Terminal osteoblast differentiation, mediated by runx2 and p27\textsuperscript{KIP1}, is disrupted in osteosarcoma

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T he molecular basis for the inverse relationship between differentiation and tumorigenesis is unknown. The function of runx2, a master regulator of osteoblast differentiation belonging to the runt family of tumor suppressor genes, is consistently disrupted in osteosarcoma cell lines. Ectopic expression of runx2 induces p27\textsuperscript{KIP1}, thereby inhibiting the activity of S-phase cyclin complexes and leading to the dephosphorylation of the retinoblastoma tumor suppressor protein (pRb) and a G1 cell cycle arrest. Runx2 physically interacts with the hypophosphorylated form of pRb, a known coactivator of runx2, thereby completing a feed-forward loop in which progressive cell cycle exit promotes increased expression of the osteoblast phenotype. Loss of p27\textsuperscript{KIP1} perturbs transient and terminal cell cycle exit in osteoblasts. Consistent with the incompatibility of malignant transformation and permanent cell cycle exit, loss of p27\textsuperscript{KIP1} expression correlates with dedifferentiation in high-grade human osteosarcomas. Physiologic coupling of osteoblast differentiation to cell cycle withdrawal is mediated through runx2 and p27\textsuperscript{KIP1}, and these processes are disrupted in osteosarcoma.

Introduction

Osteosarcoma is the most common bone cancer in young adults, and a leading cause of cancer death in this age group. Loss of differentiation has powerful prognostic significance in osteosarcoma, with well-differentiated tumors being classified as low-grade and dedifferentiated tumors usually falling into the high-grade category. In one large series, 81% of osteosarcomas were either poorly differentiated or undifferentiated (Dahlin, 1957), and a late marker of osteogenic differentiation, osteocalcin, was undetectable in (Dahlin, 1957), and a late marker of osteogenic differentiation, osteocalcin, was undetectable in 75% of osteosarcomas (Hopyan et al., 1999). In vitro data support the view that osteoblast differentiation is antagonistic to oncogenic processes. Cellular immortalization attenuates expression of osteoblast phenotype (Bodine et al., 1996; Feuerbach et al., 1997), whereas osteoblast differentiation is associated with progressive loss of proliferative capacity, culminating in terminal cell cycle exit (Stein et al., 1996; Aubin, 1998). Although it is clear that differentiation is antithetical to tumor development in osteosarcoma, the molecular basis for the tightly coupled relationship between malignant transformation and loss of differentiation remains poorly understood.

The biology of osteoblast differentiation has recently been mapped in considerable detail. Runx2 (runt-related transcription factor 2) is a key transcriptional regulator of osteogenesis (Ogawa et al., 1993; Levanon et al., 1994; Ducy et al., 1997, 1999). Mice nullizygous for RUNX2 exhibit a complete lack of ossification (Komori et al., 1997; Otto et al., 1997), whereas heterozygotes exhibit skeletal abnormalities comparable to cleidocranial dysplasia (Mundlos et al., 1997). The runt family, to which runx2 belongs, is strongly linked to human cancer (Lund and van Lohuizen, 2002). RUNXI (AML1) is mutated in human leukemia, and mice expressing loss-of-function runx1 mutants are prone to leukemia (Perry et al., 2002). RUNX3 is subject to inactivating mutations or promoter hypermethylation.
in gastric cancers (Li et al., 2002). Runx2 physically interacts with the retinoblastoma tumor suppressor protein (pRb; Thomas et al., 2001), which is mutated in up to 60% of osteosarcomas (Toguchida et al., 1989). Finally, runx2 expression varies with cell cycle status and may regulate osteoblast proliferation by unknown mechanisms (Pratap et al., 2003).

In this study, we investigated the effects of osteogenic differentiation on proliferation and growth arrest, and their disruption in osteosarcomas. Consistent with a role in suppression of proliferation, runx2 protein was absent or nonfunctional in six out of seven osteosarcoma cell lines. Both spontaneous and induced osteoblast differentiation are associated with increased p27Kip1 mRNA and protein expression. Ectopic expression of runx2 induced an Rb- and p27Kip1-dependent growth arrest. This was due in part to increased expression of p27Kip1 protein, which inhibited S-phase Cdk complexes and the dephosphorylation of pRb. Interestingly, runx2 is shown to interact preferentially with the hypophosphorylated form of pRb, a known coactivator of runx2. Although p27Kip1 expression is associated with osteoblast differentiation, loss of p27Kip1 had only a minor effect on osteoblast differentiation in vitro and in vivo. Notably, the irreversibility of both the osteogenic phenotype and terminal cell cycle exit in vitro is dependent on expression of p27Kip1.

Twelve genes, including the key osteoblast transcription factor, RUNX2, were underexpressed across all osteosarcoma cell lines relative to our osteoblastic reference. Osteocalcin, a late and specific osteoblast marker not represented on our arrays, was undetectable by RT-PCR in the osteosarcoma lines (not depicted). Overall, the average median expression of 16 osteoblast-related genes in the osteosarcoma cell lines was reduced to 38 ± 8% of the osteoblastic reference.

