

The transcription factor Sox2 is required for osteoblast self-renewal

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The development and maintenance of most tissues and organs require the presence of multipotent and unipotent stem cells that have the ability of self-renewal as well as of generating committed, further differentiated cell types. The transcription factor Sox2 is essential for embryonic development and maintains pluripotency and self-renewal in embryonic stem cells. It is expressed in immature osteoblasts/osteoprogenitors *in vitro* and *in vivo* and is induced by fibroblast growth factor signaling, which stimulates osteoblast proliferation and inhibits differentiation. Sox2 overexpression can by itself inhibit osteoblast differentiation. To elucidate its function in the osteoblastic lineage, we generated mice with an osteoblast-specific, Cre-mediated knockout of Sox2. These mice are small and osteopenic, and mosaic for Sox2 inactivation. However, culturing calvarial osteoblasts from the mutant mice for 2–3 passages failed to yield any Sox2-null cells. Inactivation of the Sox2 gene by Cre-mediated excision in cultured osteoblasts showed that Sox2-null cells could not survive repeated passage in culture, could not form colonies, and arrested their growth with a senescent phenotype. In addition, expression of Sox2-specific shRNAs in independent osteoblastic cell lines suppressed their proliferative ability. Osteoblasts capable of forming ‘osteospheres’ are greatly enriched in Sox2 expression. These data identify a novel function for Sox2 in the maintenance of self-renewal in the osteoblastic lineage.

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The development and maintenance of all tissues and organs are orchestrated by a complex interplay of signaling molecules and transcription factors, whose expression governs cell fate determination, proliferation, and differentiation. Signaling by the fibroblast growth factor (FGF) family of growth factors and cognate receptors has an important function in skeletal development by affecting the proliferation and differentiation of chondrocytes and osteoblasts, the two major cell types responsible for bone formation. Several human skeletal disorders are caused by activating mutations in FGF receptors (FGFR).^{1–3} In the osteoblastic lineage, FGF stimulates the proliferation of immature cells and inhibits their differentiation.^{4–7} By studying the mechanisms underlying the FGF response of osteoblasts, we found that FGF signaling antagonizes Wnt-induced transcription, a process, which promotes osteoblast differentiation and function.^{8,9} In these cells, treatment with exogenous FGF or the presence of activated FGFR2 strongly induces the expression of the transcription factor Sox2. Sox2 contributes to the inhibition of Wnt signaling by FGF by binding to β -catenin, and its overexpression can by itself inhibit osteoblast differentiation.^{8,9}

The transcription factor Sox2 is a member of the SRY-related, HMG box family, that has a critical function in embryonic development and in maintaining the pluripotency and self-renewal of embryonic stem (ES) cells.^{10–12} It was originally identified as a binding partner of the transcription

factor Oct-4 in ES and embryonal carcinoma cells, in which it is crucial for the expression of FGF-4,¹³ and was later shown to regulate the expression of many other ES cell-specific genes. It has also been recently shown to be a critical factor for the reprogramming of somatic cells into ‘induced’ pluripotent stem cells.¹⁴

Our knowledge about the origins, fate, and life span of cells along the osteoblast lineage is limited. Primitive osteoprogenitors arise from multipotent mesenchymal stem cells that commit and then differentiate to preosteoblasts and mature osteoblasts. Although the transcription factors Runx2 and Osterix (OSX) have been shown to be essential for commitment to the osteoblastic lineage, few factors that have a defined function in early osteoblast lineage expansion have been identified.^{15–19}

To elucidate the function of Sox2 in osteogenesis and in osteoblasts self-renewal and differentiation, we have generated a conditional Cre-mediated knockout of Sox2 in the osteoblast lineage. These mice are highly mosaic for Sox2 inactivation, but are smaller and show decreased trabecular bone density compared with their wild-type littermates. By culturing calvarial osteoblasts from these mutant mice, we were unable to recover any viable Sox2-null cells. Excision of the Sox2 gene in primary or immortalized osteoblasts in culture produced Sox2-null cells that did not survive passaging in culture, could not form colonies, and arrested their

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Abbreviations: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; CKO, conditional knockout; KO, knockout; ES, embryonic stem; GFP, green fluorescent protein; IRES, internal ribosome entry site; MSCV, murine stem cell virus; PCR, polymerase chain reaction; shRNA, short hairpin ribonucleic acid; KOH, potassium hydroxide; BrdU, bromodeoxyuridine; Rb, retinoblastoma; cdk, cyclin-dependent kinase; MSC, mesenchymal stem cell; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

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growth with a senescent phenotype. These and other results identify an earlier unknown function for this transcription factor in maintaining self-renewal of the osteoblastic lineage and suggest that Sox2 expression is required to maintain osteoprogenitor cells in a multi- or unipotent stem-like proliferative state.

Results and Discussion

Generation of SOX2 conditional knockout mice. As the Sox2 KO is lethal in early embryonic development,¹⁰ to

precisely define the function of Sox2 in osteoblast proliferation and differentiation, we created a conditional knockout (CKO) of the Sox2 gene in the osteoblastic lineage using mice with a Sox2^{lox/geo} genotype (hereafter referred to as Sox2^{lox/-}). In these mice, one of the two Sox2 alleles, is bracketed by loxP sequences, providing a target for the Cre-recombinase, whereas the other is replaced by the β -gal (geo) gene that inactivates the Sox2 gene (Figure 1a).²⁰ These mice, which are phenotypically normal, were crossed with transgenic mice expressing the Cre-recombinase under the control of the 2.3kb collagen I promoter, which is specifically expressed in the osteoblastic lineage, to elicit

