The Retinoblastoma Protein Tumor Suppressor Is Important for Appropriate Osteoblast Differentiation and Bone Development

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Abstract

Mutation of the retinoblastoma (*RB*) tumor suppressor gene is strongly linked to osteosarcoma formation. This observation and the documented interaction between the retinoblastoma protein (pRb) and Runx2 suggests that pRb is important in bone development. To assess this hypothesis, we used a conditional knockout strategy to generate pRb-deficient embryos that survive to birth. Analysis of these embryos shows that *Rb* inactivation causes the abnormal development and impaired ossification of several bones, correlating with an impairment in osteoblast differentiation. We further show that *Rb* inactivation acts to promote osteoblast differentiation *in vitro* and, through conditional analysis, establish that this occurs in a cell-intrinsic manner. Although these *in vivo* and *in vitro* differentiation phenotypes seem paradoxical, we find that *Rb*-deficient osteoblasts have an impaired ability to exit the cell cycle both *in vivo* and *in vitro* that can explain the observed differentiation defects. Consistent with this observation, we show that the cell cycle and the bone defects in *Rb*-deficient embryos can be suppressed by deletion of *E2f1*, a known proliferation inducer that acts downstream of *Rb*. Thus, we conclude that pRb plays a key role in regulating osteoblast differentiation by mediating the inhibition of E2F and consequently promoting cell cycle exit.
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Introduction

The first tumor suppressor to be cloned was the retinoblastoma gene, *RB*. *RB* is mutated in approximately one-third of all sporadic human tumors, but there is strong correlation with certain tumor types. Specifically, *RB* mutations are observed in almost all retinoblastomas (1) and also in a large percentage of osteosarcomas and small cell lung carcinomas. For patients who carry germ line *RB* mutations, osteosarcoma is the second most common tumor type after retinoblastoma (2). Overall, >70% of osteosarcomas show a molecular change or mutation at the *RB* locus (3, 4).

The gene product, pRb, belongs to a family of proteins, including p107 and p130, termed the pocket proteins, although only pRb has been shown to possess significant tumor-suppressive properties (5). The best characterized role of pRb is its regulation of cell cycle progression. Overexpression of pRb causes G_1 cell cycle arrest (6), whereas acute ablation of pRb induces cell cycle re-entry in quiescent cells (7). To execute its cell cycle–inhibitory function, hypophosphorylated pRb binds to and inhibits the E2F family of transcription factors (8). During G_1, pRb becomes hyperphosphorylated by the cyclin D-cdk4/6 complex and subsequently by cyclin E-cdk2. This phosphorylation releases the E2Fs from pRb to induce the transcription of cellular genes essential for S phase entry and cell division.

The analyses of *in vivo* mouse models and *in vitro* experiments show that pRb is required for the differentiation of specific tissues. In erythropoiesis, the loss of *Rb* results in inefficient enucleation and incomplete terminal differentiation of erythroid cells (9, 10). In skeletal muscle, pRb is required for proper cell cycle exit and differentiation (11). Conditional deletion of *Rb* in the intestine causes increased proliferation and abnormal expression of differentiation markers (12, 13). The loss of pRb affects the normal expression of differentiation genes, such as β- and γ-crystallines, in the lens (14). These deficiencies in differentiation seem to be due, at least partially, to a defect in cell cycle exit, a step believed to be required in most differentiation pathways. However, this does not rule out the possibility that pRb contributes to differentiation in a more distinct and specific manner. Notably, pRb binds to NRP/B, a protein up-regulated during neuronal differentiation and involved in neuronal process formation (15). Relevant to this, other markers of neuronal differentiation are decreased in the *Rb*-deficient embryo (16). With respect to fat cells, pRb physically interacts with CAAT/enhancer binding protein-β, and the loss of this interaction inhibits adipocyte differentiation (17).

Several studies implicate a role for pRb in osteoblast differentiation. SV40-derived large T-antigen, which targets the

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pocket proteins, prevents the differentiation of stromal cell lines into osteoblasts (18). The adenoviral E1A 12S protein also represses osteoblast differentiation, and this is dependent on a functional E1A pocket protein binding domain (19). Most striking is the finding that in immortalized cell lines, pRb physically interacts with Runx2/CBFA1, one of the transcription factors essential for osteoblast differentiation (20, 21). This latter observation suggests that pRb may play a role in osteoblast differentiation that is independent of cell cycle regulation.

Determining the role of pRb in osteoblast differentiation *in vivo* may ultimately provide some important insights concerning the high prevalence of *Rb* mutations in osteosarcoma. However, murine embryos deficient for pRb die between embryonic days 13.5 and 15.5 (22-24). This early lethality has thus far precluded the study of pRb in bone development, which primarily does not occur until embryonic day 15.5 in mice. To circumvent this problem, we generated a conditional *Rb* mouse strain that allows pRb-deficient embryos to survive until birth. This mouse model has enabled us to perform *in vitro* and *in vivo* studies to determine the effects of pRb loss in osteoblast differentiation and bone development.

Results

pRb-Deficient Embryos Exhibit Bone Defects during Development

The retinoblastoma gene, *RB*, is mutated in a large proportion of osteosarcomas. *In vitro* studies suggest that *Rb* may play a direct role in bone development (18-20), but this has not been examined *in vivo*. The germ line $Rb^{-/-}$ mice die in mid-gestation (between embryonic days 13.5 and 15.5), prior to the formation of most bones. However, recent studies show that this mid-gestational lethality results from a placental defect (25, 26). Thus, we generated a conditional mouse strain that allows *Rb* mutant embryos to develop in the presence of a wild-type placenta. Specifically, we crossed an *Rb* mutant mouse line with loxP sites flanking the third exon of *Rb* ($Rb^{c/c}$; ref. 7) with a *Mox2-Cre* transgenic line ($Mox2^{+/Cre}$) that expresses the Cre recombinase in the embryo proper, but not in the placenta, beginning approximately at embryonic day 6.5 (27). The resulting $Mox2^{+/Cre}$ conditionally null *Rb* embryos ($Rb^{c-/c-}$) survive until birth, allowing us to assess pRb's role in bone development. Importantly, we observed no difference between wild-type embryos or cells ($Rb^{+/c}$; $Mox2^{+/+}$) and heterozygous animals or cells ($Rb^{+/c-}$; $Mox2^{+/Cre}$) in any of our *in vivo* or *in vitro* experiments, and therefore have used wild-type animals as controls in our study.

Initially, we examined skeletons of wild-type and $Rb^{c-/c-}$ embryos at embryonic day 17.5 by alizarin red staining of bone and Alcian blue staining of cartilage. Compared with wild-type littermate controls, the embryonic day 17.5 $Rb^{c-/c-}$ embryos displayed less ossification in a variety of bones (Fig. 1). These include the frontal and parietal calvarial bones of the skull (Fig. 1A) that arise through intramembranous ossification and the hyoid bone (Fig. 1B) that develops by endochondral ossification. These defects were partially penetrant, as 9 of 13 $Rb^{c-/c-}$ embryos exhibited the decreased ossification. Moreover, other bones in the $Rb^{c-/c-}$ embryos, including the pterygoid bone and palatine process in the head and the xiphoid process of the sternum, were appropriately ossified but showed an abnormal structure (Fig. 1C and D). These abnormal structures were observed in all 13 $Rb^{c-/c-}$ embryos examined. Finally, several other bones such as the long bones of the forelimbs and hind limbs did not exhibit any differing phenotypes between the $Rb^{c-/c-}$ and wild-type embryos. It is possible that certain embryonic bones, such as the limbs, are less susceptible to the effects of *Rb* loss than others, perhaps due to the compensation effects of p107 and p130. Alternatively, the *Mox2-Cre* transgene may be less efficient in some settings.

