

## SOX15 and SOX7 Differentially Regulate the Myogenic Program in P19 Cells

JOSÉE SAVAGE,<sup>a</sup> ANDREW J. CONLEY,<sup>b</sup> ALEXANDRE BLAIS,<sup>a,c</sup> ILONA S. SKERJANC<sup>a</sup>

<sup>a</sup>Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, Ontario, Canada;

<sup>b</sup>Department of Biology, Biological and Geological Sciences Building, University of Western Ontario, London, Ontario, Canada; <sup>c</sup>Ottawa Institute of Systems Biology, University of Ottawa, Ottawa, Ontario, Canada

**Key Words.** P19 cells • Gene expression • Transcription • Myogenesis • Sox15 • Sox7 • Mesoderm

### ABSTRACT

In this study, we have identified novel roles for Sox15 and Sox7 as regulators of muscle precursor cell fate in P19 cells. To examine the role of Sox15 and Sox7 during skeletal myogenesis, we isolated populations of P19 cells with either gene stably integrated into the genome, termed P19[Sox15] and P19[Sox7]. Both SOX proteins were sufficient to upregulate the expression of the muscle precursor markers Pax3/7, Meox1, and Foxc1 in aggregated cells. In contrast to the P19[Sox7] cell lines, which subsequently differentiated into skeletal muscle, myogenesis failed to progress past the precursor stage in P19[Sox15] cell lines, shown by the lack of MyoD and myosin heavy chain (MHC) expression. P19[Sox15] clones showed elevated and sustained levels of the inhibitory factors Msx1 and Id1, which may account for the lack of myogenic progression

in these cells. Stable expression of a Sox15 dominant-negative protein resulted in the loss of Pax3/7 and Meox1 transcripts, as well as myogenic regulatory factor (MRF) and MHC expression. These results suggest that Sox15, or genes that are bound by Sox15, are necessary and sufficient for the acquisition of the muscle precursor cell fate. On the other hand, knockdown of endogenous Sox15 caused a decrease in Pax3 and Meox1, but not MRF expression, suggesting that other factors can compensate in the absence of Sox15. Taken together, these results show that both Sox7 and Sox15 are able to induce the early stages of myogenesis, but only Sox7 is sufficient to initiate the formation of fully differentiated skeletal myocytes. *STEM CELLS* 2009;27:1231–1243

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

During the process of vertebrate skeletal myogenesis, signaling molecules such as Wnts, bone morphogenetic proteins, and Sonic Hedgehog (Shh) are secreted from tissues surrounding the somite [1–3]. These extracellular cues activate a cascade of transcription factors that leads to the expression of the myogenic regulatory factors (MRFs) MyoD, Myf5, myogenin, and MRF4, which control the end-stages of myogenesis [4]. Induction of MRF expression in response to these signals appears to be mediated by many transcription factors including Pax3, Meox1/2, Six1, and Gli2 [5–12].

Pax3 and Meox1 are part of a regulatory network of transcription factors that regulate myogenesis [9, 11, 13]. Pax3 is expressed in the mediolateral dermomyotome and migrating limb precursors [14, 15], while the closely related Pax7, is also expressed in the dermomyotome and may have redundant functions with Pax3 [16]. Cell culture studies reveal a role for Pax3 in regulating the commitment of cells to the myogenic lineage. Ectopic expression of Pax3 in embryonic tissues is

sufficient to activate MyoD and Myf5 expression [10, 17], and overexpression in P19 cells also leads to the induction of myogenesis and MRF expression [11]. P19 cells are pluripotent embryonic carcinoma (EC) cells, derived from mouse embryonic stem cells, that can differentiate into skeletal muscle in a dimethyl sulfoxide (DMSO)- and aggregation-dependent manner [18]. Identification of a Pax3-binding site within the Myf5 regulatory region suggests direct activation of Myf5 by Pax3 in developing murine limbs [19]. Furthermore, P19 cells expressing a dominant-negative form of Pax3, termed Pax3/EnR, are unable to undergo myogenesis or express MyoD [11]. Expression of Pax3/EnR and Pax7/EnR also inhibits MyoD expression in the mouse embryo [5] and animals lacking both Pax3 and Pax7 form myotomal muscle, but do not develop primary or secondary muscle fibers [20]. Pax3/Pax7 are essential for the formation of satellite cells, suggesting that the Pax3/Pax7 pool of progenitor cells is responsible for nearly all muscle formation [20, 21].

A role for Meox1 in regulating skeletal myogenesis and Pax3 expression has been demonstrated [8, 9]. Both Meox1 and Meox2 homeobox family members are initially expressed

Author contributions: J.S.: conception and design, collection and/or assembly of data, creation of all figures in manuscript, data analysis and interpretation, manuscript writing; A.C.: collection and/or assembly of data; A.B.: collection and/or assembly of data; I.S.S.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Correspondence: Ilona S. Skerjanc, Ph.D., Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5. Telephone: (613) 562-5800, ext. 8669; Fax: (613) 562-5452; e-mail: iskerjan@uottawa.ca Received January 12, 2009; accepted for publication March 5, 2009; first published online in *STEM CELLS EXPRESS* March 5, 2009. © AlphaMed Press 1066-5099/2009/\$30.00/0 doi: 10.1002/stem.57

in the somites, and their expression becomes localized to the dermomyotome and the developing limb bud, respectively, as development proceeds [7, 22]. Mice carrying null mutations for both *Meox* genes show a loss of Pax3 and Pax7, in the somite [8]. These results place Meox1 before Pax transcription factors in the molecular hierarchy controlling myogenesis. Consistent with this, a dominant-negative Meox1 transcription factor, Meox1/EnR, downregulates Pax3 expression and ablates myogenesis in P19 cells [9]. Further, Wnt signaling can activate Pax3 expression, although it is unclear whether this activation is direct or indirect [23–26]. Although much is known about the downstream effects of Pax3 and Meox1 during myogenesis, less is understood about the factors that regulate their gene expression.

Sox transcription factors have been characterized for their involvement in muscle development. Sox8 has been identified as a marker of adult satellite stem cells, and has the ability to inhibit differentiation of cultured primary myoblasts when ectopically expressed [27]. Studies have implicated another factor, Sox15, as a regulator of myogenesis. Although Sox15 expression has not yet been examined in the somite, it is expressed at high levels in the mouse blastocyst, embryonic stem cells, and in satellite cells [28–31]. Murine Sox15 is downregulated during C2C12 differentiation and can block myotube formation when overexpressed [32]. Moreover, disruption of Sox15 in mice resulted in the loss of satellite cell formation [30] and the attenuation of adult muscle regeneration with a decrease in MyoD levels [33]. Sox15, along with Fhl3, binds and regulates Foxk1 expression, regulating adult myogenic progenitor cells [30]. Sox7, another member of the Sox family [34–37] is detected in the somite of mice as early as embryonic day 7.5 [35]. Studies in *Xenopus* have shown that xSox7 can induce the expression of mesoderm inducing genes *Xnr1-6* and *Mixer*, suggesting a role for Sox7 in regulating the cell fate of mesodermal cells [38]. In addition, Sox7 has been extensively characterized for its role in controlling arteriovenous specification in zebrafish [39–41] and *xenopus* [42].

