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Sox15 Is Required for Skeletal Muscle Regeneration

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The Sox genes define a family of transcription factors that play a key role in the determination of cell fate during development. The preferential expression of the *Sox15* in the myogenic precursor cells led us to suggest that the Sox15 is involved in the specification of myogenic cell lineages or in the regulation of the fusion of myoblasts to form myotubes during the development and regeneration of skeletal muscle. To identify the physiological function of Sox15 in mice, we disrupted the *Sox15* by homologous recombination in mice. Sox15-deficient mice were born at expected ratios, were healthy and fertile, and displayed normal long-term survival rates. Histological analysis revealed the normal ultrastructure of myofibers and the presence of comparable amounts of satellite cells in the skeletal muscles of *Sox15*$^{-/-}$ animals compared to wild-type animals. These results exclude the role of Sox15 in the development of satellite cells. However, cultured *Sox15*$^{-/-}$ myoblasts displayed a marked delay in differentiation potential in vitro. Moreover, skeletal muscle regeneration in *Sox15*$^{-/-}$ mice was attenuated after application of a crush injury. These results suggest a requirement for Sox15 in the myogenic program. Expression analyses of the early myogenic regulated factors *MyoD* and *Myf5* showed the downregulation of the *MyoD* and upregulation of the *Myf5* in *Sox15*$^{-/-}$ myoblasts. These results show an increased proportion of the Myf5-positive cells and suggest a role for Sox15 in determining the early myogenic cell lineages during skeletal muscle development.

Sox genes constitute a large family that are characterized by the presence of a specific type of DNA-binding domain called the high-mobility-group domain (HMG). According to the sequence similarity outside the HMG domain and the genomic organization, *Sox* genes can be divided into A to H subgroups (3). Analysis of expression patterns revealed that the expression of some *Sox* genes is cell-specific and restricted to definite developmental stages, whereas other genes are ubiquitously expressed. Furthermore, the expressions of different *Sox* genes overlap in many cell types and tissues (36). Gene knockouts in mouse and the identification of human mutations have demonstrated the essential role of several Sox family members in developmental processes, including sex differentiation, chondrogenesis, gliogenesis, B-cell development, and lens development (26, 36).

Murine Sox15 and its human orthologue gene, *SOX20*/*SOX15*, belong to group G of this family (26). Both proteins share 74% homology in their coding sequence. Although most of the Sox proteins are encoded by a single exon, murine and human Sox15 are the only members of the Sox family with an intron located at the 3′ end that does not interrupt the HMG domain (1). Existing reports on the expression of *SOX15* are somewhat controversial. By Northern blot analysis, SOX15 transcripts were only detected in fetal testis (15) but, by using a reverse transcription-PCR (RT-PCR) assay, human *SOX15* transcripts were amplified from a wide variety of fetal and adult tissues (35). Murine *Sox15* was cloned by using RT-PCR screening for genes that are differentially expressed between proliferating and differentiating stages of the myogenic cell line C2C12 (1). Similar to the expression pattern of the human *SOX15*, murine *Sox15* is ubiquitously expressed in different fetal and adult tissues, but transcripts have only been detected by RT-PCR (1). *Sox15* was found to be expressed in developing mouse gonads from embryonic day 11.5 (E11.5) to E13.5, with an increase in expression in the male gonad at E13.5, suggesting that Sox15 is involved in gonad development (25). Sox15 has also been postulated to play a critical role during myogenic differentiation. Studies carried out with the myogenic cell line C2C12 demonstrated that myotube formation could be blocked by overexpression of *Sox15* (1). *Sox15* is located on mouse chromosome 11 and closely linked to the *Eif4*, *Cd68*, *Supl15h*, and *Fxr2h* genes. Deletion from the genome of an ~35-kb fragment containing this gene cluster in the gene trap GT3-11 mouse line results in embryonic lethality of the homozygous mutant (19).

Skeletal muscle has an exceptional ability to regenerate after damage. This capacity for tissue repair is conferred by the satellite cells located between the basal lamina and the sarcolemma of mature myofibers. Upon injury, mitotically quiescent satellite cells reenter the cell cycle, proliferate to repopulate the satellite cell pool, and give rise to a large number of daughter myogenic precursor cells. Finally, myogenic precursor cells undergo multiple rounds of division before they are fused to existing myotubes or form new myofibers (13).

Here, we investigated the expression of the *Sox15* in primary myoblasts and after myotube formation. We found that *Sox15* expression is restricted to the nucleus of the myoblasts and downregulated during myogenic differentiation. In addition, we examined the consequences of the absence of Sox15 on gonadal and skeletal muscle development in vivo. Our results demonstrated that Sox15 does not play an essential role during development of either tissue, since mice lacking Sox15 are fully

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viable and fertile. To determine the role of Sox15 in myogenesis, we determined the differentiation potential of the primary *Sox15*$^{-/-}$ myoblasts and investigated the capacity of skeletal muscle to regenerate in *Sox15*$^{-/-}$ mice after crush injury. These experiments revealed that the differentiation of myoblasts into myotubes in vitro and the regeneration of skeletal muscle in vivo are delayed in the absence of Sox15.

MATERIALS AND METHODS

Generation of Sox15-deficient mice. A cosmid clone carrying the *Sox15* gene was isolated from a 129/Sv genomic mouse library (RZPD) by using of PCR fragment containing the cDNA sequence (1). For the determination of the restriction map of the *Sox15* locus and localization of the exonic sequences, the 4- and 10-kb XhoI genomic fragments were subcloned into the pZERO-TM-2 vector (Invitrogen) and used in Southern blot analysis. The Sox15-targeting vector was constructed by using the plasmid pPNT (34). The 5.5-kb HindIII fragment containing 5′-flanking sequence was isolated from the 10-kb XhoI subclone, inserted into SpeI/NotI-restricted pBluescript vector, and subsequently digested with XhoI and NotI. The isolated 5.5-kb XhoI/NotI fragment was then inserted into XhoI/NotI-digested pPNT vector (clone Sox15/1). The 3.5-kb KpnI/SalI fragment containing 3′ sequence of the gene was isolated from the 4-kb XhoI subclone and ligated into the EcoRI-digested clone Sox15/1 after filling in the ends with Klenow enzyme. The resulting 16-kb targeting vector (see Fig. 2A) was linearized with NotI and transfected into RI embryonic stem (ES) cells (40), and clones resistant to G418 (400 μg/ml) and 2 μM ganciclovir were isolated. Genomic DNA extracted from drug-resistant ES clones was digested with NheI, electrophoresed, and blotted onto nitrocellulose membrane. The 1.3-kb EcoRI-XbaI fragment located externally 3′ of the targeting vector (see Fig. 2A) was radioactively labeled and used to probe the Southern blots. Hybridization was carried out at 65°C overnight in solution composed of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, 0.1% sodium dodecyl sulfate (SDS), and 100 μg of denatured salmon sperm DNA/ml. Filters were washed twice at 65°C at a final stringency of 0.2× SSC–0.1% SDS. Cells from two recombinant ES clones were injected into C57BL/6J blastocysts, and these were transferred into DBA/BL6 pseudopregnant females (20). Germ line-transmitting chimeric males obtained from both lines were backcrossed to C57BL/6J and 129/Sv females. Progeny from all crosses were genotyped by PCR with three primers. A single sense primer, SF (5′-GTGTCTGTAGTGAGAAG GAAGGC-3′), was designed to amplify both wild-type and targeted loci. The first antisense primer, SR (5′-CCATGCCTCCAACCCACGAAT-3′), was designed to amplify the wild-type locus. The second antisense primer, Pgk (5′-TC TGAGCCCAGAAAGCGAAGG-3′), was designed to amplify the targeted locus (Fig. 2A). Thermal cycling was carried out for 35 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min.