To further explore the defects that were observed in the $Rb^{c-/c-}$ embryos, we examined the skeletons of mutant embryos at other developmental stages. At earlier time points, embryonic days 15.5 and 16.5, the $Rb^{c-/c-}$ embryos displayed all of the bone defects described above (data not shown). At the later time points, embryonic days 18.5 and 19.5/birth, the phenotype was altered somewhat: we still observed aberrantly developed bones, such as the pterygoid, palatine process, and xiphoid process (Fig. 1C and D; data not shown) with nearly complete penetrance (seven of eight embryonic day 18.5 $Rb^{c-/c-}$ embryos). However, we observed a similar alizarin red staining in the calvaria and hyoid bone of $Rb^{c-/c-}$ embryos versus wild-type littermate controls (Fig. 1A and B; data not shown). We considered two explanations for this latter observation. The first possibility was that pRb loss initially impaired or delayed bone differentiation, but this defect was then corrected by acceleration in the rate of bone deposition after embryonic day 17.5. The second possibility was that pRb loss impaired bone differentiation at all developmental stages, but this impairment was not apparent at later time points because the alizarin red detection method is more qualitative than quantitative. In other words, by embryonic day 18.5, there was some ossification in the appropriate regions of the $Rb^{c-/c-}$ calvaria and hyoid bone but the level of deposited bone was still lower than in the wild-type controls. To distinguish between these two possibilities, we directly assessed the rate of new bone formation after embryonic day 18.5 using calcein incorporation. Calcein is a fluorescent compound that can be injected into an animal and is then incorporated into newly forming bones. We analyzed the amount of calcein incorporation into the frontal bone of embryonic day 18.5 embryos 12 hours after the calcein injection of pregnant females. Notably, the $Rb^{c-/c-}$ frontal bones incorporated significantly less calcein compared with wild-type littermates (Fig. 1E). Similar results were obtained when calcein was injected ~12 hours prior to birth (data not shown). These data indicate that pRb loss does not cause an acceleration in frontal bone formation in the late stages of gestation. Instead, the rate of ossification remains considerably lower than that observed in wild-type embryos. Taken together, our data indicate that the loss of pRb causes a defect in the rate of ossification and/or proper formation of several bones throughout embryonic skeletal development.

The Loss of pRb Affects an Early Step in the Differentiation of Osteoblasts In vivo

Notably, pRb loss impairs the development of bones that arise through two distinct mechanisms, termed endochondral (e.g., the hyoid) and intramembranous (e.g., the calvaria)

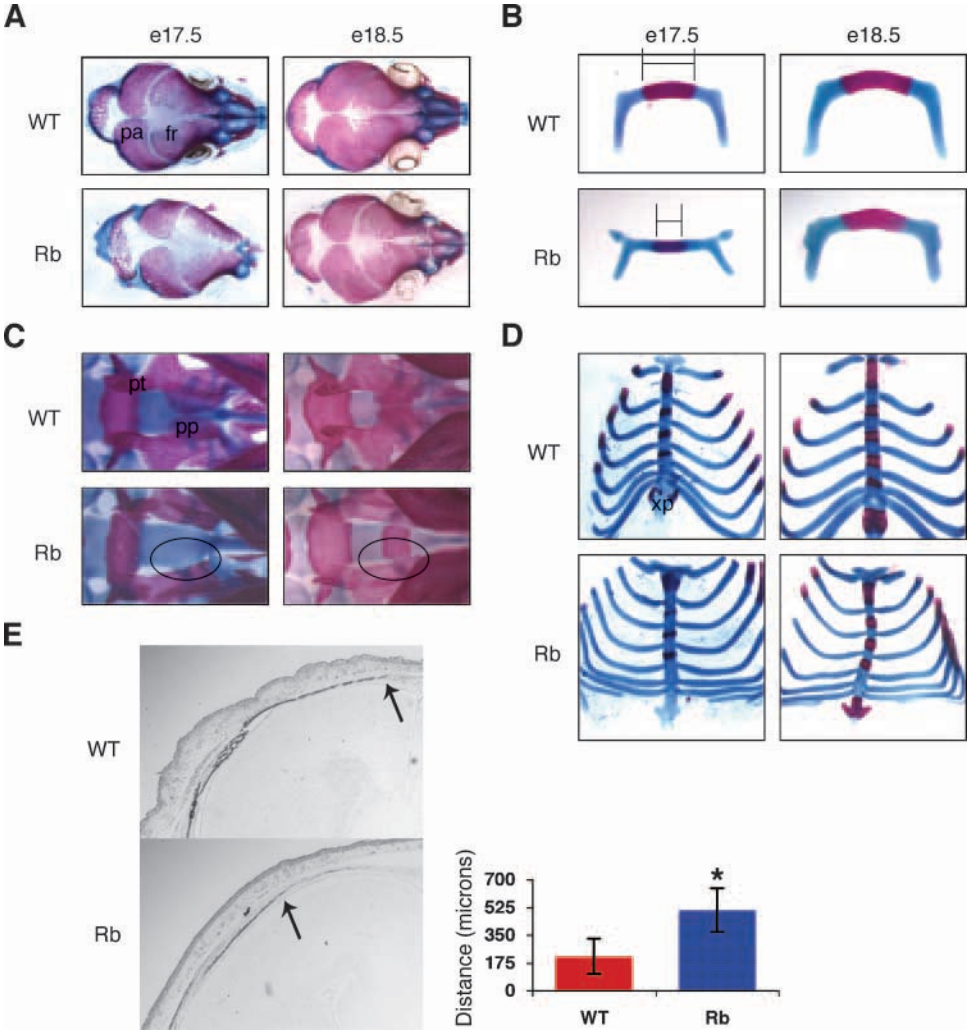


FIGURE 1. Deletion of *Rb* causes defects in embryonic bone development. **A** to **D.** Alizarin red (bone) and Alcian blue (cartilage) staining of embryos. Embryonic day 17.5 $Rb^{c-/c-}$ mice exhibit less ossification in the cranium (**A**) and hyoid bone (**B**). Bar, the difference in hyoid bone ossification at embryonic day 17.5 (**B**). $Rb^{c-/c-}$ embryos at embryonic days 17.5 and 18.5 display aberrant formation of bones in the head (ventral view of head in **C**) and sternum (**D**). The aberrantly shaped or missing palatine process in the $Rb^{c-/c-}$ embryos is circled in **C**. **E.** Pregnant mothers were injected at embryonic day 18 with calcein for 12 h. Coronal sections of the frontal bone of embryonic day 18.5 mice were analyzed for calcein incorporation. $Rb^{c-/c-}$ embryos incorporate less calcein than their wild-type littermates. Original magnification, ×2. The distance from the front of calcein incorporation (*arrow*) to the midline of the suture was measured in nine $Rb^{c-/c-}$ and nine wild-type embryo sections. Columns, mean; bars, 1 SD; *, $P < 0.001$, statistically significant difference. Abbreviations: fr, frontal bone; pa, parietal bone; pp, palatine process; pt, pterygoid bone; xp, xiphoid process. WT, $Rb^{+/c}$; $Mox2^{+/+}$; Rb, $Rb^{c-/c-}$; $Mox2^{+/Cre}$.

ossification. The former is influenced by three cell types: chondrocytes, which form an essential cartilage template; osteoblasts, which differentiate to secrete the bone matrix; and osteoclasts, which oppose bone formation by degrading and reabsorbing bone. In contrast, intramembranous ossification is influenced by osteoblasts and osteoclasts but occurs in a cartilage-independent manner. This fact, along with the apparently normal development of the cartilage skeleton within $Rb^{c-/c-}$ embryos (Fig. 1B-D; data not shown), suggests that a chondrocyte defect cannot fully account for the defective bone development. Therefore, we examined both osteoblast and osteoclast function. To assess osteoclast levels, we screened the frontal bones of embryonic day 17.5 embryos for the presence of tartrate-resistant acid phosphatase activity, an osteoclast-specific marker. There were no active osteoclasts present in either the wild-type or the $Rb^{c-/c-}$ frontal bones (Supplementary Fig. S1). Thus, the decreased ossification in $Rb^{c-/c-}$ embryos is likely not due to either cartilage defects or increased osteoclast activity.

