Sox2 induction by FGF and FGFR2 activating mutations inhibits Wnt signaling and osteoblast differentiation

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Activating mutations in fibroblast growth factor receptor 2 (FGFR2) cause several craniosynostosis syndromes by affecting the proliferation and differentiation of osteoblasts, which form the calvarial bones. Osteoblasts respond to FGF with increased proliferation and inhibition of differentiation. We analyzed the gene expression profiles of osteoblasts expressing FGFR2 activating mutations (C342Y or S252W) and found a striking down-regulation of the expression of many Wnt target genes and a concomitant induction of the transcription factor Sox2. Most of these changes could be reproduced by treatment of osteoblasts with exogenous FGF. Wnt signals promote osteoblast function and regulate bone mass. Sox2 is expressed in calvarial osteoblasts in vivo and we show that constitutive expression of Sox2 inhibits osteoblast differentiation and causes down-regulation of the expression of numerous Wnt target genes. Sox2 associates with β-catenin in osteoblasts and can inhibit the activity of a Wnt responsive reporter plasmid through its COOH-terminal domain. Our results indicate that FGF signaling could control many aspects of osteoblast differentiation through induction of Sox2 and regulation of the Wnt–β-catenin pathway.

Introduction

Human and mouse genetic studies have established that FGF signaling plays an essential role in skeletal development. Several human autosomal dominant bone disorders, such as dwarfism and craniosynostosis, are caused by missense mutations in FGF receptors (FGFR1–3), which are expressed in osteoblasts and chondrocytes, the two major cell types responsible for bone formation. These mutations cause misregulated tyrosine kinase receptor activity by producing hypersensitive receptors that respond to lower concentrations of ligand or constitutively active receptors that signal in the absence of FGF (Ornitz and Marie, 2002).

The formation of skeletal elements such as bone and cartilage is controlled by a complex network of signaling molecules that control the differentiation of multipotent mesenchymal cells into osteoblasts and chondrocytes, and then regulate their proliferation and subsequent terminal differentiation (Karsenty and Wagner, 2002). The direct conversion of mesenchymal tissue into bone without prior formation of cartilage, termed intramembranous ossification, is performed directly by osteoblasts to form the flat bones of the skull vault. Osteoblast differentiation takes place at the bone margins, or osteogenic fronts, where the surrounding mesenchymal/osteoprogenitor cells are recruited to differentiate into bone-forming osteoblasts. Before birth, calvarial bones approximate each other with sutures forming between the bone margins (Bonaventure and El Ghouzzi, 2003). Craniosynostosis, or premature suture closure, is a defining feature in craniofacial skeletal disorders such as Crouzon (CR), Apert (AP), Pfeiffer, and Jackson-Weiss syndromes, which are due to activating mutations in FGFR2 (Ornitz and Marie, 2002).

Craniosynostosis in patients carrying activating FGFR1 or FGFR2 mutations has often been interpreted as reflecting premature bone formation due to increased osteoblast differentiation (Lomri et al., 1998; Marie, 2003). However, the response of osteoblast to FGF in culture does not support such views. We and others have shown that FGFs promote proliferation of immature osteoblast osteoprogenitor cells (Debiais et al., 1998; Mansukhani et al., 2000) and that constitutive FGF signaling...
inhibits osteoblastic differentiation and dramatically increases apoptosis when cells are exposed to differentiating conditions (Mansukhani et al., 2000; Nakayama et al., 2003). Human studies also reveal that the bone of patients with craniosynostosis syndrome is often more brittle and thinner than nonaffected bone (Tholpady et al., 2004). Furthermore, recent studies on a mouse model of AP craniosynostosis showed reduced, rather than increased, bone mass in the skull, no evidence of increased expression of differentiation genes, and a highly increased rate of osteoblast apoptosis in or around the cranial sutures (Chen et al., 2003). Thus, to further understand the mechanisms underlying the response to FGF of osteoblasts and the results of constitutive FGF oversignaling in these cells, we have compared by microarray analysis the pattern of gene expression in proliferating and in differentiating conditions of an osteoblastic cell line (OB1; Mansukhani et al., 2000) with that of OB1 cells, which express the two most common FGFR2 mutations found in CR (C342Y) or AP (S252W) syndromes.

Among the many significant gene expression changes detected in AP and CR cells, we observed a striking down-regulation of many genes that have been identified as targets of Wnt signaling. Recent papers have uncovered an important role for Wnt signaling in promoting osteoblast function and bone formation (Harada and Rodan, 2003). In humans, inactivating mutations in the Wnt coreceptor LRP5 cause osteoporosis/pseudoglioma, a syndrome characterized by reduced bone density (Gong et al., 2001). Conversely, activating LRP5 mutations are linked to autosomally dominant high bone mass syndromes, which do not result from reduced bone resorption (Boyden et al., 2002; Little et al., 2002; Babij et al., 2003).

Figure 1. Gene expression changes in OB1, Crouzon (CR), and Apert (AP) cells. (A) Level of expression of selected genes normalized to OB1 in growing and differentiating [diff] conditions. The average percentage of cells positive for ALP in each set is indicated at the top. Green, down-regulation in AP and CR; gold, up-regulation in AP and CR; red underlined, Wnt target genes. Microarray analysis was performed as detailed in Materials and methods. 0.00 represents /H11021 0.01. (B) Semi-quantitative RT-PCR analysis on OB1, CR, and AP cells. The amount of PCR product obtained is visualized by ethidium bromide stain.
Mice lacking LRP5 develop low bone mass and osteoporosis, and LRP5 null-osteoblasts display reduced proliferation and differentiation (Kato et al., 2002). Thus, down-regulation of Wnt signaling could be a mechanism by which FGFs inhibit osteoblast differentiation.