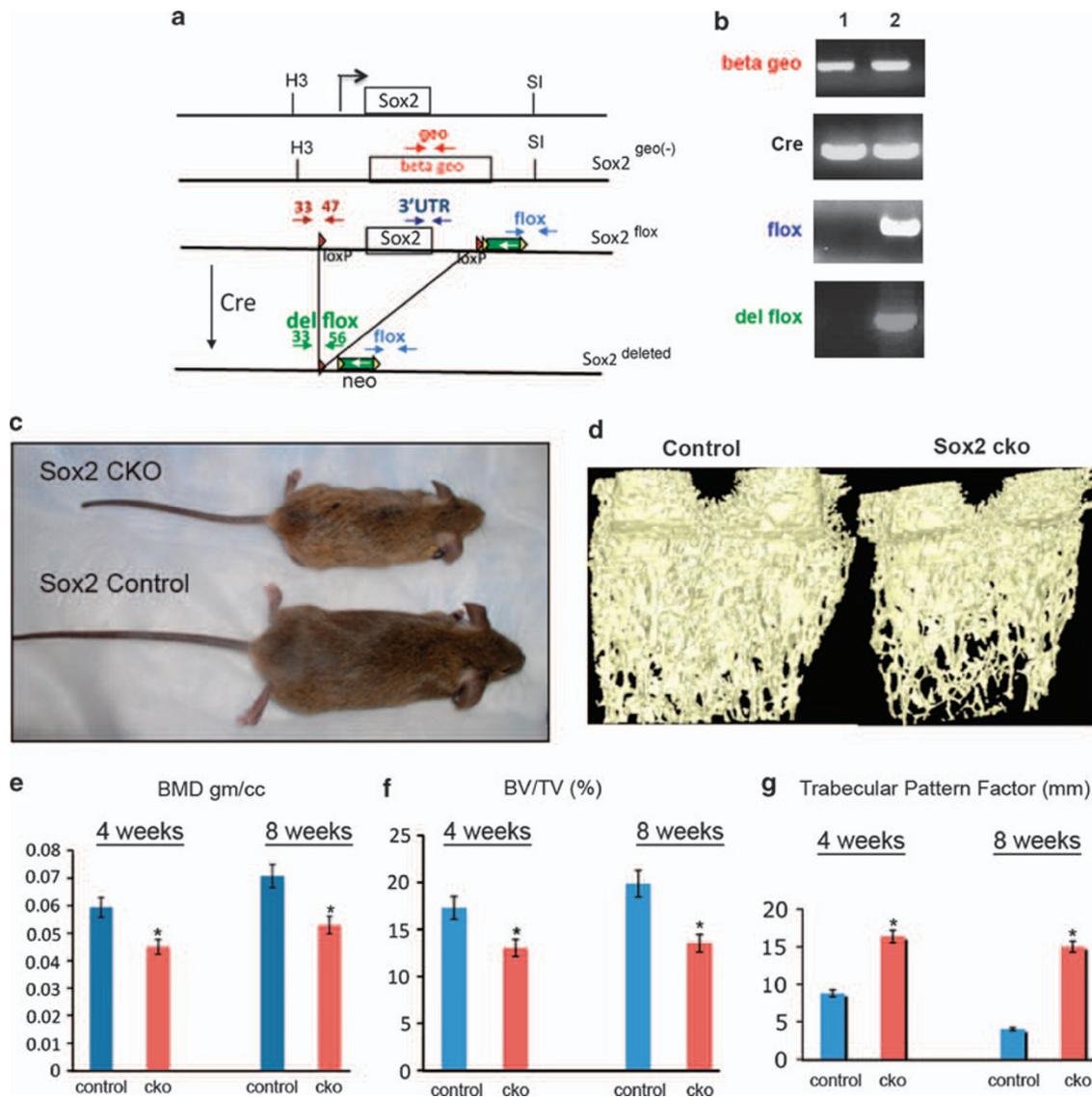


Figure 1 Osteoblast-specific Sox2 conditional knockout mice are smaller and have low bone density. (a) Schematic of Sox2, Sox2^{geo(-)}, Sox2^{lox}, and Sox2^{del} alleles. Map shows the location of diagnostic primer pairs in the Sox2 alleles; β geo is the β geo gene in the Sox2-null allele Sox2^{geo(-)}; green box is the neo gene; red triangles are the loxP sites. H3, HindIII; S1, SalI. The results obtained within the diagnostic PCR primer pairs depicted in this figure using DNA from Sox2^{lox/-} osteoblasts or Sox2^{del} osteoblasts subjected to Cre excision are shown in Supplementary Figure 1a. (b) PCR analysis of calvarial DNA. DNA from littermates was isolated using standard techniques and PCR was performed with the indicated primers. The flox primer pair detects the deleted and undeleted flox alleles. The del flox primer pair detects only the deleted Sox2 allele after Cre recombination. 1. Sox2^{+/geo};Cre (control), 2. Sox2^{lox/geo};Cre (cko). (c) Four-week-old Sox2 cko (Sox2^{lox/-}; Cre) and control (Sox2^{+/geo};Cre) littermate. (d) Reconstructed image from micro-CT analysis of 4-week-old distal femurs from Sox2 cko and control littermate. (e) Quantification of average bone mineral density, (f) bone volume per tissue volume (BV/TV), and (g) trabecular pattern factors obtained from micro-CT analysis of 4 and 8 weeks femurs of Sox2 cko and control littermate animals. * $P < 0.1$

Sox2 excision in Cre-bearing Sox2-floxed F2 offspring (Figure 1b).²¹ The resulting Sox2 CKO mice were initially difficult to study because they died at birth with esophageal atresia, most likely because of aberrant expression of the Cre-recombinase in the esophagus and trachea and the essential function of Sox2 in the development of the foregut.²² However, this phenotype has low penetrance, and at least 50% of the pups survive to adulthood. These mice are smaller than their wild-type littermates and are mostly sterile (Figure 1c). Micro-CT analysis reveals that mutant mice have reduced bone density with defects in trabecular bone. Bone mineral density and bone volume are reduced, whereas trabecular pattern factor, a parameter of micro-CT analysis that is increased in poor quality bone,²³ is higher in the Sox2 CKO mice (Figure 1d–g). Cortical bone thickness is also reduced as is the density of parietal bones in the skull (not shown). Interestingly, these effects are clearly detected even though these mice are highly mosaic for Sox2 inactivation. At birth, >50% of the calvarial osteoblasts seem to have maintained the Sox2 gene. Although in principle this could be due to inefficient expression or activity of the Cre-recombinase, this seemed unlikely, as, if anything, the collagen I Cre mice seem to express Cre more uniformly and less tissue specifically than expected. This finding suggested that Sox2-expressing cells have a growth advantage over Sox2-null osteoblasts, a notion supported by the experiments described below.

