The Short Stature Homeodomain Protein SHOX Induces Cellular Growth Arrest and Apoptosis and Is Expressed in Human Growth Plate Chondrocytes*

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Mutations in the homeobox gene SHOX cause growth retardation and the skeletal abnormalities associated with Léri-Weill, Langer, and Turner syndromes. Little is known about the mechanism underlying these SHOX-related inherited disorders of bone formation. Here we demonstrate that SHOX expression in osteogenic stable cell lines, primary oral fibroblasts, and primary chondrocytes leads to cell cycle arrest and apoptosis. These events are associated with alterations of the expression of several cellular genes, including pRB, p53, and the cyclin kinase inhibitors p21Cip1 and p27Kip1. A SHOX mutant, such as seen in Léri-Weill syndrome patients, does not display these activities of the wild type protein. We have also shown that endogenous SHOX is mainly expressed in hypertrophic/apoptotic chondrocytes of the growth plate, strongly suggesting that the protein plays a direct role in regulating the differentiation of these cells. This study provides the first insight into the biological function of SHOX as regulator of cellular proliferation and viability and relates these cellular events to the phenotypic consequences of SHOX deficiency.

Homeobox genes encode a large family of transcription factors, characterized by the presence of a 60-amino acid DNA-binding domain, the homeodomain. These proteins play fundamental roles during embryogenesis and development by regulating pattern formation and organogenesis (1, 2). They can function as transcriptional activators or as repressors that control the temporally and spatially regulated expression of target genes. Mutations of homeobox genes are associated with human disorders, including Waardenburg syndrome (PAX3) (3), aniridia (PAX6) (4), sympatho-lymphactyly (HOXD13) (5), schizencephaly (EMX2) (6), Rieger syndrome (PITX1) (7), and several types of cancer, such as alveolar rhabdomyosarcoma (PAX3) (8) and intestinal tumors (CDX2) (9). There is growing evidence that homeodomain proteins can positively and negatively regulate cellular proliferation and cell viability. Indeed, some homeodomain proteins, including HOX 11 (10), cux-1 (11), and Msx1 (12), have been shown to promote proliferation during spleen, kidney, and mammary gland development. Others, such as cdx1 (13), HOXA10 (14), and Gax (15), inhibit cell growth in intestinal epithelial, myelomonocytic, and vascular smooth muscle cells.

The short stature homeobox-containing gene (SHOX)1 is located in the pseudoautosomal region of the human sex chromosomes (16). In situ hybridization analyses of human embryos have demonstrated that SHOX is expressed in the first and second pharyngeal arches and at high levels in the developing limbs (17). High levels of SHOX expression have also been found in trabecular bone cells of adults (18), and it has been demonstrated that SHOX acts as a transcription activator in osteogenic cells (19). Together these studies suggest a role for SHOX in bone development. Direct evidence for this, however, comes from the discovery that mutations in the coding region of the SHOX gene cause idiopathic growth retardation, mesomelic short stature, and Madelung deformity in Léri-Weill dyschondrosteosis. Langer syndrome (16, 20–22), and the growth failure in Turner syndrome (16–18, 22).

To understand the role of SHOX in regulating growth and to gain insight into the mechanisms underlying SHOX-related disorders, we have investigated the influence of SHOX expression on cell proliferation and viability. Furthermore, because COOH-terminal-truncated forms of SHOX lead to Léri-Weill dyschondrosteosis and idiopathic short stature, we have compared the activity of the wild type form of SHOX with a COOH-terminal-truncated form. Our results in two different human cellular systems (osteogenic U2OS cells and primary oral fibroblasts) demonstrate that only wild type SHOX induces cell cycle arrest and apoptosis. The COOH-terminal-truncated form does not maintain these properties, suggesting that these functions are important for normal bone development. We have also shown that SHOX is expressed in human growth plate chondrocytes with the highest expression in terminally differ-

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1 The abbreviations and trivial terms used are: SHOX, short stature homeobox-containing gene; ST, U2OS cells expressing wild type SHOX; STM, U2OS cells expressing a COOH-terminal deletion mutant of SHOX; DOX, doxycycline; BrdUrd, 5-bromo-2-deoxy-uridine; FACS, fluorescence-activated cell sorting; DAPI, 4’,6-diamidino-2-phenylindole; PARP, poly(ADP-ribose) polymerase; POF, primary oral fibroblasts; PBS, phosphate-buffered saline.

