Osteoblast differentiation and skeletal development are regulated by Mdm2–p53 signaling

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Mdm2 is required to negatively regulate p53 activity at the peri-implantation stage of early mouse development. However, the absolute requirement for Mdm2 throughout embryogenesis and in organogenesis is unknown. To explore Mdm2–p53 signaling in osteogenesis, Mdm2-conditional mice were bred with Col3.6-Cre–transgenic mice that express Cre recombinase in osteoblast lineage cells. Mdm2-conditional Col3.6-Cre mice die at birth and display multiple skeletal defects. Osteoblast progenitor cells deleted for Mdm2 have elevated p53 activity, reduced proliferation, reduced levels of the master osteoblast transcriptional regulator Runx2, and reduced differentiation. In contrast, p53-null osteoprogenitor cells have increased proliferation, increased expression of Runx2, increased osteoblast maturation, and increased tumorigenic potential, as mice specifically deleted for p53 in osteoblasts develop osteosarcomas. These results demonstrate that p53 plays a critical role in bone organogenesis and homeostasis by negatively regulating bone development and growth and by suppressing bone neoplasia and that Mdm2-mediated inhibition of p53 function is a prerequisite for Runx2 activation, osteoblast differentiation, and proper skeletal formation.

Introduction

The p53 transcription factor is activated by inappropriate cell growth stimulation or by certain types of DNA damage and regulates the expression of other genes involved in cell growth arrest, DNA repair, and apoptosis (Vousden, 2000). These various p53-mediated effects suppress tumorigenesis, and mutation of the p53 gene or of the p53 signaling pathway is commonly found in most human cancers (Soussi and Beroud, 2001). Although the ability of p53 to regulate cell growth after exposure to stress has been well established, the role of p53 in regulating normal (nondamaged) cell growth and in tissue homeostasis is uncertain. Mice deleted for p53 will form tumors with 100% penetrance yet undergo normal development (Donehower et al., 1992), albeit with a reduction from the expected numbers of female births and a small percentage of embryos presenting at midgestation with exencephaly (Armstrong et al., 1995; Sah et al., 1995). In addition, transgenic mice bearing a reporter gene under transcriptional control of a p53 response element reveal little or no postnatal p53 activity in the absence of DNA damage (Gottlieb et al., 1997). These findings suggest that p53 is important in suppressing tumorigenesis but is largely dispensable for normal cell growth, cell differentiation, and development.

In contrast, a recently generated mouse model bearing a mutated p53 allele (m allele) that increased p53 activity in vitro displayed early aging-like phenotypes, including reduced mass of various internal organs, thinning of the dermis, hair loss, and osteoporosis (Tyner et al., 2002). Interestingly, the p53 m/+ mice also had reduced incidence of cancer relative to p53 heterozygous (−/+ ) mice, suggesting that the mutant allele increased p53 activity encoded by the wild-type p53 gene in the m/+ mice. Although this increase in basal levels of p53 activity offered further protection from neoplasia, the authors hypothesized that the slight increase in p53 activity also reduced stem cell proliferation in affected tissues leading to reduced tissue cellularity. These results suggest that negative regulation of p53-induced apoptosis or inhibition of cell growth might be important to maintain proper tissue homeostasis in adult mice.
Mdm2 is a key negative regulator of p53 activity in the cell. Mdm2 complexes with p53 and negatively regulates p53-induced transcription of target genes, including the Mdm2 gene (for review see Iwakuma and Lozano, 2003). During times of cellular insult, p53 activates Mdm2 gene expression by binding to a p53 response element within the first intron of the Mdm2 gene (Juven et al., 1993). Induction of Mdm2 protein levels interferes with the ability of p53 to transactivate Mdm2. Thus, gene (Juven et al., 1993). Induction of Mdm2 protein levels interferes with the ability of p53 to transactivate Mdm2. Increased Mdm2–p53 complex formation leads to an increase in Mdm2 expression because of the ability of Mdm2 to negatively regulate p53 (Wu et al., 1993). Mdm2 has an autoregulatory loop because of the ability of Mdm2 to negatively regulate p53 (Wu et al., 1993). Mdm2 has a positive feedback loop because of the ability of Mdm2 to negatively regulate p53 (Wu et al., 1993). Mdm2 has been shown to interfere with the ability of p53 to transactivate target genes by binding and sterically hindering the NH2-terminal activation domain of the p53 protein (Momand et al., 1992; Chen et al., 1995) or by altering p53 protein modifications that regulate p53 transcriptional activation (Xirodimas et al., 2004). In addition, Mdm2 can function as an E3 ligase to coordinate the ubiquitination of p53 (Honda et al., 1997) and can induce the degradation of p53 by the 26S proteasome (Haupt et al., 1997; Kubbatau et al., 1997; Li et al., 2003). Mdm2 can also assist in shuttling p53 from the nucleus into the cytoplasm (Freedman and Levine, 1998; Geyer et al., 2000). The importance of Mdm2 in negatively regulating p53 activity is perhaps best illustrated by the finding that the early (embryonic day [E] 4–5) lethal phenotype of Mdm2-null mice can be fully rescued by the concomitant deletion of p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995).

Although the requirement for Mdm2-mediated inhibition of p53 activity during early development has been well established, the role of Mdm2 in regulating p53 functions in later stages of embryogenesis or in adult tissues is unclear. However, several lines of evidence suggest that Mdm2 does function to regulate p53 activity in postnatal tissue. EuMyc transgenic mice display a delayed onset of B cell lymphoma when haploinsufficient for Mdm2, suggesting that a reduction in Mdm2-mediated suppression of p53 can reduce tumorigenesis (Alt et al., 2003). In addition, mice bearing a hypomorphic allele of Mdm2 that have ~30% of the normal endogenous levels of Mdm2 are smaller in size, have reduced numbers of hematopoietic cells, and display excess apoptosis in the lymphoid compartment (Mendrysa et al., 2003). Crossing the Mdm2 hypomorphic allele onto a p53-deficient background reversed the various phenotypes observed in these mice, demonstrating that the phenotypic effects caused by Mdm2 reduction in this model were induced by p53. These data suggest that Mdm2 is capable of negatively regulating p53 activity in hematopoietic tissues.