Sox2 inactivation impairs osteoblast proliferation. To analyze the phenotype of Sox2-null osteoblasts, we generated cultures of calvarial osteoblasts from the mutant mice. PCR analysis of DNA from freshly cultured cells showed that deletion of the floxed Sox2 allele had occurred. However, after culturing osteoblast pools from the P1 calvaria of the mutant Sox2^{fllox/-};Cre mice for 3–4 passages, the deleted Sox2 allele could no longer be detected by PCR analysis, whereas it was maintained in cultures from Sox2^{fllox/+};Cre mice, which carry a wild-type Sox2 allele that cannot be excised by the Cre-recombinase, suggesting that Sox2 deletion is incompatible with osteoblast survival (Figure 2a). To directly determine whether Sox2 inactivation was deleterious to cell survival, we infected freshly cultured primary Sox2^{fllox/-} osteoblast populations with a Cre-expressing adenovirus (also expressing GFP linked to Cre by an IRES) to induce Sox2 excision. We could show that Sox2 excision had taken place in a majority of the cells by PCR analysis, but again, after 2–3 passages in culture Sox2-negative cells could no longer be detected (Figure 2b). To better quantitate these experiments, we performed colony assays. We infected independent cultures of primary osteoblasts derived from Sox2^{fllox/-} mice with the Cre-expressing adenovirus and the corresponding control adenovirus expressing only GFP. Although cells infected in the GFP-virus yielded a very similar number of colonies, Cre-virus infection drastically reduced the colony-forming ability of these cultures. In contrast, cultures of primary osteoblasts from Sox2^{fllox/+} mice were completely resistant to the effect of Cre-virus infection (Figure 2c). Together, these results strongly suggested that Sox2 inactivation abolished the ability of osteoblasts to sustain proliferation in culture.

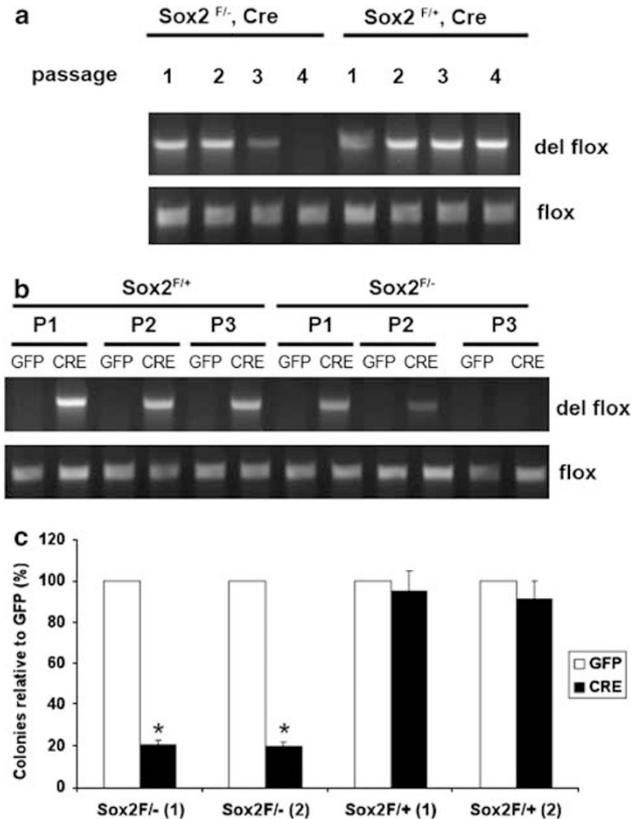


Figure 2 Sox2 inactivation impairs proliferation of primary osteoblasts. (a) PCR analysis of primary osteoblast DNA. Primary calvarial osteoblasts were prepared from Sox2^{fllox/-};Cre (cko) mice and Sox2^{fllox/+};Cre (control). On confluency, cells were passaged and DNA was extracted at each passage. The deleted floxed and the floxed allele were detected by PCR using specific primers to assess persistence of Sox2-null cells. Numbers indicate passage number after isolation. (b) PCR analysis of GFP- and Cre-virus-infected primary osteoblast DNA. Primary osteoblasts from Sox2^{fllox/+} (control) and Sox2^{fllox/-} (cko) mice were infected with GFP or Cre-adenovirus and passaged after infection. DNA was extracted from each passage and the deleted floxed allele was detected by PCR using specific primers to check for persistence of Sox2-null cells. (c) Colony assay. Primary calvarial osteoblasts from Sox2^{fllox/+} (control) and Sox2^{fllox/-} (cko) mice were infected with either GFP or Cre-adenovirus for 72 h and plated in triplicate in six-well plates. Colonies were counted after crystal violet staining. Percent of colonies obtained in Cre infection are plotted as a percentage of the colonies in the corresponding GFP infection in two independent Sox2^{fllox/-} and Sox2^{fllox/+} osteoblast pools [(1) and (2)]. **P* < 0.05. GFP Adenovirus infection of Sox2^{fllox/-} or Sox2^{fllox/+} cells produced equivalent number of colonies (~120 colonies/plate)

To facilitate biochemical and genetic experiments that are difficult to perform in primary cultures that have a limited life span, we created several clonal lines of Sox2^{fllox/-} osteoblasts by immortalizing them with polyoma large T antigen (PyLT). PyLT inactivates the Rb proteins, but not p53. As earlier reported, PyLT immortalized osteoblasts are still capable of differentiating into mature osteoblasts in culture, although this process is slightly slower than in primary osteoblasts.⁶ Several independent clones of immortalized Sox2^{fllox/-} cells were infected with a Cre-expressing retrovirus that also carries the GFP gene. Infection of such cell lines leads to deletion of Sox2 gene and reduction in Sox2 protein level (Figure 3a and c). As observed in primary osteoblasts, however, subsequent passaging of the Cre-virus-infected