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Göttingen.

Northern blots and RT-PCR. Total RNA was extracted from cell cultures and tissues by using a Qiagen RNA kit. For Northern blot analysis, 15 μg of RNA was electrophoresed in 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Amersham Pharmacia), and hybridized with the ^{32}P-labeled probe at the same conditions as used for Southern blot hybridizations.

RT-PCR assays were performed with 2 μg of total RNA and a One-Step RT-PCR kit (Qiagen). Primer sequences to amplify the *MyoD*, *Myf5*, and *myogenin* transcripts were described previously (6). PCR products were analyzed in 1.5% agarose gels. *MyoD*, *Myf5*, and *myogenin* cDNA fragments were cloned into the pGEM-T Easy vector, and the cloned fragments were used as specific probes for Northern blotting. The primers used to amplify *Sox15*, *Fxr2h*, and *Hprt* transcripts were 5′-CAACTATTCGACAGCCTACCTGCC-3′ and 5′-GTGTTT AGTGTGCATTCTGGTTCC-3′, 5′-TCATATTTGTTGGCATATTTG-3′ and 5′-CCTGAAAGAGAATCCTCTGAG-3′, and 5′-CCTGCTGGATTACATCA AAGCACTG-3′ and 5′-GTCAAGGGCATATCCTACAACAAAC-3′, respectively.

Isolation and culture of primary myoblasts. Satellite cells were isolated from 3-month-old male wild-type and homozygous mutant mice on the hybrid background by the method described previously (21). Briefly, lower hind limb muscles were dissected and dissociated at 37°C in 1% collagenase II–2.5 U of dispase (Roche Molecular Biochemicals)/ml for 45 min and triturated every 15 min. The cell suspension was filtered through 200-μm-pore-size mesh sieves and centrifuged for 5 min at 1,000 rpm. Cells were resuspended in growth medium consisting of Ham F-10 supplemented with 20% fetal calf serum, 2.5 ng of bFGF (Roche Molecular Biochemicals)/ml, 200 U of penicillin/ml, and 200 μg of streptomycin/ml and plated on collagen-coated cell culture dishes. All experiments were performed with cultures that had undergone between five and seven passages. To assay the differentiation potential, 1.0×10^5 cells from the fifth passage were plated per 35-mm dish. After 24 h, the medium was changed to differentiation medium (Dulbecco modified Eagle medium, 5% horse serum, 200 U of penicillin/ml, and 200 μg of streptomycin/ml).

Antibody generation and Western blots. A peptide corresponding to the sequence outside the HMG domain of Sox15 (residues 188 to 203 [CTFPQSDP RLQGELRP]) was synthesized, conjugated to keyhole limpet hemocyanin by using a KLH conjugation kit (Pierce Chemical Co.), and used for rabbit immunization (Eurogentec, Seraing, Belgium). The peptide was generated with an NH_2-terminal Cys residue, which was used to couple it to a matrix for affinity purification by using a Sulfolink kit (Pierce Chemical Co.). Immobilization and purification were performed according to the manufacturer's instructions.

Cell cultures were lysed in lysis buffer containing 100 mM Tris (pH 6.8), 4% SDS, 20% glycerol, and 5% mercaptoethanol. Proteins were resolved on Novex Tris-glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore). Blots were blocked with 5% dry milk in TBST (10 mM Tris [pH 7.5], 100 mM NaCl, and 0.1% Tween 20) before incubation with an anti-Sox15 antibody (dilution, 1:200) in TBST with 5% milk overnight at 4°C. After a washing step, bound antibodies were detected by using horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) and enhanced chemiluminescence (Amersham Pharmacia).

Immunohistochemistry. Myoblasts and fibroblasts were cultured in culture slides (Falcon). ES cells were dissociated with trypsin, washed with phosphate-buffered saline (PBS), and air dried on cover slides. Cells were fixed in 2% formaldehyde in PBS for 5 min and in methanol for 5 min at 4°C, washed three times with PBS, blocked in 10% normal goat serum, incubated overnight at 4°C with the primary antibodies, washed three times with PBS, incubated 1 h at 25°C with secondary antibodies, washed three times with PBS, and stained with DAPI (4′,6′-diamidino-2-phenylindole; Vector). Slides were examined with a BX60 microscope (Olympus, Hamburg, Germany) with fluorescence equipment and an analysis software program (Soft Imaging System, Münster, Germany). The primary antibodies and dilutions used were rabbit anti-Sox15 at 1:50, anti-desmin DE-U-10 (Sigma) at 1:50, anti-c-met SP260 (Santa Cruz) at 1:50, and monoclonal anti-myosin heavy-chain antibody MF20 at 1:20 (Developmental Studies Hybridoma Bank, Iowa City, Iowa). Secondary antibodies were goat anti-rabbit Cy3 and goat anti-mouse fluorescein isothiocyanate (Sigma).

Sox15-deficient and wild-type mice were perfused with PBS for 5 min, followed by treatment with fresh 4% paraformaldehyde for 15 min. Tissues were mounted in freezing medium. Cryotome sections (10 μm) were fixed with methanol for 10 min, blocked, and incubated with rabbit anti-Sox15 antibody as described above, followed by incubation with fluorescein isothiocyanate-labeled goat anti-rabbit antibody. They were then washed in PBS, stained with DAPI, and imaged as described above.

