SHOX triggers the lysosomal pathway of apoptosis via oxidative stress

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The SHOX gene encodes for a transcription factor important for normal bone development. Mutations in the gene are associated with idiopathic short stature and are responsible for the growth failure and skeletal defects found in the majority of patients with Léri–Weill dyschondrosteosis (LWD) and Langer mesomelic dysplasia. SHOX is expressed in growth plate chondrocytes where it is supposed to modulate the proliferation, differentiation and cell death of these cells. Supporting this hypothesis, in vitro studies have shown that SHOX expression induces cell cycle arrest and apoptosis in both transformed and primary cells. In this study, we further characterized the cell death mechanisms triggered by SHOX and compared them with the effects induced by one clinically relevant mutant form of SHOX, detected in LWD patients (SHOX R153L) and a SHOX C-terminally truncated version (L185X). We show that SHOX expression in U2OS osteosarcoma cells leads to oxidative stress that, in turn, induces lysosomal membrane rupture with release of active cathepsin B to the cytosol and subsequent activation of the intrinsic apoptotic pathway characterized by mitochondrial membrane permeabilization and caspase activation. Importantly, cells expressing SHOX R153L or L185X did not display any of these features. Given the fact that many of the events observed in SHOX-expressing cells also characterize the complex cell death process occurring in the growth plate during endochondral ossification, our findings further support the hypothesis that SHOX may play a central role in the regulation of the cell death pathways activated during long bone development.

INTRODUCTION

Among the multitude of genetic factors affecting growth, a major role in bone formation and development has been attributed to the short stature homeobox-containing gene (SHOX) (1,2). SHOX resides within the pseudoautosomal region of the sex chromosomes and belongs to the paired-related homeobox gene family (3,4). The homeobox-containing protein family comprises several transcription factors, all containing a characteristic 60 amino acid DNA-binding domain called the homeo-domain, that play fundamental roles during embryogenesis and development by regulating cellular proliferation and differentiation (5–7). They were shown to function as both transcriptional activators or transcriptional repressors, controlling temporally and spatially the expression of different target genes. In agreement with its role as a transcription factor, previous studies have demonstrated that SHOX is a nuclear protein which acts as a transcriptional activator (8). SHOX mutations/deletions are an important genetic cause for isolated or familial short stature (9,10). Altogether, the prevalence of SHOX mutations occurring in the general population is estimated to be as >1:1000 (1,10). Indeed, SHOX haploinsufficiency has been reported to be responsible for ~5% of the idiopathic short stature cases and for the short stature phenotype and skeletal abnormalities observed in 50–90% of individuals with Léri–Weill dyschondrosteosis (LWD) (10–12). Moreover, homozygous loss of the SHOX gene underlies the severe short stature and dysmorphic features in Langer mesomelic dysplasia (13). Consistent with the skeletal anomalies observed in LWD patients (disproportion of limbs with mesomelia, Madelung deformity, partial dislocation of ulna at wrist, elbow or both, curvature of radius and ulna/tibia, high-arched palate, scoliosis) that have
been described in many patients with SHOX defects, in situ hybridization experiments revealed a tempo-spatially restricted expression of the SHOX gene in the developing limbs and the first and second pharyngeal arches in human embryos (14).

Further supporting the role of SHOX in bone development, we and others have shown that the SHOX protein is detected in chondrocytes of the growth plate (15–17), particularly in terminally differentiated hypertrophic chondrocytes and, to a lesser extent, also in reserve and proliferating chondrocytes. These results suggest that SHOX may be involved in the processes regulating chondrocyte proliferation, differentiation and cell death. However, more detailed studies are necessary to define the SHOX-related pathways and their link to disease. These studies are hampered by the fact that SHOX gene homologues do not exist in rodents, impeding the use of knock-out rodent animal models for investigating SHOX biological functions. Use of stable cell lines expressing SHOX under an inducible system proved to be very valuable tools allowing the initial characterization of SHOX functions and identification of its target genes (8,17–20). Further supporting the role of SHOX as a regulator of cell proliferation and viability, we found that SHOX expression induces cell cycle arrest and apoptosis in osteosarcoma U2OS stable cell lines, primary oral fibroblasts and primary chondrocytes (15). At the molecular level, SHOX cell cycle arrest was associated with increased levels of the cyclin-dependent kinase inhibitors p21\(^{\text{kip1}}\) and p27\(^{\text{kip1}}\), two proteins that have been described to play important roles in the differentiation of skeletal tissues (21,22). A SHOX mutant carrying a missense mutation within the homeodomain (28) and R153L (19), and a C-terminally truncated form of SHOX (29,30), have been described in many patients with SHOX defects, in situ hybridization experiments revealed a tempo-spatially restricted expression of the SHOX gene in the developing limbs and the first and second pharyngeal arches in human embryos (14).