To determine the absolute requirement for Mdm2 during development and in adult tissues, we have recently used Cre-loxP technology to generate Mdm2-conditional mice. Gene targeting experiments in embryonic stem cells flanked the last two exons of the Mdm2 gene encoding the zinc RING (really interesting new gene) finger domains and polyadenylation signals with loxP sites. Cre-mediated recombination of the loxP sites in the conditional allele destabilizes Mdm2 transcripts and results in loss of Mdm2 message. (Steinman and Jones, 2002). Because studies of p53 mutant mice suggest that excess p53 activity might have a deleterious effect on normal bone homeostasis, we sought to determine whether Mdm2 regulates p53 activity during osteogenesis. To this end, Mdm2- or p53-conditional mice were bred with transgenic mice in which the Cre-recombinase gene has been placed under transcriptional control of a 3.6-kb fragment of the Coll1a1 promoter. These Col3.6-Cre–transgenic mice have been previously reported to express Cre in cells of the osteoblast lineage (Liu et al., 2004). Mdm2-conditional mice bearing the Col3.6 transgene have multiple skeletal defects, including fused or otherwise altered lumbar vertebrae, reduced mineralized bone, and reduced bone length. Osteoblasts deleted for Mdm2 do not undergo apoptosis but do have elevated p53 activity, increased transactivation of p53 target genes, reduced cell proliferation, and reduced levels of the osteoblast transcriptional regulator Runx2, which is essential for osteoblast differentiation (Banerjee et al., 1997; Ducy et al., 1997; Komori et al., 1997). In contrast, osteoblasts deleted for p53 display elevated Runx2 levels, enhanced cell proliferation, and...
increased maturation and mineralization. Furthermore, mice specifically deleted for p53 in osteoblast progenitor cells develop osteosarcomas. These results demonstrate that p53 is an important negative regulator of osteogenesis and that Mdm2-mediated inhibition of p53 function is a critical requirement for Runx2 activation and proper osteoblast differentiation and skeletal formation.

Results

Expression of Cre recombinase in Col3.6-Cre-transgenic mice

To understand the contribution of Mdm2 and p53 signaling to bone development, we used Mdm2-conditional mice bearing Mdm2 genes (Mdm2j101) with loxP recombination sites flanking exons 11 and 12 (Steinman and Jones, 2002). Deletion of these exons will result in loss of Mdm2 in cells expressing the P1 bacteriophage Cre-recombinase transgene under transcriptional control of a rat 3.6-kb type I collagen promoter fragment (Liu et al., 2004). This transgene has been reported to induce Cre-mediated gene excision in postnatal calvarial bone and long bone osteoblasts, as well as in skin and tendons. We initially verified the expression of Cre in skeletal elements of adult mice. RT-PCR reactions using RNA isolated from the rib and femur of adult Col3.6-Cre mice demonstrated Cre expression in these tissues, whereas Cre transcripts were not detected in other tested organs (Fig. 1 A). To identify regions in Col3.6-Cre developing embryos that express Cre and induce Cre-mediated excision, we mated the Col3.6-Cre–transgenic mice to ROSA26 (R26R) reporter mice that express β-galactosidase upon Cre-induced deletion of a floxed cassette that inhibits the reporter gene expression (Soriano, 1999). Our findings demonstrate that this promoter becomes activated at two distinct points during development. Expression of the Col3.6-Cre transgene as determined by β-galactosidase activity is first detected in whole-mount stainings of E8–19 embryos and is localized initially to the caudal portion of the embryo. Reporter gene expression is strongest in this region at E10 (Fig. 1 B). This region of the developing embryo contains both neural and mesenchymal progenitor cells that give rise to the caudal axial skeleton and spinal cord. Later in development (~E14), the Col3.6-Cre transgene undergoes robust activation in relation to the formation of connective tissue. Staining of sagital sections of R26R/Col3.6-Cre embryos for β-galactosidase activity indicates that Cre-mediated excision occurs in both the skin and developing skeletal elements (Fig. 1 C). These findings indicate that Cre transgene expression will induce Mdm2 deletion in Col3.6-Cre × Mdm2-conditional mice in the surface ectoderm and developing tail bud of E9 mice as well as in developing bone and connective tissue of these mice during midgestation.
Caudal defects in Mdm2-conditional Col3.6-Cre mice

We crossed Mdm2-conditional mice (Mdm2<sup>sjcnd1</sup>+/ or Mdm2<sup>sjcnd1/sjcnd1</sup>) to Col3.6-Cre–transgenic mice containing one conditional Mdm2 allele (Mdm2<sup>sjcnd1</sup>+/Col3.6-Cre). The resulting litters contained Mdm2<sup>sjcnd1/sjcnd1</sup>,Col3.6-Cre mice (designated as mutant), as well as Mdm2<sup>+</sup>/+,Col3.6-Cre mice and Mdm2<sup>sjcnd1/+</sup>,Col3.6-Cre mice. These last two groups of mice were indistinguishable from wild-type littermates. Skeletal preparations of wild-type neonatal mice and neonates deleted for both Mdm2 and p53 revealed no vertebral dysplasia (E) or reduction in bone length (F), indicating that the deleterious effects of Mdm2 loss on skeletal formation during development are p53 dependent.