Construction of Sox15 expression vector and generation of stable transfected cell lines. A *Sox15* cDNA containing the entire coding sequence was amplified from total myoblast RNA by using Sox15 primers (5′-AAGAACCCTGTCTCG CTGAAGC-3′ and 5′-GTGTTTAGTGTGCATTCTGGTTCC-3′). The cDNA fragment was directly cloned into pGEM-T Easy (Promega), sequenced, and subsequently transferred into a phosphatase-treated pTri-EX-Neo1.1 vector (Invitrogen) as an EcoRI fragment (pSox15-Tri). For transfection, Swiss 3T3 cells were seeded into six-well plates (Nunc) at a density of 15,000 cells/cm^2. Swiss 3T3 cells were routinely cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. After a 24-h attachment period, cells in each well were transfected with 1 μg of Sox15 construct or pTri-EX-Neo1.1 vector by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Stable transfected clones were selected with G418 (250 μg/ml) for 14 days. Colonies resistant to G418 were picked and cultured separately into the wells of a 96-well plate and then replated into the wells of a 24-well plate.

Electron microscopy. Tibialis anterior (TA) muscles were dissected and fixed in freshly prepared 1.5% paraformaldehyde–1.5% glutaraldehyde in 0.15 M HEPES buffer. Tissue blocks were osmicated, stained in 1.5% aqueous uranyl acetate overnight, dehydrated in acetone, and finally embedded in longitudinal and transversal orientation in araldite as described previously (10). Approximately 400 myofiber and satellite cell nuclear profiles in randomly chosen fields were counted from sections of wild-type and mutant mice.

Induction of muscle regeneration and histological analysis. Regeneration of skeletal muscle was induced by a single brief freeze-crush injury of the TA muscles of 12-week-old male mice as described by McGeachie and Grounds (17). At 4 and 14 days after the crush injury, the damaged TA and untreated TA

muscle tissues from the contralateral limb were dissected and fixed in Bouin's fixative, dehydrated through a graded alcohol series, embedded in paraffin, sectioned at 10 μm, and stained with hematoxylin and eosin (HE).

RESULTS

High expression of *Sox15* in ES cells and satellite cells. To study the expression pattern of *Sox15*, Northern blot and RT-PCR analyses were performed with total RNA from different tissues and primary myoblasts. In Northern blot analyses, *Sox15* was exclusively expressed in ES cells and primary myoblasts (Fig. 1A and 2C). However, *Sox15* transcripts could be detected in all studied tissues by RT-PCR (Fig. 1B). To assess the specific expression of *Sox15*, Swiss 3T3 fibroblasts, ES cells, primary myoblasts, and differentiated myogenic cells were stained with the monospecific anti-Sox15 antibody. As can be seen in Fig. 1C, specific staining of the nuclei was found in ES cells and primary myoblasts but not in the nuclei of fibroblasts and differentiated myogenic cells. These results clearly demonstrate that *Sox15* is highly expressed in satellite cell-derived myoblasts and downregulated after the fusion and differentiation of the myogenic cells. The high expression of *Sox15* in ES cells and myogenic stem cells and the ability to detect *Sox15* transcripts in different adult tissues only by using RT-PCR lead us to suggest that expression of the *Sox15* is restricted to tissue stem cells. To examine this hypothesis, frozen sections of testis and TA muscle of 3-month-old mice were probed with the Sox15 antibody. DAPI staining was used to identify all nuclei within the sections, thereby allowing the determination of Sox15-positive nuclei. No positive nuclei were detected in testicular sections (data not shown), whereas ca. 1.5% of skeletal muscle nuclei (including satellite cell nuclei and myofiber nuclei) were positive. Satellite cells make up 2 to 7% of the nuclei associated with the muscle fibers in adult mice (2). The similar percentages of Sox15-positive nuclei and satellite cells associated with myofibers suggests that *Sox15* is expressed in quiescent satellite cells.

Targeted disruption of *Sox15*. To investigate the physiological functions of Sox15 in the mouse, we generated Sox15-deficient mice by gene targeting. A *Sox15* targeting construct was designed to replace a 2.5-kb genomic fragment containing the 5′ flanking region and a part of the exon 1 sequence encoding the HMG domain by the neomycin resistance gene (*neo*^{r}) (Fig. 2A). RI E5 cells were transfected with the targeting vector and selected for homologous recombination. Drug-resistant clones were selected, and DNA was screened by Southern blot analysis with a 3′ external probe. The external probe detected a 12-kb NheI wild-type fragment and a 10.8-kb NheI recombinant fragment (Fig. 2A and B). Two *Sox15*$^{+/-}$ ES cell clones injected into C57BL/6J blastocysts gave rise to chimeric mice that transmitted the *Sox15* mutation into germ line. Chimeric mice were intercrossed with C57BL/6J or 129/Sv females to establish the *Sox15*-disrupted allele on a C57BL/6J×129/Sv hybrid and on a 129/Sv inbred genetic background. The resulting progeny from the heterozygous intercrosses displayed a normal Mendelian ratio of *Sox15*$^{+/+}$, *Sox15*$^{+/-}$, and *Sox15*$^{-/-}$ animals, indicating that *Sox15* is not essential for embryonic development. To confirm the inactivation of the *Sox15*, we performed Northern and Western blot analyses. Myoblasts were isolated from the hind-limb muscles of 3-month-old wild-type and *Sox15*$^{-/-}$ mice and then cultured. Total RNA and protein were extracted from the fourth passage of the primary myoblast cultures. Northern blot analysis failed to detect *Sox15* mRNA in *Sox15*$^{-/-}$ myoblasts (Fig. 2C). The lack of the Sox15 protein in mutant mice was investigated by Western blot anal-