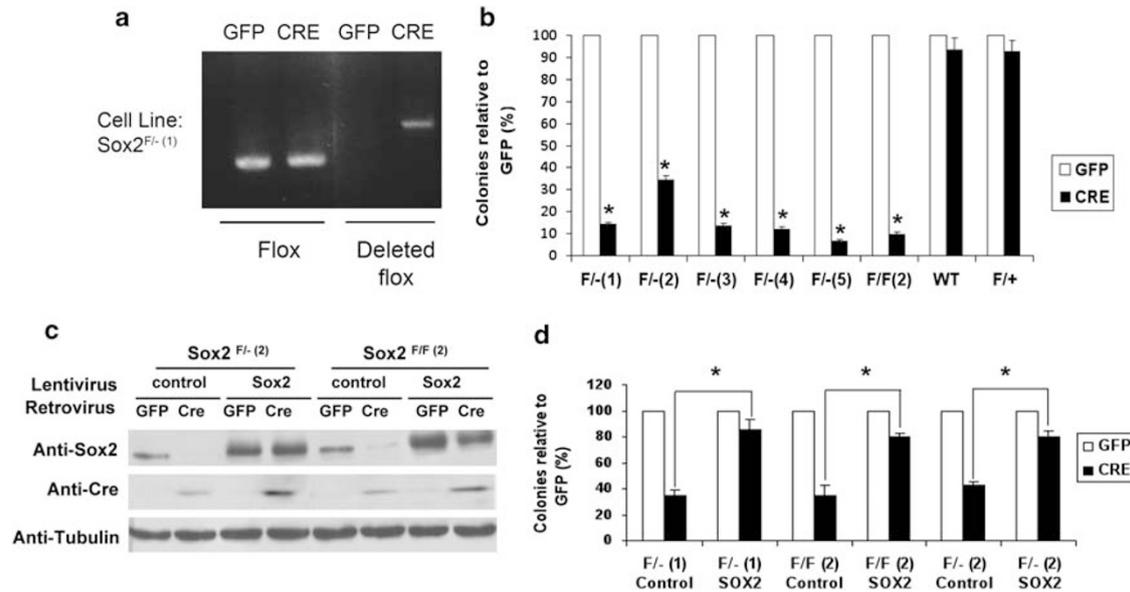


Figure 3 Transgenic expression of Sox2 rescues the lethality caused by Sox2 deletion in immortalized osteoblasts. **(a)** PCR analysis of immortalized osteoblast DNA. Representative immortalized Sox2-floxed osteoblast clone Sox2^{lox/-}(1) (referred to as Sox2^{F/-}(1)) was infected with GFP or Cre-retrovirus for 72 h. DNA was extracted and the deleted flox and flox bands were detected by PCR using specific primers to determine efficiency of *in vitro* Sox2 deletion by Cre-dependent excision. **(b)** Colony assay. Immortalized Sox2-floxed or wild-type osteoblasts were infected with either GFP or Cre-retrovirus for 72 h and plated in triplicate in six-well plates. Colonies were counted after crystal violet staining. Percent of colonies obtained in Cre infection are plotted as a percentage of the colonies in the corresponding GFP infection in five independent Sox2^{F/-} lines (F/-1–5), one Sox2^{F/F} line F/F (2), Sox2^{F/+}, and wild-type (WT) cells. **P* < 0.05. **(c)** Western analysis. Two Sox2-floxed osteoblasts clones Sox2^{F/-}(2) and Sox2^{F/F}(2) were infected with either control or Sox2-expressing lentivirus for 72 h. After infection, they were re-infected with GFP or Cre-retrovirus for 72 h. Sox2 and Cre protein were detected by immunoblotting using antibodies against Sox2 and Cre. γ -tubulin was used as a loading control. **(d)** Colony assay. Sox2-floxed osteoblasts [F/F(2)] or (F/-1) and F/- (2)] were infected as described in 3C and plated in six-well plates in triplicate. Colonies were counted after 10 days. Note that all colonies surviving Cre-virus infection in the control cells were GFP negative (therefore, not expressing Cre), whereas most of the colonies in the lenti-Sox2-expressing cells were GFP positive. **P* < 0.02

cells led to disappearance of the deleted floxed allele, indicating that cells in which Sox2 excision had occurred were incapable of survival in culture. We, therefore, performed a colony assay in which either GFP- or Cre-GFP-infected cells were plated and the number of colonies formed determined after 7 days. As shown in Figure 3b, the number of colonies obtained was drastically reduced (70–90%) in Cre-infected cells with respect to those produced by a control retroviral infection (no Cre), and all surviving clones were GFP negative, representing cells that escaped retrovirus infection. Infection of wild-type or Sox2^{lox/+} osteoblasts with the Cre-virus had no deleterious effect.

Several groups have reported that Cre expression can cause non-specific DNA-damage effects in cell culture.²⁴ Despite the lack of any effects of the Cre-recombinase in wild-type and Sox2^{lox/+} osteoblasts, we wanted to rule out the possibility that the observed cell lethality after Cre-virus expression might be due to the toxic effects of Cre. We thus isolated mouse embryonic fibroblasts (MEFs) from mouse embryos and infected them with the same Cre and GFP retroviruses. Infection of Sox2^{lox/-} fibroblasts, which do not express Sox2, produced excision of the Sox2 gene, but Sox2-null fibroblasts could be easily maintained in culture (Supplementary Figure 1b). To conclusively show that the decrease in colony formation induced by Cre-mediated excision in Sox2^{lox/-} osteoblasts was due only to Sox2 inactivation, we introduced into several clones of immortalized Sox2^{lox/-} or Sox2^{lox/flox} cells a 'transgenic' copy of the Sox2 gene using lentivirus-mediated transduction (Figure 3c). Infection with the

Cre-virus led to excision of the endogenous Sox2 gene, but no significant inhibition of colony formation was observed, showing that constitutive expression of Sox2 abolishes the detrimental effects of the inactivation of the endogenous Sox2 gene (Figure 3d). These experiments, therefore, show that the inability of Sox2-floxed osteoblasts to proliferate after Cre-virus infection is due to the lack of Sox2 expression and not to Cre-mediated cell damage.