Defects in the developing tail bud region of mutant mice were apparent as early as E10.5, and caudal runting was seen in all mutant mice throughout later stages of development (Fig. 2, A–C). Col3.6-Cre–induced deletion of Mdm2 expression in the caudal region of mutant mice resulted in a complete absence of tissue surrounding the somites at E10.5, as well as exposure of somitic mesenchyme at the surface of the embryo, absence of a tail, and a severe invagination in the posterior dorsal region encompassing the lumbar vertebrae (Fig. 2 D). Histologic analysis of mutant and wild-type embryos harvested at E10 was performed. Wild-type embryos had clearly segmented somites that will give rise to axial skeletal components, including vertebrae and ribs (Fig. 2 E, arrows). These somites are surrounded by primitive neural tissue and by developing dorsal root ganglia (Fig. 2 E, arrowheads). In contrast, mutant embryos lack developing neural tissue and posterior dorsal root ganglia, leaving caudal somites externalized (Fig. 2 F). To understand the mechanism underlying this tissue loss, we performed TUNEL staining on serial sections. Our results show that there is a dramatic increase in TUNEL-positive apoptotic cells in the caudal somites and surrounding tissue of mutant E10 embryos in comparison to wild-type littermates (Fig. 2 G).
Excision of Mdm2 in skeletal tissues results in impaired bone formation

Skeletal preparations of wild-type and Mdm2sjcnd1/sjcnd1,Col3.6-Cre embryos revealed that the apoptosis observed in the E10 caudal somatic cells resulted in highly dysplastic axial skeletal elements in the mutant embryo with fused cartilaginous lumbar vertebrae (Fig. 3, A and B). However, the appendicular skeleton and bones of the skull were unaffected by the initial Cre activation. Activation of Col3.6-Cre transgene expression in the limbs and skull did not occur until the latter half of gestation (beginning at E14.5). These skeletal elements were more porous in mutant animals (Fig. 3 C), and measurements of the long bones (tibia, radius, ulna, humerus, and femur) revealed that the total long bone length was $12.1 \pm 3.9\%$ shorter in the mutant embryos and the length of mineralized portion of long bones was $12.3 \pm 4.4\%$ shorter in the mutant embryos than in wild-type littermate embryos at E17.5 (Fig. 3 D). In contrast, skeletal preparations of embryos deleted for both Mdm2 and p53 (Jones et al., 1995) revealed no vertebral dysplasia or reduction in bone length (Fig. 3, E and F). These results reveal that the deleterious
effects of Mdm2 loss on skeletal formation during development are p53 dependent.

Appendicular and skull bones from E18.5 Mdm2<sup>y<sup>n</sup>nd1/y<sup>n</sup>nd1</sup>, Col3.6-Cre mice showed significantly less mineral deposition than wild-type littermates, as assessed by microcomputed tomography (micro-CT) scan (Fig. 4, A–C) and by silver nitrate (Von Kossa) staining (Figs. 4, D and E). Decreased bone mineralization was observed in the skull (Fig. 4 A), femur, and vertebra (Fig. 4 B and C) of Mdm2 mutant mice. Histomorphometric analysis of micro-CT scans performed on litter-matched, E18.5 embryos revealed reduced bone density in the calvaria, femur, and vertebra of Mdm2 mutant mice (Table II). However, these defects were not observed in embryos lacking both Mdm2 and p53. These results demonstrate that loss of Mdm2 in developing skeletal tissues negatively affects multiple parameters of bone quality in a p53-dependent manner. Furthermore, Von Kossa and toluidine blue staining of femur and vertebra sections (Fig. 4, D and E) revealed that the vascularized marrow cavity and other normal compartments of bone are present in the mutant mice and that Mdm2 mutant bone has a morphologically normal growth plate. These data suggest that Mdm2 mutant mice do not have a chondrogenic defect but rather a defect in ossification. However, unlike the pro-apoptotic effects of Mdm2 deletion in undifferentiated progenitor cells in E10 mutant mice, TUNEL assays performed on E14.5 skeletal rib elements from mutant embryos revealed no increase in the number of apoptotic cells (unpublished data).

Table II. Morphometric analysis and quantitative analysis of bones

| Genotype         | Skull width | Skull length | L3-5 spine | Calvaria | Femur | Vertebra |
|------------------|-------------|--------------|------------|----------|-------|----------|
| Wild type        | 8.1         | 10.1         | 2.2        | 93.0     | 28.4  | 51.7     |
| Mutant           | 5.2         | 7.7          | 1.3        | 31.5     | 12.7  | BT       |
| p53 null         | 7.8         | 10.3         | 2.2        | 88.0     | 16.4  | 40.8     |
| Mdm2/p53 null    | 8.0         | 9.9          | 2.4        | 90.3     | 18.1  | 48.4     |

Data obtained from representative (E18.5) littermate embryos. Measurements are given in millimeters. Quantitative analysis is expressed as percentage of bone density as determined by the amount of bone per tissue volume (bone volume over total volume). Below threshold (BT) means bone volume was below detectable levels.

To better understand the underlying cause of the decrease in bone quality in Mdm2-conditional Col3.6-Cre mice, calvarial osteoprogenitor cell cultures were analyzed. Examination R26R/Col3.6-Cre osteoprogenitor cells reveals that few osteoprogenitor cells had activated the transgene before isolation from the embryo and that Cre expression is induced in the osteoblast cultures during differentiation (Fig. 5 A). Because Cre-mediated recombination occurs in these cultures in maturing osteogenic nodules after reaching confluence, Mdm2 expression in Mdm2-conditional Col3.6-Cre cultures should be lost in multilayering nodules of maturing osteoblasts.