In this study, we investigated the mechanisms underlying SHOX-induced cell death by using a stably transfected U2OS osteosarcoma cell line with inducible SHOX expression as a model (15). We found that SHOX expression induces the intrinsic mitochondrial pathway of apoptosis leading to caspase activation. SHOX-induced apoptosis is associated with accumulation of intracellular reactive oxygen species (ROS) and an increase of lysosome numbers and cathepsin B protein levels. We also show that SHOX-induced apoptosis is, at least in part, mediated by partial rupture of lysosomal membranes with subsequent translocation of active cathepsin B to the cytosol.

**RESULTS**

**SHOX induces apoptosis through the mitochondrial pathway**

Using U2OS stable cell lines expressing SHOX under a tet on/tet off system (15), we previously found that the protein is able to induce apoptosis. However, the mechanisms underlying SHOX-induced apoptosis have not been investigated yet. In this study, we aimed to further characterize SHOX-mediated cell death. To this end, the activity of SHOX wild-type protein was compared with SHOX R153L [a SHOX mutant found in LWD patients carrying a point mutation within the homeodomain (28)], and with SHOX L185X, [a C-terminally truncated SHOX mutant which resembles a deletion form of the protein identified in LWD and idiopathic short stature patients (3)], whose expression failed to induce apoptosis (19).

Mitochondria play a central role in the intrinsic apoptotic pathway and mitochondrial outer membrane permeabilization (MOMP) is a hallmark of apoptosis. We addressed the question whether cell death induced by SHOX involves activation of the mitochondrial apoptotic pathway. U2OS cells were grown in the presence (SHOX induction) or absence of doxycycline for 72 h, stained with MitoTracker Red and analyzed by flow cytometry for MOMP. A time-dependent increase in MOMP, indicated by decrease in fluorescence signal emission, was observed in SHOX-expressing cells but not in cells expressing the SHOX R153L or SHOX L185X mutant forms (Fig. 1A).

We then investigated the role of caspases 3 and 8 in SHOX-induced apoptosis. Western blot analysis on whole-cell lysates revealed increased caspase 8 protein levels and the presence of active caspase 3 and 8 cleavage fragments in SHOX-induced cells (Fig. 1B). Caspase-mediated apoptosis in SHOX-induced cells was also confirmed using the CellEvent™ Caspase 3/7 green detection reagent, a substrate which becomes fluorescent upon cleavage by active caspases 3 and 7 (Fig. 1C). Altogether, these results confirm our previous findings that SHOX expression induces apoptosis in U2OS cells and show that mitochondria and caspases are involved in this event.

**SHOX induces lysosomal membrane permeabilization**

Apart from its fundamental cellular role in degradation of proteins and other macromolecules, the lysosome is now also recognized as a key point of integration of different apoptotic stimuli (29,30). Considering the importance of lysosomal destabilization in the initiation of the apoptotic pathway, we examined the potential role of SHOX in inducing lysosomal membrane permeabilization (LMP). To this end, U2OS cells were grown in the presence or absence of doxycycline for 48 and 72 h and then stained with acridine orange (AO). Besides the previously reported cell-size enlargement (15), SHOX-expressing cells displayed a progressive increase in AO staining, most likely due to a larger number of acidic vesicles (most likely lysosomes) present in these cells. Moreover, we observed a diffuse green AO staining in the cytosol and nucleus of numerous SHOX-expressing cells, suggesting a partial relocation of the highly concentrated dye from the acidic vesicles to these compartments (Fig. 2A). This effect was more prominent after 3 days of treatment. We hypothesized that the diffusion of AO staining was due to lysosomal membrane rupture and, therefore, analyzed LMP...
additionally by flow cytometry in SHOX-expressing and control
cells loaded with LysoTracker Red, a lysosome-specific fluores-
cent dye. In agreement with our data from microscopy analysis,
the percentage of cells with decreased fluorescence intensity,
reflecting lysosomal rupture, increased over time in SHOX-
expressing cells, reaching up to 33% 3 days post-induction,
whereas no shift in fluorescence emission was detected in non-
induced cells even at late time points (Fig. 2B and data not
shown). Similar LMP was observed in cells treated with stauros-
porine, a compound widely used as an inducer of apoptosis in
 cellular studies (31). In parallel, we also analyzed the potential
of SHOX R153L and SHOX L185X, which are unable to
induce apoptosis, to trigger LMP. No LMP was observed in
cells expressing the SHOX R153L or SHOX L185X mutants
(Fig. 2B). Altogether, these results provide first evidence that
SHOX-expressing U2OS cells undergo LMP, strongly suggest-
ing that lysosomal destabilization takes part in the apoptotic
cascade induced by SHOX.