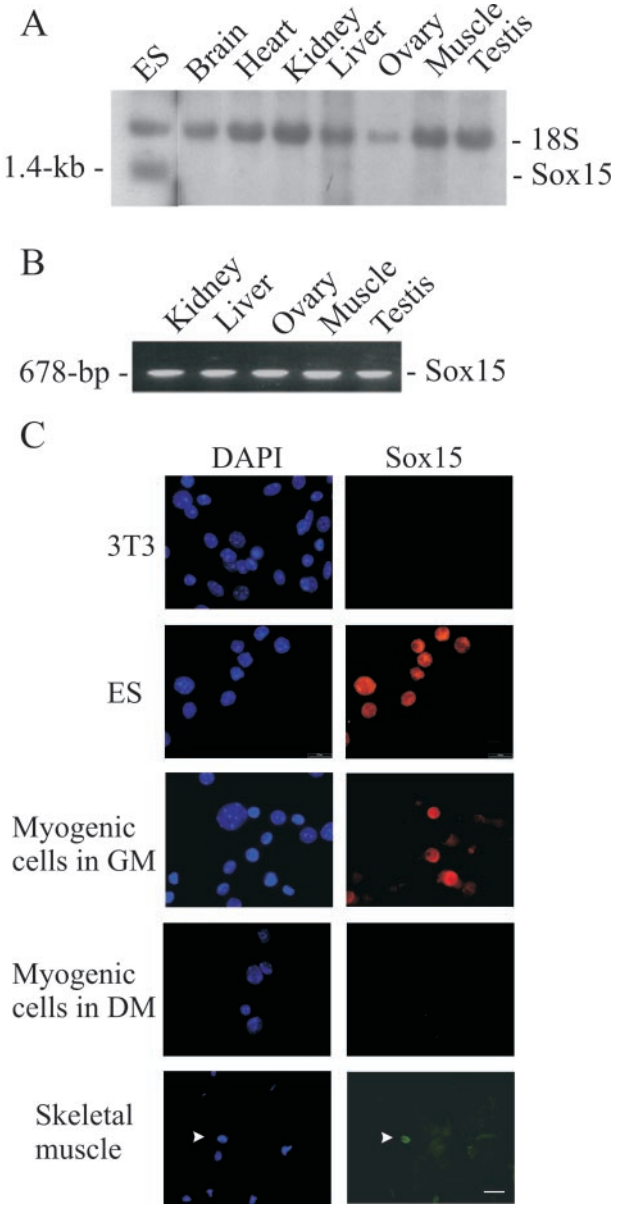

FIG. 1. Expression analyses of the *Sox15* gene. (A) Northern blot with total RNA from ES cells and different tissues hybridized with the *Sox15* cDNA revealed the 1.4-kb *Sox15* transcripts only in ES cells. The cross hybridization with 18S RNA revealed the integrity of RNA loading. (B) RT-PCR analysis with total RNA and *Sox15* specific primers showed the presence of a 678-bp amplified product in all examined tissues. (C) Immunostaining with anti-Sox15 antibodies (Sox15) revealed that expression of Sox15 is restricted to the nuclei of ES cells and to some nuclei of primary myogenic cells cultured in growth medium (GM). No Sox15 positive cells were detectable in cultures of Swiss 3T3 fibroblasts (3T3) or myogenic cells growing in differentiation medium (DM). In sections of skeletal muscle, the *Sox15* expression was associated with one nucleus (white arrowheads), which may be a satellite cell. Cells stained with Sox15 antibodies (right micrographs) were counterstained with DAPI (left micrographs) to show all nuclei. Scale bar, 100 μm.