In this paper, we show that down-regulation of Wnt target genes is observed both in osteoblasts expressing activated FGFR2 and in osteoblasts treated with exogenous FGF. We also identify a strong induction of expression of Sox2, a member of the high mobility group (HMG) domain Sox family of transcription factors, whose expression was previously thought to be limited to very early developmental stages and to the neuronal lineage. Ectopic expression of Sox2 is sufficient to inhibit osteoblast differentiation. We show that Sox2 can interfere with the transcriptional activity of β-catenin/lymphoid enhancer factor (LEF), the classical effectors of Wnt signaling, and that it associates with β-catenin in osteoblasts. Thus, Sox2 induction and antagonism of Wnt signaling play an important role in the response of osteoblasts to FGF signals, and Sox2 induction is responsible, at least in part, for down-regulation of Wnt target genes. Down-regulation of Wnt target genes by FGF signaling could provide an explanation for the inhibition of osteoblast differentiation induced by FGF.

Results

Gene expression profiles of OB1 osteoblasts and OB1 cells expressing FGFR2/AP and FGFR2/CR mutations

We have previously described the isolation and characterization of the murine OB1 osteoblast cell line, which was derived from calvarial osteoblasts by immortalization with Polyoma large T-antigen. OB1 cells are spindle-shaped immature osteoblasts that mature to a larger cuboidal phenotype when differentiating. We stained for ALP showed that AP and CR cells do not differentiate, whereas OB1 cells increase ALP expression (up to 15%) upon differentiation (Fig. 1 A). Staining for ALP showed that AP and CR cells do not differentiate, whereas OB1 cells increase ALP expression (up to 15%) upon differentiation (Fig. 1 A). Most genes known to be up- or down-regulated during osteoblast differentiation (Beck et al., 2001; Garcia et al., 2002; Qi et al., 2003) were regulated as expected in OB1 cells differentiated for 7 d, but the changes were blocked or attenuated in AP or CR cells. Matrix genes such as osf-2, osteocalcin, fibromodulin, and cadherin-11 and the transcription factor C/EBPαs and BMP-4, whose expression increased during OB1 differentiation, had a lower basal level of expression in AP and CR cells than in OB1 cells. Unexpectedly, osteoglycin and IGFBP3, which are up-regulated upon differentiation in OB1 cells, have higher basal levels in AP and CR cells. However, the extent of their induction under differentiating conditions was reduced.

Apoptosis-related genes. CR and AP cells show a high rate of apoptosis when placed under differentiating conditions. The expression of GAS6, a ligand for the AXL/ARK receptor, whose main function is to protect from apoptosis (Bellosta et al., 1997), is down-regulated in these cells both in growing or differentiation conditions. This is also the case for the WISP-1 gene, which is known to protect from apoptosis (Su et al., 2002), whereas the proapoptotic factors Bid and caspase-6 are up-regulated.

Signaling molecules. Components of several signaling pathways such as IGFs and BMPs, which are known to control bone development, were up- or down-regulated in the AP and CR cells. It has been suggested by us and others that FGFs antagonize the differentiation-inducing signals of BMPs (Minina et al., 2002; Bellosta et al., 2003). This suggestion is supported by the lower expression of BMP target genes such as noggin, d1 x 2, osf-2, decorin, and fmo1 (Vaes et al., 2002) in the AP and CR cells.

Transcription factors. Several transcription factors that play a role in craniofacial development (msx2, hmx1, dlx2, engrailed, and Pitx2) were down-regulated in the mutant receptor-expressing cells (Fig. 1 A). The strongest up-regulated gene in the AP and CR cells was the HMG-box-containing transcription factor Sox2. This finding was unexpected because Sox2 expression is usually associated with undifferentiated multipotent cells, such as ES and EC cells (Yuan et al., 1995). It has also been shown to maintain the undifferentiated state in neural progenitor cells and at later stages is a marker of the nervous system (Avilion et al., 2003; Bylund et al., 2003). As will
be discussed later, several data suggest that Sox2 induction may be a mediator of the FGF response in osteoblasts.

Wnt target genes. We noted that a significant number of Wnt target genes (www.stanford.edu/~rnusse/wntwindow.html), whether they encoded transcription factors, signaling molecules, or matrix proteins, are down-regulated in AP and CR cells (Fig. 1 A, underlined red). Although the extent of down-regulation was variable, this trend was of particular interest because of the genetic evidence linking Wnt signals with increased bone mass. The Wnts are a large family of growth factors that signal by interacting with their receptors, consisting of the Frizzled family of seven transmembrane proteins and the essential coreceptors of the low density lipoprotein-receptor-related proteins (LRP5 and LRP6). In the absence of Wnt signals, GSK3β constitutively phosphorylates β-catenin, which is then modified by ubiquitination and degraded. Activation of Wnt receptors blocks the GSK3β activity and degradation of β-catenin, which is translocated to the nucleus where it interacts with members of the T cell factor (TCF)/LEF family of HMG-domain transcription factors to activate Wnt target gene transcription (Cadiigan and Nusse, 1997; Eastman and Grosschedl, 1999). Approximately 40% (18 genes) of the known Wnt target genes that are present on the chip and that are expressed in OB1 cells were down-regulated in the AP or CR cells. In some cases, such as Id2 and osteocalcin, which have very low expression in undifferentiated OB1 cells, the down-regulation was only evident when cells were in differentiating conditions (Fig. 1).