**SHOX expression triggers relocation of cathepsins B to the
cytosol**

Recent studies have demonstrated that lysosomal destabilization
followed by the release of cathepsin proteases from lysosomes to

Figure 1. SHOX-induced cell death is characterized by mitochondrial membrane permeabilization and caspase activation. (A) U2OS-SHOX, U2OS-SHOX R153L and U2OS-SHOX L185X cell lines were grown in the presence of doxycycline (IND.) for 72 or 96 h or left untreated (NOT IND.). After 120 h from plating, cells were loaded for 30-min with MitoTracker Red before to be analyzed by flow cytometry for mitochondrial membrane permeabilization (MOMP). Columns represent the percentage of cells undergoing MOMP (left shift of red fluorescent intensity) ± SD from two independent experiments (n = 6). *** P < 0.001. (B) 20 μg of total
protein extracts from U2OS cells grown in the presence (SHOX IND.) or absence (NOT IND.) of doxycycline for 96 h were analyzed by western blotting for the presence of caspases 8 and 3 cleavage forms. Actin was used as a loading control. Ratios for each band after normalization to the actin levels are indicated. (C). U2OS-SHOX cells were seeded on glass coverslips and then induced (SHOX IND.) or not (NOT IND.) with doxycycline. After 72 h, cells were loaded with CellEvent™ Caspase 3/7 Green detection Reagent, fixed and then analyzed on a fluorescent microscope. Nuclei were visualized by DAPI staining (in blue). Positive
apoptotic cells containing active caspases 3 and 7 appear in green.
the cytosol is a necessary step for the activation of downstream events leading to cell death. Results from immunoblot analysis on total cell lysates showed a time-dependent up-regulation of both cathepsin B catalytically active fragments p27 and p30 starting 48 h after SHOX induction and increasing with time (Fig. 3A). We also noted a minor increase of the cathepsin B
Figure 3. SHOX expression triggers up-regulation of cathepsin B and its relocation to the cytosol. (A) Time-course analysis of cathepsin B protein levels in total cell lysates from cells expressing SHOX wt or R153L mutant. Ratios of cathepsin B p27 and p30 to actin were quantified and standardized to untreated cells at 24 h for SHOX wt or untreated cells and at 72 h for SHOX R153L. (B) Analysis of cathepsin L levels following SHOX wt expression by immunoblotting. Actin was used as a loading control. Ratios of cathepsin L to actin were quantified and standardized to untreated cells (0 h). (C) Detection of cathepsin B in cytosolic and lysosome-enriched subcellular fractions from U2OS cells harvested at different time points after doxycycline induction (0–72 h) by western blot. LAMP-2 was used as a lysosomal marker, and M2PK as a cytosolic marker. Blots from two different exposure times (long exposure: 1 min; short exposure: 5 s) are shown for cathepsin B. (D) Time-course monitoring of relative cathepsin B activity (cytosolic-to-lysosomal activity) in SHOX-expressing U2OS cells (average values with standard deviation from three independent experiments performed in quadruplicate). (E) Immunofluorescence analysis of lysosomal proteins LAMP-2 (lysosomal marker) and cathepsin B in doxycycline (dox)-treated (SHOX IND.) and untreated (NOT IND.) cells. Cells were grown directly on coverslip for 72 h in the presence or absence of dox before to be fixed and analyzed by confocal fluorescence microscopy. A dramatic increase of the levels of both LAMP-2 and cathepsin B proteins was found in SHOX-expressing cells.
protein levels in not induced cells which could be attributed to the leakage of the tetracycline-inducible expression system. It is noteworthy that high amounts of cathepsin B were also found in the culture medium of SHOX-expressing cells (cathepsin B was readily detected in 20 μl of the cultural medium by western blot analysis) suggesting an active secretion of the enzyme to the outside of the cells (data not shown). Expression of the R153L mutant, unlike wild-type protein, did not affect the cellular levels of cathepsin B (Fig. 3A). Parallel analysis of the protein levels of another lysosomal protease, cathepsin L, revealed that, on the contrary to cathepsin B up-regulation, the levels of cathepsin L remained unchanged up to 96 h of SHOX expression (Fig. 3B), suggesting that cathepsin B up-regulation is a specific event in SHOX-expressing cells.