The pattern of gene expression observed in osteosarcomas suggests a loss or reduction in activity of runx2, a key transcriptional regulator of osteoblast differentiation. To assess activity of the runx2 pathway in the osteosarcoma cell lines, we measured endogenous runx2 transcriptional activity using an osteoblast-specific promoter–luciferase construct, 6xoe2-luc (Ducy and Karsenty, 1995), consisting of six tandem repeats of the osteocalcin runx2 binding site. A mutant control containing a dinucleotide substitution in the runx2 binding sites was used, abolishing runx2 binding and activity (Ducy and Karsenty, 1995). The ratio of wild-type to mutant reporter activity therefore reflects endogenous runx2 function, and it was markedly reduced in all seven osteosarcoma cell lines, to levels comparable to that observed in fibroblasts (CCL-7625; Fig. 1 B). A positive control derived from an osteogenic osteoma (CCL-7672) demonstrated runx2 activity 80-fold higher than in CCL-7625 cells. Similar results were observed with native osteopontin and osteocalcin promoter–luciferase constructs (unpublished data). The effect of ectopically expressing the osteoblast-specific MASN isoform of runx2 (hereafter runx2) on reporter activity was studied (Fig. 1 C). Ectopic expression of runx2 increased reporter activity 10–60-fold in some osteosarcoma cell lines, suggesting that endogenous levels of runx2 were limiting for transcriptional activity in these cell lines.

### Results

#### Characterization of the osteoblast phenotype in a panel of osteosarcoma cell lines

We first used transcriptional profiling to objectively characterize the differentiation state of a panel of osteosarcoma cell lines (SAOS2, MG63, B143, HOS, SJSA, and G292) relative to an osteoblast-like reference. The reference consisted of primary stromal stem cells in which expression of markers of the mature osteoblast phenotype was induced by culture in the presence of ascorbic acid, dexamethasone, and inorganic phosphate (Gronthos et al., 2003). These markers include runx2, osterix, osteocalcin, and the ability to mineralize in vitro. Consistent with a transformed state, several putative oncogenes, including FOS and cyclins A1, B2, E1, and D1 (Fig. 1 A), were commonly overexpressed in osteosarcoma cell lines, whereas the Cdk inhibitors p16INK4a and p27Kip1 were relatively underexpressed. Shown in Fig. 1 A is the expression pattern of 16 bone-related genes selected from a previously published microarray study on the osteoblast phenotype (Balint et al., 2003). Twelve genes, including the key osteoblast transcription factor, RUNX2, were underexpressed across all osteosarcoma cell lines relative to our osteoblastic reference. Osteocalcin, a late and specific osteoblast marker not represented on our arrays, was undetectable by RT-PCR in the osteosarcoma lines (not depicted). Overall, the average median expression of 16 osteoblast-related genes in the osteosarcoma cell lines was reduced to 38 ± 8% of the osteoblastic reference.

The pattern of gene expression observed in osteosarcomas suggests a loss or reduction in activity of runx2, a key transcriptional regulator of osteoblast differentiation. To assess activity of the runx2 pathway in the osteosarcoma cell lines, we measured endogenous runx2 transcriptional activity using an osteoblast-specific promoter–luciferase construct, 6xoe2-luc (Ducy and Karsenty, 1995), consisting of six tandem repeats of the osteocalcin runx2 binding site. A mutant control containing a dinucleotide substitution in the runx2 binding sites was used, abolishing runx2 binding and activity (Ducy and Karsenty, 1995). The ratio of wild-type to mutant reporter activity therefore reflects endogenous runx2 function, and it was markedly reduced in all seven osteosarcoma cell lines, to levels comparable to that observed in fibroblasts (CCL-7625; Fig. 1 B). A positive control derived from an osteogenic osteoma (CCL-7672) demonstrated runx2 activity 80-fold higher than in CCL-7625 cells. Similar results were observed with native osteopontin and osteocalcin promoter–luciferase constructs (unpublished data). The effect of ectopically expressing the osteoblast-specific MASN isoform of runx2 (hereafter runx2) on reporter activity was studied (Fig. 1 C). Ectopic expression of runx2 increased reporter activity 10–60-fold in some osteosarcoma cell lines, suggesting that endogenous levels of runx2 were limiting for transcriptional activity in these cell lines.