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entiated hypertrophic cells, suggesting its involvement in the processes controlling chondrocyte differentiation and apoptosis in the growth plate. Further supporting this hypothesis and in agreement with the results obtained with the other cell culture systems, expression of SHOX in primary chondrocytes is associated with a decrease of cell proliferation and apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Retroviral Infections**—The generation of U2OS-ST and STM stable cell lines and their growth conditions were as previously described (19). Induction of SHOX expression was obtained by adding 0.5 µg ml⁻¹ of doxycycline (DOX) to the culture medium unless otherwise stated. Human chondrocytes were obtained after informed consent from patients undergoing knee joint replacement as approved by the local ethics committee. Cells were harvested from knee cartilage regions with no macroscopically evident degeneration as described previously (23). Primary human oral fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Primary chondrocytes (passage 8) were cultured in Dulbecco’s modified Eagle’s medium high glucose supplemented with 10% fetal calf serum. Retroviral infections were performed as described in Caldeira et al. (24). After infection, positive cells were selected in medium containing puromycin at the concentration of 1 µg ml⁻¹.

** Colony Formation Assay**—Colony formation assay was performed as previously described (23). Cells were selected for 10 days in Dulbecco’s modified Eagle’s medium supplemented with puromycin (500 ng ml⁻¹). Colony formation was expressed as the number of clonogenic cells per number of cells plated.

**Growth Curves**—Equal numbers of U2OS, ST, and STM cells (300,000–500,000) were plated on two different sets of 145-mm dishes. After 8 h, one set of dishes was induced with DOX. Cell numbers were calculated every day for a total of 7 days by counting the cells using a hemocytometer. Each experiment was performed in triplicate, and the average of values is given for each point.

**BrdUrd Incorporation**—Cells were grown on coverslips for 2 days before addition of BrdUrd to the culture medium for a further 20 h. Detection of labeled cells was performed according to the manufacturer’s instructions using the 5-bromo-2-deoxyuridine labeling and detection kit (BD Biosciences). Only after induction with DOX were both the wild type and mutant SHOX proteins detected by Western blot analysis on total cellular extracts. The expression of the protein was proportional to the amount of DOX added to the cultured medium (Fig. 1B).

**Immunohistochemistry**—The human growth plate sections used in this study were obtained from a normal fetus of 22 weeks gestation (humeral growth plate), from 12- and 15-year-old boys (tibial growth plates) subjected to surgery intervention because of abnormal leg overgrowth, and a 13-year-old girl (hip growth plate) subjected to surgery intervention because of hip reconstruction. Immunohistochemistry on paraffin sections of growth plates was performed as previously described (26) with the only exception that the digestion step was carried out using 5 µg ml⁻¹ of proteinase K (Invitrogen) in 100 mM Tris-HCl, pH 8.0, 50 mM EDTA for 10 min at 37 °C. The SHOX antibodies used for the immunohistochemistry were SHOX-β (19) and SHOX-γ (this study) used at 1:50 and 1:25 dilution, respectively.

**RESULTS**

**SHOX Expression Induces Cell Cycle Arrest in U2OS Cells**—To investigate the cellular function of SHOX, we established stable osteosarcoma cell lines expressing SHOX in a tetracycline-inducible manner (19). ST cells express wild type SHOX, whereas STM cells express a COOH-terminal deletion mutant that resembles SHOX mutations observed in patients with idiopathic short stature and Léri-Weil syndrome (4) (Fig. 1A). Only after induction with DOX were both the wild type and mutant SHOX proteins detected by Western blot analysis on total cellular extracts. The expression of the protein was proportional to the amount of DOX added to the cultured medium (Fig. 1B).

Expression of wild type SHOX was associated with dramatic morphological changes in ST cells and a marked decrease in growth that became evident 2 days after induction. By day 4, virtually all cells were growth-arrested (Fig. 1C). SHOX-induced ST cells showed an enlarged and apparently more differentiated phenotype. A significant portion of cells (~30%) displayed nuclear segmentation resulting in two nuclei of equal size (Fig. 1C). Doxycycline titration experiments (from 5 to 0.005 µg ml⁻¹) showed that very low levels of SHOX are sufficient to cause the phenotype (data not shown). In contrast to ST cells, the induced STM cells expressing the SHOX mutant protein did not display any evident morphological changes and grew like the non-induced cells (Fig. 1C, lower panel).