Given these findings, we next screened embryonic day 17.5 frontal bones for the presence of osteoblast-specific markers. Two early markers of differentiating osteoblasts are alkaline phosphatase (ALP) activity and Collagen1a1 (*Col1*) mRNA expression. The activity and expression, respectively, of these two markers were significantly decreased in the $Rb^{c-/c-}$ frontal bone compared with those in wild-type sections (Fig. 2). Moreover, the expression levels of osteopontin (*OPN*), an early to mid-differentiation marker, were also typically down-regulated in the $Rb^{c-/c-}$ embryos relative to wild-type controls (data not shown). These data indicate that osteoblast differentiation is perturbed in $Rb^{c-/c-}$ embryos at the earliest stages of the pathway.

pRb-Deficient Osteoblasts Differentiate to a Greater Extent than Wild-type Cells In vitro

Our *in vivo* data show that an early step in osteoblast differentiation is affected. One possibility is that pRb regulates osteoblast differentiation directly. For example, it has been reported previously that pRb can interact with and coactivate *Runx2/CBFA1*, one of the transcription factors essential for osteoblast differentiation (20, 21). To further dissect the role of pRb in osteoblast differentiation, we used a well-defined and often used *in vitro* osteoblast differentiation system. Specifically, primary cells were isolated from the calvaria of wild-type

and $Rb^{c-/c-}$ embryos and expanded. Two hundred and fifty thousand cells were plated onto 3-cm tissue culture dishes and then induced to differentiate upon confluency. In this system, bone-like calcium deposits are secreted by fully differentiated osteoblasts and can be analyzed by alizarin red staining. Based on our *in vivo* data and previous *in vitro* differentiation studies with fibroblasts (20), we anticipated that $Rb^{c-/c-}$ osteoblasts would differentiate to a lesser extent than wild-type cells. Contrary to this hypothesis, however, the $Rb^{c-/c-}$ osteoblasts secreted a greater number of calcium deposits than wild-type osteoblasts based on the alizarin red staining (Fig. 3A).

We then used quantitative real-time PCR (RT-PCR) to analyze the mRNA levels of several osteoblast markers during the differentiation of these cells. Although the transcriptional levels of *Alp* and *Col1* were unchanged, the $Rb^{c-/c-}$ osteoblasts exhibited significantly greater levels of expression for several other osteoblast genes compared with the wild-type cells (Fig. 3B). Notably, *Runx2* and osterix (*OSX*), two transcription factors that are necessary to induce osteoblast differentiation (28-30), were up-regulated in the $Rb^{c-/c-}$ cells from the earliest stages of the differentiation process (Fig. 3B). *Runx2* and *OSX* have been shown to induce the transcription of downstream osteoblast differentiation genes (28, 30, 31). In accordance with these findings, we observed the increased expression of the early/mid- and late-differentiation markers, osteopontin (*OPN*) and osteocalcin (*OC*), respectively, in the $Rb^{c-/c-}$ osteoblasts.

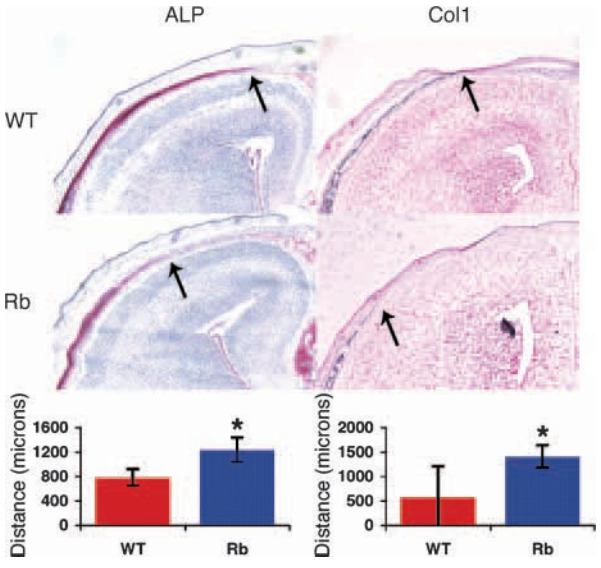


FIGURE 2. pRb-deficient frontal bones display decreased levels of osteoblast markers. Coronal sections of frontal bones from embryonic day 17.5 embryos were assessed by histochemical analysis of alkaline phosphatase activity (*left column*) and *in situ* analysis of Collagen1a1 mRNA (*right column*). $Rb^{c-/c-}$ frontal bone sections (*bottom row*) exhibit decreased levels of both markers compared with wild-type (*top row*). Original magnification, ×2. The distance from the front of activity or expression (*arrows*) to the midline of the suture was measured in at least 8 embryo pairs for *Col1* and in 12 pairs for ALP. Columns, mean; bars, 1 SD; *, $P < 0.01$, statistically significant difference. WT, $Rb^{+/c}; Mox2^{+/+}$; Rb, $Rb^{c-/c-}; Mox2^{+/Cre}$.

Together, these data suggest that osteoblasts deficient for pRb differentiate to a greater extent than wild-type cells *in vitro*, and this correlates with the increased transcriptional levels of *Runx2, OSX*, and their downstream targets.

Acute Ablation of pRb Promotes the Differentiation of Osteoblasts In vitro

The wild-type and $Rb^{c-/c-}$ osteoblasts were prepared on embryonic day 17.5, when there was a significant difference in the degree of calvarial differentiation (Fig. 1A). This raised the possibility that the increased *in vitro* differentiation of the $Rb^{c-/c-}$ versus wild-type cells simply reflected the presence of a larger pool of progenitor osteoblasts in the $Rb^{c-/c-}$ versus wild-type calvaria. To address this hypothesis, we isolated conditional $Rb^{c/c}$ osteoblasts. These cells were brought to confluence and then infected with either a control adenovirus containing green fluorescent protein (Adeno-GFP) or one expressing the Cre recombinase gene (Adeno-Cre). This strategy yielded parallel populations of control and $Rb^{c-/c-}$ osteoblasts that had identical starting numbers of progenitors. Consistent with previous studies (7), we found that the Adeno-Cre was sufficient to acutely ablate pRb within 2 days of infection (data not shown). Therefore, 2 days post-infection (denoted day 0 in Figs. 3C and D and Figs. 4D-F) we placed the confluent wild-type and $Rb^{c-/c-}$ cells in differentiation media. The acutely ablated $Rb^{c-/c-}$ osteoblasts differentiated to a greater extent than the control-infected $Rb^{c/c}$ cells, just as we had observed with the germ line $Rb^{c-/c-}$ osteoblasts (compare Fig. 3C and A). Moreover, the acutely ablated $Rb^{c-/c-}$ cells expressed increased levels of *Runx2, OSX, OPN*, and *OC* relative to the Adeno-GFP–infected cells in a comparable manner to that observed in the germ line $Rb^{c-/c-}$ osteoblasts (compare Figs. 3D and B). These data show that loss of pRb acts in an intrinsic manner to increase the differentiation of primary osteoblast cultures *in vitro*.

Depletion of pRb in Progenitor Osteoblasts Causes Cell Cycle Exit Defects In vitro

We aimed to understand the molecular changes that accompanied this increased differentiation. One possibility is that pRb possesses a cell cycle–independent repressive function in osteoblast differentiation. In this manner, loss of pRb would allow for the deregulated increase in osteoblast genes such as *Runx2* and *OSX*. We have attempted several experiments to test the potential contribution of this interaction, including conducting chromatin immunoprecipitations of Runx2 at osteoblast-specific promoters in wild-type, $Rb^{c-/c-}$, and $Rb^{c-/c-}$; $E2f1^{-/-}$ calvarial preparations (data not shown). These studies did not yield any evidence that *Rb* loss altered Runx2 promoter-binding activity. Moreover, we did not detect any pRb binding to the *Runx2* and *OSX* promoters. This latter, negative chromatin immunoprecipitation result is not particularly informative because pRb chromatin immunoprecipitation works poorly in murine cells. However, the *Runx2* and *OSX* promoter both lack conventional E2F binding sites. Thus, although these observations do not rule out a direct, repressive role for pRb in osteoblast differentiation *in vitro*, we have no data to support this model.