Based on the evidence in the literature showing that Sox15 and Sox7 are involved in controlling the cell fate of mesodermal derivatives, the present study aims to further characterize the role of SOX transcription factors in regulating the expression of skeletal muscle precursor genes. The P19 cell model was chosen because the differentiation of these cells follows early embryonic pathways [11, 26, 43] and the DMSO-dependence of the differentiation allows for both gain- and loss-of-function studies. Here we show that while both Sox15 and Sox7 can induce an early skeletal muscle mesoderm phenotype, expressing Pax3/7, Meox1, and Foxc1, only Sox7 can initiate the entire pathway leading to skeletal muscle formation.

## MATERIALS AND METHODS

### DNA Constructs

Expression constructs of PGK-Puro, PGK-LacZ, B17, PGK-Pax3, PGK-Meox1, CMV-Gli2, and activated CMV- $\beta$ -catenin have been described previously [9, 11, 26, 44]. CMV-Sox15 was kindly provided by F. Béranger [32]. PGK-Sox7 was created by excision of the Sox7 ORF from the pCMVScript vector (kind gift from Y. Hayashi, Japanese Science and Technology Agency). The ends of the excised insert were blunted to form compatible ends for cloning into the SmaI site of the PGK vector, which has been described previously [45].

The dominant-negative Sox15/EnR fusion protein was created by polymerase chain reaction (PCR) amplification of the engrailed (EN-2) repressor domain using the following oligonucleotides: EnR-F 5'-AACTCGAGAGAGGAGAAGGATTC-CAAGCCC and EnR-R 5'-TTGAATTCCTAGCCCA-GAGTGGCGCTGGCTT. Restriction sites for *XhoI* and *EcoRI* were introduced for cloning purposes and are italicized in the sequences. The activation domain of Sox15 was removed by digesting the pRK5-Sox15 vector using the restriction enzymes *XhoI* and *EcoRI* (removes nucleotides 376–696 from the Sox15 cDNA) and replaced by ligation with the engrailed repressor domain. DNA sequencing was performed to ensure that the resulting chimeric protein was in-frame.

The 1.6 kb fragment upstream of the Pax3 transcriptional start site [46] was amplified by PCR from 50 ng of genomic DNA, isolated from P19 cells [47]. To amplify the promoter region, the 5' oligonucleotide utilized was AAAGCTAGC-GAGCTCTAATGCTCCTCC and the 3' oligonucleotide was AAACCTGAGCACCAGTGCAGGGATCC, at 0.25  $\mu$ M each. Amplification (30 cycles) was performed with annealing at 56°C, in the presence of Q solution (Qiagen, Mississauga, ON). The amplified fragment was cloned into the luciferase reporter vector pGL3-basic (Promega, Madison, WI, <http://www.promega.com>) via the *NheI* and *XhoI* sites, respectively (italicized in the oligonucleotide sequences) and sequenced.

### Cell Culture and Transfections

P19 EC cells were cultured as described previously [48] in  $\alpha$ -minimal essential media (Invitrogen, Burlington, ON, Canada) supplemented with 5% cosmic calf serum (Hyclone, Logan, UT, <http://www.hyclone.com>) and 5% fetal bovine serum (FBS) (CanSera, Rexdale, ON, Canada). P19[control], P19[Sox15], P19[Sox15/EnR], and P19[Sox7] cell lines were created as described previously [9, 11, 26]. Cells were differentiated in the presence or absence of DMSO as described previously [44]. RNA was harvested on the days indicated for quantitative (Q)-PCR analysis and cells were fixed for immunofluorescence. Seven clonal populations were examined for the P19[Sox15] cell line, three for P19[Sox15/EnR], four for P19[Sox7], and four for P19[control] cells. Little variability was observed between clonal populations for a given cell line.

### Luciferase Reporter Assays

For reporter analysis, 200,000 cells/well were plated into 6-well plates and transiently transfected 24 hours post-plating. Transfection mixtures were composed of 0.2  $\mu$ g *Renilla* luciferase, 0.5  $\mu$ g Pax3 promoter-luciferase, and 0.5  $\mu$ g CMV-Sox15 and/or 2.5  $\mu$ g CMV-Sox15/EnR, as indicated. Cells were harvested 20–24 hours following transfection using the Dual Luciferase Kit, according to the manufacturer's protocol (Promega) and samples were analyzed using an LmaxII384 luminometer (Molecular Devices). All transfections were done in duplicate, and results shown are from four independent experiments. To determine the statistical differences among means, statistical analysis was done using the student's *t* test.

### Immunofluorescence

Myosin heavy chain (MHC) expression was detected using the MF20 monoclonal antibody supernatant as described previously [44]. A 1:100 dilution of goat anti-mouse Cy3-linked antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, <http://www.jacksonimmuno.com>) in phosphate-buffered saline (PBS) was used as a secondary antibody. For Pax3 and Meox1 detection, coverslips were fixed at  $-20^{\circ}$ C in

acetone for 10 minutes before rehydration in PBS. Sox15 and Myc staining were performed on cells fixed in 3.7% paraformaldehyde for 20 minutes at room temperature. Following permeabilization in 0.5% Triton X-100/PBS, cells were blocked using a 10% FBS, 0.1% bovine serum albumin, and 0.1% Triton X-100 solution in PBS at room temperature for 1 hour. The coverslips were incubated overnight using a 1:50 dilution of Meox1 antibody (sc-10185, Santa Cruz Biotechnology, Santa Cruz, CA, <http://www.scbt.com>), a 1:50 dilution of Pax3 antibody (clone 274212, R&D Systems, Minneapolis, MN, <http://www.rndsystems.com>), a 1:100 dilution of a Sox15 antibody (kind gift from Ibrahim M. Adham) or a 1:100 dilution of a myc antibody (clone 9E10, Santa Cruz Biotechnology, Santa Cruz, CA) followed by a 1 hour room temperature incubation with a 1:100 dilution of donkey anti-goat Cy3-linked antibody (Jackson ImmunoResearch Laboratories) in PBS, or a 1:100 dilution of goat anti-mouse Cy3-linked antibody (Jackson ImmunoResearch Laboratories) in PBS. Immunofluorescence was detected using a Zeiss Axio-scope microscope. Images were captured on a Sony 3CCD camera and processed with Axiovision, Adobe Photoshop7 and Canvas 8. For cell counting experiments, 10 fields of view per coverslip were counted using the 20 $\times$  objective. Two coverslips for each treatment were counted, and the results shown are the average of three independent differentiations  $\pm$  SEM.

### Reverse Transcription and Q-PCR

RNA was isolated using the RNeasy Mini Kit, according to the manufacturer's protocol (Qiagen, Mississauga, ON, Canada). One microgram of the purified RNA was used during the first strand DNA synthesis reaction using the Quantitect Reverse Transcription Kit, as per the manufacturer's specifications (Qiagen, Mississauga, ON, Canada). For the PCR reaction, 1/20th of the reverse transcription was used as a template for quantitative PCR amplification, using the FastStart SYBR Green kit from Roche (Roche Applied Sciences, Laval, QC, Canada). All reactions were performed and analyzed using the ABI 7,300 system and SDS analysis software (Applied Biosystems, Streetsville, ON, Canada). Primers used for quantitative detection of gene expression are listed in supporting information Table 1.