Toluidine blue staining of calvarial osteoprogenitor cells cultured from wild-type and Mdm2-conditional Col3.6-Cre mice revealed that both wild-type and Mdm2 mutant–derived osteoprogenitor cultures achieved confluence simultaneously (Fig. 5, B and C, left), and BrdU incorporation indicated no difference in the rates of cell proliferation in these cultures before confluence (not depicted). However, upon reaching confluence and upon robust induction of Cre expression, wild-type osteoprogenitor cells cultured from wild-type and Mdm2-conditional Col3.6-Cre cultures were unable to form a significant number of multilayered nodules. Subsequently, only a small fraction of the mutant cells were able to undergo osteoblast differentiation as reflected by alkaline phosphatase activity (Fig. 5 C). Consistent with our in vivo findings of reduced mineralization in Mdm2 mutant bone, wild-type osteoblasts ultimately formed heavily mineralized nodules in culture, whereas mutant cells failed to deposit significant mineral in the extracellular matrix (Fig. 5, B and C) as determined by silver nitrate staining (right).

Quantitative analysis by real-time PCR of osteogenic gene expression revealed that both wild-type and mutant cultures began to activate early osteogenic genes type I collagen and alkaline phosphatase upon reaching confluence. However, osteogenic gene expression was abrogated in Mdm2 mutant cultures shortly after achieving confluence (Fig. 5 D). Real-time PCR analysis revealed that Mdm2 and Runx2 are expressed at low levels in wild-type cultures during the period of proliferation but are strongly activated in postconfluent cultures during multilayering and differentiation. However, as expected, Mdm2 up-regulation is abrogated in osteoprogenitor cultures derived from Mdm2-conditional Col3.6-Cre mice during the course of differentiation (Fig. 5 E). Interestingly, both Runx2 protein and message levels were lost in mutant cultures concomitantly with the loss of Mdm2 expression (Fig. 5, E and F). Furthermore, although the G1/S ratio of preconfluent osteoprogenitor cells

Negative regulation of p53 by Mdm2 is required for proper osteoblast differentiation

To better understand the underlying cause of the decrease in bone quality in Mdm2-conditional Col3.6-Cre mice, calvarial osteoprogenitor cells were isolated from E19 wild-type and mutant embryos as well as from R26R/Col3.6-Cre embryos to visualize the pattern of Cre-mediated excision in these cultures. Calvarial osteoprogenitors were induced to undergo osteogenic differentiation ex vivo by allowing cultures to proliferate for several days followed by the addition of ascorbic acid and inorganic phosphate to the media postconfluence. The addition of ascorbic acid to the cultured osteoprogenitors stimulates postconfluent proliferation, resulting in the formation multilayered nodules that later become mineralized (Owen et al., 1990). Examination R26R/Col3.6-Cre osteoprogenitor cells reveals that few osteoprogenitor cells had activated the transgene before isolation from the embryo and that Cre expression is induced in the osteoblast cultures during differentiation (Fig. 5 A). Because
was unchanged in Mdm2-conditional Col3.6-Cre cells (before induction of Cre expression), postconfluent cycling of mutant cells deleted for Mdm2 was strongly inhibited as determined by BrdU uptake assays, with a G1/S ratio of 7.5:4.2 for nonmutant cells. In addition, no differences in apoptotic cell numbers were detected by TUNEL assays during differentiation of cultured osteoblast progenitor cells after Cre-mediated deletion of Mdm2 (unpublished data). Collectively, our results indicate that Mdm2 activity is required for postconfluent cell proliferation and nodule formation in osteoblast cultures and the subsequent activation of the master osteoblast transcriptional regulator Runx2. Failure of cultures lacking Mdm2 to activate the Runx2 gene ultimately results in inhibition of osteoblast differentiation and inactivity of osteoblast phenotypic genes. To confirm that reduced Runx2 expression in the Mdm2 mutant cells is the underlying cause of the maturation defect, recombinant adenovirus vectors were generated to transduce either lacZ (control) or Runx2 cDNA into the osteoblast progenitor cultures. Addition of exogenous Runx2 into Mdm2 mutant cells induced maturation of these progenitor cells and partially or fully restored the expression of mature osteogenic genes such as collagen type 1, alkaline phosphatase, and osteocalcin (Fig. 5 G).

Examination of p53 levels in ex vivo osteoblast cultures revealed no change in total p53 protein levels in cultures undergoing deletion of Mdm2 (Fig. 6 A). However, a difference in the amount of activated p53 transcription factor present in the cultures was detected using a phospho-Ser15 specific antibody, with a marked induction in P-Ser15 p53 levels observed in mutant cultures relative to levels in wild-type cells (Fig. 6 B), suggesting that Mdm2 negatively regulates p53 activity but not overall p53 protein levels in differentiating osteoblasts. Furthermore, real-time PCR analysis revealed up-regulation of Ptprv and p21 gene expression (Fig. 6 C and D) in postconfluent osteoblasts. Collectively, our results indicate that Mdm2 regulates p53 activity but not overall p53 protein levels in differentiating osteoblasts.

Figure 5. Mdm2 is required for proper osteoblast differentiation. Calvarial osteo-


generators were isolated and cultured ex vivo from E19 Col3.6-Cre-transgenic em-

bryos bearing the R26R reporter gene, from E19 wild-type and Mdm2-conditional Col3.6-