Inactivation of Sox 2 in cultured osteoblasts causes a senescent-like growth arrest. Examination of Sox2-depleted cell populations 6–7 days after infection showed extensive morphological changes with the appearance of large cells with a 'flat,' senescent-like morphology, and substantial inhibition of proliferation. (Figure 4a). DNA synthesis was greatly decreased by 48 h after Cre-virus infection (Figure 4b), but no evidence of increased apoptosis, as measured by TUNEL assay or caspase activation, was observed. As expected, Sox2-transduced cells experienced no reduction in DNA synthesis. As Sox2 overexpression inhibits differentiation in immortalized osteoblasts⁸ as well as in primary cells (Supplementary Figure 2), we tested the expression of several markers of osteoblast differentiation, including alkaline phosphatase (ALP), Runx2, and OSX expression.¹⁷ We did not observe any increased expression of ALP by 7 days after Cre-infection, and only a minor increase in the expression of Runx2 and OSX RNAs. Instead, we noticed a clear increase in the expression of cdk inhibitors p16 and p27 (Figure 4c). Cdk inhibitors

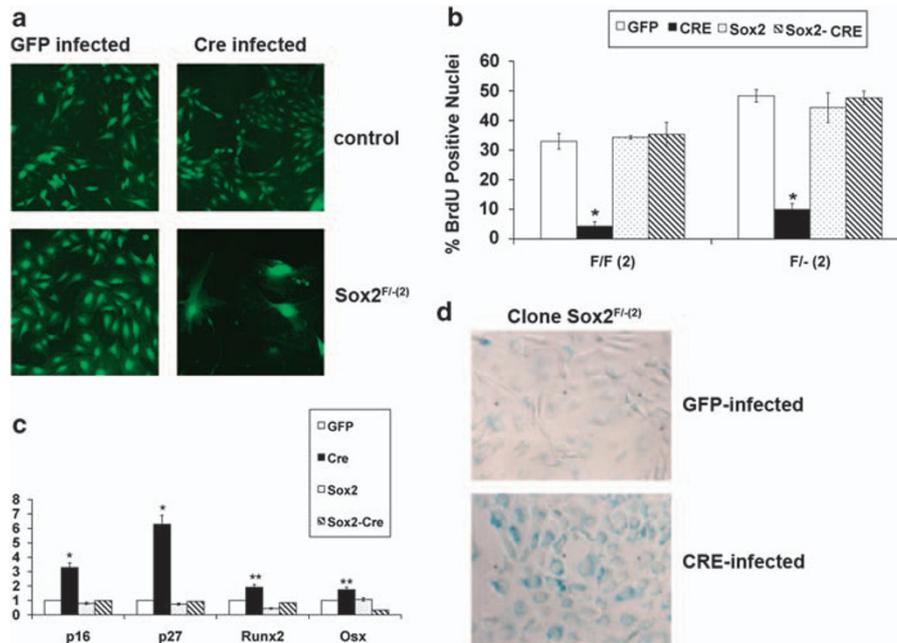


Figure 4 Inactivation of Sox2 in immortalized osteoblasts causes a senescent-like growth arrest with p16 and p27 up-regulation. (a) Morphology of colonies. Live colonies of GFP and Cre-infected control and Sox2^{F1-2} immortalized osteoblast clones were examined under a fluorescence microscope ($\times 10$ magnification) 1 week after infection. (b) BrdU incorporation of GFP and Cre-infected Sox2-floxed osteoblasts. Two independent clones (Sox2^{F1-2} and Sox2^{F2}) were infected as described in Figure 3c. BrdU incorporation 48 h after infection was assessed by labeling cells with 4 μ g/ml BrdU for 3 h and staining with an anti-BrdU antibody. GFP/BrdU-positive cells were counted using a fluorescence microscope. At least 10 independent fields were counted and each experiment was repeated at least twice. * $P < 0.01$. (c) Gene expression analysis. Sox2^{F1-2} was infected as described in Figure 3c and mRNA was extracted. Gene expression of p16, p27, Osterix, and Runx2 was analyzed after 72 h of infection by qRT-PCR using specific primers. All values are normalized to actin as an internal control and are expressed relative to GFP-infected control cells. * $P < 0.01$, ** $P < 0.05$. (d) Senescence-associated β -galactosidase (SABG) expression. SABG activity in Sox2-floxed osteoblast clone Sox2^{F1-2} was determined after 96 h of infection. Slides were mounted in DAKO mounting medium and photographed under $\times 40$ magnification

expression is elevated in senescent cells,²⁵ and this together with the cell morphology of Cre-infected osteoblasts made us assay for the presence of senescence-associated β -galactosidase (SABG) that is considered to be a hallmark of senescence.²⁶ Cre-infected osteoblasts stained positive for SABG, whereas Sox2-transduced cells did not, indicating that loss of Sox2 might produce a phenotype akin to senescence in osteoblasts (Figure 4d). It is relevant to mention here that the endogenous β -gal from the Sox2-null allele is not expressed in these cells, probably because of the deletion of a ~ 2 kb DNA region²⁰ that seems to contain osteoblast-specific regulatory elements.