### Microarray Analysis

For microarray analysis using the mouse genome 430 v2.0 Affymetrix array, P19[Sox15] and P19[control] cells were differentiated in the absence of DMSO, and RNA was extracted on day 2 using the RNeasy Mini Kit (Qiagen, Mississauga, Canada), according to the manufacturer's instructions. All procedures including labeling, hybridization, and scanning were performed at the Ottawa Genome Centre (Ottawa, ON, Canada). Data analysis was performed with the dChIP program [49], using the PM-only method and filtering out probes with near-background expression levels in both samples. A probe representing the Sox15 mRNA was among the most induced genes in this analysis.

### RNA Interference

Complementary DNA sequences targeting nucleotides 269–287 (5'-TTTGGATGAAGAGAAGCGACCCTTTCAAGAGAAGGGTCGCTTCTTTCATCGCTTTTT-3' and 5'-CTAGAAAAGCGATGAAGAGAAGCGACCCTTCTTTGAAAGGGTCGCTTCTTTCATC-3') and nucleotides 514–533 (5'-TTTGCCTGGCAGTTACACCTTCTTCAAGAGAAAGAGGTGTAAGTCCAGGCATTTTT-3' and 5'-CTAGAAAAATGCCTGGCAGTTACACCTTCTTCTTGAGAAGAGGTGTAAGTCCAGG-3' of mouse Sox15 (NM\_009235) were

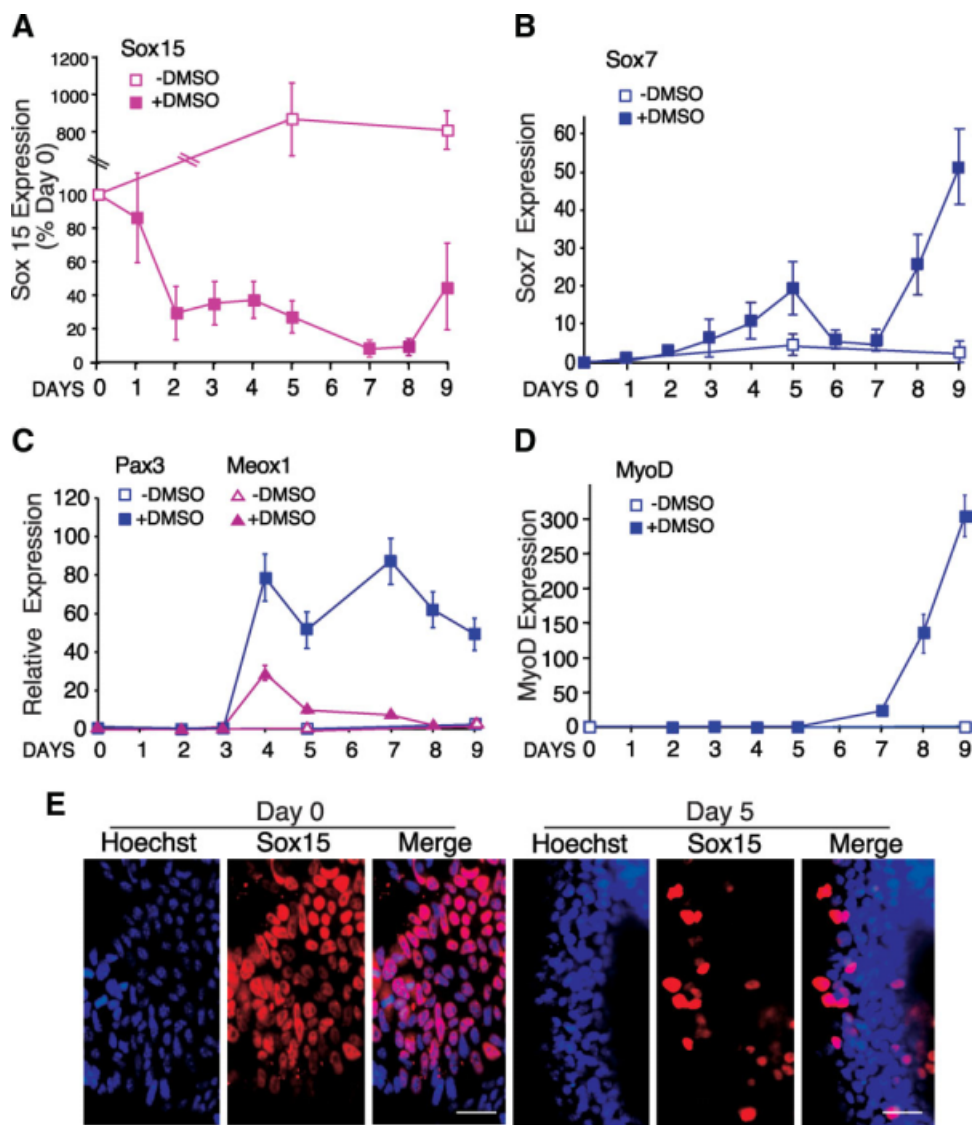
annealed and cloned into mU6pro using *BbsI* and *XbaI* restriction enzymes. A control vector was created using a scrambled sequence that is not complementary to any sequences in the mouse genome (5'-TTTGGCTAAGCGAGCTGCTAGAGTTCAAGAGACTCTAGCAGCTCGCTTAGCTTTTT-3' and 5'-CTAGAAAAAGCTAAGCGAGCTGCTAGAGTCTCTGAACTCTAGCAGCTCGCTTAGC-3'). The mU6pro vector was a generous gift from Dave Turner (University of Michigan, Ann Arbor, MI) and has been described previously [50]. For the generation of stable cell lines expressing the short hairpin constructs, 0.8  $\mu$ g of either shSox15 or shScrambled was transfected into P19 cells as described previously [44]. Following puromycin selection, individual clones were either picked and expanded for further analysis or pooled together and further analyzed.