Cre-transgenic embryos. Calvarial osteo-


generators were induced to undergo osteogenic differentiation ex vivo, resulting in proliferation (toluidine blue staining for total cell number), the formation of multilayered nodules and activation of alkaline phosphatase, and min-

eralization as detected by silver nitrate staining. (A) Examination R26R/Col3.6-Cre osteopro-

genitor cells stained for β-galactosi-


dase activity reveals that Mdm2 expression in Mdm2-conditional Col3.6-Cre cultures will be lost in multilayering nodules of maturing osteoblasts. (B) Upon reaching confluence, wild-
type osteogenitor cell cultures underwent robust nodule formation and mineralization. (C) Mdm2-conditional Col3.6-Cre-transgenic cultures were unable to form a significant num-

ber of multilayered nodules, and subsequently only a small fraction of these cells were able to undergo differentiation, activate alkaline phosphatase activity, or induce mineraliza-


tion. (D) Quantitative analysis by real-time PCR of the expression of early osteogenic genes type I collagen (Coll) and alkaline phosphatase (AP) and of osteocalcin (OC) at vari-


ous stages of osteoblast maturation. Solid lines depict the relative transcript levels of genes in wild-type cell cultures, and dashed lines represent the levels of expression of the various genes in Mdm2-conditional Col3.6-Cre cell cultures. Expression levels of early- and late osteogenic genes are reduced in Mdm2-conditional Col3.6-Cre-transgenic cells. (E) Real-time PCR analysis of Mdm2 and Runx2 expression in wild-type (WT) and Mdm2-conditional Col3.6-Cre-transgenic (MT) culture osteoblast progenitor cells during maturation. Solid and dashed lines depict the relative transcript levels of Mdm2 and Runx2 in wild-type or Mdm2-conditional Col3.6-Cre-transgenic cultures, respectively. As expected, Mdm2 levels do not increase in Mdm2-conditional Col3.6-Cre-transgenic cells during maturation. Similar induction of Runx2 and Mdm2 expression is observed in wild-type cells during maturation. (F) Western blot analysis of Runx2 protein levels in wild-type or Mdm2-conditional Col3.6-Cre-transgenic culture osteoblast progenitor cells during maturation and mineralization. Lamin-b is shown as a loading control. Decreased amounts of Runx2 protein are observed in Mdm2-conditional Col3.6-Cre-transgenic cells. (G) Quantitative PCR was performed on reverse-transcribed RNA isolated from osteoblast progenitor cells transduced with Runx2 or lacZ (negative control). Exogenous Runx2 up-regulated the expression of osteogenic maturation genes in Mdm2 mutant cells, including collagen 1, alkaline phosphatase, and osteocalcin. Error bars indicate SD.
mice (dashed lines) relative to expression levels observed in Col3.6-Cre cells containing wild-type Mdm2 alleles (solid lines). These genes are targets of p53 transactivation known to be involved in regulating the progression of primary cells from G1 into S phase of the cell cycle (Deng et al., 1995; Doumont et al., 2005). This increase in Ptprv and p21 expression is consistent with the inhibition of postconfluent osteoprogenitor cell growth observed in the Mdm2-conditional Col3.6-Cre cultures. Interestingly, the expression levels of Bax and Puma, two pro-apoptotic p53 response genes, were not significantly altered in osteoblast progenitor cells after deletion of Mdm2 (unpublished data), consistent with the unaltered level of apoptosis in Mdm2 mutant and wild-type cells.

### Discussion

The rescue of Mdm2-null mice from peri-implantation lethality by deletion of p53 demonstrates that Mdm2 plays an important role in early development by negatively regulating p53 activity (Jones et al., 1995; Montes de Oca Luna et al., 1995).
but little is known about the requirement for Mdm2 throughout embryogenesis and in postembryonic tissues. We previously documented that the growth characteristics of p53-null primary fibroblasts and the tumorigenic potential of p53-null mice are indistinguishable in the presence or absence of Mdm2 (Jones et al., 1996). Thus, if Mdm2 was important in the latter stages of development, in organogenesis, or in postnatal cell growth control, it is likely due to the ability of Mdm2 to down-regulate p53 activity and not to p53-independent effects of Mdm2. In support of a role for Mdm2 in regulating p53 during the latter stages of development, recent studies of mice that contain reduced amounts of Mdm2 relative to wild-type levels indicate that Mdm2 regulates p53 activity in hematopoietic development and in B cell tumorigenesis (Alt et al., 2003; Mendrysa et al., 2003). However, the effect of complete ablation of Mdm2 activity on cellular differentiation and in organogenesis is unknown.

Figure 7. Regulation of osteoblast progenitor differentiation and osteosarcoma formation by p53. Calvarial osteoprogenitor cells were cultured ex vivo from E19 wild-type (WT) and p53-null embryos. (A) Calvarial osteoprogenitors were induced to undergo osteogenic differentiation, resulting in proliferation (toluidine blue), maturation and the formation multilayered nodules and activation of alkaline phosphatase, and mineralization as detected by silver nitrate staining. Osteoprogenitor cells deleted for p53 displayed far more proliferation than wild-type cells cultured at the same initial density, increased alkaline phosphatase, and increased mineralization. (B) Proliferation was documented by BrdU uptake in postconfluent cultures of wild-type and p53-null progenitor cells just before cell maturation. The percentage of cells in each phase of the cell cycle was determined by FACs analysis after propidium iodide staining of the harvested cells. Postconfluent p53-null cells had more cells in S phase than wild-type cells. (C) Real-time PCR analysis of Runx2 expression in wild-type and p53-null cultures of osteoblast progenitor cells during maturation. Runx2 transcript levels are strongly up-regulated in p53-null cells during maturation. (D) No difference was observed in the robust osteoprogenitor cell differentiation in p53-null cells that contained (top) or lacked (bottom) Mdm2. (E) Tumorigenesis in mice deleted for p53 in osteoblasts. Col3.6-Cre-transgenic mice heterozygous for a conditional p53 allele [p53-cond/wild type] or homozygous for the p53-conditional allele (p53-cond/cond) were assayed for spontaneous tumor development. A majority of the mice presented with osteosarcomas, with a mean time to tumorigenesis of 40 wk for Col3.6-Cre, p53-cond/cond mice and 57 wk for Col3.6-Cre, p53-cond/wild-type mice. (F) Hematoxylin and eosin stains of osteosarcomas harvested from Col3.6-Cre, p53-cond/cond mice. Samples PT2, -50 (showing invasion into the liver), and -38 are all more differentiated than sample PT58, which displays a spindle-like morphology in addition to some osteoid cells. (G) Analysis of Runx2 protein levels in representative primary tumor samples from p53-conditional Col3.6-Cre mice. Runx2 levels were readily detected in representative osteosarcomas (lanes 1–3: samples PT2, -38, and -58, respectively) but reduced in fibrosarcoma (lane 4), lymphoma (lane 5), or hemangiosarcoma (lane 6) samples.
little is known regarding the role of p53 in this process, previous studies of mice bearing a hypermorphic p53 mutation revealed that mice with increased amounts of p53 activity exhibit symptoms of rapid aging, including osteoporosis (Tynery et al., 2002). Furthermore, a subset of mice haploinsufficient for functional p53 in all tissues develop osteosarcomas (Harvey et al., 1993b). These data suggest that p53 may regulate normal bone growth and that alterations in the levels of p53 activity can contribute to abnormal bone phenotypes.