Several of the changes seen in the microarray experiment were validated by Northern blotting or by RT-PCR analysis on selected genes. Fig. 1 B shows that the Wnt target genes Wisp1, Wisp2, Dlx2, and mxs2 were down-regulated in CR and AP cells as was the Wnt receptor frizzled-4 (Fig. 1 B, Fzd4). In contrast, as indicated by the microarray analysis, the transcription factor Sox2 was strongly up-regulated (Fig. 1 B). We found that the level of total β-catenin mRNA remained constant in all samples and during differentiation and was used as an internal control in subsequent real-time PCR experiments.

Wnt target genes are down-regulated by exogenous FGF in osteoblasts

Among the changes in gene expression observed in the AP and CR cells, we found down-regulation of Wnt target genes and up-regulation of Sox2 particularly striking. Wnt signaling has been recently shown to promote osteoblast differentiation and function (Harada and Rodan, 2003), and other members of the Sox family have been shown to interfere with Wnt signaling in Xenopus laevis by binding to β-catenin and inhibiting the transcriptional activity of the β-catenin–TCF/LEF complex (Zorn et al., 1999). To demonstrate that such changes in gene expression were not due to clonal selection in our AP or CR lines, or specific effects of mutant FGFR2 forms, we treated OB1 cells with exogenous FGF1 for 1, 3, 6, 10, and 24 h and performed microarray analysis on duplicate RNA samples as before. The details of this analysis will be described elsewhere; however, the great majority of genes whose expression was elevated or reduced in the AP and CR cells relative to OB1 were similarly regulated by exogenous FGF treatment. Of the 18 Wnt target genes that were down-regulated in the AP and CR cells (Fig. 1 A), all but 4 (msx2, pitx2, BMP4, and IGF-1) were also down-regulated by exogenous FGF treatment of OB1 cells. Furthermore, three additional Wnt target genes, Frizzled-1, sfrp-2, and islr, that were unchanged in AP and CR cells were down-regulated by exogenous FGF, whereas expression of the Wnt targets c-myc, fra-1, and twist, which were not down-regulated in AP and CR cells, were all transiently induced early by FGF treatment (unpublished data).

The down-regulation of the expression of Wnt target genes Dlx2, engrailed-1, cadherin-11, Wisp1, and Wisp2 was confirmed by real-time RT-PCR analysis (Fig. 2). Fig. 2 also shows that Sox2 expression is strongly induced by 1 h of FGF treatment, before significant down-regulation of Wnt target genes.

Sox2 is induced by FGF in osteoblasts

To further analyze the FGF-mediated induction of Sox2 in osteoblasts, we treated OB1, AP, and CR cells with FGF1 for various times and examined the expression of Sox2 mRNA by Northern analysis. Fig. 3 A shows that Sox2 mRNA was strongly induced by FGF treatment in the OB1 cells, whereas the induction is modest in AP and CR cells that have a high basal level. Treatment of AP cells with an inhibitor of FGFR kinase activity (SU5402) drastically reduced the level of Sox2 mRNA, indicating that Sox2 expression is dependent on FGF signaling (Fig. 3 B). We examined whether or not this response was specific to osteoblasts. The induction of Sox2 mRNA by
FGF was not observed in NIH3T3 fibroblasts (Fig. 3 A), RCS chondrocytes, or in mouse embryo fibroblasts (not depicted). To ensure that the specificity of Sox2 induction was not a peculiarity of OB1 cells, we also determined that Sox2 mRNA is induced by FGF treatment of primary murine calvarial osteoblasts (Fig. 3 C).

Fig. 3 D shows that also Sox2 protein is expressed in OB1 osteoblasts treated with FGF, whereas AP cells have a high level even in the absence of FGF. Sox2 protein was also induced by FGF in OB5 cells, a spontaneously immortalized osteoblastic cell line derived from p53+/− murine calvaria (Fig. 3 D). FGF treatment also induced Sox2 in both U2OS osteosarcoma cells and in murine primary calvarial osteoblasts (Fig. 3 E). These data clearly show that Sox2 is an FGF-inducible gene in osteoblasts that is constitutively expressed in osteoblasts expressing activated FGFR.

We performed in situ hybridization on calvarial sections of P1 mice using Sox2 as a probe. Fig. 3 F shows that Sox2 expression is already detected in osteoblasts at the osteogenic bone fronts, where FGFR2 expression is high (Iseki et al., 1999). Thus, the in vivo expression pattern of Sox2 strongly indicates that the in vitro findings are of physiological relevance.

Constitutive expression of Sox2 blocks osteoblast differentiation

To test whether Sox2 could interfere with osteoblast differentiation, we transfected OB1 cells with a murine Sox2 expression plasmid and isolated individual hygromycin-resistant clones as well as pools of resistant cells. Clones or pools were tested for their ability to differentiate and compared with a pool transfected with the empty vector (OB1/vector). Cells were plated and stained after 2 and 25 d for ALP (Fig. 4 A). Although at day 2 all the cells expressed very little ALP, after 25 d much fewer ALP-positive cells were visible in the Sox2-expressing cells relative to the control (Fig. 4 A). OB1/vector cells displayed the multilayering typical of differentiating cells and stained strongly for ALP. OB1/Sox2-pool cells that have a low level of Sox2 show much less differentiation than OB1/vector at day 25. The OB1/Sox2-cl2 cells that express high levels of Sox2 mRNA do not express ALP, do not form multilayers, and maintain the phenotype of undifferentiated cells (Fig. 4 A). In other clones that expressed relatively high levels of Sox2 protein, the block in differentiation was evident as measured by ALP staining (Fig. 4 B).