#### Lack of correlation between runx2 protein levels and transcriptional activity

There was little or no correlation between runx2 protein levels and transcriptional activity. Immunoblot analysis of endogenous runx2 protein demonstrated low levels in five out of seven cell lines (Fig. 1 D, top and middle). The upper band represents the MRIPV isoform of runx2, whereas the faster migrating band in SAOS2 represents the osteoblast-specific MASN isoform (unpublished data). The MRIPV isoform strongly induces AP activity but not osteocalcin, whereas the MASN isoform more potently activates osteocalcin but not AP (Harada et al., 1999). Consistent with the results of the transcriptional assays, no runx2 protein was detectable by Western blot in G292 cells, which showed the greatest induction of transcription by ectopic runx2 (Fig. 1 D). In contrast, abundant endogenous runx2 protein was present in SAOS2 cells. The striking contrast between protein levels and intrinsic activity of runx2 in SAOS2 cells is highlighted by comparison with protein levels in the osteoblastic control, although osteoblast gene expression is 5.6 ± 2.9-fold higher in the reference than in SAOS2 cells. We have confirmed that no mutations exist in the genomic sequences of runx2 in SAOS2 cells or any other cell line in this study. Thus, some osteosarcomas, such as G292, appear to lack functional runx2, whereas others, such as SAOS2, appear unable to activate transcription of runx2-dependent genes even when ectopic runx2 is supplied. Interestingly, we observed that all lines responsive to ectopic runx2 express pRb, whereas those that fail to respond express no or low levels of this known runx2 coactivator. Furthermore, some pRb-positive cell lines show only limited activation in response to runx2 expression, suggesting...
that additional factors influence the activity of runx2 in osteosarcoma cell lines (Fig. 1 D). Together, these data confirm gathering evidence that runx2 activity is critically dependent on cofactors or posttranslational modifications, and that oncogenic transformation results in consistent dysregulation of runx2 activity by multiple mechanisms.

Runx2 inhibits cell growth through p27KIP1 and pRb

It appears that normal runx2 function is incompatible with malignant transformation of osteoblastic cells. To determine why, we examined the effect of reexpression of runx2 in G292 cells. As shown in Fig. 1 (B and C), in G292 cells runx2 levels appeared to be rate limiting for transcriptional activity. This suggests that the molecular apparatus for full runx2 activity, including any potential tumor suppressor functions, exists in G292 cells. Consistent with this idea, ectopic expression of runx2 suppressed cell growth (Fig. 2 A). In contrast, this effect was not seen in SAOS2 cells, in which forced expression of runx2 had little effect on transcriptional activity. Because SAOS2 lacks functional pRb, whereas G292 has wild-type pRb, we hypothesized that the lack of pRb may account for the inability of runx2 to reduce the proliferative capacity of these cells. Indeed, when overexpressed in wild-type and RB-/- 3T3 cell lines, runx2 suppressed colony numbers of 3T3 cells by 60–90%, an effect dependent on pRb (unpublished data). To study this effect further, we used two runx2 constructs containing transactivation domains. One of these (27ala) lacks transcriptional activity, whereas the other (3ala) possesses wild-type activity (Thirunavukkarasu et al., 1998). Introduction of these constructs into 3T3 cells showed that the colony suppression activity of runx2 is due to a G1 cell cycle arrest dependent on transcriptional activity and pRb (Fig. 2 B). Runx2 was ectopically expressed in primary human fibroblasts (CCL-211 and IMR90) and osteosarcoma cell lines (U2OS and SAOS2), using adenoviral vectors. As expected, runx2 inhibited the S-phase fraction of fibroblastic but not osteosarcoma cells. Initial studies of cell cycle protein expression in these transfected cells revealed a specific induction of p27KIP1 protein but no effect on p21CIP1 (Fig. 2 C). Coimmunoprecipitation from CCL-211 cells showed that p27KIP1 was strongly associated with cyclin A and Cdk2 (Fig. 2 D), suppressed in vitro kinase activity of cyclin A–Cdk2 complexes (Fig. 2 E), and was accompanied by dephosphorylation of endogenous pRb (Fig. 2 E). We have shown previously that pRb binds and coactivates runx2 (Thomas et al., 2001). We hypothesized that a feed-forward loop, integrating progressive cell cycle withdrawal and differentiation, would be completed if runx2 specifically interacted with the hypophosphorylated form of pRb. This is the case (Fig. 2 F). Collectively, these data are consistent with the transcriptional induction of growth arrest by runx2 through an Rb- and p27KIP1-dependent mechanism that is reinforced by coactivation of runx2 by direct interactions with the hypophosphorylated form of pRb.

A role for p27KIP1 in osteogenic differentiation

The data described above suggest a role for p27KIP1 and pRb in mediating runx2-dependent proliferation arrest. We have previously reported a role for pRb in runx2-dependent expression of differentiation-related genes, and so wished to determine the physiologic significance of p27KIP1 in osteoblast differentiation. As reported previously (Drissi et al., 1999), osteoblast differentiation in vitro is associated with increased expression of p27KIP1 (Fig. 3 A). Bone morphogenetic proteins (BMPs) are powerful osteoinductive agents whose effects are mediated by runx2 (Tsuji et al., 1998; Gori et al., 1999). Treatment of murine embryonic fibroblasts (MEFs) with a synthetic BMP4/7 fusion protein induced osteoblast differentiation (and cell cycle
arrest), concomitant with expression of runx2 and p27KIP1 mRNA (Fig. 3 B). Similarly, BMP2 induced a G1 cell cycle arrest in MEFs that was dependent in part on the presence of both pRb and p27KIP1 (Fig. 4 A). This is consistent with a role for runx2 in inhibition of cell proliferation and induction of differentiation by BMPs. To confirm these observations in a pre-osteoblast cell model, we used siRNA to knockdown p27KIP1 in MC3T3E1 cells (Fig. 4 B). The level of knockdown achieved was >75%, which we consider significant because p27KIP1 is believed to act as a haploinsufficient tumor suppressor gene (Fero et al., 1998). Consistent with observations that runx2-null osteoblasts have increased rates of proliferation (Pratap et al., 2003), we observed an increased rate of proliferation in MC3T3E1 cells in which p27KIP1 was reduced. BMP2 treatment of MC3T3E1 cells expressing a control siRNA vector resulted in a 42% reduction in S-phase cells, compared with a 17% reduction in cells expressing the p27KIP1 siRNA (Fig. 4 C). Furthermore, as observed in G292 cells and 3T3 fibroblasts, ectopic expression of runx2 suppressed growth of MC3T3E1 cells, and this effect was abolished in cells expressing siRNA for p27KIP1 (Fig. 4 D; Chi square P < 0.001). These data suggest that p27KIP1, like pRb, plays a role in regulating basal rates of proliferation in preosteoblasts and contributes to the growth arrest associated with osteoblastic differentiation.