To further explore a role for p53 in osteogenesis and to determine whether Mdm2–p53 signaling is important in bone growth and development, we bred Mdm2-conditional mice with Col3.6-Cre–transgenic mice. Deletion of Mdm2 upon expression of the Col3.6-Cre transgene resulted in midgestational caudal defects, including loss of tissue surrounding the somites, exposure of somitic mesenchyme at the surface of the embryo, absence of a tail, and a severe caudal invagination. Furthermore, TUNEL staining of serial sections of mutant embryos revealed increased apoptosis in the caudal somites and surrounding tissue of mutant embryos, suggesting that these defects arose through unregulated p53 apoptosis. Harvests of embryos from timed matings of Mdm2-conditional Col3.6-Cre–transgenic mice revealed a marked decrease in the recovery of Mdm2+/−/+;Col3.6-Cre embryos at E13.5, coincident with the robust activation of the Cre transgene in developing skeletal elements. Skeletal preparations of wild-type and of Mdm2+/−/−;Col3.6-Cre embryos documented numerous skeletal defects during the latter stages of development, including a reduction in mineralized bone and in length of appendicular bone, abnormal bone architecture, and an increase in bone porosity. However, analysis of skeletal preparations of embryos deleted for both Mdm2 and p53 revealed no skeletal defects, and no difference was observed in the growth and maturation of cultured calvarial cells deficient for p53 or for both Mdm2 and p53, indicating that the effects of Mdm2 loss on skeletal formation and osteoblast maturation are p53 dependent. In contrast to what was observed in the caudal mesoderm of E10 mutant embryos, TUNEL assays performed on E14.5 bone isolated from Mdm2-conditional Col3.6-Cre embryos and on osteoprogenitor cells cultured from E19 Mdm2-conditional Col3.6-Cre embryos revealed no increase in the number of apoptotic cells. This finding indicates that deletion of Mdm2 does not induce p53-mediated apoptosis in these cells but rather induces p53-mediated effects that block osteoblast proliferation or differentiation. To confirm that p53 plays a role in regulating osteoblast differentiation, we harvested osteoblast progenitor cells from the calvarial of p53-null mice just before birth (E19). Analysis of the growth and development of these cultured cells revealed that p53-null osteoprogenitor cells proliferated far faster than wild-type progenitor cells and underwent more robust differentiation, confirming that p53 functions to negatively regulate osteoblast maturation and mineralization.

Surprisingly, the overall level of p53 protein did not change in osteoblast cells during differentiation in the presence or absence of Mdm2; however, deletion of Mdm2 did result in an increase in the level of activated p53 as judged by the increased levels of phosphorylated p53. Furthermore, the message levels of Protr and the cyclin-dependent kinase inhibitor p21, two p53 target genes involved in regulating cell cycle progression from G1 to S phase, were increased in osteoblast cells after Mdm2 deletion. These results indicate that Mdm2 regulates p53 activity during osteoblast differentiation not by altering p53 stability but by inhibiting p53-mediated transactivation of genes involved in regulating osteoblast growth and differentiation.

Runx2 is a critical inducer of osteoblast differentiation in vitro and in vivo (Stein et al., 2004). Interestingly, levels of Runx2 message and protein were reduced in cells deleted for Mdm2, as were the message levels of Runx2 target genes type I collagen and alkaline phosphatase. In addition, expression of osteocalcin, a marker of late osteoblast differentiation, was not activated in cultures deleted for Mdm2 during differentiation, providing further molecular evidence for a block in osteoblast development upon deletion of Mdm2. In contrast to the reduction in Runx2 levels observed in osteoblasts deleted for Mdm2, Runx2 message levels were found to be greatly elevated in maturing osteoblasts deleted for p53. As Runx2 is a well-established master regulator of osteoblast differentiation, it is possible that p53 directly controls osteoblast maturation by negatively regulating Runx2 expression. However, analysis of the Runx2 promoter sequences failed to identify any p53 canonical binding sites, and there is no evidence present in the literature to suggest that Runx2 expression is directly regulated by p53. Therefore, we hypothesize that proper osteoblast differentiation and bone development require Mdm2 to inhibit a p53-mediated block on osteoprogenitor cell division. By permitting the postconfluent proliferation of osteoblasts through the down-regulation of p53 activity, Mdm2 indirectly facilitates Runx2 induction and osteoblast maturation. In support of this hypothesis, expression levels of mature osteogenic genes were found to be elevated in Mdm2 mutant osteoblasts after restoration of Runx2 expression.