We also tested the ability of Sox2 to block differentiation of OB5 osteoblasts that lack Polyoma Large T-antigen. OB5 cells differentiate more uniformly than OB1 cells, and mineralization of the matrix can be seen by 7–10 d of differentiation upon staining with Alizarin red. Fig. 4 C shows that also in OB5 cells, expression of Sox2 delays the appearance of ALP and mineralization is not detected even at 16 d. Thus the block to osteoblast differentiation induced by constitutive FGF signals can be reproduced by ectopic expression of the FGF-inducible gene Sox2.

We used microarray analysis to determine the expression of genes known to be regulated during osteoblast differentiation (Beck et al., 2001; Garcia et al., 2002; Qi et al., 2003). We chose the OB1/Sox2#16 clone because it expressed the highest level of Sox2 (Fig. 4 B). The analysis was done, as described in Materials and methods, on cRNA prepared from duplicate samples of OB1/Sox2#16 that were either untreated or maintained in differentiation medium for 7 d. The data from this analysis was normalized against the data from OB1 cells (Table I).

Table I shows that the expression of most genes that are strongly up or down-regulated during OB1 differentiation was modestly altered or unchanged under differentiating conditions in Sox2#16 cells. Osteocalcin, C/EBPα, and IGFBP3 were strongly induced in differentiating OB1 cells, but were not in-
duced in Sox2#16 cells. Decorin and fibromodulin, which are matrix genes that were increased during OB1 differentiation, were strongly reduced in Sox2#16 cells under proliferation and differentiation conditions. Cadherin-11, osteomodulin, osf-2, and BMP-4 were induced in differentiation medium much more weakly in Sox2#16 cells than in the control OB1 cells. Early induced genes (c-fos, Junb, Krox-20, and Krox-24) and S phase genes such as PCNA and thymidine kinase, which are down-regulated in differentiation in primary osteoblasts and in OB1 cells, were not down-regulated in Sox2#16 cells. Most of these changes had also been observed in the CR and AP cells. Thus, constitutive expression of Sox2 can, like FGF signaling, block osteoblast differentiation as measured by morphological changes, by staining for ALP and mineralization, and by the level of expression of differentiation genes.

**Constitutive expression of Sox2 down-regulates Wnt signaling**

The down-regulation of several Wnt target genes together with the strong induction of Sox2 in the AP and CR cells, as well as by FGF treatment of OB1 cells, suggested the hypothesis that FGF may be interfering with Wnt signals in osteoblasts via increased levels of Sox2. Furthermore, because Sox2 expression blocks differentiation and Wnt signals promote differentiation, we reasoned that Sox2 may block differentiation via the down-regulation of Wnt signaling. We have seen that treatment of OB1 with LiCl, which mimics the activation of Wnt signals by inhibiting GSK3 activity, increases ALP staining. In fact, LiCl is also able to induce ALP in AP cells and enhance mineralization in OB5 cells (Figs. S1 and S2, available at http://www.jcb.org/cgi/content/full/jcb.200409182/DC1). These, along with genetic data, indicate that FGF and Wnt signals have opposing effects in osteoblasts.

Therefore, we used the microarray analysis described in the previous section to examine the expression of Wnt target genes in Sox2#16 cells. Fig. 5 A shows that out of 18 Wnt target genes in Fig. 1 A that were down-regulated in AP or CR cells, 12 were also down-regulated in the Sox2#16 cells. It is interesting to note that the expression of the Wnt target gene fra-1, which was up-regulated in the AP or CR cells because this gene is a direct FGF target, was unchanged in the Sox2-expressing osteoblasts, whereas the Wnt targets jun and twist, which were
unchanged in AP and CR cells, are down-regulated in Sox2#16 cells (Fig. 5 A). Some of the Wnt target genes whose expression is not changed in Sox2#16 cells were also not strongly down-regulated in the AP and CR cells (BMP-4 and noggin). The validity of the microarray data was verified by performing real-time RT-PCR of a few strongly down-regulated Wnt target genes (Fig. 5 B). Thus, constitutive Sox2 expression causes down-regulation of the expression of a large number of Wnt target genes, while inhibiting osteoblast differentiation.

Sox2 interferes with a β-catenin-dependent, Wnt-inducible promoter

To test whether or not Sox2 could repress Wnt–β-catenin–induced gene expression, we assessed the effect of increasing amounts of Sox2 on the expression of the TOPFLASH Wnt reporter plasmid. When TOPFLASH is cotransfected with a plasmid encoding a constitutively active form of β-catenin (ΔN89), basal luciferase activity was strongly increased in 293 cells (Fig. 6 A). Cotransfection with increasing amounts of the Sox2 encoding plasmid reduced the β-catenin–induced activity of TOPFLASH in a dose-dependent manner, whereas Sox2 alone had no effect on the basal activity of TOPFLASH (Fig. 6 A). A control plasmid (FOPFLASH) containing mutated TCF/LEF sites was unaffected by cotransfection of active β-catenin or Sox2 (unpublished data).