### Chromatin Immunoprecipitation

Five 150 mm dishes of day 5 P19[Sox15] aggregates were fixed using 1% formaldehyde at room temperature for 45 minutes, and quenched by adding 0.125 M glycine. Cells were washed in ice-cold PBS and resuspended in lysis buffer one (50 mM HEPES-KOH pH 7.4, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100 and complete protease inhibitor cocktail; Roche Applied Sciences, Laval, QC) and incubated at 4°C for 10 minutes with rocking. Cells were then resuspended and incubated for an additional 10 minutes at room temperature in lysis buffer two (200 mM NaCl, 1 mM EDTA [pH 8.0], 0.5 mM EGTA [pH 8.0], 10 mM Tris [pH 8.0], and protease inhibitor cocktail) before resuspension in sonication buffer (1 mM EDTA [pH 8.0], 0.5 mM EGTA [pH 8.0], 10 mM Tris [pH 8.0], and protease inhibitors). Cells were sonicated using a Sonic Dismembrator (Fisher Scientific International, Hampton, NH, <http://www.fisherscientific.com>) for a total of 15  $\times$  30-second pulses (1-minute rest between pulses) and lysates were cleared by centrifugation at 13,000 rpm for 30 minutes at 4°C. The lysates were pre-cleared by incubation with protein-G sepharose beads for 1 hour, and the chromatin was separated into three aliquots supplemented with 1% TritonX-100, 0.1% sodium deoxycholate, 1 mM EDTA, and 1 mM AEBSF. An input sample of 1% of the total chromatin was set aside. For immunoprecipitation, 5  $\mu$ g of Sox15 antibody (sc-17354, Santa Cruz Biotechnology, Santa Cruz, CA) or 5  $\mu$ g of IgG antiserum (Zymed Laboratories, CA) was used. Following overnight incubation with the antibodies, the immune complexes were captured by addition of protein-G sepharose beads for 1 hour, followed by 10-minute washes in each of the following buffers: low-salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high-salt (low-salt buffer with 500 mM NaCl) and LiCl (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris pH 8.1), and TE. Protein/DNA complexes were eluted using 50 mM Tris pH 8.0, 1 mM EDTA, 1% SDS, 50 mM NaHCO<sub>3</sub> and crosslinks reversed overnight at 65°C by the addition of 200 mM NaCl. Samples were treated with 20  $\mu$ g RNase A and 40  $\mu$ g Proteinase K and DNA was purified using Qiagen's PCR Purification Kit (Qiagen, Mississauga, ON). Relative enrichment of binding sites compared with the IgG negative control immunoprecipitation were analyzed using SYBR Green real-time PCR, as described earlier (supporting information Table 1).

### Statistical Analysis

Statistical differences between means were calculated using the Student's *t* test. *p* values of at least *p* < .05 were considered significant.



**Figure 1.** Sox15 expression is downregulated in a population of cells during myogenesis in P19 cells. (A–D): P19 cells were differentiated in the presence and absence of DMSO, and RNA was harvested daily for 9 days for analysis by quantitative polymerase chain reaction. The data was normalized to glyceraldehyde 3-phosphate dehydrogenase, and results were expressed relative to day 0, except for the Sox15 graph, in which results are expressed as a percentage of day 0 expression. Error bars represent average  $\pm$  SEM, and the reactions were performed in triplicates. The experiments shown in (A) and (B) represent  $n = 3$  and (B) and (D) are representative of a typical differentiation. (E): The expression pattern of Sox15 protein on day 0 and 5 was confirmed by immunofluorescence using an anti-Sox15 antibody (red). Nuclei were stained by Hoechst (blue). Scale bar = 20  $\mu$ m. Abbreviation: DMSO, dimethyl sulfoxide.

## RESULTS

### Sox15 Expression Is Downregulated in a Population of Cells During Myogenesis in P19 cells

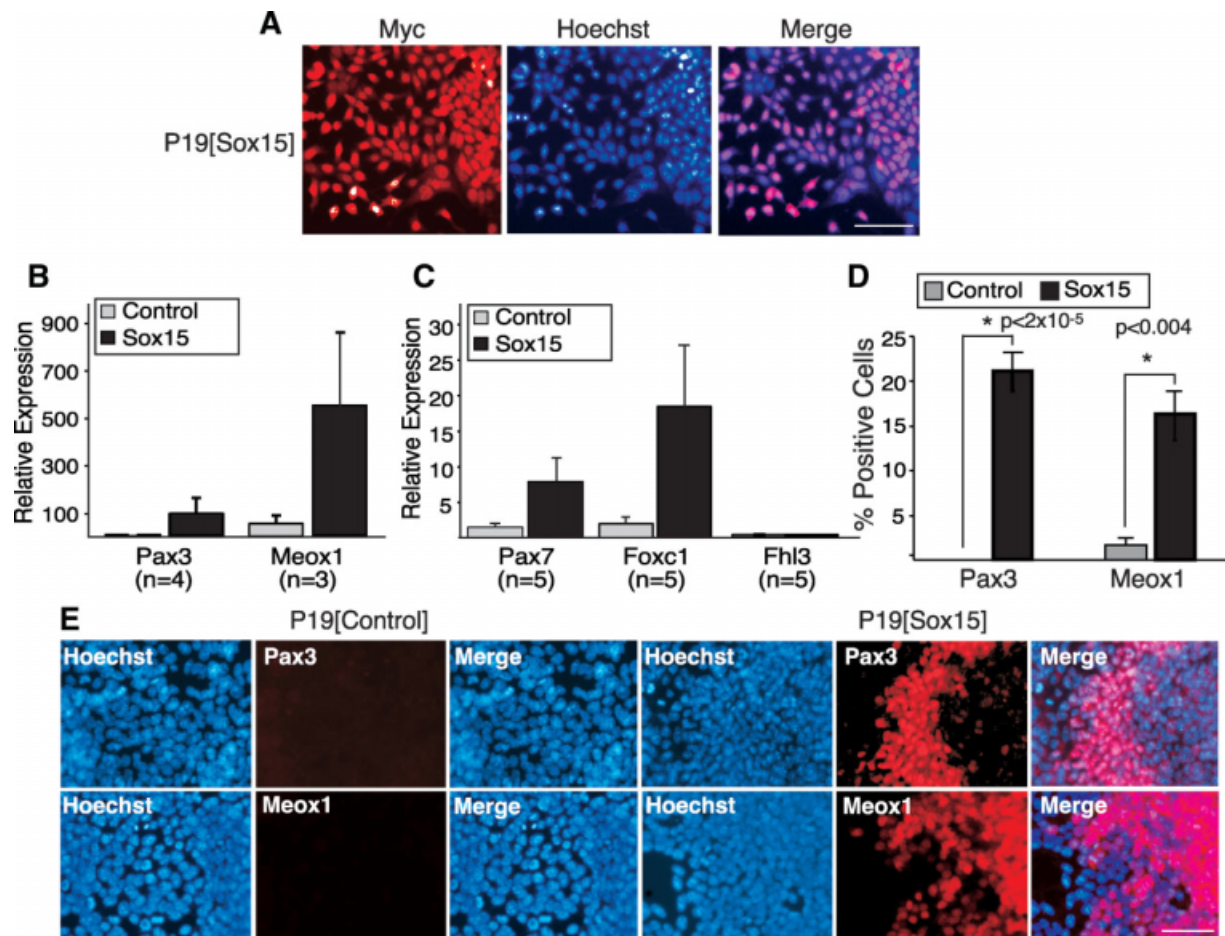
To determine whether Sox15/Sox7 regulate the molecular program controlling muscle precursor cell formation, the temporal pattern of Sox15 and Sox7 mRNA expression was established in differentiating P19 cells by Q-PCR. Cellular aggregation in the presence and absence of DMSO was performed, utilizing conditions that did and did not support skeletal myogenesis, respectively. Sox15 transcripts were highly abundant in undifferentiated stem cells and transcript levels decreased during differentiation, but were still detectable throughout the entire time course of differentiation and showed a small peak of expression on day 4 (Fig. 1A). After aggregation in the absence of DMSO, Sox15 transcript levels remained high (Fig. 1A). Sox7 mRNA levels, on the other hand, showed a peak of expression on day 5 of differentiation, (Fig. 1B) and maximal expression on day 9 of the time course. Both Sox7 and Sox15 were expressed on days 4 and 5, which correspond to peaks in Pax3 and Meox1 expression (Fig. 1C). This overlap in expression patterns indicates that

Sox7/15 is expressed at a time consistent with their potential role in initiating muscle precursor formation during skeletal myogenesis in P19 cells. The myogenic commitment of cells in this experiment was confirmed by the detection of MyoD transcripts (Fig. 1D). During aggregation in the absence of DMSO, upregulation was not observed for Sox7, Pax3, Meox1, and MyoD transcripts, indicating the lack of entry into the skeletal muscle lineage (Fig. 1B–1D).