A threshold of Sox2 is necessary to maintain osteoblast survival. Although our findings with osteoblasts from Sox2 mutant mice highlighted the function of Sox2 in maintaining proliferation potential in osteoblasts, all of our experiments had been performed with osteoblasts from mutant mice carrying a floxed Sox2 allele, and it was of interest to be able to extend these observations to independent osteoblastic cell lines. Furthermore, we wished to rule out the effect of excision of any Sox2 flanking sequences such as those that may encode regulatory micro-RNAs. To address these questions, we used two immortalized osteoblast cell lines that our laboratory had earlier characterized: OB1, a PyLT immortalized immature osteoblast line, and OB5, a spontaneously immortalized cell line derived from p53^{+/-} calvaria.^{6,27} We also tested OB1-Apert cells, an OB1-derived

clone carrying the activated FGFR2 bearing the S252W mutation found in Apert syndrome, that express high levels of Sox2, at least 10-fold more than the parental OB1 cells.⁸ We infected these cell lines with pBabe-hygro retrovirus expressing three shRNAs directed against murine Sox2, and a scrambled shRNA control and selected for hygromycin-resistant cell clones. These three shRNAs were found to differentially reduce Sox2 expression in the OB1-Apert cells (Figure 5a), ranging from 60 to 90% inhibition of Sox2 protein expression. As seen in Figure 5b, infection of OB1 or OB5 cells with Sox2 shRNA vectors completely abrogated colony formation. On the other hand, OB1-Apert cells were still capable of forming colonies, with an efficiency ranging between 25 and 85% of the colonies produced by the cells expressing the scrambled control shRNA (Figure 5b). The differential ability of the shRNAs to inhibit Sox2 protein expression was reflected in the ability to form colonies, as the shRNA (1043) that caused the least reduction in Sox2 levels produced the highest number of colonies. The OB1-Apert cells expressing Sox2 shRNA 900 and 998 that could form colonies grew considerably more slowly than the parental cells (Figure 5c), whereas the growth of cells expressing shRNA 1043 was not affected, as expected. The proliferation of NIH3T3 mouse fibroblasts, that do not express Sox2, was unaffected by Sox2 shRNAs as infection with all Sox2-specific shRNA viruses resulted in a number of hygromycin-resistant colonies essentially identical to that produced by the scrambled shRNA control (not shown). These results show

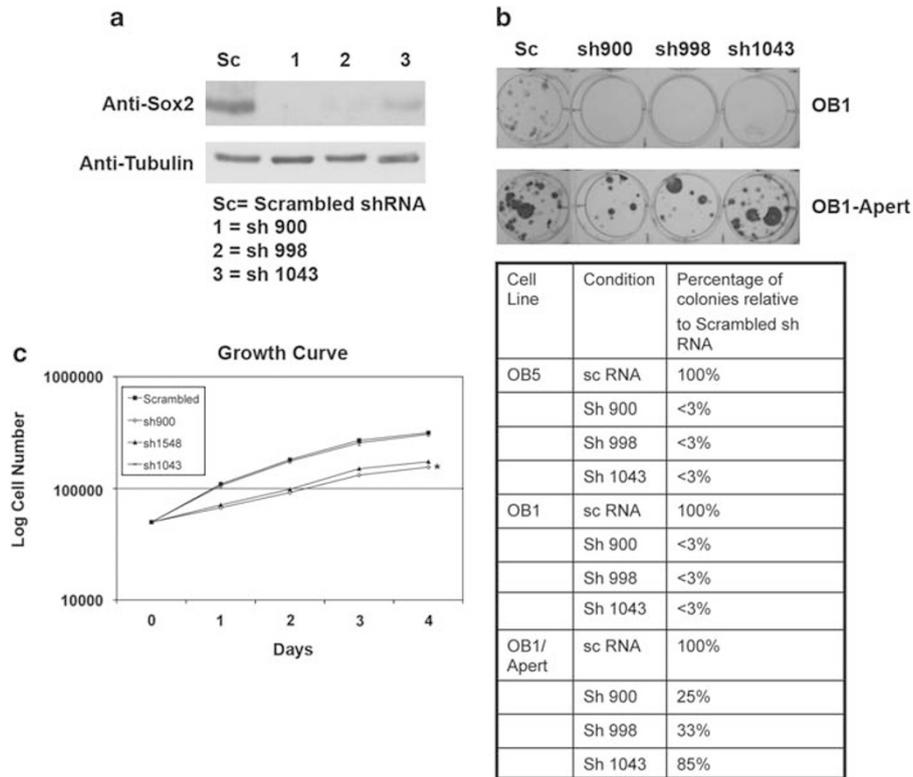


Figure 5 A threshold of Sox2 is required to maintain osteoblast survival. (a) Western blot for Sox2 expression. OB1-Apert cells were infected with pBabe-hygro retrovirus expressing the indicated shRNAs against Sox2 or scrambled (sc) shRNA. Pools of cells were selected in Hygromycin B and protein was extracted. Sox2 expression was determined by immunoblotting. (b) Colony formation by Sox2 shRNA-expressing osteoblasts. OB5, OB1, and OB1-Apert cells infected with pBabe-hygro retrovirus expressing either scrambled (sc) or sh900, sh998, and sh1043 were plated in triplicate and selected in Hygromycin B for 7–10 days followed by staining with crystal violet. Pictures of representative plates of OB1 and OB1-Apert cells expressing shRNA are shown and quantified in adjoining table. (c) Growth of Sox2 shRNA-expressing OB1-Apert osteoblasts. OB1-Apert cells (0.1×10^6 cells/well) stably expressing either scrambled (sc) or sh900, sh998, or sh1043 were plated in triplicate and growth of cells assessed by counting cells in a Z1 Beckman Coulter counter. * $P < 0.05$

that the requirement for Sox2 expression is not limited to osteoblasts from Sox2 mutant mice and suggest that a threshold level of Sox2 expression is necessary to maintain osteoblast self-renewal.

Sphere-forming osteoblasts are enriched for Sox2 expression. The results presented so far show that Sox2 is essential for osteoblast self-renewal and suggest that its expression may mark a population of uncommitted or unipotent osteoblasts. To investigate this hypothesis, we have used a technique that has been successful in identifying such stem-like cells in the nervous system, mammary gland, skin, and tumor tissues, that is the ability of these cells to form ‘spheres’ of unattached cells in suspension culture. Indeed, a recent report has shown that calvarial osteoblasts are capable of forming ‘osteospheres’ in suspension, representing progenitor/multipotent cell populations.²⁸ In line with this observation, we found that osteoblasts isolated from calvaria of P1 pups are capable of forming osteospheres. We found that Sox2 expression is greatly enriched in the osteosphere fractions as compared with the total adherent cell population (Figure 6a). On the other hand, the expression of OSX, Runx2, and Collagen1-a1, markers of committed osteoblasts, was down-regulated in the spheres, confirming that they indeed represent a precursor cell population (Figure 6b–d). Treatment with FGF, that

induces Sox2 expression, increased osteosphere formation by primary osteoblasts 5–6-fold (Figure 6e). As expected, we also found that osteoblasts made from calvaria of Sox2^{fllox/-};Cre mice, that are a mosaic of Sox2 positive and Sox2-null cells, show reduced osteosphere-forming ability as compared with the osteoblasts isolated from control littermate mice, indicating that Sox2 is necessary to maintain a pool of osteoprogenitor cells (Figure 6f).