A second potential cause of the observed increase in osteoblast differentiation *in vitro* upon pRb loss may be related to cell cycle defects. Notably, the increased density of osteoblast cultures is known to enhance their differentiation (32, 33). We hypothesized that loss of pRb may affect the normal confluence arrest of the calvarial cells, leading to an increase in proliferation and consequently, an increase in cell density. Thus, we compared the proliferation of wild-type versus germ line $Rb^{c-/c-}$ cells throughout the differentiation process. At all time points, we found that a higher proportion of the $Rb^{c-/c-}$ osteoblast nuclei incorporated 5-bromo-2-deoxyuridine (BrdUrd) compared with the wild-type controls (Fig. 4A and B). In agreement with these findings, the $Rb^{c-/c-}$ osteoblasts showed elevated levels of cyclin A and cyclin E mRNAs (Fig. 4C). Finally, total cell counts during the initiation of differentiation showed an increase in the total number of cells present in $Rb^{c-/c-}$ confluent cultures compared with wild-types (Table 1). Similar results in all of these assays were observed in the analyses of osteoblasts acutely ablated for pRb (Fig. 4D-F; Table 1). Thus, we conclude that pRb loss increases the proliferation, and consequently, the density of confluent osteoblast cultures, thereby leading to an increase in primary calvarial osteoblast differentiation *in vitro*. Notably, the increased proliferation in $Rb^{c-/c-}$ cultures is not perpetual, as the percentage of proliferating cells does decrease to almost zero by day 35 (Fig. 4; data not shown). This suggests that compensatory mechanisms, perhaps through the pocket proteins p107 and p130, exist to eventually enable cell cycle exit in the osteoblasts.

The Loss of Rb *Prevents Osteoblasts from Properly Exiting the Cell Cycle* In vivo

Having established a likely basis for the increased differentiation of pRb-deficient osteoblasts *in vitro*, we wished to determine whether a similar mechanism could explain the impaired bone development *in vivo*. Specifically, because appropriate cell cycle exit is important for the early stages of osteoblast differentiation *in vivo*, we hypothesized that pRb loss might impair cell cycle exit *in vivo* and cause a negative effect on bone formation. Thus, to assess cell cycle progression *in vivo*, we analyzed coronal sections of embryonic day 17.5 frontal bones for BrdUrd, which incorporates into newly synthesized DNA during S phase. Embryos deficient for pRb exhibited a significantly greater percentage of osteoblast nuclei that incorporated BrdUrd compared with the wild-type embryos (Fig. 5A). We also tested frontal bone sections for protein expression of proliferating cell nuclear antigen (PCNA), a known proliferation marker. Consistent with our BrdUrd data, we observed a greater number of $Rb^{c-/c-}$ osteoblast nuclei that stained positively for PCNA compared with wild-type nuclei (Fig. 5B). Interestingly, at the apex of the frontal bone (the midline of the skill) where most of the osteoprogenitors were still proliferating, we did not observe a difference in BrdUrd or PCNA staining between the wild-type and $Rb^{c-/c-}$ embryos (data not shown). This would indicate that the loss of pRb does not affect the proliferation rate of osteoprogenitors but does affect their ability to properly exit the cell cycle and to remain outside of the cell cycle. We did not observe any proliferative differences between $Rb^{c-/c-}$ and wild-type forelimbs (data not shown), corresponding with our finding that there was no difference in the forelimbs based on alizarin red staining.

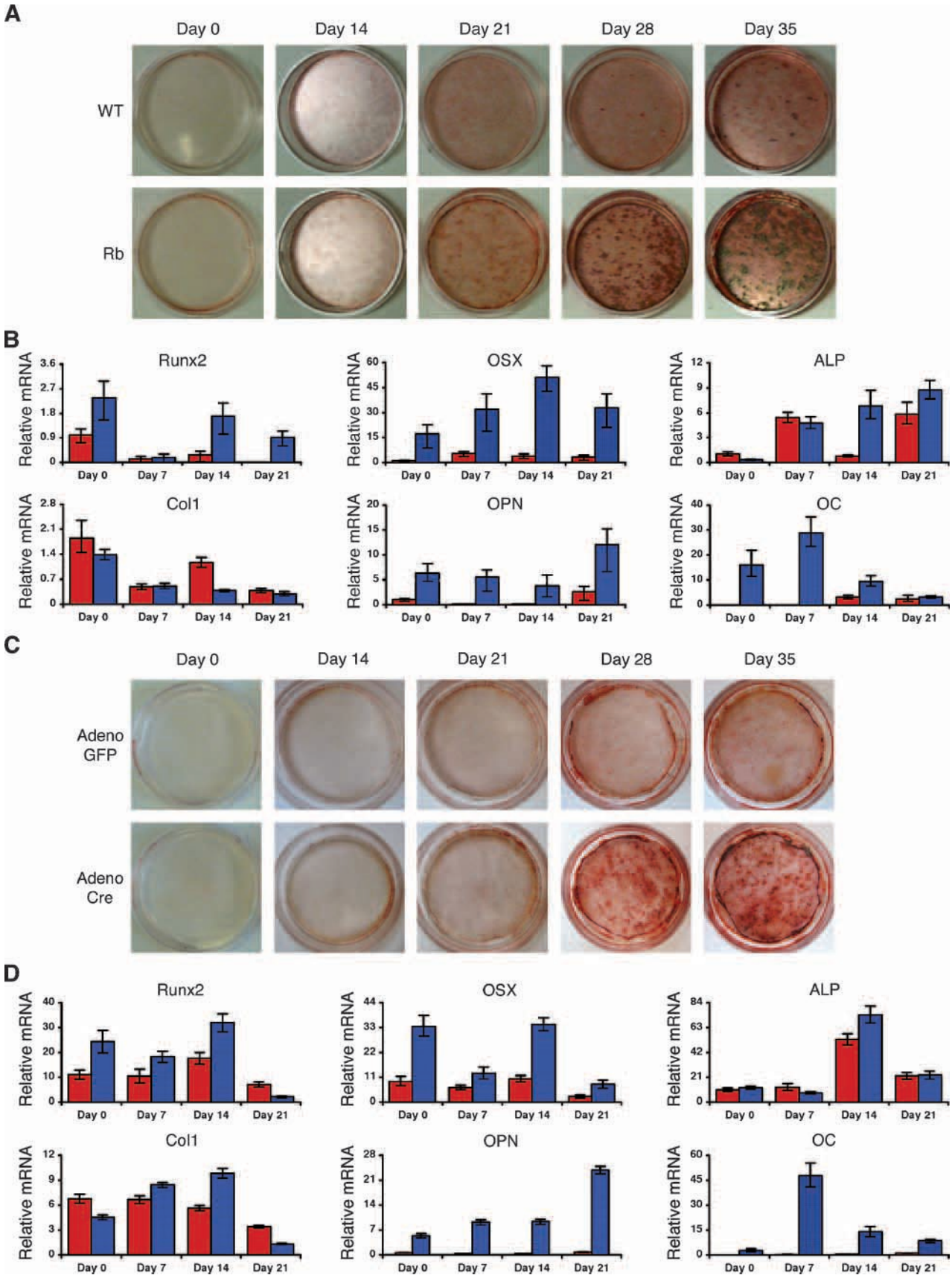
We also extracted RNA from the calvaria of $Rb^{c-/c-}$ and wild-type embryos to examine the transcript levels of cyclin A and cyclin E. Like PCNA, these transcripts are specifically induced in proliferating cells. $Rb^{c-/c-}$ calvaria typically expressed greater mRNA levels of cyclin A and cyclin E than wild-type skulls (Fig. 5C). Importantly, the unrestricted cell cycle progression in $Rb^{c-/c-}$ frontal bones was not associated with an apoptotic response, as determined by terminal nucleotidyl transferase–mediated nick end labeling staining (data not shown). These data suggest that pRb deficiency impairs osteoblasts from exiting the cell cycle *in vivo* at the appropriate developmental stage.