To determine whether the decrease in Sox15 mRNA is a global decrease in transcript numbers in all cells, or a selective decrease in a subpopulation of cells, we performed localization studies using an antibody against Sox15. Immunofluorescence imaging indicated that Sox15 expression was detected in the majority of undifferentiated P19 cells, and that protein expression became restricted to a subpopulation of cells as differentiation progressed (Fig. 1E).

### Sox15 Upregulates the Expression of Muscle Precursor Genes

To ascertain the role of Sox15 during the early stages of myogenesis, we performed gain-of-function studies by isolating clonal populations of cells stably expressing myc-tagged Sox15, termed P19[Sox15]. Immunofluorescence was



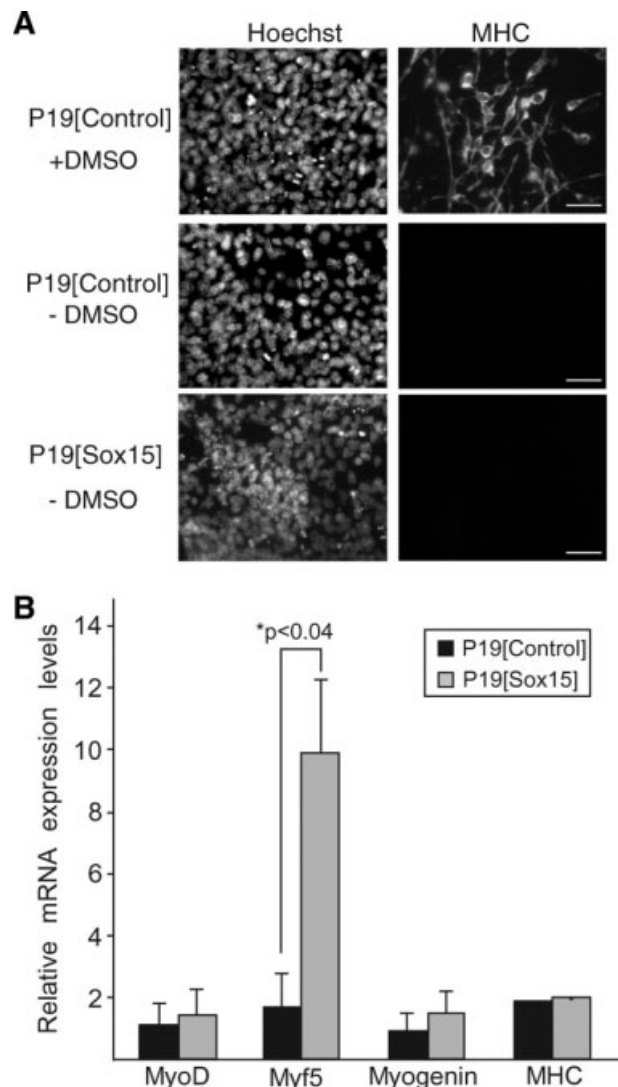
**Figure 2.** Sox15 induces the expression of muscle precursor genes. (A): P19[Sox15] cells grown in monolayer were stained using anti-Myc antibodies (red), to detect the presence of exogenous Sox15 and Hoechst (blue) to detect nuclei. Scale bar = 20  $\mu$ m. (B, C): P19[control] and P19[Sox15] stable cell lines were differentiated in the absence of dimethyl sulfoxide and analyzed for the expression of Pax3/7, Meox1, Foxc1, and Fhl3 using quantitative polymerase chain reaction. For this experiment, the data has been normalized to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase, and is expressed relative to day 0 P19[control] cells. Error bars represent average  $\pm$  SEM, of three independent differentiations of two separate clones. (D): For quantification of positive cells from (E), 10 fields of view were counted per coverslip, in duplicates, for a total of three independent differentiations. Error bars represent the average  $\pm$  SEM. Statistical analysis was performed using the Student's *t* test (vs. control cells), with a *p* value of at least  $p < .05$  considered statistically significant. (E): Immunofluorescent staining was performed using an anti-Pax3 or anti-Meox1 antibody to detect the number of cells expressing these two transcription factors on day 5 (red). The nuclei were visualized by Hoechst dye (blue). Scale bar = 20  $\mu$ m.

performed with an anti-myc antibody to show that virtually every cell expressed the exogenous Sox15, as expected (Fig. 2A). To determine if Sox15 can, in part, replace the DMSO requirement for myogenesis, and to identify the transcription factors that may be regulated by Sox15, RNA from P19[control] and P19[Sox15] clonal populations, aggregated without DMSO, was harvested on day 0 and 5 and subjected to quantitative gene expression analysis by Q-PCR (Fig. 2B, 2C). Sox15 was overexpressed an average of (79–187)-fold ( $n = 6$ ) over background (Fig. 7A). In the absence of DMSO, ectopic expression of Sox15 upregulated Pax3 and Meox1 transcript levels ( $92 \pm 66$ )-fold and ( $546 \pm 310$ )-fold, respectively, compared with a ( $2 \pm 1$ )-fold change in Pax3 expression and a ( $55 \pm 33$ )-fold change in Meox1 expression observed in the P19[control] cell lines (Fig. 2B). Additionally, we noted an ( $8 \pm 3$ )-fold increase in mRNA expression for Pax7 and an ( $18 \pm 9$ )-fold upregulation of Foxc1, both markers of pre-skeletal mesoderm (Fig. 2C). Interestingly, Fhl3, a Sox15 coactivator important for FoxK1 expression in the limb [30], was not upregulated by Sox15 (Fig. 2C),

although Fhl3 was upregulated during P19 cell myogenesis (data not shown).

To differentiate between an increase in transcript copy number per cell and an increase in the number of muscle precursors actually formed, we performed immunofluorescent staining using antibodies against Pax3 and Meox1. Both Pax3 and Meox1 protein levels were upregulated in P19[Sox15] cells when compared with P19[control] cells (Fig. 2E). Quantification of the Pax3 and Meox1 positive population revealed that  $21\% \pm 2\%$  ( $n = 5$ ) of the total cell population was Pax3 positive and  $16\% \pm 3\%$  ( $n = 4$ ) was Meox1 positive (Fig. 2D). These results suggest that expression of Sox15 is sufficient to enhance the proportion of cells specified to the myogenic lineage.