To determine whether cathepsin B relocates from lysosomes into the cytosol, we examined its subcellular distribution in SHOX-expressing cells compared with uninduced cells. Cell lysates were collected at different time points and were subjected to cell fractionation to obtain cytosolic and lysosome/mitochondria-enriched subcellular fractions. Immunoblot analysis of these fractions revealed that both cathepsin B active fragments were detected in high amounts after 48 h of protein induction, and increased over time (Fig. 3C). Importantly, a significant fraction of the cathepsin B protein was also detected in the cytosol, providing evidence that SHOX-induced apoptosis in U2OS cells is associated with both overexpression and cytosolic translocation of cathepsin B. We then assayed cytosolic and lysosome/mitochondria-enriched cell fractions for subcellular cathepsin B activity using the fluorogenic cathepsin B-specific substrate Z-Arg-Arg-AMC (32). Consistent with our immunoblot data, a 13-fold increase in relative cytosolic cathepsin B activity was observed in SHOX-expressing cells after 48 h compared with control cells, and even higher increase was measured after 72 h of expression (Fig. 3D).

To confirm the cytoplasmic relocation of cathepsin B, we performed immunofluorescence confocal microscopic analysis of SHOX-expressing U2OS cells labeled for LAMP-2, a lysosomal marker, and cathepsin B (Fig. 3E). The results from this analysis revealed an increase in lysosomal volume and numbers after SHOX expression, as indicated by the increased LAMP-2 staining, when compared with control untreated cells. This higher lysosome number in SHOX-induced cells correlated with an increase in the cathepsin B staining, confirming that SHOX expression is associated with the overexpression of this protein. Although most of the cathepsin B protein remained associated with lysosomes and co-localized with LAMP-2, a minor but considerable amount was detected in the cytosol, suggesting lysosomal leakage of cathepsin proteases into the cytosol and participation of active cathepsin B in SHOX-mediated cell death. To validate the contribution of cathepsin B in SHOX-mediated cell death, we treated SHOX-induced cell death with Ca074-Me which inactivates cathepsin B and other cysteine proteases (33). Use of the inhibitor partly protected the cells from SHOX-induced apoptosis further supporting the hypothesis that cathepsin B redistribution in the cytoplasm plays a key role in SHOX-induced cell death (Fig. 4). Additionally, cathepsins have been associated with necrotic and autophagic cell death (34). However, we did not observe any manifestations of these types of cell death upon SHOX expression in U2OS cells (data not shown).

**Figure 4.** Cathepsin B chemical inhibition rescues cells from SHOX-induced cell death. Ca074-Me inhibitor (1 or 2 μM) was added to SHOX induced (black bars) or not induced (white bars) cells for 5 days. After fixation, cells were stained with propodium iodide and analyzed by flow cytometry for the apoptotic subG1 cell population. Data represent average values with standard deviation bars from a representative experiment performed in triplicate. *P < 0.05; **P < 0.001.

**Reactive oxygen and nitrogen species are important mediators of SHOX-induced cell death**

One of the principal factors leading to LMP is oxidative stress due to elevated cellular levels of reactive oxygen and nitrogen species (ROS and RNS). For this reason, we examined the extent of oxidative stress in SHOX-expressing cells and its role in the cell death cascade triggered by the protein. Cellular levels of ROS and RNS were measured by flow cytometric analysis after labeling with an oxidation-sensitive fluorescent dye—DCFH-DA (35). Our analysis indicated that SHOX expression in U2OS cells was associated with accumulation of ROS and RNS (Fig. 5A). The oxidative stress was strongly reduced by either the antioxidative glutathione precursor N-acetyl-L-cysteine (NAC) or the peroxynitrite decomposition catalyst FeTPPS. Similar experiments using SHOX R153L- and SHOX L185X-expressing cells showed that these SHOX mutants are unable to induce ROS and RNS accumulation (Fig. 5A). Notably, antioxidative treatment with non-toxic doses of either NAC, reduced glutathione (GSH) or FeTPPS significantly reduced both SHOX-mediated lysosomal instability (Fig. 5B) and SHOX-induced cell death (Fig. 5C). These findings provide important evidence for the involvement of oxidative stress in the cell death cascade triggered by SHOX.