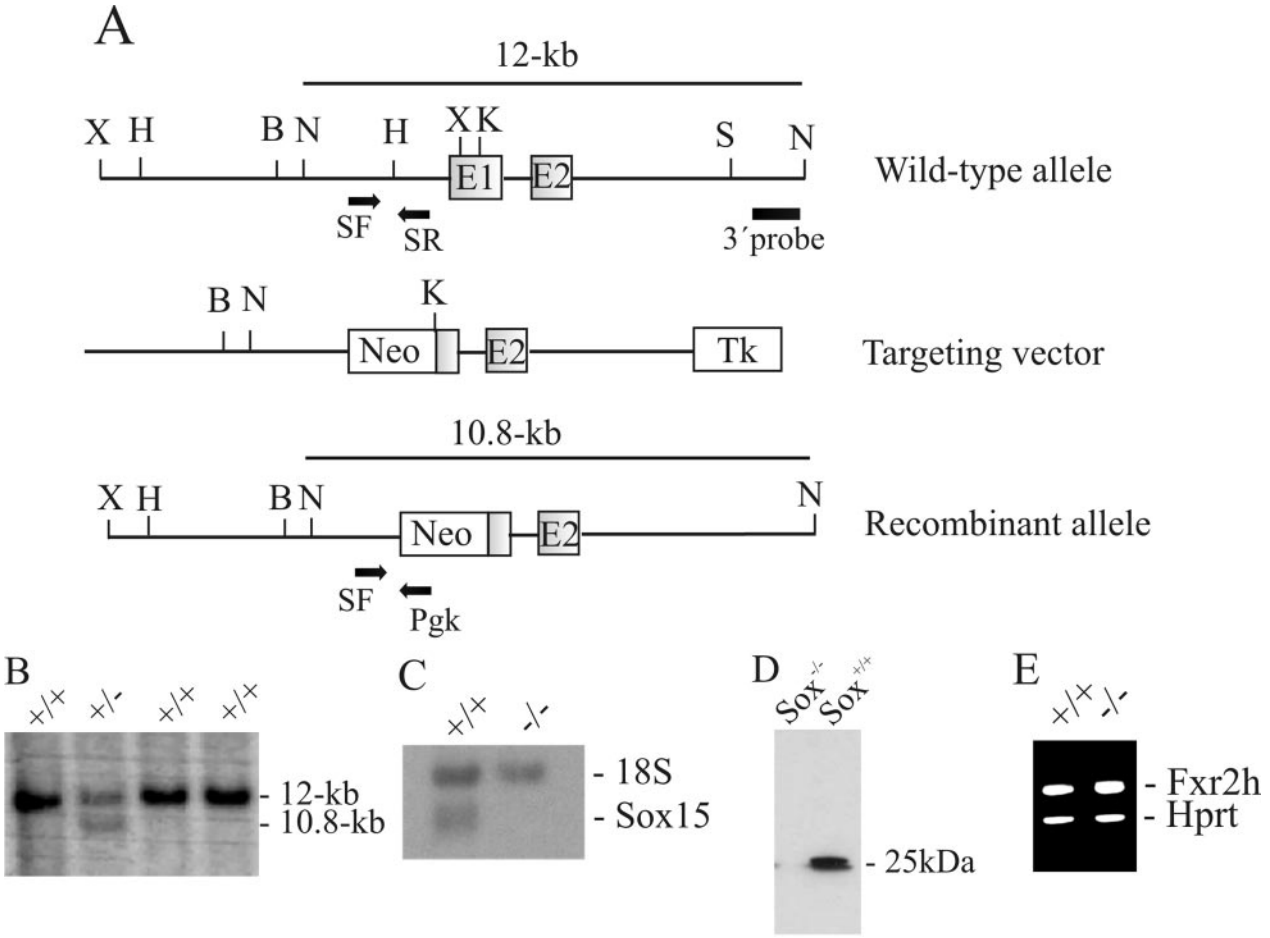

FIG. 2. Targeted disruption of the *Sox15* gene. (A) The structures of the wild type, the targeted vector, and the recombinant allele are shown together with the relevant restriction sites. A 2.5-kb HindIII/KpnI fragment containing 5′flanking region and a sequence encoding the HMG domain was replaced by a *pgk-neo* selection cassette (NEO). The 3′external probe used and the predicted length of NheI restriction fragments in Southern blot analysis are shown. The primers SF, SR, and Pgk used to amplify the wild-type and mutant allele by PCR are indicated. Abbreviations: TK, thymidine kinase cassette; B, BamHI; H, HindIII; K, KpnI; N, NheI; S, SalI; X, XhoI. (B) Southern blot analysis of the recombinant ES cell clones. Genomic DNA extracted from ES cell clones was digested with NheI and probed with the 3′ probe shown in panel A. The wild-type *Sox15* allele generated a 12-kb NheI fragment, whereas the targeted allele yielded a 10.8-kb NheI fragment, as indicated in panel A. (C) Northern blot analysis with total RNA isolated from $Sox15^{+/+}$ and $Sox15^{-/-}$ myogenic cultures was hybridized with the *Sox15* cDNA. The cross hybridization with 18S RNA revealed the integrity of RNA loading. (D) Western blot with proteins extracted from primary $Sox15^{+/+}$ and $Sox15^{-/-}$ myoblasts was probed with purified anti-Sox15 antibodies. The immunoreactive 25-kDa Sox15 was detectable in wild-type but not in $Sox15^{-/-}$ myoblasts. (E) RT-PCR analysis revealed the presence of the *Fxr2h* gene in the brains of $Sox15^{-/-}$ mice.

ysis with an antibody raised against an epitope mapping to the C-terminal tail of the protein. In Western blots, an antibody directed against the Sox15 recognized a band of the appropriate size in lysates of wild-type myogenic cells but not in lysates of $Sox15^{-/-}$ cells (Fig. 2D). *Sox15* is located downstream of the *Fxr2h* gene and in the same transcriptional orientation. The 2.5-kb deleted region in the genome of $Sox15^{-/-}$ mice is located in the 3′ flanking region of *Fxr2h*. To confirm that the deleted region did not disrupt the expression of the *Fxr2h*, an RT-PCR assay with total RNA from brain was performed. As shown in Fig. 2E, *Fxr2h* transcripts containing the sequence of exons 11 to 16 were detected in the brains of $Sox15^{-/-}$ mice.

Sox15 is not essential for gonad development and fertility. Previous reports have shown that *Sox15* is expressed at high levels in the sexually undifferentiated gonad and during the early stages of testis development in human and mouse (15, 25), suggesting that Sox15 is involved in gonad differentiation and/or testis development. The ratio of males and females in the progeny of heterozygous breedings was not significantly different from the expected sex ratio (1:1). Furthermore, PCR analysis for the Y-linked gene *Sry* showed that all analyzed $Sox15^{-/-}$ females ($n = 20$) were genetically females (data not shown). We have kept the $Sox15^{-/-}$ animals in our lab for over 16 months. Both males and females are as fertile as their wild-type counterparts, producing similar numbers of pups per litter. Histological examination of testis and ovary of adult mutants showed no obvious abnormalities in tissue architecture (data not shown). These results demonstrate that Sox15 is dispensable for gonad development.

***Sox15*$^{-/-}$ mice exhibit normal skeletal muscle development.** Heterozygous and homozygous mutants in both genetic backgrounds C57BL/6J×129/Sv and 129/Sv exhibited no visible

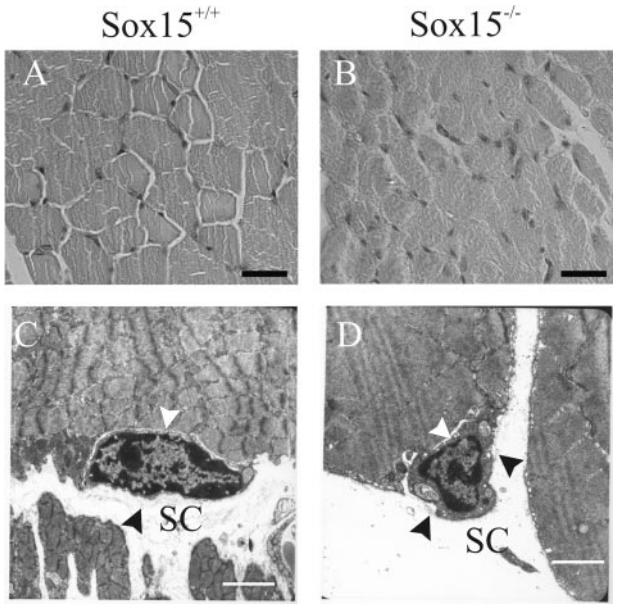

FIG. 3. Normal skeletal muscle development in *Sox15*$^{-/-}$ mice. Cross-sections of HE-stained TA muscles of 3-month-old wild-type (A) and Sox15-deficient (B) mice revealed a normal histological appearance of *Sox15*$^{-/-}$ skeletal muscle. (C and D) Electron microscopic examination of sections revealed the presence of morphological normal satellite cells in *Sox15*$^{-/-}$ TA muscle (D). Quiescent satellite cell in wild-type (C) and *Sox15*$^{-/-}$ (D) TA muscles showing characteristically condensed heterochromatin in the nucleus and little cytoplasmic fraction. The cell was in close contact between the mature myofiber and the continuous basal lamina (black arrowheads). Scale bars: A and B, 200 μm; C, 1.1 μm; D, 0.6 μm.

signs of impaired mobility or weight differences, suggesting that skeletal muscle development occurred normally in the absence of Sox15.