We examined the proliferation capacity of these cell populations by counting cell numbers for 7 days (Fig. 2A). This showed that, in the absence of DOX, ST and STM cells have growth rates comparable with the parental U2OS cell line. The addition of DOX (0.5 µg/ml) to the culture medium did not affect the growth of U2OS cells and had only a marginal effect on the growth of STM cells. In contrast, ST cells expressing SHOX rapidly stopped growing. Moreover, after 3 days we observed a decrease in cell number, indicating that expression of SHOX also influences cell viability (Fig. 2A).

**Antibody Generation**—Polyclonal rabbit anti-human SHOX-γ antibodies used for immunohistochemistry experiments were generated against keyhole limpet hemocyanin or bovine serum albumin-conjugated peptides (NH₂-FKHDVNDKELKEF-COOH) corresponding to amino acids 71–85 of the SHOX protein sequence. Sera were collected at 10 weeks after the second boost. SHOX-γ antibodies were affinity purified using the original peptide bound to Sepharose 4B columns (Pineda Antibody Service, Berlin, Germany). Specificity of antibody was demonstrated by Western blot analysis using cellular extract derived from ST cells, grown in the presence or absence of DOX, where the antibody recognizes a single band of the equivalent molecular size of SHOX expressing cells (data not shown).

**Flow Cytometric Analysis**—Cells were grown on coverslips for 2 days before addition of BrdUrd to the culture medium for a further 20 h. Detection of labeled cells was performed according to the manufacturer’s instructions using the 5-bromo-2-deoxyuridine labeling and detection kit (BD Biosciences). Only after induction with DOX were both the wild type and mutant SHOX proteins detected by Western blot analysis on total cellular extracts. The expression of the protein was proportional to the amount of DOX added to the cultured medium (Fig. 1B).

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To confirm the SHOX-induced cell cycle arrest, we assessed the percentage of cells undergoing de novo DNA synthesis by BrdUrd incorporation experiments in U2OS, ST, and STM cells. Cells were grown for 2 days, either in the presence or absence of DOX, before BrdUrd was added to the medium for 20 h. As shown in Fig. 2B, expression of SHOX was associated with a strong decrease in the number of ST cells incorporating BrdUrd (from ~80 to 10%), confirming a reduced cell cycle.
activity in the induced ST cell line.
To define in which phase of the cell cycle SHOX blocks proliferation, we measured the DNA content of both uninduced and induced ST and STM cells by FACS of propidium iodide-labeled cells in time course experiments. In agreement with the BrdUrd experiments, analysis of the cell cycle profiles showed
that the expression of SHOX causes a decrease in the percentage of cells in S phase with an increase of the two peaks in G1 and G2/M phase (Fig. 2C, 2 days). At the time when cellular growth is no longer apparent (Fig. 2A, days 3 and 4), and consistent with the observation that a significant fraction of cells becomes binucleated, the reduction of S phase is accompanied by an accumulation of cells predominantly in G2/M phase (Fig. 2C, 3 and 4 days). However, the fact that the relative number of G1 cells remains similar and that the FACS profiles do not reveal a total accumulation of cells in G2/M phase suggests that G1 cells also do not proceed through S phase. Therefore, expression of SHOX seems to induce a double block in both G1 and G2/M phase of the cell cycle in U2OS cells (Fig. 2C). No cell cycle arrest is detected following induction of the COOH-terminal-truncated SHOX in STM cells, strongly suggesting that the COOH-terminal portion of SHOX is essential for these functions (Fig. 2D). These data were confirmed by using different clones of each cell type, demonstrating that the cellular changes caused by SHOX are not clone-specific but truly dependent on protein expression. Taken together, these results demonstrate that SHOX induces cell cycle arrest in U2OS cells.