To determine which domains of Sox2 were required for the inhibition of TOPFLASH activity, we used mutants of Sox2 lacking the NH₂ or COOH terminus of the protein. A deletion mutant of Sox2 (31–319 aa), which lacks the first 31 amino acids, can efficiently repress the β-catenin–dependent TOPFLASH activity, whereas the 1–129 mutant, which contains the HMG box and nuclear localization signals (Sudbeck and Scherer, 1997) but is missing the COOH-terminal portion, cannot. Furthermore, a Sox2 fragment (121–319 aa) containing only the COOH-terminal portion of the protein could repress TOPFLASH promoter activity when fused to the DNA binding domain of the LexA protein, which contains a nuclear localization signal (Fig. 6 B; Rhee et al., 2000). These experiments show that the ability of Sox2 to interfere with a β-catenin responsive promoter containing TCF/LEF binding sites resides within the COOH-terminal portion of Sox2 outside of the DNA binding HMG domain. A similar result was obtained in OB1 osteoblasts.

Table I. Expression changes in differentiation-associated genes in OB1 cells constitutively expressing Sox2 (clone Sox2#16)

| Gene      | OB1 | OB1/diff | Sox2#16 | Sox2#16/diff |
|-----------|-----|----------|---------|--------------|
| osteocalcin | L24431 | 3.45 | 0.63 | 0.71 |
| decorin | X53929 | 4.01 | 0.01 | 0.23 |
| osteomodulin | AB007848 | 15.57 | 2.93 | 6.80 |
| osteoglycin | D31951 | 26.67 | 2.77 | 26.13 |
| C/EBPα | M62362 | 8.17 | 0.37 | 0.56 |
| IGFBP3 | X81581 | 67.94 | 1.36 | 0.78 |
| BMP4 | X56848 | 6.76 | 2.70 | 3.22 |
| fibromodulin | X94998 | 12.41 | 0.38 | 0.09 |
| cadherin-11 | D21253 | 9.39 | 1.14 | 3.77 |
| osf2 | D13664 | 125.3 | 1.25 | 6.23 |
| c-fos | V00727 | 0.06 | 0.09 | 0.18 |
| PCNA | X57800 | 0.21 | 1.83 | 1.89 |
| TK | X60980 | 0.14 | 1.77 | 0.82 |
| junB | U20735 | 0.06 | 0.67 | 0.51 |
| Krox-20/egr-2 | M24377 | 0.22 | 0.18 | 0.33 |
| Krox-24/egr-1 | M28845 | 0.09 | 0.08 | 0.22 |

Biotinylated cRNAs were prepared from duplicate samples of undifferentiated and differentiated Sox2#16 cells. Samples were hybridized to U74Av2 mouse chips and scanned using the GeneArray scanner. Numbers represent the level of gene expression normalized to that of OB1 cells. Fold changes were calculated by the GeneSpring software averaged from the separate samples of OB1, OB1/diff, and Sox2#16 and Sox2#16/diff. Normalizations were performed as described in Materials and methods. Because the normalization corrects for hybridization intensity across a set of chips in each experiment, the values produced for OB1/diff samples in this experiment vary slightly from the values in Fig. 1 A.

Figure 5. Wnt target genes are down-regulated in Sox2#16 expressing osteoblasts. (A) Level of expression of Wnt target genes in Sox2#16 osteoblasts normalized to OB1. These data are derived from the same microarray analysis described in Table I. Normalizations were done as described in Materials and methods. (B) Real-time PCR analysis of Wnt target genes in osteoblasts expressing Sox2. Total RNA from OB1, OB1/vector, and OB1/Sox2#16 cells was reverse transcribed amplified by real-time PCR using SYBR green I for detection. For each PCR reaction, crosspoint values for engrailed1 and connexin 43 were normalized using β-catenin values, and relative expression level obtained for OB1 osteoblast (black bar) was set as 100%. Each bar represents the mean of three independent experiments ± SD.
Thus, although Sox2 HMG domain shares homology with TCF/LEF factors and could potentially compete for DNA binding, the repressive effect of Sox2 on a Wnt-inducible promoter does not require its DNA binding activity.

Endogenous Sox2 associates with β-catenin in osteoblasts
We determined if Sox2 associates with β-catenin in osteoblasts. We performed coimmunoprecipitation experiments on lysates prepared from OB1, AP, or CR cells that were untreated (−) or treated (+) with FGF. The cellular lysates were subjected to immunoprecipitation with antibodies directed against β-catenin or Sox2. Fig. 6 D shows that Sox2 is detected in the immunoprecipitate with β-catenin in OB1 cells that are treated with FGF, whereas in AP lysates, Sox2 is detected in the β-catenin immunoprecipitate both in untreated and FGF-treated cells. In the reverse experiment, β-catenin was detected in immunoprecipitates of Sox2 only in OB1 cells treated with FGF, whereas in AP cells it is seen in both FGF-treated or untreated samples. Thus, Sox2 coprecipitates with β-catenin in osteoblasts, an association that is clearly detected when Sox2 expression is induced by FGF. These data strongly suggest that Sox2 interferes with Wnt responsive genes in osteoblasts by sequestering nuclear β-catenin and preventing its binding to TCF/LEF factors.