To determine a possible role of Sox15 in postnatal skeletal muscle growth, cross sections from mutant mice were analyzed and compared to the wild type. There was not a visible difference in the total skeletal muscle mass or in the diameter of myofibers compared to those of the wild type by light microscopic analysis of HE-stained TA muscle. Furthermore, skeletal muscles of *Sox15*$^{-/-}$ did not display centrally nucleated myofibers that are characteristic of regenerated skeletal muscle (Fig. 3A and B). Likewise, the thicknesses of diaphragms were indistinguishable between mutant and wild-type animals (data not shown). Next, we performed transmission electron microscopy to compare the TA muscle of wild-type and *Sox15*$^{-/-}$ mice at the ultrastructural level and to count the number of quiescent satellite cells. No differences in ultrastructure of myofibers were observed between wild-type and *Sox15*$^{-/-}$ (Fig. 3C and D). The satellite cells reside between sarcolemma and basal lamina of muscle fibers and have nuclei with a characteristic heterochromatic appearance. Analysis of these sections clearly revealed the presence of morphologically normal satellite cells in TA muscle of wild-type and *Sox15* mutants (Fig. 3C and D). We have examined ~400 myofiber and satellite cell nuclear profiles in sections of each genotype and found that 1.2% of counted nuclei were located in satellite cells compared to 1.0% in wild-type sections. These results exclude the role of Sox15 in the development of satellite cells.

***Sox15*$^{-/-}$ myogenic cells display a differentiation delay in vitro.** To test whether the differentiation program of myogenesis is normal in Sox15-deficient myoblasts, primary myogenic cells were isolated from the TA muscles of 3-month-old mice, cultured in growth medium for five passages, and then induced to differentiate by a shift to a differentiation medium containing a low concentration of mitogens. Under these conditions, myoblasts fused, formed multinucleated myotubes, and stained for myosin heavy chain (MHC). To confirm that the derived cells in the primary culture represent myogenic cells and to determine the developmental status of isolated myogenic cells, we assessed the expression of myogenic specific markers. As expected, the myogenic cells derived from *Sox15*$^{-/-}$ animals showed no detectable Sox15 protein (Fig. 4D). In contrast, >95% of the wild-type cells were Sox15 positive (Fig. 4B). c-met, which is a specific marker for quiescent and activated satellite cells in vivo and in vitro, was expressed in >93% of primary cells derived from wild-type and *Sox15*$^{-/-}$ animals (Fig. 4F and H). The expression of the intermediate filament, desmin, which is known to be expressed in myogenic precursor cells in vivo and in myoblasts in vitro but not in satellite cells (2, 11), was expressed at a low level in only 4.7% of the *Sox15*$^{-/-}$ cells (Fig. 4M). In contrast, ca. 84% of the wild-type cells expressed high levels of desmin (Fig. 4K). A terminal differentiation marker of myogenesis such as MHC was expressed in 12 and 6% of the primary wild-type and *Sox15*$^{-/-}$ myogenic cells (Fig. 4O and Q), respectively. These results suggest that cultured *Sox15*$^{-/-}$ myogenic cells represent an intermediate developmental stage between satellite cells and myogenic precursor cells.

After 3 days of culture of myogenic cells in differentiation medium, the proportion of differentiated myocytes and myotubes, which were immunostained with the monoclonal MHC antibody, reached >65% of cells in wild-type culture (Fig. 4S). In contrast, most *Sox15*$^{-/-}$ myocytes that were detected at the third day in differentiation medium contained one nucleus (Fig. 4U). However, differentiation potential to myocytes and myotubes was found to be comparable for primary wild-type and Sox15-deficient cells after 6 days of culture in differentiation medium. These results and the preferential expression of *Sox15* in undifferentiated myogenic cells suggest that the transition of the *Sox15*$^{-/-}$ myogenic cells from proliferation to differentiation is delayed.

Downregulation of *MyoD* and upregulation of *Myf5* in *Sox15*$^{-/-}$ myogenic cells. MyoD and Myf5, which are known to regulate the early myogenic program, are expressed exclusively in skeletal muscle, and their ectopic expression in nonmyogenic cultured cells induces the myogenesis program (37). To evaluate whether deregulation of *MyoD* and *Myf5* is responsible for the delayed differentiation of *Sox15*$^{-/-}$ myogenic cells, total RNA extracted from primary cultures in growth medium was used for Northern blot analysis. *MyoD* was expressed at high levels in wild-type cells and was downregulated in the *Sox15*$^{-/-}$ myoblasts. In contrast, *Sox15*$^{-/-}$ myogenic cells expressed higher levels of the *Myf5* than wild-type cells (Fig. 5). The expression of the myogenic differentiation factor myogenin could not be detected in *Sox15*$^{-/-}$ and wild-type myoblasts by Northern blot analysis; however, comparable amounts of myogenin transcripts were found in both cells by RT-PCR assay (data not shown). The downregulation of *MyoD* and the