Deletion of E2f1 *Suppresses the Cell Cycle and Ossification Defects in* $Rb^{c-/c-}$ *Embryos*

The cell cycle regulatory activity of pRb is known to be at least partially dependent on its ability to suppress the E2F transcription factors and prevent the activation of genes such as *PCNA, cyclin A* and *cyclin E* that control cell cycle progression. E2F1 is an archetypal member of the E2F family. It is bound to and inhibited by pRb in arrested cells, and it contributes to the activation of target genes once pRb is inactivated by either mitogenic signaling in wild-type cells or genetic lesions in tumor cells. Previous work has shown that the loss of E2F1 can suppress the ectopic cell cycles arising from the loss of *Rb* in other tissues (34). We found that *Rb* and *E2f1* are both expressed in the calvaria (Supplemental Fig. S2). Thus, we crossed a mouse possessing a deletion of *E2f1* into our conditional *Rb* model, and we then examined the compound mutant embryos to determine if E2F activity contributes to the excess proliferation and ossification defects arising in the $Rb^{c-/c-}$ embryos.

First, we assessed the level of cellular proliferation in the embryonic osteoblasts through analysis of both BrdUrd

FIGURE 3. $Rb^{c-/c-}$ primary osteoblasts differentiate to a greater extent than wild-type. **A.** Terminal differentiation of primary calvarial osteoblasts was determined by alizarin red staining of secreted calcium deposits from 0 to 35 d. $Rb^{c-/c-}$ osteoblasts (*bottom row*) secrete a greater number of calcium deposits than wild-type osteoblasts (*top row*). **B.** Quantitative RT-PCR results of bone marker expression levels from wild-type (*red columns*) and $Rb^{c-/c-}$ (*blue columns*) osteoblasts during differentiation. $Rb^{c-/c-}$ osteoblasts express greater mRNA levels of *Runx2*, osterix, osteopontin, and osteocalcin but not alkaline phosphatase or Collagen1a1 compared with wild-type osteoblasts. Ubiquitin was used as an internal control to normalize for RNA levels within the samples. Each time point is an average of four reactions. Columns, results from a representative littermate pair; bars, 1 SD. WT, $Rb^{+/c}$; $Mox2^{+/+}$; Rb, $Rb^{c-/c-}$; $Mox2^{+/Cre}$. **C.** $Rb^{c/c}$ primary calvarial osteoblasts were infected with adenovirus expressing either the Cre recombinase enzyme or green fluorescent protein 2 d prior to differentiation. Terminal differentiation was assessed by alizarin red staining. $Rb^{c/c}$ osteoblasts acutely ablated for pRb (*bottom row*) secrete a greater number of calcium deposits than control-infected osteoblasts (*top row*). **D.** Quantitative RT-PCR analysis done as described in **B**. Osteoblasts acutely ablated for pRb (*blue columns*) express greater mRNA levels of *Runx2*, osterix, osteopontin, and osteocalcin but not alkaline phosphatase or Collagen1a1 compared with control-infected osteoblasts (*red columns*).



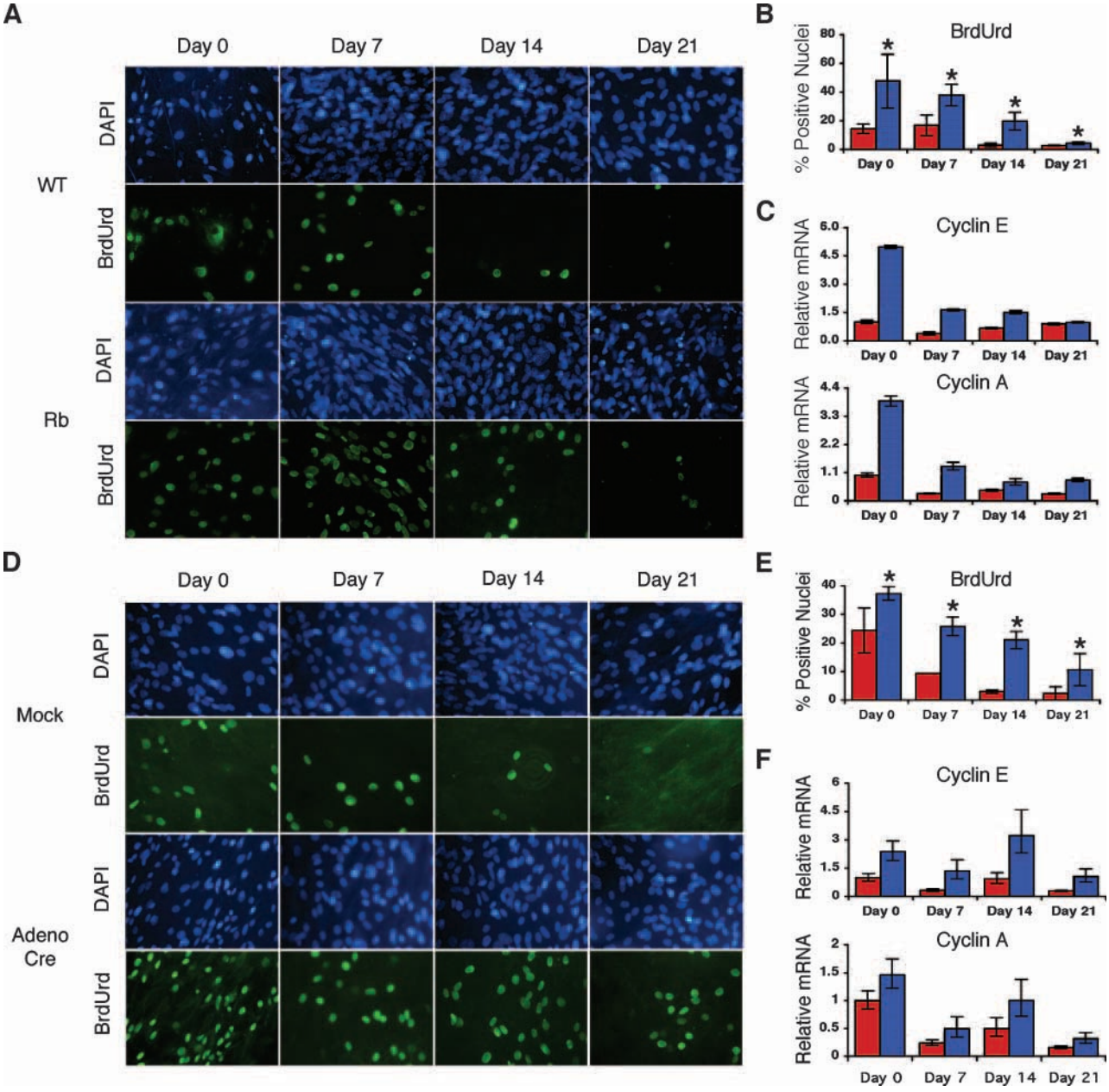


FIGURE 4. Confluent osteoblasts *in vitro* exhibit excess proliferation upon loss of pRb. **A.** Immunofluorescence analysis of BrdUrd incorporation in differentiating osteoblasts. Wild-type (*top two rows*) and $Rb^{c-/c-}$ (*bottom two rows*) osteoblasts were treated with BrdUrd (*green*) for 24 h at the indicated time points during differentiation *in vitro*. Nuclei are stained with 4′,6-diamidino-2-phenylindole (*blue*). Original magnification, ×20. **B.** Quantitation of the immunofluorescence analysis in **A**. A minimum of 250 cells was counted from each of three or more separate images for each sample. A greater percentage of $Rb^{c-/c-}$ osteoblasts incorporate BrdUrd compared with wild-type cells at all time points. **C.** Quantitative RT-PCR analysis was done as described in Fig. 3. $Rb^{c-/c-}$ osteoblasts (*blue columns*) express greater mRNA levels of *cyclin E* and *cyclin A* relative to wild-type osteoblasts (*red columns*) during *in vitro* differentiation. **D.** Nuclei of mock-infected (*top two rows*) and acutely ablated (*bottom two rows*) $Rb^{c/c}$ osteoblasts were stained for BrdUrd (*green*) and 4′,6-diamidino-2-phenylindole (*blue*). Original magnification, ×20. **E.** Quantitation of the immunofluorescence analysis in **D**. A greater percentage of $Rb^{c/c}$ osteoblast nuclei acutely ablated for pRb (*blue columns*) stain positively for BrdUrd incorporation than control nuclei (*red columns*). **F.** Quantitative RT-PCR shows that acutely ablated $Rb^{c/c}$ osteoblasts (*blue columns*) express greater mRNA levels of *cyclin E* and *cyclin A* compared with mock-infected osteoblasts (*red columns*). Bars, 1 SD. *, $P < 0.05$, a statistically significant difference. WT, $Rb^{+/c}$; $Mox2^{+/+}$; Rb, $Rb^{c-/c-}$; $Mox2^{+/Cre}$.