**SHOX Alters the Expression of Several Cell Cycle Regulatory Proteins**—Cell cycle progression is dependent on the successive activation of different cyclin-dependent kinases. Their activity is tightly regulated by several mechanisms, which include transient associations with cyclin regulatory subunits and binding of small proteins, the cyclin-dependent kinase inhibitors. To investigate the mechanism underlying the SHOX-induced events, we examined the expression of several cell cycle regulatory proteins by Western blot analysis. Experiments were performed on parallel cultures of ST cells maintained under inducing and non-inducing conditions. Total protein extracts were prepared at 48 and 72 h. Immunoblotting analysis revealed that the levels of two cyclin-dependent kinase inhibitors, p21Cip1 and p27Kip1, were strongly increased upon induction of SHOX (Fig. 3A). Interestingly, the levels of p53, which plays a central role in controlling cell cycle and apoptosis, were...
also elevated in SHOX-expressing cells (Fig. 3A). The pocket proteins, pRb, p107, and p130, are targets of the cyclin-dependent kinase inhibitors, and their phosphorylation is essential for cell cycle progression. Hypophosphorylated forms of the pocket proteins are often associated with cell cycle arrest. Consistent with the elevated levels of p21(Cip1) and p27(Kip1) in SHOX-expressing cells, pRb was present at lower levels and was completely converted to the faster migrating hypophosphorylated forms as shown by Western blot analysis (Fig. 3B). Similarly, p107 levels were also greatly reduced. In contrast, p130 was up-regulated with an accumulation of its hypophosphorylated forms (Fig. 3B). These changes in the pocket proteins are associated with a G0/G1 cell cycle arrest and are consistent with the G1 block detected by FACS analysis (see Fig. 2C). In addition, protein levels of both cdc2 and cyclin B1, which regulate G2/M transition, were either slightly (cdc2) or strongly (cyclin B1) reduced by SHOX expression, providing additional proof of the SHOX-mediated cell cycle arrest (Fig. 3C). Together, these data confirm the multiple effects of SHOX on cell proliferation at the biochemical level.

SHOX Expression Leads to Apoptosis in U2OS Cells—The reduction in cell numbers following prolonged SHOX expression indicates that SHOX influences cell viability (Fig. 2A). To test whether this event in ST cells expressing SHOX can be attributed to apoptosis, we used four independent methods. First, nuclei of uninduced and induced ST cells were stained with the cell-permeable dye DAPI and analyzed by fluorescence microscopy. A fraction of cells expressing SHOX showed markedly condensed chromatin and nuclear fragmentation characteristic of apoptotic cells. These features were not observed in uninduced cells (data not shown).

We then assessed the occurrence of DNA fragmentation in these cells by flow cytometric analysis, calculating the percentage of cells with a DNA content lower than 2n (sub G1 population). Uninduced and induced ST cells were collected at different time points, fixed, and stained with propidium iodide. The presence of an apoptotic cell population was already detectable at days 2 and 3 postinduction, and the percentage increased constantly with the time of SHOX expression. After 7 days of induction, ~30% of cells were undergoing apoptosis. Conversely, uninduced ST cells had a normal DNA content (Fig. 4A). In a third approach, we performed terminal deoxynucleotidyltransferase-mediated dUTP-fluorescein nick end labeling (TUNEL) assays. In agreement with the results shown above, and compared with uninduced cells, SHOX-expressing cells displayed a larger number of TUNEL-positive nuclei (Fig. 4B).

Quantification of TUNEL by FACS analysis confirmed that ~30% of cells underwent apoptosis after 7 days of SHOX expression (Fig. 4C). Finally, we performed immunoblot analysis for the caspase-cleaved fragment of poly(ADP-ribose) polymerase (PARP) that is associated with apoptosis (27). Lysates were prepared from ST cells cultured either in the absence or in presence of DOX for 3 days. Levels of cleaved PARP were considerably higher in SHOX-induced cells than in uninduced cells (Fig. 4D). No apoptosis was detected in STM cells (Fig. 5 and data not shown). To verify the results derived from U2OS cells in a physiologically more relevant setting, we also expressed SHOX in human POF (non-transformed cells, where cell cycle checkpoints are intact and no culture stress has accumulated). SHOX was cloned in the pBABE-puro vector, which contains the promoter/enhancer sequence of the Moloney murine leukemia virus and expresses exogenous genes at low levels (28). POF cells were infected with recombinant retrovirus containing pBABE-SHOX. After puromycin selection, cells expressing SHOX displayed morphological changes and grew more slowly than the mock-infected cells (Fig. 6A). Colony formation assays and BrdUrd incorporation experiments confirmed that the expression of SHOX strongly reduces cellular growth (Fig. 6, B and C). FACS analysis of propidium iodide-stained cells demonstrated that POF cells expressing SHOX were arrested in G1 phase (Fig. 6D). The G2/M block detected in U2OS cells was not observed in the primary cells, suggesting cell type specificity, most likely dependent on U2OS alterations in G1-specific cell cycle regulatory pathways (29). Thus, in human primary cells where the cell cycle checkpoints are intact, the G1 arrest becomes predominant. Next, we analyzed the protein levels of various cell cycle regulators by Western blot analysis of total cellular lysates. As previously demonstrated for ST cells, in POF cells the expres-
Fig. 4. Expression of SHOX leads to apoptosis in ST cells. A, FACS analysis. Cells were harvested at the times indicated and processed for flow cytometric analysis. A typical experiment is shown. The percentage of apoptotic cells is shown for each cell cycle profile as the sub-G₁ population and was calculated using Cell Quest software. B, TUNEL assay. The photographs show one example of TUNEL assay analyzed by fluorescence microscopy. Cells were grown on coverslips for 7 days in the presence or absence of DOX before being processed. Position of the nuclei was determined by DAPI staining. C, TUNEL assay. The graph shows the percentages of TUNEL-positive cells as quantified by flow cytometry. D, Western blot analysis of caspase-cleaved PARP. Twenty micrograms of lysate were used for the analysis. (−), ST not induced, (+), ST after 72 h of induction. A specific antibody that recognizes only the 87.5-kDa PARP fragment was used. β-tubulin was used as loading control.
Expression of SHOX is also associated with a significant increase of p53, p21<sup>WAF1</sup>, and p27<sup>Kip1</sup> protein levels. Consistent with the up-regulation of the cyclin-dependent kinase inhibitors and the G1 arrest observed by flow cytometric analysis, pRB is converted in the hypophosphorylated form (Fig. 6E).