Discussion
The experiments presented in this paper were undertaken to provide a mechanistic explanation for the response of osteoblasts to FGF signaling, a response that must underlie the skeletal pathologies caused by excessive FGF signaling in these cells. The most distinctive responses of osteoblasts in culture to sustained FGF treatment or to the expression of activating FGFR mutations are inhibition of differentiation and a high rate of apoptosis when exposed to differentiating conditions. These effects are osteoblast-specific as GGFs are generally mitogenic and antiapoptotic growth factors. Therefore, we
thought that the response of osteoblasts to FGF could be due to a combination of direct FGF signals, a cell type–specific ability to regulate the expression of particular genes, and cross-talk with other signaling pathways.

Therefore, we analyzed the profiles of gene expression under growing or differentiating conditions in an osteoblastic cell line, OB1, and two isogenic derivatives expressing constitutively active FGFR2. We also extended this analysis to cells treated with exogenous FGF. Our results show that FGF signaling in osteoblasts induces several changes in gene expression that could explain, at least in part, the complex biological response of these cells to FGF stimulation. Among these changes we focused our attention on the down-regulation of Wnt target genes and the induction of the transcription factor Sox2, and on how these two events could influence osteoblast differentiation.

Expression of genes involved in osteoblast differentiation

The gene expression profiles of AP and CR cells compared with OB1 in growing and differentiating conditions were generally consistent with the block in differentiation observed in these cells by morphological criteria and ALP staining. For example, it appears that the IGF signaling system, which is known to induce osteoblast differentiation, is down-regulated in AP and CR cells compared with OB1. Although this appears to be the case also for BMP signaling, which also drives osteoblast differentiation, the effects of FGF on expression of genes regulated by BMP signals in the microarray are less straightforward, and some BMP antagonists are also down-regulated. Also, some BMP target genes may be indirectly affected due to the differentiation block. The expression of several apoptosis-related genes is altered in AP and CR cells consistent with their increased apoptosis. Although it is still unclear how increased apoptosis relates to osteoblast differentiation and bone formation, there are clear indications that it is increased in craniosynostosis and must play a role in modulating these processes (Chen et al., 2003; Marie, 2003).

Of the transcription factor genes that have been shown to be important for osteoblast physiology and pathologies, we found that the expression of Cbfα1 (runx2), which is essential for osteoblast commitment and differentiation (Karsenty and Wagner, 2002), is unchanged in the AP and CR cells, both at the RNA and protein level (unpublished data). Furthermore, we observed down-regulation of msk2 mRNA, whose gain-of-function mutations are linked to Boston-type craniosynostosis (Wilkie, 1997). We also found no change in expression of the transcription factor twist. Haplosufficiency of twist is responsible for Saethre-Chotzen type craniosynostosis, and it has been suggested that FGF signaling and twist may lie in the same pathway and that twist may regulate FGFR2 expression as well as that of several FGF ligands (Wilkie, 1997; Rice et al., 2000). Our results do not support the simple hypothesis that excessive FGF signaling may produce craniosynostosis through up-regulation of msk2, down-regulation of twist, or down-regulation of Cbfα/Runx2 expression. Thus, craniosynostosis may be an endpoint that can be achieved by several independent mechanisms.

Down-regulation of Wnt signaling by FGF

A significant proportion (40%) of Wnt target genes present on the microarray chip are down-regulated in the AP and CR cells, and treatment of OB1 cells with exogenous FGF elicited a similar down-regulation. Because the expression of most genes is subject to multiple transcriptional controls, it is not surprising that not all Wnt target genes are down-regulated. Down-regulation of some Wnt target genes could be counteracted by the activation of other factors by FGF. Indeed, Wnt target genes such as c-myc, fra-1, and cyclinD1, which are not down-regulated in the AP and CR cells, are induced by FGF with early kinetics in many cell types, including OB1 (unpublished data). Additionally, the targets of Wnt–β-catenin signals may vary depending on the cell type and FGF signals may down-regulate a much higher proportion of Wnt target genes in osteoblasts.

Wnt signals are regulators of cell proliferation, differentiation, and adhesion and are required to maintain the pluripotent state of a variety of embryonic stem cells (Cadigan and Nusse, 1997; Lee et al., 2004). As discussed in the Introduction, recent studies from human genetics and animal models have uncovered a role for Wnt signaling in promoting osteoblast function and bone formation, and Wnt signals cooperate with BMPs to induce osteoblast differentiation (Rawadi et al., 2003), an effect that is opposite to that of the FGFs. Thus, down-regulation of Wnt signaling could be an important mechanism by which FGF inhibits osteoblast differentiation.

The FGFs and Wnts initiate signaling cascades that can interact in a cooperative or antagonistic manner, depending on the cell and tissue type. Cooperation between the FGF and Wnt pathways is seen in mesoderm patterning, the developing limb, neural crest induction, and the tooth bud (Ciruna and Rossant, 2001; Kawakami et al., 2001; Kratochwil et al., 2002). In contrast, in bone development, FGFs and Wnts appear to have opposing roles. Wnt signals promote osteoblast differentiation and bone formation, whereas FGF signals inhibit osteoblast differentiation. Furthermore, activation of Wnts protects from apoptosis (Longo et al., 2002), and therefore, FGFs may increase apoptosis in osteoblasts by antagonizing Wnt signals. Although the mechanisms underlying these interactions are unknown, they could be due to cell type–specific factors that mediate the cross-talk between these signaling pathways. We identified one such mediator, the FGF-inducible transcription factor Sox2, that can block osteoblast differentiation and interfere with Wnt signaling.

