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Hicham Drissi
University of Massachusetts Medical School

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The Cell Cycle Regulator p27kip1 Contributes to Growth and Differentiation of Osteoblasts

Hicham Drissi, Dennet Hushka, Fauzia Aslam, Que Nguyen, Elizabeth Buffone, Andrew Koff, André J. van Wijnen, Jane B. Lian, Janet L. Stein, and Gary S. Stein


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. The first family of p27, 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 their activating subunits, the cyclins (1, 2). Cdk activity by the Cip/Kip proteins provides a mechanism for linking to exit from the cell cycle and/or expression of phenotypic defects. 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 cdk3 and their activating subunits, the cyclins (1, 2). Cdk's mediate phosphorylation of regulatory factors that contribute to control of transitions between sequential phases of the cell cycle (3). Different combinations of cyclins and cdk's 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 p21Cip1 (also designated WAF1; Refs. 5–9), p27kip1 (10, 11), and p57kip2 (12, 13). The second family of p27, 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 D1, D2, or D3. 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, p21Cip1 expression is induced in response to DNA damage to promote cell cycle arrest (5, 18), whereas p27kip1 induction is observed in several tissues after growth inhibition, mediated either by the growth factor TGF-β or by serum deprivation (10, 11, 19).

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 p21Cip (21). In situ hybridization of developing mouse embryos demonstrates that p21 mRNA is localized to tissues that primarily contain postmitotic differentiated cells (22). p27kip1 may be required for restriction point control to exit the cell cycle; the levels of p27kip1 are increased in fibroblasts deprived of serum mitogens and consequently arrested in G1 (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). p57kip2 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 p27kip1 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 cdk's 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.
MATERIALS AND METHODS

Cell Cultures. Primary cell cultures were established from animals main-
tained 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 supple-
mented with ascorbic acid (50 µg/ml) and β-glycerophosphate (10 mM; Sigma
Chemical Co.; Ref. 32). These ROBs were treated with 100 pm TGF-β or 10-8 M
1,25(OH)2D3 (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-µm filter before seeding at 3 × 106 cells/well into
6-well plates. Cells were maintained in culture with α 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 µM
ascorbic acid and 10 mM β-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 × 104/100-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 × 105/100 mm-dish and harvesting at confluency.

AP. AP activity was assessed cytchemically 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% parafor-
maldehyde 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 soni-
cated. 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 meas-
ured at excitation and emission wavelengths of 350 nm and 455 nm, respec-
tively. Bovine thymus DNA was used as standard.

Western Blot Analysis. Cells were harvested in ice-cold PBS and centri-
fuged 10 min at 2500 × g. Cell pellets were then resuspended in EBC lysis
buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% NP40, 1 mM EDTA, 10 µg/ml
trypsin inhibitor, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 20 µg/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 × g. The supernatants were collected for
Western analysis. After total protein concentrations were quantified by Brad-
ford assay, 25 or 30 µg 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 manufactur-
er’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, deamineralized 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 devel-
opmental stages of growth and differentiation: the proliferation period
(3 days of culture), MM period (11 days), and the terminally differ-
entiated 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 differenti-
ation, 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-β treatment of osteoblasts during the proliferation period
results in loss of competency for differentiation at later postconfluent
stages. TGF-β initially arrests growth and then exerts a mitogenic
effect on osteoblasts (37–39). To examine the mechanisms associated
with TGF-β blockade of osteoblast differentiation, we assessed the
effect of TGF-β on expression of p21 and p27 CDIs. TGF-β 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-β treatment (40). Although low levels of p27 are
found in proliferating cells, TGF-β has a slight inhibitory effect on
p27 levels in proliferating osteoblasts. In contrast, in postproliferative
mature osteoblasts, TGF-β 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)2D3 to
observe the effects on p21 and p27 expression. Vitamin D3 sig-
nificantly 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 proliferat-
ing cells and, to a lesser extent, in differentiated cells. These
observations are consistent with the known antiproliferative effect of
1,25(OH)2D3 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
p27<sup>Kip1</sup> Regulates the Number of Osteoprogenitor Cells in BM.

To address an involvement of p27 in the development of the pre-osteoblast phenotype, we examined the competency of osteoprogenitor cells from p27<sup>−/−</sup> mice compared with p27<sup>+/+</sup> 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 β-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<sup>−/−</sup> mice, there is an increased representation of AP-stained colonies compared with cultures from WT mice (Fig. 4A). These differences between the two populations of cells persist after 20 days of culture (Fig. 4B) 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup>-derived cells after 7 days and 20 days of culture. Fig. 6 shows that at 7 days, p27<sup>−/−</sup>-derived cell cultures contain significantly higher amounts of DNA compared with the WT, indi-
ating 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 non-osseous 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 extra-cellular matrix competent for mineral deposition, and end their differentiation as osteocytes in a mineralized matrix. In this study, we have demonstrated that the CDI p27Kip1 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)2D3, 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,
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)2D3, a differentiation-promoting hormone and the absence of an effect on p27 levels by TGF-β, which delays differentiation of osteoblasts. In epithelial cells, TGF-β 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-β 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.
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