**DISCUSSION**

Previous work has demonstrated the ability of SHOX to trigger apoptotic signaling in different cell types (15). In this study, we investigated the cell death mechanisms underlying SHOX-induced cell death and the cytotoxic factors involved. For many years, caspases have been recognized as the universal group of proteases that mediate the regulation and execution of apoptotic cell death (36,37). The ability of SHOX to induce apoptosis was confirmed in this study by the detection of the active cleaved form of caspases 3 and 7 in SHOX-expressing cells. In addition, we also observed mitochondrial membrane permeabilization arguing for the activation of the intrinsic pathway of apoptosis. We also noticed activation of caspase-8 in SHOX-expressing cells. Although caspase-8 is the paradigm
Figure 5. Oxidative stress in SHOX-expressing U2OS cells. (A) Doxycycline-induced (SHOX IND.) or not induced (NOT IND.) cells expressing SHOX wt, SHOX R153L or SHOX L185X were grown for 72 h, then loaded for 1 h with 10 μM of DCFH-DA and analyzed by flow cytometry for intracellular levels of reactive oxygen (ROS) and nitrogen (RNS) species. In addition, cells expressing SHOX wt were grown in the presence or absence of N-acetyl cysteine (NAC) (5 mM) or FeTPPS [5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato iron(III) chloride; 40 μM]. The use of antioxidants N-acetyl cysteine and FeTPPS counteracted SHOX-induced ROS and RNS accumulation. (B) After 72 h of SHOX expression, cells grown in the presence or absence of NAC (5 mM) or reduced glutathione (GSH; 5 mM) were stained with LysoTracker Red and analyzed by flow cytometry. The percentage of cells with decreased fluorescence intensity signal, associated with LMP, are indicated. (C) Prevention of SHOX-induced cell death by N-acetyl cysteine (5 mM), glutathione (5 mM) or FeTPPS (40 μM). Cells were grown for 5 days in the presence (black bars) or absence (white bars) of doxycycline and treated with the indicated antioxidants or left untreated. After propidium iodide staining for 1 h, cells were analyzed by flow cytometry for DNA content, and percentages of cells displaying DNA fragmentation (subG1 cell population) are represented here. Data presented indicate means ± SD from a representative experiment out of three independent experiments. **P < 0.01; ***P < 0.001.
initiator caspase of the extrinsic apoptotic pathway, evidence has been accumulated demonstrating its activation independently of death receptors, acting downstream of mitochondrial depolarization as an amplifying executioner caspase (38–40).

Although caspases play a central role in the execution of the apoptotic program, recent studies have argued for the existence of other non-caspase death initiators, including cathepsins, calpains, granzymes and caspase-independent pathways (34,41–43). Among these, the lysosomal proteases cathepsins have been recognized as potent inducers of programmed cell death (44). Currently, accumulating evidence suggests that lysosomal proteases, when released to the cytosol upon stimulation or cell damage, may induce apoptosis, either directly by procaspase activation, or indirectly by acting on the mitochondria to release pro-apoptotic factors (30). We provide strong evidence that cathepsin B is involved in SHOX-induced apoptosis as (i) the protease was found in its catalytically active forms to relocate from the lysosomes to the cytosol of SHOX-expressing cells and (ii) treatment with Ca074-Me, a cathepsin B inhibitor [with some ability to inactivate also other cysteine proteases, e.g. cathepsin L (33)], partly protected the cells from undergoing cell death. However, the fact that Ca074-Me did not completely protect the cells argues for the involvement of other mediators (e.g. other cathepsins simultaneously released from damaged lysosomes which are not efficiently inactivated by Ca074-Me or ROS/RNS themselves) in SHOX-induced cell death whose identification warrants future investigation.