Role of Sox2 in osteoblast differentiation and down-regulation of Wnt target genes

Given the documented role of Sox2 in maintaining the undifferentiated state of cells, and that it is expressed in the neural crest (Wakamatsu et al., 2004), from which calvarial osteoblasts are derived, we reasoned that the induction of Sox2 by FGF could be important in inhibiting osteoblast differentiation. The induction of Sox2 by FGF occurs in variety of osteoblastic cells, but not in fibroblasts or chondrocytes. This cell-type restriction may be due to osteoblast-specific signal transduction pathways, unique transcription factors, and/or to an “active”
chromatin configuration at the Sox2 locus in osteoblasts. The induction of Sox2 by FGF has also recently been reported in lens regeneration in the newt (Hayashi et al., 2004). We have shown that ectopic expression of Sox2 in OB1 or OB5 osteoblasts inhibits their ability to differentiate. Previous analysis of Sox2 expression in the developing mouse embryo (Avilion et al., 2003) did not report Sox2 expression in cranial osteoblasts. Here, we show that Sox2 is expressed in the calvarial bone osteogenic fronts, an area that corresponds to high FGFR2 activity. Furthermore, we have obtained primary calvarial osteoblasts from Sox2+/− heterozygous animals (Avilion et al., 2003) and found that they differentiate faster than osteoblasts from littermate control animals (unpublished data). Thus, osteoblast differentiation may be accelerated by a reduction in the level of Sox2.

The mechanism by which Sox2 inhibits osteoblast differentiation appears to reside, at least in part, in its ability to inhibit Wnt–β-catenin signaling. We showed that Sox2 associates with β-catenin in FGF-stimulated osteoblasts and can repress the activity of a reporter plasmid driven by β-catenin–TCF/LEF binding sites. Furthermore, osteoblasts constitutively expressing Sox2, like cells expressing activated FGFR2, down-regulate the expression of multiple Wnt target genes.

In mammals, the Sox family of transcription factors comprises 20 members, which share a single HMG domain that binds specifically to variations of a consensus DNA sequence. In its function as a transcription factor, Sox2 synergizes with different protein partners to direct cell type-specific gene expression (Ambrosetti et al., 2000; Kamachi et al., 2000; Dailey and Basilico, 2001). Thus, it is possible that Sox2 may down-regulate Wnt genes by partnering with unidentified cofactors, as is the case for Sox17, which partners with β-catenin to activate transcription of endoderm-specific genes (Sinner et al., 2004). Sox factors are distantly related to the TCF/LEF-1 family, which bind to similar consensus sequences, and thus Sox factors could compete directly for DNA binding with TCF/LEF (Haremaki et al., 2003).

An alternate function for Sox factors in inhibiting Wnt signaling was demonstrated by Zorn et al. (1999), who showed that Sox3 and Sox17 could repress β-catenin–stimulated gene expression in X. laevis and physically interact with β-catenin in vitro. Subsequently, other Sox factors have been reported to interfere with β-catenin–induced transcription (Kan et al., 2004). The inhibitory activity of Sox3 and Sox17 on a Wnt-inducible reporter gene was contained in the COOH terminus (Zorn et al., 1999). We also found that the region of Sox2 that inhibited the β-catenin–inducible TOPFLASH promoter was contained in the COOH terminus and did not require the Sox2 DNA-binding domain, supporting the notion that the major mechanism by which Sox2 inhibits Wnt signaling does not involve its classical function as a transcription factor.

Although Sox17 shares little homology with Sox2 in the COOH terminus, it is conceivable that they both bind to the armadillo repeats of β-catenin that can interact with diverse ligands at conserved noncontiguous epitopes (Daniels and Weis, 2002). This motif is also present in the Sox2 COOH terminus (unpublished data). In chondrocytes, Sox9 inhibits Wnt signaling by competing with TCF/LEF for β-catenin binding and enhancing the degradation of β-catenin (Akiyama et al., 2004). However, the effect of Sox2 on Wnt signaling in osteoblasts does not appear to be due to the degradation of β-catenin.

**Implications for FGF-induced craniosynostosis**

Craniosynostosis is a heterogeneous developmental skeletal disorder that can lead to a variety of abnormalities including abnormal head shape, proptosis, and mental retardation. The results presented in this paper suggest a mechanism by which excessive FGFR2 signaling, through a combination of direct and indirect effects, could induce premature suture fusion. The initial response to FGF of immature osteoblast/osteoprogenitor cells is increased proliferation. This response would cause an expansion of the cell population entering the differentiation pool and accelerate suture closure. As these cells mature and differentiate, they would have to modulate the expression of the genes involved in osteoblast function, many of which are under the control of Wnt signaling, as well as slow down their growth. The block to Wnt signaling induced by FGF would prevent such events, and thus osteoblast differentiation. As also shown by our data, this block is not complete, and thus stochastically, the expanded osteoblast population would still be able to lay down bone matrix and create bone, a process facilitated by the down-regulation of FGFR2 expression that occurs behind the osteogenic fronts, as cells differentiate (Iseki et al., 1999). The high rate of apoptosis observed in vitro and in vivo in differentiating osteoblasts with constitutive FGFR2 signaling could represent the response of cells that have lost their ability to arrest proliferation when subjected to growth-inhibiting signals. Apoptosis could be promoted by the modulation of the expression of apoptosis-regulating molecules such as GAS6, wisp1, and bid (Fig. 1) that are induced by down-regulation of Wnt signaling and/or constitutive FGF signaling.