P19[Sox15] cells were also analyzed for their ability to induce myogenic differentiation in the absence of DMSO, conditions which do not normally support differentiation in culture. As expected, P19[control] cells aggregated with DMSO formed MHC-positive skeletal myocytes, while P19[control] cells aggregated without DMSO did not form



**Figure 3.** Sox15 does not induce P19 cell differentiation into skeletal myocytes. P19[control] and P19[Sox15] stable cell lines were differentiated with and without DMSO, as indicated. (A): Skeletal muscle differentiation was confirmed by immunofluorescence using an antibody against MHC, and nuclei were visualized with Hoechst dye. Scale bar = 20  $\mu$ m. (B): Quantitative polymerase chain reaction analysis of gene expression reveals a lack of MyoD, myogenin, and MHC mRNA expression (data has been normalized to glyceraldehyde 3-phosphate dehydrogenase and is expressed relative to P19[control] day 0). Error bars represent average  $\pm$  SEM, of three independent differentiations of two separate clones. \* $p < .04$ , compared with P19[control] (Student's  $t$  test). Abbreviations: DMSO, dimethyl sulfoxide; MHC, myosin heavy chain.

myocytes, as seen by the lack of MHC-positive staining (Fig. 3A). P19[Sox15] cells aggregated in the absence of DMSO and fixed on day 9 also did not differentiate into skeletal muscle, as noted by the lack of MHC staining, indicating that Sox15 could not bypass the DMSO requirement for myogenesis (Fig. 3A). In terms of gene regulation, MyoD, myogenin, and MHC mRNA transcripts were not induced in P19[Sox15] cultures, consistent with the phenotype of the undifferentiated P19[control] cells (Fig. 3B). However, we did note a statistically significant ( $10 \pm 2$ )-fold over background increase in Myf5 expression for the P19[Sox15] clonal populations, compared with the ( $2 \pm 1$ )-fold seen in P19[control] cells

(Fig. 3B,  $n = 3-4$ ). These observations suggest that while Sox15 is able to increase the proportion of cells specified to the myogenic lineage, it is not sufficient to induce skeletal myogenesis in aggregated P19 cells.

The upregulation of Pax3/7, Meox1, and Foxc1 at early stages, but not MyoD, myogenin or MHC at later stages suggests a positive role for Sox15 in enhancing muscle precursor formation and possibly a negative role in regulating the differentiation of myoblasts to myocytes.

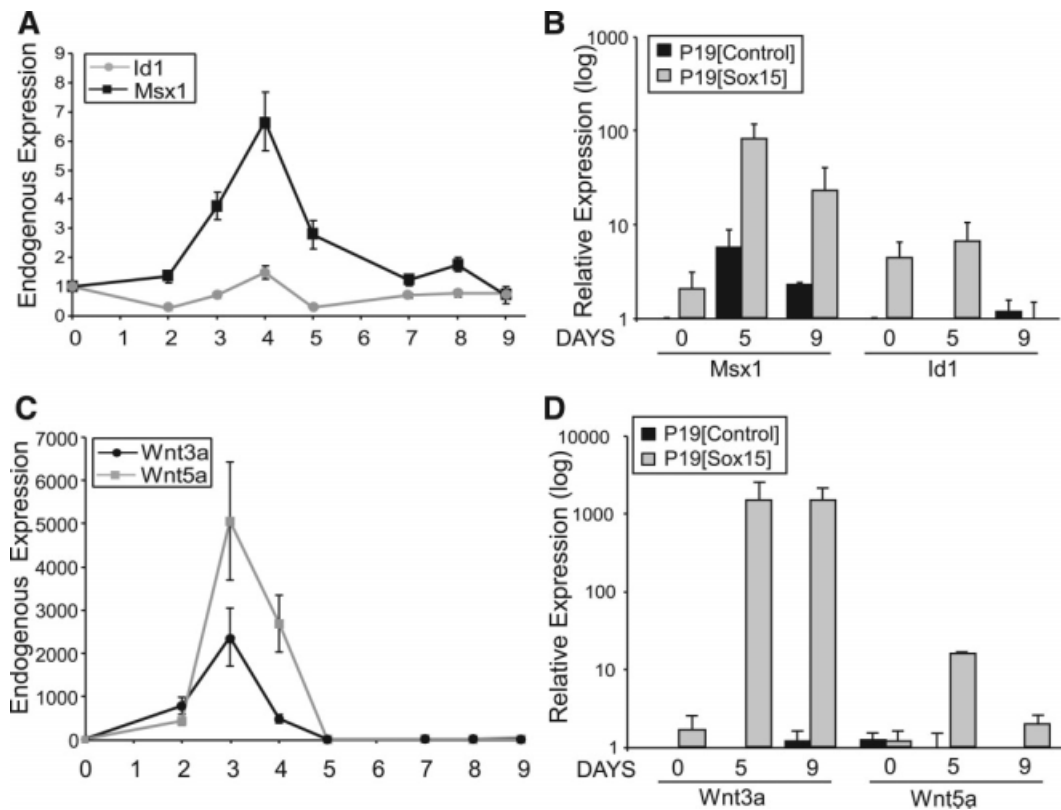
### Sox15 Upregulates the Expression of Negative Regulators of Myogenic Differentiation

In an attempt to interpret the lack of progression of P19[Sox15] cell lines past the muscle precursor stage, microarray analysis was performed to identify genes that are differentially regulated by expression of Sox15 (data not shown). Our screen detected the upregulation of Msx1 and Id1, which are known inhibitors of myogenic differentiation [51–56]. The expression levels of both Msx1 and Id1 peaked on day 4 of DMSO-induced P19 cell differentiation, and were downregulated to basal levels as differentiation progressed (Fig. 4A). However, in P19[Sox15] cell lines, both Msx1 and Id1 were elevated on day 5, compared to P19[control] cells, after aggregation without DMSO (Fig. 4B). These levels were also substantially higher than the endogenous levels observed during EC cell differentiation (Fig. 4A). Therefore, elevated and sustained Msx1, and elevated Id1 levels could contribute to the block observed in the myogenic progression of P19[Sox15] cells. As Id1 functions by inhibiting MRFs, it could inhibit the function of Myf-5 in this system, preventing subsequent differentiation. In addition, we observed elevated and sustained levels of Wnt3a, and to a lesser extent, Wnt5a, in the presence of Sox15 (Fig. 4C, 4D), factors which are correlated with proliferating mesodermal populations [57]. Therefore, it is plausible that a combination of elevated levels of Msx1/Id1 and Wnt3a/Wnt5a contribute to the failure of P19[Sox15] cells to progress along the myogenic pathway.

### Myogenesis is Disrupted in the Presence of a Dominant-Negative Sox15 Mutant

A dominant-negative Sox15 mutant was created by replacing the activation domain of Sox15 with the repressor domain of the mouse EN-2 protein, as described previously [9, 11, 26]. This approach creates an active dominant-negative mutant and results in a repressive chromatin structure surrounding Sox15 binding sites. This cannot be bypassed by other Sox family members or by other transcription factors that bind near to Sox15 binding sites. In transient promoter assays using a Pax3 promoter region driving luciferase [46], Sox15/EnR repressed the ability of Sox15 to activate transcription (Fig. 5A). Thus, Sox15/EnR was shown to function as a dominant-negative mutant.