To determine whether SHOX expression also leads to apoptosis in primary human fibroblasts, we monitored DNA fragmentation in SHOX-expressing cells as sub-G1 population by flow cytometric analysis. As shown in Fig. 7A, a higher percentage of apoptotic cells was observed in SHOX-expressing cells compared with control. Similarly, high levels of the PARP-cleaved form were detected in SHOX-expressing cells, confirming the induction of apoptosis in these cells (Fig. 7B). In summary, these results demonstrate that SHOX also retains the ability to promote cell cycle arrest and apoptosis in primary cells.

**SHOX Is Expressed in Human Growth Plate Chondrocytes**—Because mutations in the SHOX gene cause defects in bone growth and development, it is reasonable to assume a role for the protein in the growth plate where, as suggested by the results described above, it may play a role in controlling the proliferation, differentiation, and/or apoptosis processes. To address the putative function of SHOX in the growth plate, we examined the SHOX expression pattern on human fetal (22 weeks gestation) and pubertal (13 years) growth plate sections by immunohistochemistry using specific antibodies. The growth plate is organized in horizontal zones of column-wise-oriented chondrocytes. The reserve zone contains the resting chondrocytes, the proliferating zone the proliferating chondrocytes, and the hypertrophic zone contains the chondrocytes, which enlarge in size and terminate differentiation with apoptosis, leaving a scaffold for new bone formation. We found that in humeral fetal growth plate, SHOX is expressed in the chondrocytes of the reserve, proliferating, and hypertrophic zones. Interestingly, the intensity of the immunostaining increases with the progression of chondrocytes toward a terminally differentiated stage, with the highest expression of SHOX detected in the hypertrophic zone (Fig. 8A) where chondrocytes are presumed to undergo apoptosis. Immunohistochemistry performed on pubertal tibial growth plates revealed a more pronounced SHOX protein localization within the hypertrophic chondrocytes with less staining detected in the other zones of the growth plate (Fig. 8B). Analysis of other pubertal (12 and 15 years) growth plate specimens confirmed strong SHOX distribution in hypertrophic chondrocytes (data not shown). A second antibody raised against a different domain of the SHOX protein demonstrated identical staining patterns (data not shown). We did not observe any staining using preimmune sera (Fig. 8A and B), further confirming the specificity of the immunostaining. SHOX was not detected in other cell types present in the sections analyzed, such as osteoblasts and osteoclasts. These results constitute the first demonstration that SHOX is expressed in the chondrocytes of the growth plate. The fact that the protein is preferentially expressed in terminally differentiated hypertrophic chondrocytes, in combination with the results described previously, suggests that SHOX is involved in the regulation of chondrocyte differentiation and apoptosis in the growth plate.