Next, we wished to determine whether p27KIP1 is required for the osteoblast phenotype. Loss of p27KIP1 partially attenuated BMP2-induced AP activity (Fig. 5 A), and both basal and BMP2-induced expression of osteocalcin, osteopontin, and type I collagen mRNA were reduced (but not abolished by) the absence of p27KIP1 (Fig. 5 B). To determine the net effect of loss of p27KIP1 in vivo, the long bones of murine wild-type and p27KIP1/–/– littermates were analyzed by histomorphometry. There was a minor effect of loss of p27KIP1 on the formation of...
These data are consistent with a very minor effect of loss of function is unlikely to account for the reduction in osteoid. Alkalized osteoid (Chambers et al., 1985), any defect in osteoclast (unpublished data). Because osteoclasts do not resorb unmineralized bone (osteoid), both as a function of total bone volume and as measured by osteoid thickness (Fig. 5 C). This effect was not a result of accelerated mineralization caused by loss of p27KIP1, because there was no evidence of altered mineral apposition rate as measured directly by dual calcine labeling. Osteoblast and osteoclast numbers were unchanged (unpublished data). Because osteoclasts do not resorb unmineralized osteoid (Chambers et al., 1985), any defect in osteoclast function is unlikely to account for the reduction in osteoid. These data are consistent with a very minor effect of loss of p27KIP1 on osteoblast differentiation and function.

\[ \text{Figure 4. Growth arrest due to expression of runx2 or treatment with BMP2} \]

\[ \text{BMP2 is reduced by knockdown of p27KIP1.} \]

\[ \text{(A) Primary MEFs of the indicated genotypes were treated with 100 ng/ml BMP2 for 48 h followed by flow cytometric analysis of DNA content. Data shown are the change in G1 fraction due to treatment. This experiment was repeated twice with similar results.} \]

\[ \text{(B) Western blot for p27KIP1 in MC3T3E1 cells infected with a retrovirus expressing either control siRNA or siRNA for p27KIP1.} \]

\[ \text{(C) DNA analysis using flow cytometry of cultures of cells derived as described in B.} \]

\[ \text{(D) Colony suppression assay of cells as described transfected with empty vector or runx2. Each experiment was performed in triplicate.} \]

\[ \text{p27KIP1 is needed for terminal cell cycle exit in osteosarcoma cells (Alexander and Hinds, 2001). Because terminal differentiation is associated with permanent cell cycle withdrawal, we tested whether MEF cultures lacking p27KIP1 could reenter the cell cycle after culture under prolonged differentiating conditions. There was no prior difference in cell cycle profile of littermate wild-type or p27KIP1-/- preconfluent early passage MEFs (unpublished data). However, although wild-type MEFs postdifferentiation grew poorly after passage, MEFs lacking p27KIP1 proliferated robustly (Fig. 6 A). This suggests a role for p27KIP1 in mediating the irreversibility of the postconfluent state. However, wild-type undifferentiated MEFs also failed to reenter the cell cycle, probably because p27KIP1 also accumulated in untreated cultures upon confluence (Hirano et al., 2001; and unpublished data). Consistent with an additional effect of BMP2 upon p27KIP1, the degree of growth inhibition after passage was greater in cultures treated with BMP2 (Fig. 6 A). Moreover, even postdifferentiated MEFs lacking p27KIP1 demonstrated a reduction in cell growth when passaged after BMP2 treatment, indicating that p27KIP1 contributes to (but is not solely responsible for) terminal growth arrest. We have not observed compensatory increases in p21 or p57KIP2 in p27KIP1-null cultures (unpublished data), and the mechanisms accounting for the residual effects are not known.} \]

Reentry into the cell cycle of BMP2-treated cultures was associated with loss of differentiation. Compared with wild-type cultures, BMP2-treated p27KIP1-null cultures rapidly lost expression of osteocalcin within 2 d of passage (Fig. 6 B). Additionally, the number of AP-positive cells was >20-fold greater in wild-type compared with p27KIP1-/- cultures (4.8 ± 1.8% [n = 263, 10 high-power fields], compared with 0.2 ± 0.1% AP-positive cells [n = 2039]). This suggests that terminal cell cycle exit, in this case dependent on p27KIP1, is required for maintenance of the differentiated state.

