal stem cells into osteoprogenitors are regulated in the BM cultures from $p27^{-/-}$ and WT littermates. BMCs from $p27^{-/-}$ animals proliferate at a faster rate, as demonstrated by DNA measurements up to day 7. An increase in the number of stromal colonies was observed. At this point in time, colonies also exhibit osteogenic properties, becoming positive for AP, an early marker of osteoblast differentiation. AP expression is induced in postproliferative cells and is enhanced by secretion and formation of the collagenous extracellular matrix (32, 55). In the $p27^{-/-}$ marrow cultures, this first phase of osteogenesis is accelerated by the increased number of cells that are promoted to the osteogenic phenotype. Subsequent to this initial stage of early differentiation in the $p27^{-/-}$ cultures, mineralization of the extracellular matrix occurs because of an adequate amount of matrix is produced by larger osteogenic colonies in $p27^{-/-}$ cultures compared with WT. Thus, the second stage of osteoblast differentiation proceeds at a normal rate.

Although p27 null animals present tumors in some tissues (26) due to compromised control of proliferation, the enhanced growth of the animals is not followed by development of osteosarcomas. The increase in bone cell proliferation does not abrogate competency for differentiation. In addition to signaling pathways arising from extracellular matrix formation that contribute to differentiation, we found that p21 expression is increased in $p27^{-/-}$ bone cells compared with WT. Notably, Kiyokawa *et al.* (24) examined p21 levels in soft tissues of p27 null mice and found no differences compared with WT. Thus, the enhanced p21 expression in osteoblasts may provide a compensatory mechanism contributing to exit from the cell cycle in the absence of p27, leading to differentiation and accounting for the absence of tumors in bone. *In vivo* studies of double mutant $p21^{-/-}$, $p57^{-/+}$, and $p27^{-/-}$ with $p57^{\pm}$ show that the CDIs function redundantly to control cell differentiation (56, 57). In these studies, we provide evidence for $p27^{Kip}$ as a key regulator in the transition from a proliferating osteoprogenitor to a postproliferative osteoblast. Thus, it is possible that p57, implicated in skeletal development (28), may also provide redundant activity in the regulation of osteoblast differentiation.

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