incorporation and PCNA expression in frontal bone sections from embryonic day 17.5 embryos (Fig. 6A). These two assay methods yielded highly concordant results. First, there was no significant difference in the levels of either BrdUrd- or PCNA-positive nuclei in the wild-type versus the $E2f1^{-/-}$ osteoblasts. Thus, loss of *E2f1* alone seems insufficient to perturb osteoblast proliferation. Second, consistent with our prior analysis, proliferating osteoblasts were present at significantly higher levels in the $Rb^{c-/c-}$ frontal bone compared with the wild-type and $E2f1^{-/-}$ controls. Finally, the deletion of *E2f1* was suf-

ficient to almost fully suppress the excess proliferation arising in the $Rb^{c-/c-}$ embryos. The loss of *E2f1* on its own or in the $Rb^{c-/c-}$ background did not affect proliferation at the apex of the frontal bone (data not shown), suggesting that the rate of progenitor proliferation remained unaffected. Therefore, we conclude that inappropriate activation of E2F1 contributes to the inability of pRb-deficient osteoprogenitors to properly exit the cell cycle *in vivo*.

We then assessed whether *E2f1* inactivation modulated the $Rb^{c-/c-}$ embryonic skeletal defects observed at embryonic day 17.5 (Fig. 6B). Consistent with the absence of any proliferation defects, the deletion of *E2f1* alone did not cause any detectable defects in skeletal development. As observed previously, *Rb* deficiency caused decreased ossification in the skull and hyoid, and aberrant formation of the xiphoid process, palatine process, and pterygoid bone. Notably, in almost all $E2f1^{-/-}$; $Rb^{c-/c-}$ double mutant embryos (12 of 13), the reduced ossification was partially or completely ameliorated (Fig. 6B, *first two columns*). Moreover, ~40% (5 of 13) of the double mutants exhibited normal formation of the palatine process, pterygoid bone, and the xiphoid process was completely normal (Fig. 6B, *latter two columns*). Taken together, these data show that deletion of *Rb* causes defects in embryonic skeletal development that are due, at least in part, to the inappropriate release of E2F1.

Discussion

The *RB* locus is mutated or altered in >70% of all osteosarcomas (3, 4). Moreover, several *in vitro* studies implicate pRb and the pocket proteins in osteoblast differentiation (18-21). Given these observations, we used the $Mox2^{+/Cre}$ transgene to conditionally inactivate *Rb* in the $Rb^{c/c}$ embryo proper, but not in the placenta, and thereby generate pRb-deficient embryos that survive until birth. This conditional strategy allows us to assess pRb's role in bone development *in vivo* and primary osteoblast differentiation *in vitro*. Our analyses reveal a role for pRb in the promotion of osteogenesis via the regulation of proper cell cycle exit.

In the developing embryo, the loss of pRb impaired bone formation in a manner that caused two types of defects. Some bones, such as the pterygoid bone, palatine process, and xiphoid process, developed abnormally and were misshapen, whereas the skull and hyoid bone exhibited decreased bone formation. The decreased ossification in the $Rb^{c-/c-}$ frontal bone was accompanied by reduced alkaline phosphatase activity and decreased levels of *Col1* and *OPN* mRNA. Previous studies have shown that deletion of the pRb-related proteins, p107 and p130, or overexpression of E2F1 affect chondrocyte differentiation and development (35-37). Although our data do not rule out a role for pRb in cartilage development, they clearly show that pRb plays a role in bone development that is independent of chondrocytes. Specifically, $Rb^{c-/c-}$ skeletons did not show any apparent defects in cartilage formation, and several of the affected bones formed via intramembranous ossification, a process that does not involve chondrocytes. Moreover, the bone defects in the $Rb^{c-/c-}$ frontal bone, and presumably in other affected bones, were not the result of increased osteoclast activity or apoptosis. Therefore, our data suggest that the loss of *Rb* impairs osteoblast differentiation *in vivo* at the earliest stages of the pathway.

One caveat of the *in vivo* studies is that they do not prove that pRb's requirement for osteoblast differentiation is cell autonomous. To address this issue, we determined how the loss of pRb affects the differentiation of primary osteoblasts *in vitro*. Given our *in vivo* defects and the prior observation that pRb-deficient MEFs were impaired in their ability to undergo osteogenesis (20), we anticipated that primary osteoblasts isolated from $Rb^{c-/c-}$ embryos would display an impaired differentiation phenotype *in vitro*. However, the exact opposite was observed: the $Rb^{c-/c-}$ osteoblasts differentiated to a greater extent than the wild-type controls. Importantly, we found that the acute ablation of *Rb* in confluent osteoblasts was sufficient to trigger increased differentiation. These data show that loss of pRb acts in a cell autonomous manner to promote osteoblast differentiation *in vitro*.

Our study shows that two distinct molecular changes accompany the improved *in vitro* differentiation upon loss of pRb. First, we observe a dramatic up-regulation of osteoblast genes, such as *Runx2* and *OSX* in differentiating pRb-deficient osteoblasts to levels that are sometimes not reached by wild-type cells. At this time, we do not know if the extreme up-regulation in $Rb^{c-/c-}$ cultures is due to an increased ability of individual cells to induce osteoblast genes, an increased percentage of terminally differentiated cells in the culture, or both. Interestingly, in these *in vitro* assays, pRb loss clearly

Table 1. Cell Numbers at Day 0 of Differentiation

Genotype	Germ line		Conditional	
	Wild-type	$Rb^{c-/c-}$	Adeno-GFP	Adeno-Cre
Cell count (×1,000)	481 ±16.5	656 ± 14.1	483 ± 24.5	579 ± 17.6

NOTE: Two hundred and fifty thousand cells were plated onto a 3-cm tissue culture dish and allowed to reach confluency (typically 4 days later). For "germ line" cells, this confluency arrest constituted day 0 of differentiation, and the number of cells was ascertained. For "conditional" cells ($Rb^{c/c}$) at confluence, adenovirus containing either green fluorescent protein or Cre recombinase was added to the medium. Two days after adenovirus addition (designated as day 0 of differentiation) cells were counted. Average cell counts from at least three separate experiments ± SD are shown.

Table 2. Quantitative RT-PCR Primer Pairs

Gene	Primer sequence
Alkaline phosphatase	For: TCT CCA GAC CCT GCA ACC TC Rev: CAT CCT GAG CAG ACC TGG TC
Collagen 1a1	For: CGA GTC ACA CCG GAA CTT GG Rev: GCA GGC AGG GCC AAT GTC TA
Cyclin A	For: AGT TTG ATA GAT GCT GAC CC Rev: TAG GTC TGG TGA AGG TCC
Cyclin E	For: TGT TTT TGC AAG ACC CAG ATG A Rev: GGC TGA CTG CTA TCC TCG CT
Osteocalcin	For: CTC TGT CTC TCT GAC CTC ACA G Rev: CAG GTC CTA AAT AGT GAT ACC G
Osteopontin	For: TGC TTT TGC CTG TTT GGC AT Rev: TTC TGT GGC GCA AGG AGA TT
Osterix	For: GCA AGG CTT CGC ATC TGA AA Rev: AAC TTC TTC TCC CGG GTG TGA
Runx2	For: TGA GAT TTG TGG GCC GGA Rev: TCT GTG CCT TCT TGG TTC CC
Ubiquitin	For: TGG CTA TTA ATT ATT CGG TCT GCA T Rev: GCA AGT GGC TAG AGT GCA GAG TAA