Four clonal P19[Sox15/EnR] cell lines and two P19[control] cell lines were aggregated in the presence of DMSO to determine if Sox15/EnR can inhibit DMSO-induced myogenesis. P19[Sox15/EnR] cells were found to express high levels of Sox15/EnR throughout the differentiation (Fig. 5B). Sox15/EnR clones showed an  $84\% \pm 5\%$  and  $97\% \pm 2\%$  reduction in the levels of Meox1 and Foxc1 transcripts, respectively, on day 5 of differentiation when compared with P19[control] cultures. Further, there were statistically significant decreases in the expression of Pax3, Pax7, and Sox7 ( $49\% \pm 23\%$ ;  $68\% \pm 9\%$ ;  $56\% \pm 29\%$  remaining), respectively, in P19[Sox15/EnR] cell lines, compared with P19[control] cells (Fig. 5C,  $n = 4$ ). Interestingly, Msx1 and Id1 levels were not significantly



**Figure 4.** Sox15 upregulates the expression of genes known to inhibit myogenesis. RNA was harvested on day 0–9 from P19 cells differentiated in the presence of dimethyl sulfoxide (DMSO), and analyzed for the expression of Id1/Msx1 (A) and Wnt3a/Wnt5a (C). Error bars represent average  $\pm$  SEM, for reactions performed in triplicate. P19[Sox15] and P19[control] cells were differentiated in the absence of DMSO, and RNA isolated on day 0, 5, and 9 for gene expression analysis by quantitative polymerase chain reaction. Error bars represent average  $\pm$  SEM, of three independent differentiations of two separate clones (B, D). For all of the experiments, the data was normalized using the internal control glyceraldehyde 3-phosphate dehydrogenase, and is expressed relative to day 0.

altered in P19[Sox15/EnR] cultures (data not shown), indicating that additional pathways may regulate Msx1 and Id1.

Myoblast formation and terminal differentiation were impaired in P19[Sox15/EnR] cultures, as demonstrated by the loss of MyoD ( $92\% \pm 5\%$ ), Myf5 ( $87\% \pm 11\%$ ), and MHC ( $75\% \pm 13\%$ ) expression, compared with control cultures (Fig. 5D,  $n = 3$ ). The decrease observed in MHC mRNA expression translated into a loss of MHC protein, and an overall decrease in the number of myocytes observed in P19[Sox15/EnR] (Fig. 5E). Quantification of the number of MHC-positive cells revealed a threefold decrease in overall myogenesis in the absence of a functional Sox15 transcription factor (Fig. 5F). These results suggest that Sox15, or genes that are bound by Sox15 are required for efficient myogenesis to occur in P19 cells.

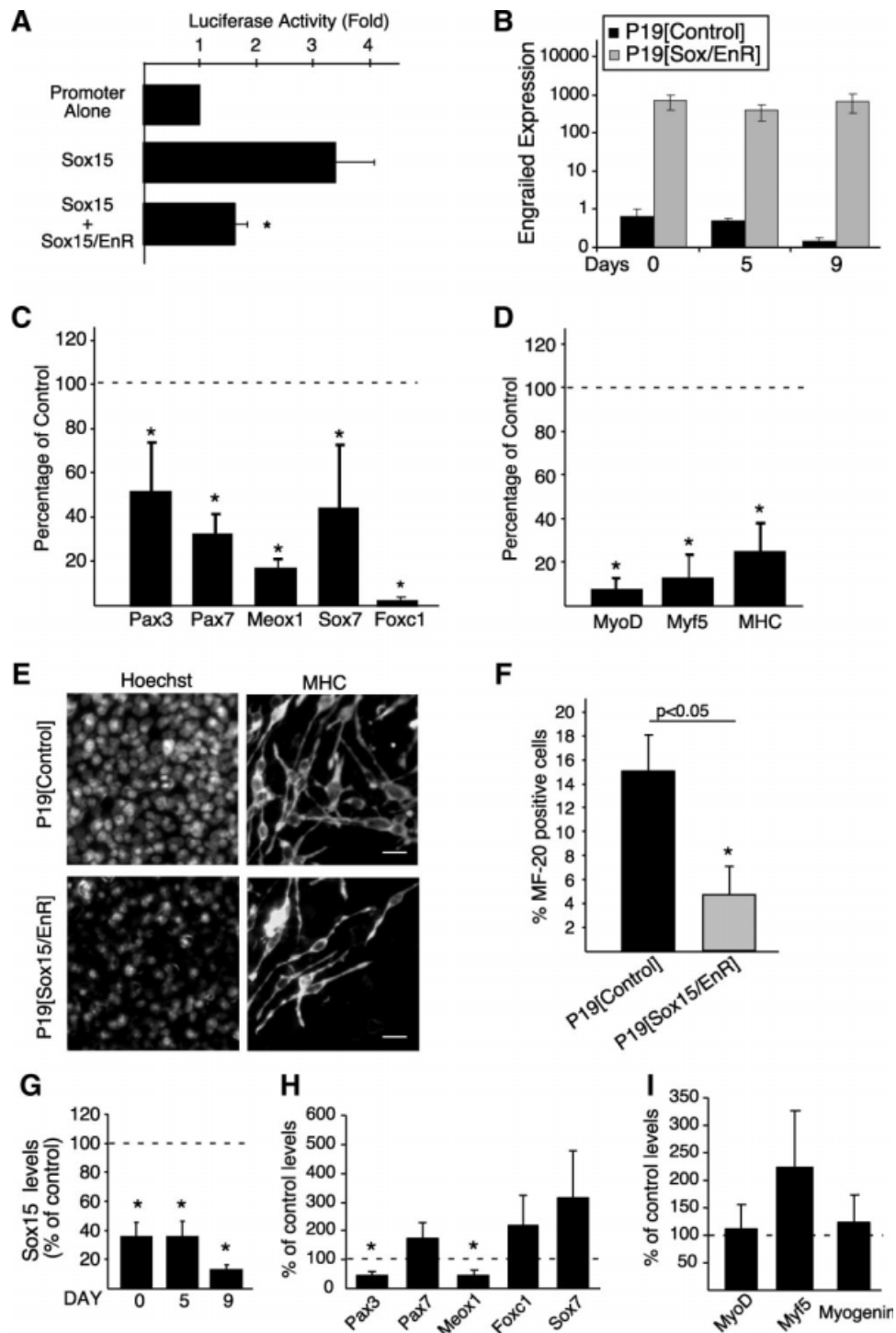
#### Loss of Endogenous Sox15 Expression Does Not Affect Overall Myogenesis

To assess the role of endogenous Sox15 during myogenesis, we silenced the expression of Sox15 using RNA interference. In this approach, other Sox family members or other transcription factors can functionally compensate for the loss of Sox15. Cell lines were generated that stably express a short-hairpin construct targeting one of two different regions of the Sox15 open reading frame (shSox15), or a scrambled sequence with no homology to any sequences in the mouse genome (shScrambled). Individual clones were picked and expanded for further analysis, or pooled together and analyzed. For the purpose of these studies, one pooled experi-