**Expression of SHOX Leads to Cell Cycle Arrest and Apoptosis in Human Primary Chondrocytes**—To provide additional evidence supporting the hypothesis of SHOX being a regulator of proliferation and differentiation of chondrocytes, we investigated whether SHOX also induces cell cycle arrest and apoptosis in primary chondrocytes. These cells (passage 8) expressed several typical markers of chondrocytes, such as collagen 11A1, fibromodulin, biglycan, decorin, lumican, and aggrecan (data not shown). Primary chondrocytes were infected with retrovirus carrying either pBABE empty vector or pBABE-SHOX.
After puromycin selection, we consistently observed that SHOX-expressing cells grew slower than control cells and a large number of them died (Fig. 9A). BrdUrd incorporation experiments confirmed a reduced proliferative capacity of the cells expressing SHOX compared with mock-infected cells (Fig. 9B). Western blot analysis on total cellular extracts showed that, similar to U2OS and POF cells, SHOX expression in primary chondrocytes was associated with higher protein levels.
Growth Arrest and Apoptosis Induced by SHOX

of p21\(^{Cip1}\) (Fig. 9C), whereas protein levels of p53 and p27\(^{kip1}\) were below detection limits. Finally, TUNEL assays revealed higher levels of apoptosis in SHOX-expressing chondrocytes compared with control cells (Fig. 10). These experiments show that expression of SHOX also leads to cell cycle arrest and apoptosis in primary chondrocytes, further supporting a role for SHOX in chondrocyte differentiation.

DISCUSSION

Long bone formation is a dual phase process within the growth plate in which chondrogenesis and osteogenesis are tightly coupled. During chondrogenesis a coordinated balance of chondrocyte proliferation, hypertrophy, and apoptosis results in the formation of a cartilage anlage that serves as a template for osteogenesis. In the second phase, osteoblasts and osteoclasts replace degenerated chondrocytes to form the final bone. This process is influenced by a wide variety of hormonal and local factors whose relative concentration and specific expression pattern vary during development. Alterations in this highly coordinated system can have dramatic consequences on bone formation (30). Based on our current knowledge, the transcription factor SHOX is primarily involved in skeletal development, bone formation, and linear growth. Indeed, individuals harboring SHOX mutations show short stature and severe abnormalities in bone development. However, the underlying mechanisms and the detailed biological functions of SHOX in these processes have hitherto been unclear. These studies have also been hampered by the fact that no orthologous genes exist in mouse or rat (17), impeding the establishment of rodent knock-out models. For this reason, to examine the cellular functions of SHOX we used three different human cell culture systems, namely osteogenic U2OS stable cell lines, primary oral fibroblasts, and primary chondrocytes.

The results presented in this study demonstrate that SHOX expression leads to an inhibition of cellular growth and apoptosis. Primary fibroblasts expressing SHOX are arrested in the G\(_1\) phase of the cell cycle, whereas in U2OS cells the block occurs in both G\(_1\) and G\(_2\)/M phases. We reasoned that the different cell cycle arrest detected in U2OS may be explained by U2OS defects in the G\(_1\)-specific pathways involved in cell cycle control (29). In addition, the presence of multinucleated cells in SHOX-expressing U2OS cultures could also account for an increase in the G\(_2\) peak (DNA content = 4n), where a binucleated G\(_1\) cell would mimic a G\(_2\) cell. In fact, the nuclei of these cells show decondensed chromatin, appearing to have completed nuclear division without completing cytokinesis. Moreover, the general mechanisms activated by SHOX with accumulation of several cell cycle regulators, such as p21\(^{Cip1}\), p27\(^{kip1}\), p53, and hypophosphorylated pRB, are similar in both systems and consistent with a G\(_1\) biochemical status.

In all the cellular systems studied, prolonged SHOX expression leads to apoptosis. Interestingly, Western blot analysis revealed an increase in p53 protein levels in SHOX-expressing cells. Because p53 plays a critical role in regulating cell growth and apoptosis and is activated by a series of stress signals, including DNA damage, oncogene activation, and deregulation of normal growth (31), the up-regulation of p53 suggests that the protein could take part in the SHOX-induced events. This hypothesis is currently under investigation.

The capacity of SHOX to induce cell cycle arrest and apoptosis is shared with other members of the homeodomain protein family. For example, Cdx1 inhibits proliferation of intestinal epithelial cells (13) and HOXA10 stimulates p21\(^{Cip1}\) transcription, resulting in cell cycle arrest and differentiation in myelomonocytes (14). Similarly, Gax negatively regulates cardiomyocyte proliferation in a p21\(^{Cip1}\)-dependent manner (15) and induces apoptosis in vascular smooth muscle cells (32). Our results showing an antiproliferative role of SHOX further support the concept of homeodomain proteins fulfilling fundamental roles during development by regulating the cell cycle and cell viability.