Permanent cell cycle withdrawal is a feature of both senescence and terminal differentiation (Goldstein, 1990; Sellers et al., 1998). Postdifferentiated wild-type MEFs assumed a binucleated, enlarged, flattened morphology reminiscent of replicative senescence. Whether differentiated or not, significant numbers of postconfluent wild-type MEFs stained for senescence-associated β-galactosidase activity (Dimri et al., 1995). This effect was greater in cultures that had been treated with BMP2 (Fig. 6 D). In contrast, MEFs lacking p27KIP1 did not stain for senescence-associated β-galactosidase and morphologically resembled undifferentiated cultures. Although the effects of both BMP2 and p27KIP1 were independently statistically significant, the interaction between these factors failed to reach significance, perhaps because of the powerful effect of confluence on accumulation of p27KIP1 in both treated and control cultures. Together, these data suggest that culture conditions required for differentiation of MEFs (including both BMP2 treatment and prolonged confluence), as well as expression of p27KIP1, contribute to the entry of MEFs into a senescence-like state.
Expression of p27<sup>KIP1</sup> is lost in osteosarcoma

We finally wished to determine whether these observations have relevance to human osteosarcoma. p27<sup>KIP1</sup> expression appears to be key for cell cycle withdrawal and terminal differentiation in osteoblasts, and integrates the functions of BMPs, pRb, and runx2 in these processes. Regardless of the nature of the defect in the pRb–runx2 pathway in osteosarcoma cells, the net effect will be loss of growth restraint due to diminished expression of p27<sup>KIP1</sup>. Consistent with this, we found negligible expression of p27<sup>KIP1</sup> protein in high-grade osteosarcoma cells, although p27<sup>KIP1</sup> was clearly seen in osteoclasts as reported previously (Okahashi et al., 2001; Fig. 7, A and D), correlating inversely with expression of proliferating cell nuclear antigen (PCNA; Fig. 7, D and F, arrows). These high-grade osteosarcomas demonstrated frequent mitotic figures and little differentiation, as evidenced by osteoid production and osteocalcin expression (Fig. 7, B and E). High-grade tumor cells expressed high levels of PCNA, consistent with a high S-phase fraction (Fig. 7, C and F). In contrast, in lower grade tumors with mineralizing osteoid and lower cellularity with more normal osteoblastic morphology, expression of both p27<sup>KIP1</sup> and osteocalcin is evident, especially in terminally differentiated (PCNA negative) osteocytes embedded within bone (Fig. 7, G and H, arrows). Critically, there was a significant relationship between expression of p27<sup>KIP1</sup> protein and osteoblast differentiation scored by osteoid production in a panel of 100 osteosarcomas (Fig. 7 J, P<sub>H11021</sub> 0.05).

This effect was independent of proliferative rate, because there was no significant relationship between PCNA expression and p27<sup>KIP1</sup>. These data support the view that the loss of differentiation of osteosarcomas, which conveys adverse prognostic significance, is associated with loss of expression of p27<sup>KIP1</sup>.

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**Figure 5.** Role of p27<sup>KIP1</sup> in osteoblast function. (A) MEFs of the indicated genotypes were differentiated in the presence of 100 ng/ml BMP2, ascorbic acid, and β-glycerophosphate for 7 d and analyzed for AP activity. Data shown are means ± SEM of fold change relative to untreated wild-type controls (n = 4 experiments using independently derived littermate-matched cultures, each in triplicate). Genotype effect significant by analysis of variance (ANOVA; P < 0.01). (B) RT-qPCR analysis of gene expression in MEFs of the indicated genotypes, normalized to ARPP<sub>H9252</sub>. Data shown are means ± SEM. Genotype effect significant for BMP2 induction of osteocalcin (P < 0.01) and type I collagen (P < 0.05, ANOVA), but not osteopontin. (C) Histomorphometric analysis of long bones in mice between 8 and 12 wk old of the indicated genotypes. Data shown are means ± SEM. Bold-faced data are significantly different from wild-type littermates (P < 0.05).

**Figure 6.** p27<sup>KIP1</sup> is required for terminal growth arrest in vitro. MEFs were cultured in the presence or absence of 100 ng/ml BMP2, ascorbic acid, and β-glycerophosphate for 14 d after confluence. Cells were then passaged. (A) 5 × 10<sup>4</sup> cells were grown under standard culture conditions for 3 d, and then counted. Data shown are means ± SEM. (B) RT-qPCR analysis of osteocalcin gene expression in MEFs of the indicated genotypes 2 d after passage. Data shown are means ± SEM of cycle number (ΔΔCT) normalized to ARPP<sub>H9252</sub>, and expression before passage. The interaction between BMP2 and genotype was significant (P < 0.01, ANOVA). (C) Photomicrograph of senescence-associated β-galactosidase–stained cultures. (i) Wild-type untreated cultures; (ii) p27<sup>KIP1</sup>/−/− untreated cultures; (iii) wild-type differentiated cultures; and (iv) p27<sup>KIP1</sup>/−/− differentiated cultures. (D) Cells were stained for senescence-associated (SA) β-galactosidase activity after passage and counted (>200 cells in triplicate cultures). Data shown are means ± SEM. The effect of BMP2 and genotype was significant (P < 0.01), although there was no interaction by ANOVA.
Discussion