Partial lysosomal rupture has been associated with a variety of apoptotic signals, such as oxidative stress, serum withdrawal, lysosomotropic agents, DNA damage etc. (45–48). In particular, ROS have been shown to damage lysosomes via activation of phospholipase A2 and/or peroxidation of membrane phospholipids (49). Our results point to a critical role of ROS and RNS leading to lysosomal destabilization in SHOX-induced cell death. This idea is supported by the finding that antioxidant treatment partly protects SHOX-expressing cells from undergoing cell death, suggesting a primary function of reactive species in the initiation of this process. Moreover, antioxidant treatment significantly decreased the extent of LMP, strongly suggesting that increased ROS levels are responsible for lysosomal damage and consequently the release of lysosomal proteases to the cytosol in the course of SHOX-induced cell death. The mechanisms through which wild-type SHOX protein is able to induce oxidative stress currently remain unknown. One of the mutant SHOX form used in this study, R153L, has been identified in a patient with LWD (28) and was present in three affected individuals of this family and absent in any of the analyzed controls (Human SHOX Allelic Variant Database; www.shox.uni-hd.de). It carries a mutation within the homeodomain that impairs its transcriptional activity and renders it incapable of inducing apoptosis (19). The fact that we did not observe any of the wild-type SHOX cytotoxic activities (MOMP, LMP, ROS accumulation, cathepsin B cytosolic release) in R153L-expressing cells supports the idea of the involvement of SHOX-mediated transcriptional regulation in triggering these events.

A potential source of intracellular reactive species is the iron-catalyzed generation of hydroxyl radicals from H2O2. Lysosomes, which are the principal reservoir of chelatable iron from autophagic degradation of iron-containing cellular proteins, are thus able to accumulate high amounts of free radicals that could induce the peroxidation of lipids and thus compromise the integrity of the lysosomal membrane (50,51). One of these highly reactive pro-oxidants is peroxynitrite, a potent oxidant with a wide range of biological targets (52). The inhibition of SHOX-induced cell death by blocking the synthesis of peroxynitrite by FeTPPS treatment suggests that peroxynitrite could be also involved in the SHOX-induced oxidative stress leading to lysosomal membrane destabilization.

The ability of SHOX to trigger apoptosis has been initially demonstrated in a SHOX-inducible U2OS osteosarcoma cell line, the same model used also in this study, and later on has been confirmed in primary cells, including primary chondrocytes (15). Thus, although U2OS cells do not resemble the physiological interactions and complexity of the growth plate, they have proved useful as a valuable tool for an initial characterization of the SHOX cellular functions. SHOX expression in the hypertrophic chondrocytes of the growth plate, which undergo cell death during endochondral ossification, support an important role of SHOX in regulating chondrocyte survival, differentiation and death during long bone formation. Currently, it is a matter of debate which intracellular signaling pathways regulate the cell death of hypertrophic chondrocytes. A growing body of evidence suggests that elevated concentrations of extracellular inorganic phosphate and nitric oxide (NO) production are involved in this process. Inorganic phosphate induces apoptosis through rapid increase in NO and mitochondrial dysfunction in hypertrophic chondrocytes of avian growth plates (53–55), and by activating NO production and JNK-MAP kinase signaling in mammalian chondrocytes (56). Hypertrophic chondrocytes display a dramatic increase in the endoplasmic reticulum and the Golgi apparatus (57), with destruction of cellular material within autophagic vacuoles and potential involvement of lysosomal proteases. Noteworthy, during endochondral ossification cathepsin B has been detected by immunohistochemistry in growth plate sections from different species (58,59) and is believed to participate in the degradation of extracellular matrix components (60,61). Thus, we may speculate that SHOX could play an important role in regulating cathepsin B activity also in the context of growth plate chondrocytes during endochondral ossification.

Up to date, little is known about the role of reactive oxygen species in the life cycle of growth plate chondrocytes. A previous study has demonstrated that increased ROS levels are present in hypertrophic chondrocytes and, importantly, are responsible for inhibiting proliferation and inducing the hypertrophic phenotype, as treatment with NAC protects the cells from oxidative stress and inhibits chondrocytes hypertrophy (62). Additionally, Kim et al. have shown that ROS generation is required for the early stage of chondrogenic differentiation (63). Factors, such as IGF-1, FGF and TGF-B1, are known to modulate the intracellular ROS levels in articular chondrocytes (64). Our data provide hints for the critical involvement of oxidative stress in SHOX-mediated cell death. We speculate that SHOX-induced oxidative stress may be also relevant to the physiological cell death of hypertrophic chondrocytes, a hypothesis that warrants further investigation.