Although the tissue culture systems used in our study cannot completely reproduce the complexity of the in vivo situation, e.g. within the growth plate, our results suggest that SHOX could be part of the intricate program that assures the correct balance between proliferation and apoptosis during bone development. This is supported by immunohistochemical analysis of the fetal and pubertal growth plates that reveals strong SHOX expression in chondrocytes. Significantly, endogenous SHOX was not detected in other cell types of the growth plate, such as osteoblasts and osteoclasts, suggesting a specific role of the protein in the chondrocytes of the growth plate. In the growth plates of pubertal individuals the protein is predominantly expressed in terminally differentiated hypertrophic chondrocytes, cells that are prone to undergo apoptosis, whereas in the

Fig. 7. SHOX induces apoptosis in human primary oral fibroblasts. A, flow cytometric analysis. 7 days after selection, cells were harvested, ethanol fixed, and stained with propidium iodide. Detection and quantification of apoptotic cells were performed as described in the legend of Fig. 8. B, Western blot analysis. 100 μg of total cellular extract were analyzed for the presence of apoptotic cleavage form of PARP and β-tubulin (loading control).
fetal growth plate we localized SHOX protein both in hypertrophic chondrocytes and to a lesser extent in resting and proliferating chondrocytes. We hypothesize that the observed differences between fetal and pubertal growth plates may reflect differential SHOX distribution at the subsequent stages of human development. We also speculate that SHOX may be present in proliferating chondrocytes of the fetal growth plate in an inactive form. For instance, it is known that the activity of homeodomain proteins is often regulated by posttranslational modifications (33) or by their ability to form complexes with other molecules (34). Our experiments using primary chondrocytes in which overexpression of SHOX leads to cell cycle arrest and apoptosis are in agreement with the proposed role of SHOX in the regulation of the cell cycle and apoptosis in growth plate chondrocytes. Interestingly, we have also shown that SHOX expression is associated with an up-regulation of

**Fig. 8. SHOX is expressed in chondrocytes of the human growth plate.** Immunohistochemistry performed on fetal humeral growth plates (22 weeks gestation) (A) and on pubertal (13 years) tibial growth plates (B). Staining was performed with preimmune serum (negative control) and SHOX-specific antibodies.
Both p21Cip1 and p27Kip1 have been previously described to regulate chondrocyte differentiation and are particularly highly expressed in the terminally differentiated chondrocytes (35, 36) where we observe maximum SHOX expression.

Remarkably, we have shown that the expression of a COOH-terminal-truncated version of SHOX, frequently observed in Léri-Weill dyschondrosteosis, has no detectable effects on cell cycle progression and apoptosis. Based on these results, we deduce that the abnormal skeletal development and growth impairment associated with SHOX deficiency can be attributed to the lack of these properties. Interestingly, the same mutant, although maintaining the nuclear localization and the ability to bind DNA, is unable to activate transcription in U2OS cells (19), suggesting a link between SHOX transcriptional activities and SHOX-mediated cellular effects. How might SHOX haploinsufficiency (e.g. in Léri-Weill and Turner syndromes) or the complete loss of SHOX function (e.g. in Langer syndrome) account for the abnormal skeletal development in short stature patients? We propose that the absence of wild type SHOX would promote atypical proliferation of the chondrocytes combined with defective differentiation. This may lead to retarded longitudinal bone growth by altering the balance between proliferation and subsequent differentiation of the chondrocytes in the growth plate of the long bones. A decreased rate of chondrocyte differentiation leading to dwarfism has been reported in mice overexpressing the parathyroid hormone-related peptide (37) and in humans with Jansen-type metaphyseal chondrodysplasia (38). Further work will be necessary to define the SHOX-related pathways and their link to disease as well as to...
Fig. 10. SHOX induces apoptosis in human primary chondrocytes. TUNEL assay. After selection (4 days), cells carrying the indicated plasmids were seeded and grown on coverslips for an additional 24 h, and then TUNEL assay was carried out according to the manufacturer’s protocol. Position of the nuclei was ascertained by staining with DAPI dye. Numbers indicate the percentage of apoptotic cells calculated by counting positives on a total of 300 random nuclei.

decipher the mechanisms that regulate SHOX activities during bone development.

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