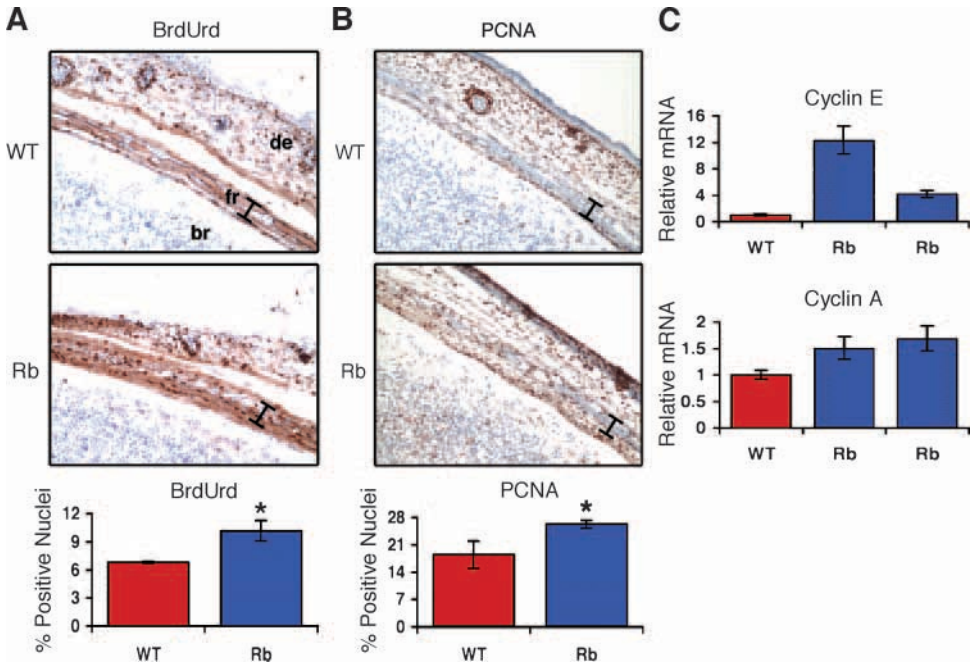


FIGURE 5. pRb-deficient osteoblasts do not properly exit the cell cycle *in vivo*. **A** and **B.** Immunohistochemical analysis of BrdUrd incorporation (**A**) or PCNA protein expression (**B**) in coronal sections of frontal bones from embryonic day 17.5 embryos. Pregnant females were injected with BrdUrd for 2 h. $Rb^{c-/c-}$ frontal bones (*bottom*) exhibit a greater number of nuclei positively staining for BrdUrd or PCNA than wild-type littermates (*top*). Original magnification, ×20. Frontal bones (*bar*). Columns, quantified results from four pairs of $Rb^{c-/c-}$ and wild-type frontal bone sections; bars, 1 SD; *, $P < 0.05$, statistically significant difference. **C.** Quantitative RT-PCR analysis of *cyclin E* (*top*) and *cyclin A* (*bottom*) mRNA levels from $Rb^{c-/c-}$ and control littermates. mRNA was isolated from the calvaria of embryonic day 16.5 embryos. Analysis done as described in Fig. 3. $Rb^{c-/c-}$ calvaria (*blue columns*) express increased levels of *cyclin A* and *cyclin E* relative to wild-type littermates (*red columns*). Bars, 1 SD. Abbreviations: br, brain; de, dermis; fr, frontal bone. WT, $Rb^{+/c}$; $Mox2^{+/+}$; Rb, $Rb^{c-/c-}$; $Mox2^{+/Cre}$.

induces some (e.g., *Runx2* and *OSX*) but not all (*ALP* and *Col1*) osteoblast genes. The reason for this differential response is unclear. However, we note that even prior to the induction of differentiation, the *ALP* and *Col1* mRNAs are present at much higher levels in the cultured osteoblasts than in the endogenous calveria. This suggests that the *in vitro* culture somehow induces *ALP* and *Col1* expression or that it selects for a subpopulation of the calverial cells that are committed to the osteoblast lineage and therefore have high *ALP* and *Col1* expression.

The second molecular change that accompanies the improved *in vitro* differentiation of pRb-deficient osteoblasts is an increase in the fraction of cells that are proliferating and the sustained presence of proliferating cells at later time points in the differentiation process. Because the density of osteoblasts has been reported to correlate positively with their ability to differentiate *in vitro* (32, 33), we believe that the increased proliferation of the pRb-deficient osteoblasts contributes to their improved differentiation by increasing the density of the confluent cells. We tried two distinct approaches to directly test this model. First, we attempted to maintain the $Rb^{c/c}$ osteoblasts in the presence of antiproliferative drugs prior to the ablation of pRb. However, the experiment requires several days of drug treatment to which the cells faired poorly. Second, because our *in vivo* data indicate that deletion of *E2f1* suppresses excess proliferation due to the loss of *Rb*, we analyzed the consequence of *E2f1* deficiency in acutely ablated and germ line–deleted $Rb^{c-/c-}$ osteoblasts. Unfortunately, the loss of *E2f1* did not suppress the cell cycle defects of osteoblasts in this *in vitro* setting. Thus, we have been unable to prove that a cell cycle exit defect can account for the increased differentiation of *Rb*-depleted osteoblasts *in vitro*. Despite this limitation, our *in vivo* studies provide strong support for this model. Specifically, we find that osteoblasts of the $Rb^{c-/c-}$ frontal bone fail to exit the cell cycle at the appropriate stage of development, and we can completely suppress both the proliferation defect and the decreased ossification of the skull and hyoid bones through inactivation of *E2f1*, a known pRb target and proliferation inducer.

If a cell cycle exit defect is the major underlying cause of both the *in vitro* and *in vivo* defects, how does this account for the apparently opposing effects on bone differentiation seen in the two settings? One possibility is that this is an aberrant consequence of the *in vitro* culture that somehow enables the *Rb*-deficient cells to overcome their differentiation defect. The alternative possibility, which we favor, is that pRb loss affects cells at early and late stages of osteoblast differentiation in a differential manner, and the *in vivo* and the *in vitro* studies highlight the defects in these distinct populations. Specifically, we hypothesize that pRb loss leads to ectopic proliferation that prevents early progenitors from entering osteoblast differentiation but concomitantly enhances the differentiation of late stage osteoblasts. In this model, the *in vitro* cultures could favor analysis of the late stage osteoblasts, thereby showing that pRb loss promotes osteoblast differentiation. In contrast, the *in vivo* phenotype would be more complex.

Specifically, our data clearly show ectopic proliferation of $Rb^{c-/c-}$ cells in the developing frontal bone, but we cannot know whether these represent uncommitted early progenitor cells or differentiating osteoblasts that are proliferating inappropriately. In fact, we believe that both populations coexist. In this event, at early time points in the bone differentiation process, the shortage of committed osteoprogenitors would initially impair bone formation—exactly as we observe in the late stage embryos. However, as the committed osteoblasts accumulate, their increased proliferation would eventually allow, and perhaps ultimately enhance, bone differentiation—as we observe in the *in vitro* assays. Unfortunately, because the $Rb^{c-/c-}$ animals die at birth, we cannot determine whether their osteoblast density and bone deposition ultimately exceeds that seen in wild-type animals.

There is considerable evidence to suggest that pRb plays a direct role in regulating the transcriptional programs that control osteoblast differentiation. Most compelling is the finding that pRb can positively regulate Runx2 *in vitro* (20, 21). Our findings do not discount the possibility that pRb plays a direct role in bone differentiation through Runx2, or some other mechanism, or that this might contribute to the bone defects we observe *in vivo*. However, they argue that the primary role of pRb in bone differentiation is to inhibit E2F1 and thereby facilitate cell cycle exit. Given that *Rb* inactivation is observed in a large proportion of osteosarcomas, it will be important to develop additional models that allow a comparison of the mechanisms by which loss of *Rb* affects bone development versus osteosarcoma formation.

Materials and Methods

Animal Maintenance and Histologic Preparations

The generation of $Rb^{c/c}$ and *Mox2-Cre* mice has been described previously (7, 27). $Rb^{c/c}$ and $E2f1^{-/-}$ mice were provided by Tyler Jacks. *Mox2-Cre* mice were purchased from The Jackson Laboratory. Gestation was dated by detection of a vaginal plug. Pregnant mice were injected with 10 μL/g body weight of 5 mg/mL BrdUrd in PBS 2 hours prior to tissue collection. For calcein incorporation, pregnant mice were injected with 10 μL/g body weight of 2.5 mg/mL calcein 12 or 24 hours prior to tissue collection. Collected embryonic tissue was fixed in 4% paraformaldehyde and embedded in optimal cutting temperature. Frozen sections were cut at 6 to 8 μm except for those for *in situ* analysis, which were cut at 10 to 12 μm. The morphology of the brain and presphenoid bone were used to ensure that equivalent planes of the frontal bone were analyzed in all samples.