In summary, these results indicate that FGF signaling activates or represses a network of transcriptional events in osteoblasts, which includes induction of Sox2 and down-regulation of Wnt signaling. Both of these effects are likely to be important mechanisms by which FGF affects the ability of these cells to differentiate. Our results also suggest that early pharmacological interventions that would boost Wnt signaling could perhaps ameliorate the craniofacial defects of patients with FGFR2 mutations.

**Materials and methods**

**Cell culture and preparation of primary osteoblasts**

Cells were grown in DMEM (GIBCO BRL) containing 10% FCS. Primary calvarial osteoblasts were prepared as previously described (Mansukhani et al., 2000). For differentiation of osteoblasts, cells were cultured for up to 21 d in growth media containing 50 μg/ml ascorbic acid and 4 mM ß-glycerophosphate and the medium was changed every 3 d. Sox2 mutant osteoblasts were a gift of S. Nicolis (University of Milan-Bicocca, Milan, Italy).

**Microarray analysis**

Total RNA was prepared from three independent replicates of each sample using Trizol (GIBCO BRL). Biotinylated cRNA was prepared from 10 μg RNA as detailed according to the protocol (Affymetrix, Inc.) hybridized to U74Av2 mouse genome array, and scanned by the GeneArray...
Scanner (Affymetrix, Inc.) at the Columbia University Microarray Facility (New York, NY). Metrics files were generated from each chip using Microarray Suite 5.0 (Affymetrix, Inc.). The U74Av2 arrays contain probes for ~9,400 genes including ~3,700 EST clusters. Further analysis was performed in the GeneSpring 5.0 (Silicon Genetics) as detailed previously (Dailey et al., 2003). Genes that did not exhibit a minimal raw signal of 100 in at least one sample were filtered out. Genes were classified as expressed in a given experiment if they were flagged as present in at least one sample. The value for each gene’s expression was normalized across all of the chips using 50% of all measurements as a positive control. The measurement for each gene was divided by this value (per chip normalization). Background correction was made using the lower 10% (per gene normalization). The values from multiple points in each sample were averaged and used for comparative analysis. Genes with ~2.5-fold changes in gene expression were identified using Venn Diagrams of expressed genes in each sample.

ALP and Alizarin red staining
Cells were fixed with acetone/methanol for 1 min at RT, and histochemical ALP staining was performed according to manufacturer’s instructions (Sigma-Aldrich). To detect mineralized nodules, Alizarin red S (75 μg/ml) was left on the plates overnight and washed with water.

PCR analysis
Total RNA was extracted using TRIzol Reagent (Invitrogen), treated with DNAase, and purified using the Rneasy mini kit (Qiagen) according to the manufacturer’s protocol. 0.5–2 μg of purified RNA was reverse transcribed at 42°C for 1 h using 50 U of SuperScript II RT and oligo(dT)15 as a primer in a final volume of 20 μl. 1–2 μl of cDNA was used as a template for amplification by PCR. Real-time quantitative PCRs were performed in a Light Cycler Instrument using the DNA Master SYBR Green I dye intercalation assay (Roche). Primers were designed using the Oligo Primer Analysis software. Primers were generally chosen to amplify 150–250-bp amplicons in the 3′ untranslated region of the gene. For each amplified cDNA, crossing point values for different sample were determined by fluorescent monitoring and transformed to relative amounts of mRNA by comparison with the crossing point value obtained for the control OB1 cells. In some experiments, β-catenin expression levels were used as reference for normalization.

Immunoprecipitation and Western blot analysis
Cells were treated with 10 ng/ml FGF1 and 5 μg/ml heparin for 24 h in DME containing 10% FCS. Cells were rinsed in PBS and lysed in RIPA buffer (10 mM Tris/HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Na Deoxycholate, and 1% Triton X-100) containing protease and phosphatase inhibitors. 0.75–1 mg of protein extracts were immunoprecipitated with specific antibodies, and proteins were visualized as previously described (Mansukhani et al., 2000). Antibodies against Sox2 were purchased from Chemicon International and anti-β-catenin from BD Biosciences.

Expression of Sox2 in OB1 cells and reporter assays in 293 cells
Full-length Sox2 and Sox2 deletion mutants expression plasmids have been previously described (Ambrosetti et al., 2000). Transfection in OB1 or OB5 cells was done using Lipofectamine 2000 (Gibco BRL). Colonies were selected in 200 μg/ml hygromycin (GIBCO BRL). TOPFLASH, FOPFLASH (Korinek et al., 1997), and ΔN89β-catenin were a gift from L. Howe [Starg Cancer Prevention Center, New York, NY]. Typically, HEK293 cells in 24-well plates were transfected with 100 ng TOPFLASH, 20 ng ΔN89β-catenin, and up to 40 ng of Sox2-expressing plasmids using Superfect Transfection Reagent (Qiagen); HEK293 as described by the manufacturer. After 48 h, cell lysates were prepared and assayed for luciferase activity using the Promega Luciferase Assay System.

In situ hybridization
In situ hybridization was performed as described by Holmes and Niswander (2001) using RNA antisense riboprobes labeled with digoxigenin-UTP.

Image acquisition
Images were viewed on Telaval 31 (for plates; Carl Zeiss Microlmaging, Inc.) or Axioplan 2 [for slides; Carl Zeiss Microlmaging, Inc.] using 5 or 40× objectives. Digital images were acquired with an Axioimcer camera and AxioVision 2.0 software (Carl Zeiss Microlmaging, Inc.).

Online supplemental material
Figs. 1 and 2 show that UCI treatment promotes differentiation of OB1, OB1/AP, and OB5 osteoblasts. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.20040918/DC1.