The inverse relationship between proliferation and differentiation in osteoblasts has been carefully documented for many years, although the mechanisms have not been delineated. These observations have lead to the proposition that full expression of the osteoblast phenotype is necessarily associated with terminal cell cycle exit (Stein et al., 1996; Aubin, 1998). In accord with these observations, osteoblasts lacking functional Runx2 appear to lose a growth restraint (Pratap et al., 2003). Our data provide a mechanistic basis for these observations (Fig. 8). Osteogenic differentiation in culture imposes a growth restraint and, eventually, a terminal cell cycle exit resembling senescence, through Runx2-dependent induction of p27KIP1. We and others show that osteogenic differentiation in vitro is associated with increased expression of p27KIP1 (Drissi et al., 1999). This leads to a pRb-dependent growth arrest through inhibition of S-phase cyclin complexes. We have shown previously that interactions between Runx2 and pRb enhance Runx2-dependent transcriptional activity (Thomas et al., 2001). Because it is the hypophosphorylated form of pRb that binds Runx2, the induction of p27KIP1 will enhance the transactivation of Runx2 by pRb, leading to progressive growth arrest and expression of the mature osteoblast phenotype (Fig. 8). It is likely that loss of function of any component of this feed-forward loop will disrupt both differentiation and a restraint on cell growth. Although mutations have been documented in pRb in osteosarcoma, the molecular events that affect Runx2 function in cell lines in which pRb is not affected remain unknown.

We observed a very minor defect in osteoid synthesis in p27KIP1−/− mice, accompanied by defects in BMP2-induced differentiation in vitro, consistent with apparently normal skeletal patterning in mice nullizygous for p27KIP1 (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996; and unpublished data), we found that p27KIP1 was required to maintain a growth-arrested state in differentiated cells. p27KIP1 has been suggested previously to be a part of the normal timer that determines the cessation of proliferation and commitment to differentiation of oligodendrocyte precursors (Casaccia-Bonnefil et al., 1997; Durand et al., 1998). The Drosophila melanogaster p27KIP1 homologue, dacapo, initiates terminal cessation of cell division and differentiation, an effect that interacts genetically with pRb (de Nooij et al., 1996; Lane et al., 1996). These data suggest that p27KIP1 may act as a “fate switch” that, once expressed at sufficient levels, commits the osteoblast to a postmitotic state. Irreversible cell cycle exit is a feature of senescence, in which p27KIP1 plays a role and which may represent a defense to oncogenic transformation (Serrano et al., 1997; Sellers et al., 1997).

The induction of p27KIP1 leads to more than a simple proliferative arrest. Although the proliferation of early passage MEFs was not strikingly affected by loss of p27KIP1 (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996; and unpublished data), we found that p27KIP1 was required to maintain a growth-arrested state in differentiated cells. p27KIP1 has been suggested previously to be a part of the normal timer that determines the cessation of proliferation and commitment to differentiation of oligodendrocyte precursors (Casaccia-Bonnefil et al., 1997; Durand et al., 1998). The Drosophila melanogaster p27KIP1 homologue, dacapo, initiates terminal cessation of cell division and differentiation, an effect that interacts genetically with pRb (de Nooij et al., 1996; Lane et al., 1996). These data suggest that p27KIP1 may act as a “fate switch” that, once expressed at sufficient levels, commits the osteoblast to a postmitotic state. Irreversible cell cycle exit is a feature of senescence, in which p27KIP1 plays a role and which may represent a defense to oncogenic transformation (Serrano et al., 1997; Sellers et al., 1997).

Figure 7. Expression of p27KIP1, osteocalcin, and proliferating cell nuclear antigen (PCNA) in human osteosarcoma samples. [A–I] High-power photomicrographs of parallel sections from two high-grade (A–C and G–I) and one low-grade (G–I) human osteosarcomas were stained for p27KIP1 (A, D, and G), osteocalcin (B, E, and H), and PCNA (C, F, and I). Arrows in D–F indicate multinucleated osteoclasts; arrows in G–I indicate osteocytes. Bar, 50 μm. (J) Blinded quantitation of staining for p27KIP1 and PCNA in tumors with evidence of osteoblast differentiation (osteoid production) compared with dedifferentiated tumors. Error bars represent SEM. *, P < 0.05.

Figure 8. A model for interactions between cell cycle proteins and Runx2 in osteoblasts. The interaction of hypophosphorylated pRb with Runx2 completes a positive feedback loop, promoting cell cycle withdrawal and expression of the osteoblast phenotype. See Discussion section.
et al., 1998; Alexander and Hinds, 2001; Thomas et al., 2001). Clearly, terminal cell cycle exit, whether in response to differentiation or to oncogenic events, is fundamentally inconsistent with oncogenic transformation.