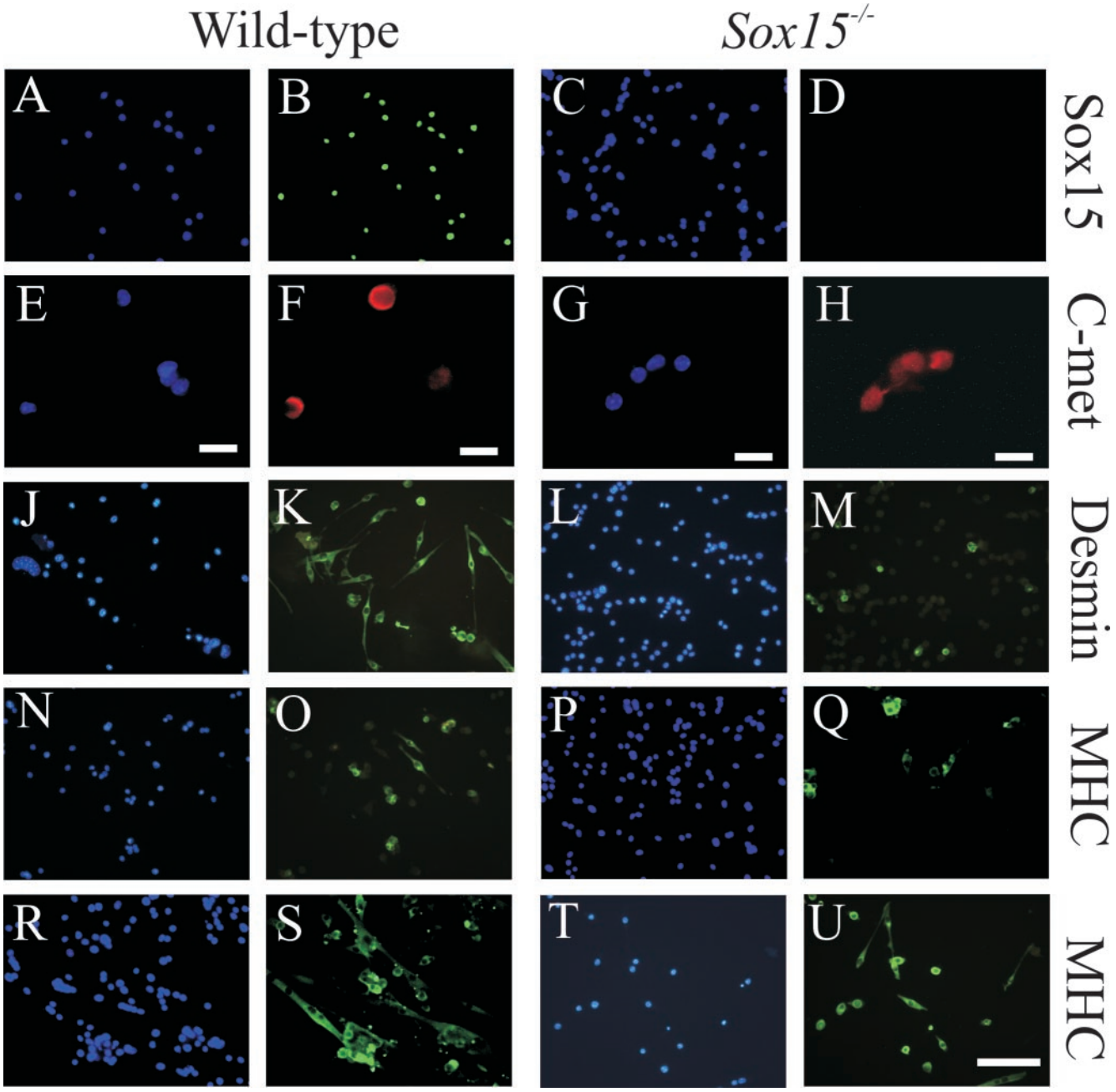

FIG. 4. Expression of myogenic markers in primary myoblasts of wild-type and Sox15-deficient mice. Primary myogenic cultures under growth conditions were analyzed by immunocytochemistry with anti-Sox15 (B and D), anti-c-met (F and H), anti-desmin (K and M) and anti-myosin heavy chain (MHC) (O and Q) antibodies. Differentiation potential of wild-type and *Sox15*$^{-/-}$ myogenic cells were determined after 3 days of culture in differentiation medium by immunostaining with anti-MHC antibody (S and U). Cells stained with antibodies were counterstained with DAPI (left micrographs). Scale bars: G and H, 200 μm; other panels, 1,000 μm.

upregulation of *Myf5* in primary cultures of *Sox15*$^{-/-}$ myoblasts suggests that the Sox15 is involved in the initiation of Myf5- and MyoD-dependent pathways during myogenesis. It has been shown that two myogenic lineages result from activated satellite cells. One expresses MyoD alone, and the other expresses Myf5 alone (6). Consistent with this hypothesis, we suggest that elevated expression of *Myf5* is due to an increase in the proportion of Myf5-positive cells among the *Sox15*$^{-/-}$ myoblasts. To evaluate whether the ectopic expression of *Sox15* in nonmyogenic cells induces the myogenic program, a Swiss 3T3 cell line was transfected with the *Sox15* expression vector, and stable clones expressing *Sox15* were examined by immunostaining with the Sox15 antibody. The importance of Sox15 for converting Swiss 3T3 fibroblasts into myogenic pathway was addressed by immunoassay with antibodies to MHC (MF20), a marker for skeletal muscle conversion, and by Northern blot and RT-PCR analyses of the expression of *MyoD* and *Myf5*. Sox15 stably transfected cells were not posi-

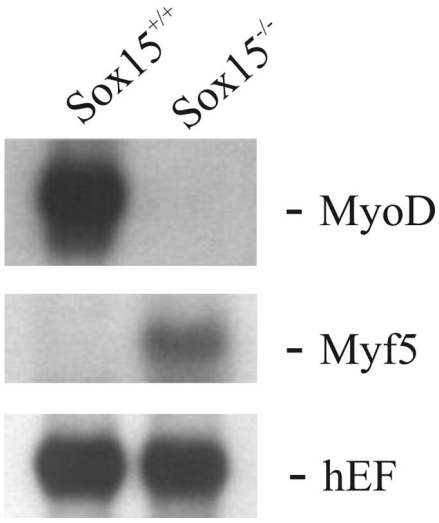

FIG. 5. Expression of myogenic determination factors *MyoD* and *Myf5* in primary *Sox15*$^{-/-}$ myoblasts. Northern blots with total RNA from wild-type and *Sox15*$^{-/-}$ cells revealed the downregulation of the *MyoD* and upregulation of *Myf5* in the *Sox15*$^{-/-}$ myoblasts. Rehybridization with human elongation factor1 (*EF1*) confirmed equal amounts of RNA.

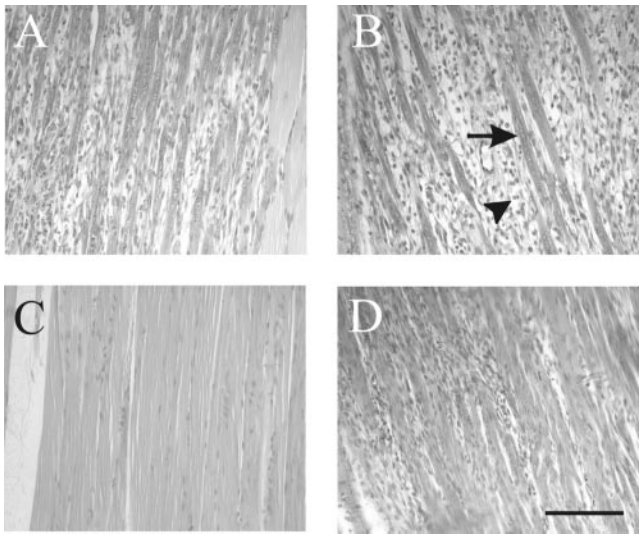

FIG. 6. Attenuated regeneration in *Sox15*$^{-/-}$ TA muscles after crush injury. HE staining of injured TA muscles of wild-type (A and C) and mutant mice (B and D) is shown. (A and B) Sections of damaged muscle at 4 days after crush injury displayed a significant accumulation of mononucleated cells (arrowhead) in the injured area. Newly formed myotubes with central nuclei (arrow) were smaller in *Sox15*$^{-/-}$ muscle (B) than in wild-type muscle (A). (C) At 2 weeks after injury, a complete restoration of damaged muscle was observed in the wild type. (D) In *Sox15*$^{-/-}$ muscle, however, a large number of mononuclear cells and limited regeneration were visible. Scale bar, 1,000 μm.

tive for MHC staining, and *MyoD* and *Myf5* transcripts are not detectable in transfected cells by Northern and RT-PCR assays (data not shown). These results demonstrate that the ectopic expression of *Sox15* in a Swiss 3T3 cell line is not able to induce the expression of early myogenic factors in this cell line.