Our results indicate that up-regulation of p53 activity due to Mdm2 deletion induces a block in bone differentiation and mineralization and causes profound skeletal defects in the developing embryo. Furthermore, deletion of p53 in osteoblasts induces hyperproliferation, greatly elevated levels of Runx2 expression, and increased bone maturation in vitro. These findings indicate that p53 is an important negative regulator of bone growth and development. Interestingly, loss of cell differentiation and reduced expression of mature osteogenic genes such as Osteocalcin are prognostic indicators in human osteosarcomas, with poorly differentiated or dedifferentiated tumors usually associated with the high-grade category (Hopyan et al., 1999). In addition, Runx2 is down-regulated in various human osteosarcoma cell lines, suggesting a link between loss of Runx2 expression, dedifferentiation, and cancer (Thomas et al., 2004). However, we observed increased osteoblast differentiation and elevated Runx2 expression in osteoblast progenitor cells derived from p53-null mice. Therefore, we examined the ability of p53 to suppress tumorigenesis in osteoprogenitor-derived cells by crossing Col3.6-Cre–transgenic mice with p53-conditional mice (Marino et al., 2000). Our results indicate that loss of p53 in osteoblasts induces a fairly rapid tumorigenesis in mice. Interestingly, Col3.6-Cre–transgenic mice heterozygous or homozygous for the p53-conditional allele display kinetics of
tumor onset similar to those that have been previously documented for p53 knockout heterozygous or homozygous mice, though the tissue specificity of tumorigenesis was greatly altered. Mice deleted for p53 in all tissues died predominantly from lymphomas, chiefly of the thymus, and only occasionally will present with bone tumors (Harvey et al., 1993b). Depending on the genetic background of the mice, between 3 and 8% of p53-null mice develop osteosarcomas (Harvey et al., 1993a; Jones et al., 1996). However, this tumor spectrum may reflect the critical importance of p53 in suppressing thymic lymphomas in relatively young mice and not a reduced role for p53 in suppressing bone cancer, as osteosarcomas do constitute approximately one third of all tumor types observed in the longer lived p53 heterozygous mice (Harvey et al., 1993b). A majority of the Col3.6-Cre–transgenic, p53conditional heterozygous mice or p53-conditional homozygous mice in our study developed osteosarcomas. Furthermore, Runx2 expression was elevated in primary osteosarcoma samples harvested from these mice, in agreement with our finding of increased Runx2 expression in the p53-null calvarial cell cultures.

The results of our p53-conditional Col3.6-Cre–transgenic mouse cross confirm that p53 is a critical tumor suppressor in bone tissue and indicate that osteosarcoma formation does not require loss of Runx2 expression. Instead, we propose that p53 inhibition of osteoblast cell proliferation is the mechanistic basis for suppression of bone osteosarcomas in this model. As we have demonstrated that Mdm2 is a key regulator of p53 activity in osteoblasts, disrupting the ability of Mdm2 to downregulate p53 activity in these cells may prove to be a useful therapeutic strategy in treating osteosarcomas.

Materials and methods

Mouse lines

Mdm2-conditional mice were developed in our laboratory using standard gene targeting techniques in embryonic stem cells. The resulting allele contains two loxp sites flanking exons 11 and 12. Upon Cre-mediated recombination, exons 11 and 12 are excised, rendering the allele inactive (described in Steimann and Jones [2002]). Mdm2 excision was mediated by crossing Mdm2-conditional (Mdm22loxPloxP) mice to transgenic mice in which the 3.6-kb type I collagen promoter governs the expression of the Cre recombinase enzyme (Col3.6-Cre). Visualization of tissues in which the Cre recombinase activity has recombined target alleles was facilitated by mating Col3.6-Cre–transgenic mice to R26R reporter mice (The Jackson Laboratory) in which expression results in the removal of a 2.3-kb reporter DNA segment that prevents expression of a β-galactosidase reporter gene (described in Steinman and Jones [2002]). Mdm2 excision was mediated by crossing Mdm2-conditional (Mdm2sjcnd1) mice to transgenic mice in which expression results in the removal of a 2.3-kb reporter DNA segment that prevents expression of a β-galactosidase reporter gene (described in Steinman and Jones [2002]). Mdm2 excision was mediated by crossing Mdm2-conditional (Mdm2sjcnd1) mice to transgenic mice in which expression results in the removal of a 2.3-kb reporter DNA segment that prevents expression of a β-galactosidase reporter gene (described in Steinman and Jones [2002]). Mdm2 excision was mediated by crossing Mdm2-conditional (Mdm2sjcnd1) mice to transgenic mice in which expression results in the removal of a 2.3-kb reporter DNA segment that prevents expression of a β-galactosidase reporter gene (described in Steinman and Jones [2002]).

Whole-mount and histological analysis

Embryos were harvested from timed pregnant mothers at various time points during gestation followed by fixation in 4% paraformaldehyde. Tissues destined for histological sectioning were dehydrated in a graded series of ethanol and xylene, followed by infiltration with paraffin wax. Tissues from R26R/Col3.6-Cre crosses were fixed in 4% paraformaldehyde, equilibrated overnight in 30% sucrose, and embedded in optimal cutting temperature for cryosectioning. Some embryos were fixed in 4% paraformaldehyde followed by whole-mount staining for β-galactosidase activity. Paraffin sections were cut at 7 μm, stained with hematoxylin and counterstained with eosin. frozen sections were cut at 10–12 μm, stained for β-galactosidase activity, and counterstained with eosin. β-Galactosidase activity was visualized by staining whole embryos, cryosections, or calvarial cultures in a solution containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2, 0.2% NP-40, 0.01% sodium deoxycholate, and 1 mg/ml X-Gal in PBS, pH 7.4, at 37°C for 1–6 h. Calvarial cultures were stained for mineral content using the method of Von Kossa. In brief, sections were exposed to a solution of 3% silver nitrate under direct sunlight for 15 min, after which mineral deposits were visualized as black precipitate under brightfield microscopy. Alkaline phosphatase activity in calvarial cultures was visualized by colorimetric enzymatic reaction to a solution containing 0.5 mg/ml naphthol AS-MX phosphate disodium salt (Sigma-Aldrich), 2.8% NaN3 dimethylformamide (Sigma-Aldrich), 0.1 M Tris-maleate buffer, pH 8.4, and 1 mg/ml fast red salt (Sigma-Aldrich). The reaction was performed at 37°C for 10 min. Decalcified femora and vertebrae were embedded in a mixture of methyl methacrylate: glycol methacrylate, sectioned at 2 μm, stained with Von Kossa, and counterstained with toluidine blue. TUNEL staining was performed on dewaxed paraffin sections using a fluorescein-conjugated in situ cell death detection kit (Roche) according to the manufacturer’s protocol.