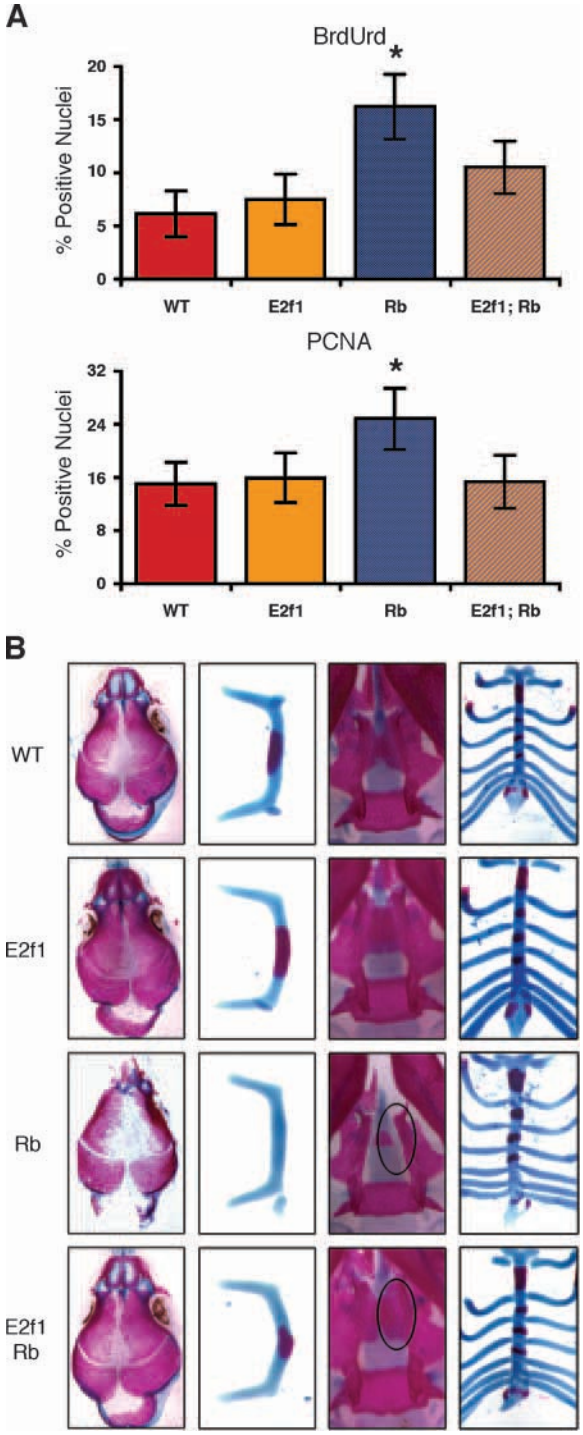


FIGURE 6. Deletion of *E2f1* suppresses the bone defects due to the loss of pRb. **A.** Immunohistochemical analysis of BrdUrd incorporation (*top*) or PCNA protein expression (*bottom*) in coronal sections of frontal bones from embryonic day 17.5 embryos, done with four to six samples of each genotype. Deletion of *E2f1* suppresses the increased BrdUrd incorporation and PCNA expression observed in $Rb^{c-/c-}$ frontal bone osteoblasts. **B.** Skeletal staining of embryonic day 17.5 embryos as described in Fig. 1. Deletion of *E2f1* suppresses the decreased ossification found in the $Rb^{c-/c-}$ calvaria (*first column*) and hyoid bone (*second column*). Deletion of *E2f1* also suppresses the aberrant formation of the palatine process and pterygoid bone (*third column*) and xiphoid process (*fourth column*) observed in $Rb^{c-/c-}$ skeletons. An aberrant palatine process in the $Rb^{c-/c-}$ and a suppressed palatine process in the double mutant are circled (*third column*). Bars, 1 SD; *, $P < 0.05$, statistically significant difference between $Rb^{c-/c-}$ and wild-type, $E2f1^{-/-}$, or $Rb^{c-/c-}; E2f1^{-/-}$. WT, $Rb^{+/c}; Mox2^{+/+}; E2f1^{+/+}$; E2f1, $Rb^{+/c}; Mox2^{+/+}; E2f1^{-/-}$; Rb, $Rb^{c-/c-}; Mox2^{+/Cre}; E2f1^{+/+}$; RbE2f1, $Rb^{c-/c-}; Mox2^{+/Cre}; E2f1^{-/-}$.

Histologic Analyses

Enzymatic ALP assays were done on unfixed frozen sections. Briefly, 0.06 g of sodium nitrite was dissolved into 1.5 mL of water and added to 600 μL of 50 mg/mL of new fuchsin (Sigma) in 2 mol/L of HCl. This solution was added to 210 mL of Tris buffer (pH 9.0). Finally, 1.8 mL of 83.3 mg/mL naphthol AS-Bi-phosphate (Sigma) in DMF (Sigma) was added. Sections were incubated with this overall solution for 15 min, washed in PBS and counterstained with hematoxylin. Immunohistochemical analyses were done using antibodies against BrdUrd (1:50 347580; BD Biosciences) and PCNA (1:2,000 sc56; Santa Cruz) as previously described (38). For Collagen1a1 *in situ*, digoxigenin-11-UTP–labeled single-strand riboprobe was prepared (probe was a gift from B. Olsen), and hybridization was carried out overnight in 50% formamide at 55°C. Washing, detection, staining, and mounting of slides were carried out as described previously (39). Statistical significance was determined using the two-sample Student's *t* test with two-tailed distribution and unequal variance.

Skeletal Staining

Embryos were sacrificed, skinned, and eviscerated. The remaining tissue was fixed in 95% ethanol for 4 days, transferred to acetone for 3 days, and subsequently transferred to staining solution [final volume of 0.015% Alcian blue 8GX (Sigma), 0.005% alizarin red S (Sigma), and 5% glacial acetic acid in ethanol] at 37°C for 2 days and at room temperature for a 3rd day. Tissue was cleared in 1% potassium hydroxide for several days and ultimately stored in glycerol.

Calvarial Preparations and Culture

Calvaria from embryonic day 17.5 embryos were removed, treated with several rounds of collagenase/trypsin digests at 37°C, and plated onto six-well plates. Cells were grown and expanded in αMEM with 10% fetal bovine serum and penicillin/streptomycin. For differentiation, 250,000 cells were plated onto 3-cm tissue culture plates. Upon reaching confluence, calvarial osteoblasts were treated with medium supplemented with 50 μg/mL of ascorbic acid and 10 mmol/L of β-glycerol-phosphate. Adenovirus (University of Iowa Gene Transfer Vector Core) was added to the medium at 100 plaque-forming units per cell and washed away 24 h later. To assay for calcium deposits, plates were stained with 1% alizarin red S solution (pH 5.0).

Immunofluorescence

For BrdUrd incorporation, osteoblasts were plated onto coverslips prior to achieving confluence. BrdUrd was added to the medium (final concentration of 10 μmol/L) and incubated for 24 h prior to 4% paraformaldehyde fixation. Antigen was detected using antibody against BrdUrd (1:50 347580; BD Biosciences) with Texas red-X goat anti-mouse secondary (1:1,000; Invitrogen). Statistical significance was determined using Student's *t* test.

Quantitative RT-PCR

RNA was isolated from differentiation plates using the Qiagen RNeasy kit. First-strand cDNA was transcribed from 1 μg of RNA using Superscript III reverse transcriptase (Invitrogen) following the instructions of the manufacturer. Quantitative RT-PCR with 20 to 100 ng cDNA was done using SYBR Green (Applied Biosystems). Reactions were run on the ABI Prism 7000 Sequence Detection System and analyzed using the 7000 SDS software. Primers are listed in Table 2.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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