Delayed regeneration of skeletal muscle of *Sox15*$^{-/-}$ mice. Postnatal growth of skeletal muscle is accompanied by the proliferation of satellite cells. Some satellite cells remain as undifferentiated myogenic stem cells. After muscle injury, satellite cells proliferate and fuse to form new muscle fibers. To determine whether the satellite cells are able to participate in the regeneration process in the absence of Sox15, crush injuries were performed on *Sox15*$^{-/-}$ and wild-type mice. Examination of HE-stained longitudinal sections of *Sox15*$^{-/-}$ TA muscle 4 days after crush injury revealed the significant accumulation of mononuclear cells and the presence of few myofibers (Fig. 6B). In contrast, the damaged muscle in wild-type exhibited well-advanced regeneration and contained long myotubes crossing the entire wound site (Fig. 6A). At 2 weeks after injury, wild-type skeletal muscle had a complete restoration of skeletal muscle architecture (Fig. 6C). In *Sox15*$^{-/-}$ mice, however, high numbers of mononuclear cells and limited regeneration were visible, as evidenced by the presence of thin myofibers with centrally located nuclei at the site of injury (Fig. 6D). These results indicate that the skeletal muscle regeneration is attenuated in the *Sox15*$^{-/-}$ mutant.

DISCUSSION

We show here that targeted disruption of the *Sox15* has no deleterious effect on gonadal and skeletal muscle development. The preferential expression of the *Sox15* in satellite cells in vivo and in primary myoblasts in vitro suggests a possible role for maintenance and proliferation of satellite cells or regulation of myogenesis. Electron microscopic analysis revealed no changes in the ultrastructure of myofibers and the presence of comparable amounts of satellite cells in skeletal muscles of *Sox15*$^{-/-}$ animals compared to wild-type mice. These results exclude the role of Sox15 in the development of satellite cells. However, cultured *Sox15*$^{-/-}$ myoblasts displayed a marked delay in differentiation potential in vitro. Moreover, skeletal muscle regeneration was attenuated after application of an injury to the TA muscle (Fig. 6). These results suggest a requirement for Sox15 in the myogenic program.

Activated satellite cells in adult skeletal muscle give rise to two myogenic lineages: one expresses MyoD but not Myf5 (MyoD$^+$), and the other express Myf5 alone (Myf5$^+$). These two myogenic lineages were found to develop distinct skeletal muscle sublineages in the embryo, suggesting that the expression of either MyoD or Myf5 might be sufficient for complete myogenesis (5, 8, 16, 32). However, the myogenic regulation factors that are involved in the initiation of MyoD- or Myf5-dependent pathway remain unknown (7). Gene knockout experiments have shown that MyoD and Myf5 play a redundant role in myogenesis. Deletion of either gene alone has no effect on muscle development, whereas deletion of both genes results in the complete absence of skeletal muscle (5, 22, 23). The elevation of *Myf5* and the absence of *MyoD* expression in primary cultures of *Sox15*$^{-/-}$ myoblasts reflects the increased proportion of the Myf5$^+$ cells in the cell population and suggests that the Sox15 is one of the early myogenic factors that regulate the cell fate of the myogenic precursor cells derived from the activated satellite cells. However, the ectopic expression of the *Sox15* in Swiss 3T3 fibroblasts did not induce the myogenic program as does ectopic expression of MyoD and Myf5 in fibroblast cell lines (4, 33, 39). The inability of Sox15

to induce the myogenesis in stable transfected fibroblasts can be attributed to the lack of other factors whose interaction with Sox15 is required for initiation of the MyoD- or Myf5-dependent pathway.

The primary *Sox15*$^{-/-}$ myoblasts display some features that are known for *MyoD*$^{-/-}$ cells. Like *Sox15*$^{-/-}$ myoblasts, the majority of MyoD-deficient cells are Myf5^{+} and do not express a detectable level of desmin, a muscle-specific intermediate filament protein. Moreover, cultures of *MyoD*$^{-/-}$ myoblasts in differentiation medium revealed a deficit in the differentiation potential (24, 38). In normal muscle, MyoD^{+} cells rapidly exit the cell cycle and differentiate (12), whereas Myf5^{+} cells have increased intensity for stem cell self-renewal rather than progression through myogenesis (18, 24). The difference between both lineages in proliferation and differentiation suggests that the continued proliferation of *Sox15*$^{-/-}$ myogenic cells under conditions that induce terminal differentiation contributes to the observed differentiation delay. Delay of the myotube formation in cultures of desmin-deficient myoblasts (*Des*$^{-/-}$) and in regenerating *Des*$^{-/-}$ skeletal muscle led other groups to suggest that the lack of desmin is a cause for the delayed fusion of the *MyoD*$^{-/-}$ myoblasts during myogenesis (30, 38). Therefore, it can be concluded that the downregulation of MyoD and desmin results in delayed differentiation of *Sox*$^{-/-}$ myogenic cells.

There now exist many examples in which deletion of a single member of a gene family has minimal consequences but simultaneous elimination of multiple members has a severe impact on development or adult physiology (9, 14, 31). For example, inactivation of *Sox5* and *Sox6* in the mouse revealed redundant roles for both genes in chondrogenesis. Whereas *Sox5*$^{-/-}$ and *Sox6*$^{-/-}$ mice are born with minor cartilage defects, *Sox5*$^{-/-}$ *Sox6*$^{-/-}$ double-knockout embryos have severe defects in notochord development (28, 29). Sox members such as *Sox8*, *Sox9*, and *Sox11* are expressed in the myogenic cell line C2C12. Interestingly, the expression of *Sox8* in skeletal muscle is restricted to the satellite cells. Like Sox15, Sox8-deficient mice are viable with no gross anatomic abnormalities (27). These observations suggest that functional redundancy of Sox8 and Sox15 is the most likely explanation for the mild phenotype in skeletal muscle development in *Sox15*$^{-/-}$ mice. Generation of *Sox8*/*Sox15* double-knockout mice should allow us to understand the compensation between them and the biological function of the Sox15.

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