Together, these results support the notion that, while Sox2 expression is necessary for the survival of the entire osteoblast population, high levels of Sox2 correlate with the ability to form osteospheres, and mark a subpopulation of immature, progenitor cells.

In this report, we describe the novel finding that the transcription factor Sox2 has an essential function in the expansion of the osteoblastic lineage. Progenitor stem-like cells are responsible for the development and maintenance of most tissues and organs. Osteoblast populations in culture represent a mixture of osteoprogenitor cells and immature osteoblasts, and proliferation in culture selects for more immature multi- or unipotent stem-like cells.^{28,29} Our data show quite clearly that inhibition of Sox2 expression, whether by excision of the endogenous Sox2 gene or by RNA interference, abolishes the ability of both primary and immortalized osteoblasts to proliferate. These conclusions are also supported by the observation that mice we have

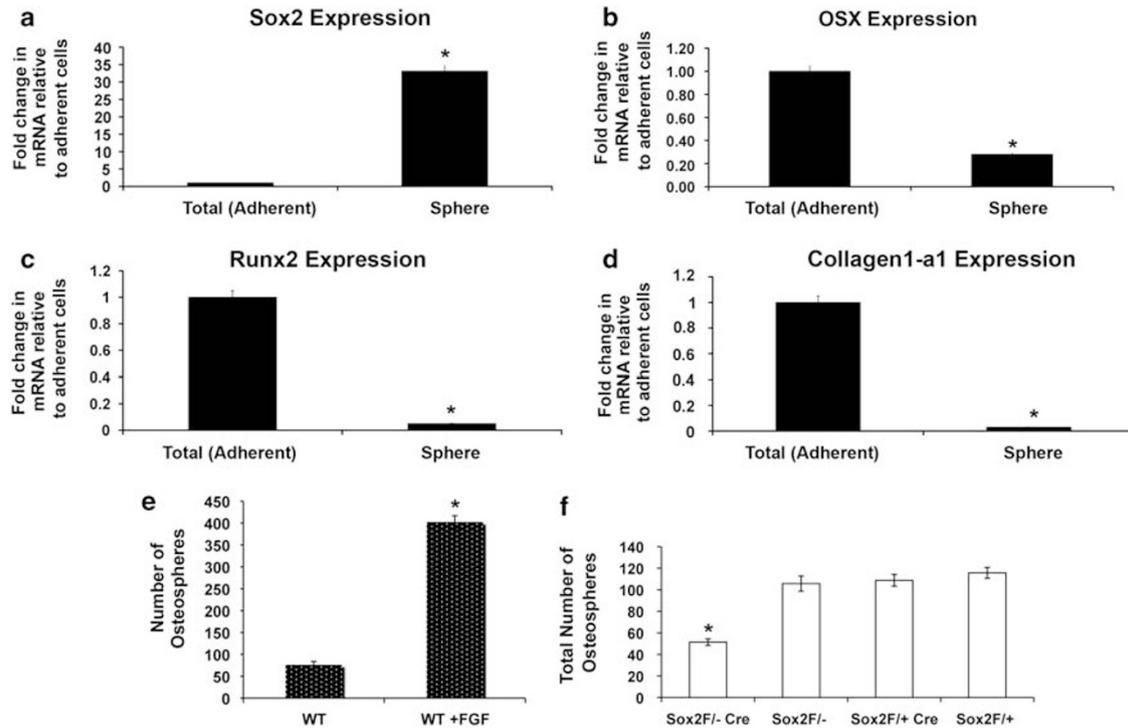


Figure 6 Sox2 is enriched in osteospheres and is required for osteosphere formation. (a–d) Osteospheres from osteoblasts derived from P1 calvaria of wild-type mice were collected by straining through a 40 μ M strainer and RNA was extracted from the spheres. Gene expression of Sox2 (a), Osterix (b), Runx2 (c), and Collagen1-a1 (d) was analyzed by qRT-PCR using specific primers. All values are normalized to actin as an internal control and are expressed relative to total (adherent) cells in each case. * $P < 0.05$. (e) Primary calvarial osteoblasts were plated in suspension culture in the presence or absence of 10 ng/ml recombinant FGF1. Spheres were counted after 7 days. * $P < 0.05$. (f) Primary P1 calvarial osteoblasts from control (Sox2^{flox/+};Cre, Sox2^{flox/-}, and Sox2^{flox/+}) and cko (Sox2^{flox/-};Cre) littermates were plated in triplicate in 6 cm Corning ultra-low attachment plates. Total number of osteospheres was counted after 7 days. * $P < 0.05$

generated with a CKO of Sox2 in the osteoblastic lineage have reduced bone density, in spite of being highly mosaic for Sox2 inactivation. Although a full description of the phenotype of these mice is beyond the scope of this report, clearly this phenotype is less severe than what could have been expected from the behavior of Sox2-null osteoblasts in culture. A likely reason for this is that only ~50% of the osteoblasts in the skull (and presumably also in other bones) have lost the Sox2 gene, probably because the collagen I promoter-driven Cre used in these experiments is expressed rather late during osteoblastic lineage development,^{21,30,31} or is inefficient, allowing cells escaping Cre-mediated Sox2 excision to proliferate at the expense of Sox2-null cells. Another possible explanation is that Sox2's function could be redundant with that of other Sox factors *in vivo*, such as Sox4, whose inactivation has been also shown to impair bone development.³² However, such redundancy does not seem to occur in cell cultures, and would not account for the mosaicism of the Sox2 CKO mice.