In conclusion, in this study we have further confirmed that SHOX expression induces apoptosis in U2OS cells and we have characterized the cellular mechanisms underlying this event. We discovered that SHOX is able to induce oxidative stress. This discovery provides important insights into the possible roles of SHOX in the regulation of cell death during the formation of long bones.
stress leading to lysosomal membrane rupture with subsequent cathepsin B relocation to the cytosol, MOMP and finally caspase activation (Fig. 6). Further investigations are required in order to address whether SHOX plays a similar role in chondrocytes of the growth plate.

MATERIALS AND METHODS

Cell lines and cell culture

The human osteosarcoma-inducible cell lines U2OS T-Rex (R712–07; Invitrogen) expressing either wild-type protein SHOX (8), R153L mutant (19) or L185X mutant (15) were maintained in DMEM medium supplemented with 10% tetracycline-free fetal bovine serum and selective antibiotics (50 μg/ml hygromycin B1, 200 μg/ml zeocin). For induction of SHOX expression, cells were treated with 1 μg/ml of doxycycline for the indicated time periods.

Analysis of lysosomal membrane integrity

Identification of acidic compartments by lysosomotropic agents was performed to estimate lysosomal stability in SHOX-expressing and control cells. AO is a metachromatic fluorophore that accumulates in active, intact acidic vesicles (including lysosomes) on the basis of proton trapping and emits green or orange-to-red fluorescence at low or high concentrations, respectively. For microscopic visualization of lysosomal leakage, U2OS cells were seeded into six-well plates at a density of 10^5 cells per well and were induced with 1 μg/ml of doxycycline for 48 or 72 h, or left uninduced. After harvesting, cells were incubated for 3 min at RT with 10 μg/ml of AO, rinsed in complete medium and analyzed on a Leica inverted microscope.

For flow cytometric analysis of LMP, SHOX-induced and control cells were harvested by trypsinization and incubated with 500 nM LysoTracker Red for 1 h at 37°C. Cells were then washed twice in PBS, passed through a mesh, and analyzed by flow cytometry, collecting the fluorescence intensity of at least 10,000 cells per sample. Loss of lysosomal LysoTracker red fluorescence emission measured on the FL2 channel was used as an indication for the extent of lysosomal membrane destabilization.

Analysis of reactive oxygen species and antioxidant treatment

Intracellular oxidative stress was assessed using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) that emits high fluorescence after its conversion to DCF through oxidation by ROS/RNS (65). Cells grown in the presence or absence of doxycycline were incubated with 10 μM DCFH-DA for 1 h at 37°C before harvesting. Then cells were washed twice, resuspended in 500 μl of PBS, and directly analyzed by flow cytometry. The antioxidants NAC (5 mM), glutathione (GSH; 5 mM) and FeTPPS (40 μM) were added to the culture medium at the time of doxycycline treatment (SHOX induction).

Apoptosis assay

For analyzing the subG1 apoptotic cell population, cells were trypsinized, washed with PBS and fixed for 16 h at 4°C in 70% ethanol. After incubation with RNase (100 μg/ml) and propidium iodide (25 μg/ml) for 1 h at 4°C, cells were analyzed by flow cytometry using Cell Quest Pro software package (BD Biosciences) as previously described (15).

Detection of caspases 3 and 7 was performed using the CellEvent™ Caspase 3/7 Green Detection Reagent (Invitrogen); 15,000 cells were seeded on glass cover slips (three wells, 14 mm diameter) and after 24 h induced with doxycycline (1 μM). After additional 72 h each well was loaded with 10 μM CellEvent™ Caspase 3/7 Green Detection Reagent for 30 min at 37°C. Cells were then washed and fixed in 3.7% paraformaldehyde for 15 min, followed by 4,6-diamidino-2-phenylindole (DAPI) staining. Positive cells were analyzed on a fluorescence microscope (KEYENCE BZ-9000E, Japan) with the BZ-II Analyzer software.

Analysis of MOMP

Loss of mitochondrial membrane potential, a characteristic of apoptosis, was assessed after staining with MitoTracker Red CM-H2XRos (Molecular Probes), a fluorescent dye that passively diffuses across the plasma membrane and stains mitochondria. Permeabilization of the mitochondrial membrane is associated with reduction of the red fluorescence emitted by the dye. For each cell line, three sets of six-well plates (10^5 cells per well) were prepared: one set was induced with doxycycline after 24 h, the second after 48 h, while the third was left untreated. After 120 h from plating, cells were harvested, washed and resuspended in 100 μl of 100 mM MitoTracker Red staining solution. After 1 h incubation at 37°C, cells were PBS-washed twice and analyzed on the FL2 channel of a flow cytometer.
Flow cytometry
A FACScan (argon-ion lamp, 488 nm excitation laser line; BD Biosciences) was used for the evaluation of lysosomal integrity, mitochondrial membrane potential and apoptosis induction in SHOX-induced versus control cells by flow cytometric analysis. Data were acquired on homogeneous populations, gated according to cell size (forward scatter values) and granularity (side scatter values) for LMP and MOMP, or un gated for the evaluation of subG1 population, and further processed with the CellQuest and Flow Jo (Tree Star, Inc.) software.