Does p27KIP1 act as a tumor suppressor in bone? In animal models, loss of p27KIP1 is associated with infrequent spontaneous pituitary tumors and intestinal adenomas but accelerates the rate of tumor formation when combined with carcinogen exposure (Fero et al., 1998) or mutations to TP53 (Philipp-Staheli et al., 2004). Interestingly, osteosarcomas were observed in this latter study, albeit at low frequency. Consistent with a heli et al., 2004). Interestingly, osteosarcomas were observed in this latter study, albeit at low frequency. Consistent with a role for p27KIP1 in osteosarcoma, the protooncogene c-Fos, which causes osteosarcoma in mice (Grigoriadis et al., 1993), induces cyclin A–Cdk2 activity and represses p27KIP1 in osteoblasts (Sunters et al., 2004). Unusually, p27KIP1 appears to act as a haploinsufficient tumor suppressor in the mouse (Fero et al., 1998), and, where human tumors have undergone a loss-of-heterozygosity event, silencing of the remaining allele is rare (Kawamata et al., 1995; Ponce-Castaneda et al., 1995). This may be due to the dual role of p27KIP1 as an assembly factor for G1-phase cyclin complexes as well as a stoichiometric inhibitor of S-phase cyclin complexes (Sherr and Roberts, 1999). Importantly, decreases in p27KIP1 protein expression have been found in 60% of human carcinomas (Slingerland and Pagano, 2000), and are associated in breast cancer with poor prognosis (Fredersdorf et al., 1997). Our clinical studies reveal an association between p27KIP1 expression and loss of differentiation in human osteosarcomas, independent of rates of proliferation per se (Fig. 7). Loss of differentiation in sarcomas in general is a marker of high-grade status, which in turn is associated with worse prognosis.

Disruption of runx2-dependent transcriptional activity is common in osteosarcoma cell lines and leads in a clinically measurable fashion to loss of both differentiation and expression of p27KIP1 in human osteosarcomas. Although the specific mechanisms contributing to the loss of function of runx2 are not understood, global demethylation of osteosarcoma cell lines results in reactivation of differentiation concomitant with reversion of transformation (unpublished data). Methylation is a well-described, common method of silencing tumor suppressor pathways (Baylin and Herman, 2000), and the restoration of differentiation by demethylation further supports the notion that tumors gain a selective survival advantage by silencing differentiation-related growth inhibitory processes. We hope that identifying targets of methylation-induced silencing will shed additional light on the interactions between differentiation and cell cycle exit.

Materials and methods

Cell lines and reagents

The osteosarcoma cell lines were maintained in DME (GIBCO BRL) containing 15% FCS. CCL-7625 and CCL-7672 cells were obtained from American Type Culture Collection. MC3T3E1 cells were maintained in MEM supplemented with 10% FCS. R8+/− 3T3 and NIH3T3 cells were infected with pBABEpuro retrovirus (Morgenstern and Land, 1990) expressing mutant constructs of runx2 (Thirunavukkarasu et al., 1998), and with pooled clones selected with puromycin. Stable expression of constructs was confirmed by immunoblot (unpublished data). Wild-type and R8+/− MEFs with the described genotypes (wild-type and R8+/−) were derived from matched littermates (gifts of T. Jaks, Massachusetts Institute of Technology, Boston, MA), and p27KIP1+/− mice were obtained from J. Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA) (Fero et al., 1996). Mineralization was induced by culture in the presence of 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid for 3 wk after confluence (Thomas et al., 2001). For colony suppression assays, cells (107/10 cm plate) were transfected with constructs as indicated in Figs. 2 A and 4 D. After 24 h, cells were selected in the presence of antibiotic (2 μg/ml puromycin or 1–5 μg/ml neomycin) for 14–21 d. Colonies were visualized with crystal violet. Doses of 5-aza-2-deoxycytidine were titrated in preliminary studies for each cell line to achieve growth arrest without significant cell death, usually between 2 and 5 μM.

SV-Rb and SV-HARb were used for the expression of full-length pRb (Hinds et al., 1992). Expression constructs for runx2 were cloned into pBABEpuro (Morgenstern and Land, 1990). The retrovirus was amplified and purified in amphotrophic packaging cell line Phoenix 293 (courtesy of G. Nolan, Stanford University, Palo Alto, CA), according to the method of Pear et al. (1993). Plasmids were transfected into cells with the use of FuGene, according to the manufacturer’s instructions (Roche Pharmaceuticals). Adenoviral constructs expressing pRb and runx2-FLAG were generated as reported previously (Thomas et al., 2001).

Cell-based assays

Luciferase assays were performed according to manufacturer’s instructions (Promega). Where indicated (Fig. 1, B and C), results were normalized for transfection efficiency with β-galactosidase activity or protein content. AP activity was assayed as described previously (Sellers et al., 1998). Assays for mineralization were performed as described previously (Thomas et al., 2001). Quantitation was performed by dissolving stained mineralized cultures in 10% cetylpyridinium chloride, followed by spectrophotometric analysis at 540 nm. Both AP activity and mineralization were normalized to protein content (Bio-Rad Laboratories). Flow cytometry for DNA content was performed as described previously (Thomas et al., 2001).

RT-PCR analysis of gene expression

RNA was extracted with the use of TRIzol (Invitrogen), according to the manufacturer’s instructions. cDNA was produced from 1 μg of total RNA with the use of a commercially available kit (SUPERSCRIPT Choice system for cDNA synthesis; GIBCO BRL). Semiquantitative PCR analysis was performed after optimization. Primer sequences and PCR conditions are available on request. For quantitative RT-PCR, expression of each target gene was normalized to expression of ARPP, with the use of an ABI Prism 7700 Light Cycler and SYBR Green. Optimal PCR conditions were established for each gene in preliminary experiments.