Skeletal preparations

Skeletally matured and used in accordance with the University of Massachusetts Laboratory (in which the 3.6 kb type I collagen promoter governs the expression of the Cre recombinase enzyme (Col3.6-Cre). Visualization of tissues in which the Cre recombinase activity has recombined target alleles was facilitated by mating Col3.6-Cre–transgenic mice to R26R reporter mice (The Jackson Laboratory) in which Cre expression results in the removal of a loxP-flanked DNA segment that prevents expression of a β-galactosidase reporter gene. The p53-conditional mouse model (Marino et al., 2000) was obtained from A. Berns (Netherlands Cancer Institute, Amsterdam, Netherlands). All animals were maintained and used in accordance with the University of Massachusetts Animal Care and Use Committee.

Bone micro-CT

embryos were fixed overnight in 4% paraformaldehyde, rinsed three times with PBS, and scanned on a Skyscan 1072 instrument (Skyscan). Image acquisition of the head was performed using 25× magnification (50× for the limb) at 45 kV and 222 μA, with a 0.45° rotation between frames to obtain two-dimensional images. Three-dimensional reconstruction and quantitative analyses were performed on a computer (Dell) using the NRecon, ANT, and CTAn software supplied with the Skyscan instrument.

Calvarial osteoblast preparations

Calvarial osteoblasts were isolated from E19 embryos by enzymatic digestion of calvarial bones. In brief, calvaria were minced and subjected to three sequential digestions [8, 10, and 26 min] with collagenase P (Roche) at 37°C. Osteoblasts in the second and third digest were collected and resuspended in α-MEM supplemented with 10% FBS (HyClone). Cells were plated at a density of 106 cells/6-well plate (Owen et al., 1990). Differentiation was initiated after confluence by the addition of ascorbic acid and β-glycerol phosphate. Cultures were harvested at various time points and stained for β-galactosidase activity, mineral content, alkaline phosphatase activity, or total cellularity using toluidine blue. All osteoblast differentiation experiments were performed a total of three times, and each experiment used embryos of different genotypes harvested on the same day (E19) from the same litter. The proliferation, confluence, maturation, and mineralization stages of differentiation are defined as days 5, 10, 14, and 20 in culture, respectively, except for Fig. 6 C, where confluence, maturation, and mineralization stages of differentiation were reached on days 8, 12, and 17 of culture, respectively.

Image acquisition

Embryonic and postnatal stages were imaged with a fluorescence microscope (Eclipse E400; Nikon) connected to a Nikon digital camera (Coolpix 4500) or a Sony digital camera (Cybershot DSC-M5). Histologic sections of embryos (Fig. 1 C and Fig. 2, A and F), skeletal preparations (Fig. 3), and stained osteoblast cultures (Fig. 5, A–C, and Fig. 7, A and D) were obtained using a stereoscope (MZ8; Leica) with either a 1× or 0.63× reduction lens and a digital camera (3008 Prog/Res; Jenoptik) coupled to a computer (G4; Macintosh), using Photoshop 4 software (Adobe). Bone histology images (Fig. 4, D and E) were captured using a Nikon digital camera (2000) and Adobe Photoshop 7 software.
Transduction of Runx2 into calvarial cultures

Adenoviral infection of primary mouse osteoblasts was performed at day 10 of culture (confluence) with either a vector expressing Xpress-tagged mouse Runx2 under transcriptional control of the cytomegalovirus CMV5 promoter (pAd/CMV5/Xpress-Runx2/IRES/GFP) or a control vector expressing LacZ (pAd/CMV5/LacZ/IRES/GFP). Infections were performed at a multiplicity of infection of ~100 in αMEM containing 5% FBS (HyClone). 12 h after infection, the media was replaced with αMEM containing 10% FBS and ascorbic acid to initiate osteogenic differentiation. Cultures were harvested 7 d after infection, and quantitative RT-PCR was performed on total RNA isolated from each sample.

RNA isolation and analysis

RNA was isolated from tissue or cell cultures using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. After purification, 5 μg of total RNA was DNase treated using a DNA-free RNA column purification kit (Zymo Research). 1 μg RNA was then reverse transcribed using Oligo-dT primers and a first-strand synthesis kit (SuperScript; Invitrogen) according to the manufacturer’s protocol. Gene expression was assessed by semiquantitative [Cre, Mdm2, and gelsolin] membrane. Membranes were blocked in PBS Tween 20 (PBST) containing 2% nonfat dry milk (Bio-Rad Laboratories) before incubation with antibodies. Antibodies were incubated with membranes in the presence of PBST containing 2% nonfat dry milk for 1 h at room temperature. Excess primary antibody was removed with three 10-min washes of PBST. Secondary antibodies were incubated with membranes for 1 h at room temperature followed by three 10-min washes with PBST to remove excess antibody. Proteins were visualized on the membrane by exposure to Western light- ning chemiluminescent reagent. The Runx2 antibody was a gift from Y. Ito (National University of Singapore, Singapore). Total p53 was detected using a 50:50 mix of ab-1 and 3 (Oncogene Research Products). Activated p53 was detected using an antiSer15 p53 antibody (Cell Signaling Technology). All antibodies were used at a concentration of 50 ng/ml.

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