Cell fractionation and cathepsin B activity assay

3 × 10⁷ cells were seeded in 15-cm-diameter culture dish. After different periods of incubation with doxycycline, cells were washed twice, collected by scraping with a rubber scraper and pelleted (5 min at 180g, 4°C). Then cells were resuspended in 0.6 ml of hypotonic buffer [0.25 M glucose, 50 mM HEPES pH 7.4, 1 mM EDTA, protease inhibitor cocktail (Roche)] and homogenized in a cell cracker (bead diameter of 8.006 mm, 10–15 strokes). Nuclei and heavy mitochondria were excluded by centrifugation at 2000g for 10 min at 4°C, and supernatant was further centrifuged at 21 000g for 40 min at 4°C. The second supernatant was kept as a cytosolic extract and the lysosomal and mitochondria-enriched pellet was resuspended in 125 mM sucrose, 25 mM MES, 0.5 mM EDTA, 1 mM NAC and 1 mM of the fluorogenic cathepsin B-specific substrate Z-Arg-Arg-AMC (Calbiochem). The reaction was monitored for 1 h on a Fluoroskan FL spectrofluorimeter (Thermolabsystem) by measuring fluorescence at emission wavelength of 455 nm and an excitation wavelength of 360 nm.

Protein extraction and immunoblot analysis

Total cell extracts were prepared as previously described (15). Proteins from total cell lysates or subcellular fractions were size-separated by SDS–PAGE and then transferred onto a Hybond-P membrane (Amersham Biosciences). Membranes were blocked for 1 h at RT in PBS, 0.05% Tween 20, 5% non-fat dry milk before overnight incubation at 4°C with the following primary antibodies: anti-SHOX-β rabbit antibody [1:2000; ((15)]; anti-cathepsin B antibody [1:5000; (66)]; anti-cathepsin L antibody (1:1000; Santa Cruz); anti-caspase 8 antibody (1:1000; BD Pharmingen); anti-active caspase 3 antibody (1:1000; BD Pharmingen); anti-LAMP-2 antibody (1:1000; Santa Cruz); anti-M2PK antibody (1:1000; ScheBo Tech); anti-LC3 antibody (1:1000; Biozol); anti-actin antibody (1:10.000; MP Biomedicals); anti-β-tubulin antibody (1:5000; Sigma). After washing, membranes were probed with peroxidase-conjugated secondary goat anti-mouse or goat anti-rabbit antibodies (dilution 1:3000) for 1 h at RT. Proteins were visualized with the ECL detection kit. Band intensities were quantified using the ImageJ software.

Immunofluorescence

Cells were directly seeded on glass coverslips. After indicated treatments cells were fixed in PBS, 4% paraformaldehyde for 10 min and then permeabli zed for another 10 min in PBS, 0.2% Triton X-100. After a brief wash, cells were incubated for additional 20 min with normal goat serum (dilution 1:200; Santa Cruz Biotechnology) to block unspecific binding. Incubation with primary antibodies anti-LAMP-2 (1:100) and anti-cathepsin B (1:200) were carried out for 1 h in a dark humidified chamber. Cells were then PBS-washed three times and incubated for 1 h with Alexa Fluor 594-conjugated goat anti-mouse or Oregon Green 488-conjugated goat anti-rabbit secondary antibodies (1:200; Invitrogen). After extensive washing with PBS, cell nuclei were stained with 10 g/ml Hoechst 33342 (Sigma) for 2 min at RT. Coverslips were mounted onto glass slides with Elvanol (ICN) and images were acquired using a laser scanning confocal microscope (TCS SP2 scanning head and inverted DMIRBE microscope, 40× oil immersion HCX PL APO objective, Leica Confocal Scan software; Leica).

Statistical analysis

Data from multiple experiments performed in triplicates are expressed as the means ± SD. Differences between groups were assessed using an unpaired two-tail t-test.

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Conflict of Interest statement. None declared.

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