Analysis of protein expression and kinase assays

Nuclear extracts and immunoblot analyses were performed as described previously (Thomas et al., 2001). The following antibodies were used: anti-FLAG antibody (M2; Sigma-Aldrich); human pRB: monoclonal antibody 245 (BD Biosciences); runx2: M-70 (Santa Cruz Biotechnology, Inc.); p27KIP1: K25020 (Transduction Laboratories); and cyclin E: HE12, cyclin A: H432, and Cdk2: M2 (Santa Cruz Biotechnology, Inc.). Horseradish peroxidase–conjugated secondary antibodies were used (Jackson Immunoresearch Laboratories) and signal was detected by ECL (NEF Life Science Products). The GST-runx2 pulldown studies shown in Fig. 4 D were performed as described previously (Thomas et al., 2001). Kinase assays were performed as described previously (Alexander and Hinds, 2001). Cyclin A was immunoprecipitated using agarose-conjugated antibody 6F683 (Upstate Biotechnology).

Immunohistochemistry

25 paraffin-embedded osteosarcoma samples were obtained from the pathology archives at St. Vincent’s Hospital Melbourne, with approval from the Human Research Ethics Committee. 2-mm cores were punched and then assembled into a tissue microarray. Sections were cut at 3 μm and mounted onto Superfrost Plus slides. Primary antibodies were incubated for 30 min. For p27KIP1, clone SK5308 (DakoCytomation) was used at 1:50. A predilute monoclonal antibody to human osteocalcin (OC-1; Biogenex) was used at 1:4. For PCNA, clone PC10 (DakoCytomation) was used at 1:500. The primary antibody was detected with the mouse Envision+ system (DakoCytomation). Immunoreactivity was visualized with AEC+ chromogen (DakoCytomation), using hematoxylin as a counterstain. Samples for p27KIP1 and osteocalcin were incubated in 10 mM boiling sodium citrate buffer, pH 6.0, for 2 min before staining.
Slides were imaged using a microscope (Axioskop 2; Carl Zeiss MicroImaging, Inc.) with a Plan-Neofluar objective (40×/0.75 NA), and a cooled color digital camera (RT Slider SPOT; Diagnostic Instruments) and software (SPOT V4.0.2 for Windows). Subsequent processing of TIFF files was undertaken in Adobe Photoshop (V7.0.1). Images were cropped, labeled as indicated in Fig. 7, and assembled into composites for high-quality paper requirement adjustments for contrast and color balance were applied to all parts of each image.

Microarray analysis
Total RNA from osteosarcoma and common reference cell lines was isolated using phenol-chloroform extraction (TRizol; Invitrogen) and purified by column chromatography (RNeasy; QIAGEN). The common reference RNA, containing pooled RNA from 11 human tumor cell lines, was prepared as described previously (Pollack, 2002). Total RNA (40–50 μg) was reverse transcribed with Moloney Murine Leukemia Virus Reverse Transcriptase (Promega), in the presence of amino-allyl (AA)–modified dUTP (Sigma-Aldrich). AA-dUTP cDNA was labeled by coupling to Cy3 and Cy5 (reference and sample, respectively) monoaxerous dyes (Amersham Biosciences). cDNA arrays containing ~10,5 K Elements representing 9,381 unique cDNA (Unigene build 172) were produced at Peter MacCallum Cancer Centre Microarray Core facility on superamine slides (Telechem), with a robotic arrayer (Virtek/Bio-Rad Laboratories). Labeled probe was hybridized to the array in 3.1 × SSC and 50% formamide at 42°C for 1–4 h in a humidified and temperature-controlled chamber (Hybaid). Slides were washed at room temperature with 0.5 × SSC/0.1% SDS for 1 min, then with 0.5 × SSC for 3 min, and finally with 0.06 × SSC for 3 min. Scanning was performed with an Agilent G2565AA Microarray Scanner and data was extracted with GenePix Pro 4.1 software (Axon Instruments, Inc.). All array experiments, including cell culture, were performed independently twice. A complete list of genes is available from the authors on request. Data from each independent experiment were averaged, and then the median obtained from all six cell lines was used in the data shown in Fig. 1. Data were analyzed with GeneSpring software (Silicon Genetics), and samples were normalized with LOWESS (Yang et al., 2002).

Histomorphometry
Tibiae were collected from male p27Kip1−/− and wild-type littermates at 16 wk of age, fixed in cold 4% paraformaldehyde in PBS overnight, and embedded in methylmethacrylate (Sims et al., 2000). Double fluorochrome labeling to quantity mineral appositional rates was performed as described previously (Sims et al., 2000). S-μm sections were stained with toluidine blue or analyzed unstained for fluorochrome labels according to standard procedures in the proximal tibia using the Osteomeasure system (Osteometrics, Inc.). Tibial cortical thickness and periosteal mineral appositional rates were measured as described previously (Sims et al., 2000).

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