The results presented here strengthen the notion that Sox2 expression is required for the maintenance of a stem cell progenitor-like state in several tissues. Indeed, a similar requirement for Sox2 expression has been reported for neural and retinal progenitor cells.^{20,33,34} Our results also show that Sox2 inactivation causes growth arrest by inducing a senescent-like phenotype. Runx2-deficient osteoblasts have been reported to bypass senescence,³⁵ but we found that the level of Runx2 in Sox2-null cells is not appreciably altered.

Sox2-null cells do not seem to be in a terminally differentiated osteocyte-like state as DMP1, a marker of osteocytes is not expressed in Sox2-null cells (data not shown). This suggests that Sox2 inactivation targets specific osteoblast genes that may be important for their proliferation. Although we have shown that Sox2 can interfere with Wnt/ β -catenin signaling by binding to β -catenin, such an effect requires relatively high levels of Sox2 such as those induced by FGF,^{8,9} whereas the basal levels of Sox2 expression in osteoblasts are quite low. We, therefore, do not think the main function of Sox2 in the osteoblast lineage is, in the absence of exogenous Wnt stimulation, that of inhibiting Wnt signaling. Rather, we interpret the ability of Sox2 overexpression to inhibit differentiation as reflecting its function in maintaining osteoblasts in an immature, unipotent state. This is further supported by our observation that Sox2 expression is higher in osteosphere-forming cells as well as by the finding that Sox2 expression is enhanced in osteogenic cells in which differentiation is impaired.³⁶ Although further work will be required to conclusively answer these questions, it is interesting to note that forced Sox2 expression in bone marrow-derived mesenchymal stem cells (MSC) has been reported to promote their expansion and ability to differentiate into the osteoblastic lineage.³⁷ Furthermore, several groups have described an anabolic effect of FGF treatment *in vivo* on bone formation, a finding that still awaits a mechanistic explanation.^{38–40} MSCs persist in adult organisms and are thought to contribute to the replacement of osteoblasts in bone turnover and fracture

healing. It is tempting to speculate that the bone anabolic effect of FGF *in vivo* results from its ability to induce Sox2 and expand the pool of osteoprogenitor cells or of MSCs that commit to the osteoblastic lineage. If this hypothesis is correct, compounds that mimic or induce Sox2 expression in the osteoblastic lineage could be useful bone anabolic agents.

Materials and Methods

Generation of Sox2-floxed mice. The Sox2-floxed mice have been recently described elsewhere.²⁰ Primers used for PCR genotyping of such mice are available on request.

Analysis of skeletal phenotype of mutant mice. Mice were euthanized by carbon dioxide narcosis and defleshed in 2% KOH for 7 days. Quantitative micro-computed tomography (micro-CT) analysis was conducted at the Center for Bone and Periodontal Research, McGill University, Montreal, Quebec, Canada. Data acquisition and analysis was on a SkyScan T-1072 micro-tomograph using software supplied by SkyScan.

Cell culture. Osteoblasts from P1 calvaria were isolated by serial collagenase digestion and immortalized as described.⁶ MEFs were isolated from E16.5 embryos. All cells were maintained in DMEM supplemented with 10% fetal bovine serum.

Viral vectors. For primary cells, an adenovirus expressing either eGFP (control) or Cre (linked to an IRES-GFP to detect expression) was obtained from the Vector Development Laboratory at Baylor College of Medicine, Houston, TX, USA, and used at an MOI of 50 in 2% FBS containing DMEM for 2 h. For immortalized cells and MEFs, a retrovirus either encoding eGFP (MSCV-eGFP) or CRE (MSCV-CRE-eGFP) was used at an MOI of 10 to infect cells in the presence of 8 μ g/ml polybrene for 24 h. For Sox2 lentiviral transduction experiments, a lentivirus, expressing full-length human Sox2, was used at an MOI of 10 for infection in the presence of 8 μ g/ml polybrene for 24 h.

Colony assay. GFP or Cre-GFP virus-infected osteoblasts were plated at a density of 500–5000 cells/well in six-well plates. GFP-positive colonies were visualized and counted under a fluorescence microscope after 7–10 days. Colonies were fixed in 20% methanol for 15 min at room temperature, and counted after staining with 0.5% crystal violet in 20% methanol.

Detection of SABG activity. Cells were plated on coverslips and infected with GFP or CRE retrovirus, and recovered for 96 h. SABG activity was detected at pH 6.0 as described.²⁶

Gene expression analysis by quantitative real-time RT-PCR and western blotting. mRNA was prepared using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription and real-time PCR analysis was carried out as described earlier³ using specific primers. Actin was used as a normalization control. Antibodies used in the study were Sox2 (Cell Signaling, Danvers, MA, USA), Cre (Chemicon, Temecula, CA, USA), and γ -tubulin (Sigma, St. Louis, MO, USA).

DNA synthesis assay. BrdU incorporation assay was carried out on cultured cells as described earlier.⁶

Colony-formation assay with Sox2 shRNA. shRNAs against murine Sox2 were designed using the RNAi oligo retriever program (<http://katahdin.cshl.org/siRNA/RNAi.cgi?type=shRNA>). For each target sequence, a U6 promoter/hairpin cassette was generated by a PCR-based strategy, and then cloned in the Bam HI/Eco RI sites of retroviral vector pBabe, which carries resistance to hygromycin. The shRNAs used in this study are as follows: Sox2 1043 – AAA AAC ATA CCG GGA ATG CCC TGC TGC GAG TAG G, Sox2 900 – AAA AAC ATT GAG GCC CAG GTG CTG CGG GTA GCC C, and Sox2 998 – AAA AAC AAA CCG TTC ATG TAG GTC TGC GAG CTG G.

Osteosphere assay. Osteosphere assay was carried out as described in Gutierrez *et al.*²⁸ Briefly, 1×10^5 cells were plated in triplicate in 6 cm Corning ultra-low attachment plates for 7 days. Osteospheres were collected by sieving through a

40 μ m strainer and RNA was extracted from the spheres for gene expression analysis. Spheres were counted in each plate under a dissecting microscope.

Conflict of interest

The authors declare no conflict of interest.

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