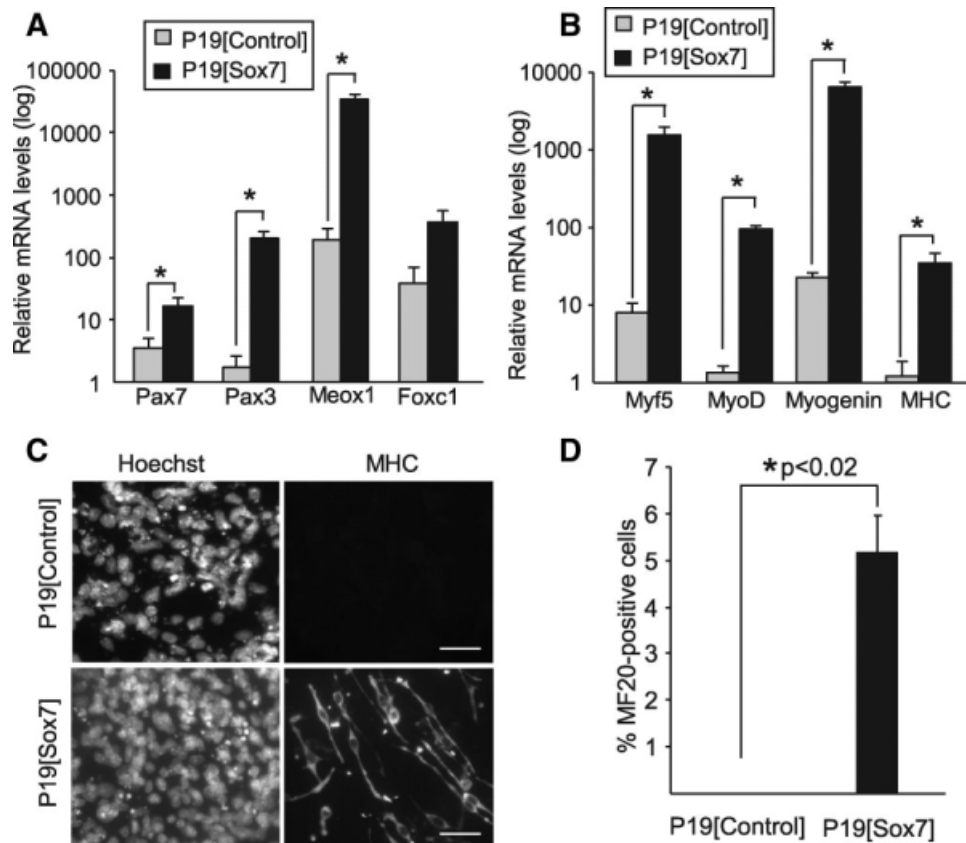
ment was considered as one clone. Analysis of 12 clones indicated a  $64\% \pm 10\%$  to  $87\% \pm 3\%$  knockdown of Sox15 expression, over the time course of differentiation, compared with P19[shScrambled] cell lines (Fig. 5G). Analysis of gene expression in P19[shSox15] cell lines revealed a  $46\% \pm 8\%$  decrease in Pax3 and a  $47\% \pm 14\%$  decrease in Meox1 mRNA expression, when compared to P19[shScrambled] cells (Fig. 5H,  $n = 12$ ). Surprisingly, Pax7 and Foxc1 were unaffected by the knockdown of Sox15, while Sox7 appeared slightly upregulated (Fig. 5H). Moreover, MRF expression remained unaltered, and even appeared slightly increased for Myf-5 in the P19[shSox15] cells, as compared with P19[shScrambled] cells (Fig. 5I). From these observations, we can conclude that while Sox15 appears to be required for efficient Pax3 and Meox1 expression, it is dispensable for Pax7 and Foxc1 expression. It is possible that other factors can compensate for Sox15 and regulate downstream gene expression in its absence.

#### Sox7 Induces Myogenesis in Aggregated P19 cells

Given our previous observations that Sox7 is expressed during the precursor stage of myogenic differentiation in P19 cells (Fig. 1), and that Sox7 is downregulated by Sox15/EnR (Fig. 5C) and slightly upregulated by Sox15 knockdown (Fig. 5H), we were interested in determining whether Sox7 could also modulate muscle differentiation. Cell lines that stably expressed Sox7, termed P19[Sox7], were generated and differentiated in the absence of DMSO. Q-PCR was used to measure the changes in gene expression that were induced by the



**Figure 5.** Interference of Sox15 function and expression causes a decrease in Pax3 and Meox1 gene expression. (A): Sox15/EnR repressed the ability of Sox15 to activate a Pax3 promoter region. Pax3-luciferase was transfected alone or with Sox15 and/or Sox15/EnR, transiently into P19 cells. The resulting luciferase activity was expressed relative to the promoter alone ( $n = 4$ ). (B–F): P19[control] and P19[Sox15/EnR] cell lines were differentiated in the presence of dimethyl sulfoxide (DMSO) and RNA was harvested for quantitative polymerase chain reaction (PCR) analysis. RNA was harvested on day 5 for the detection of Sox15/EnR, Pax3/7, Meox1, Foxc1, and Sox7 on day 9 for Sox15/EnR, myogenic regulatory factors, and MHC expression (B–D). Myocytes were detected in these cell lines by immunofluorescence using an antibody against MHC (E). The percentage of cells that was MHC-positive was quantified by counting 10 fields of view per coverslip from two independent differentiations. Error bars represent average  $\pm$  SEM (F). Stable cell lines expressing shRNA targeted to Sox15 were differentiated in the presence of DMSO, and RNA was harvested on day 0, 5, and 9. Quantitative polymerase chain reaction was performed to determine the efficiency of Sox15 knockdown throughout the differentiation (G) and for the expression of the indicated genes on day 5 (H) and day 9 (I) of differentiation. The error bars represent average  $\pm$  SEM,  $n = 12$  clones. The data was normalized using glyceraldehyde 3-phosphate dehydrogenase as an internal control and expressed as a percentage of the expression in control cell lines, for each respective day. Statistical analysis was performed using the Student's *t* test (compared with the control group), with a *p* value of at least  $p < .05$  considered statistically significant. Abbreviation: MHC, myosin heavy chain.



**Figure 6.** Sox7 upregulates genes found in preskeletal mesoderm, myoblasts, and myocytes, resulting in the induction of skeletal myogenesis. P19[control] and P19[Sox7] cell lines were differentiated in the absence of dimethyl sulfoxide. (A, B): Quantitative polymerase chain reaction analysis of mRNA levels was performed on day 5 for the detection of Pax3/7, Meox1, and Foxc1, and on day 9 for the detection of the myogenic regulatory factors and MHC. The data was analyzed by normalizing to glyceraldehyde 3-phosphate dehydrogenase and expressing the results relative to day 0 of P19[control] cells. The error bars represent the average  $\pm$  SEM, for 2 P19[control] and 3 P19[Sox7] clones from two independent differentiations. Statistical analysis was performed using the Student's *t* test, with a *p* value of at least  $p < .05$  considered statistically significant. (C): Immunofluorescence was performed by fixing cells on day 9 of differentiation and staining the coverslips using an anti-MHC antibody. Scale bar = 20  $\mu$ m. (D): Ten fields of view per coverslip (in duplicate) were counted for four P19[Sox7] cell lines and three P19[control] cell lines. Statistical significance of  $p < .02$  was calculated using the Student's *t* test. Abbreviation: MHC, myosin heavy chain.

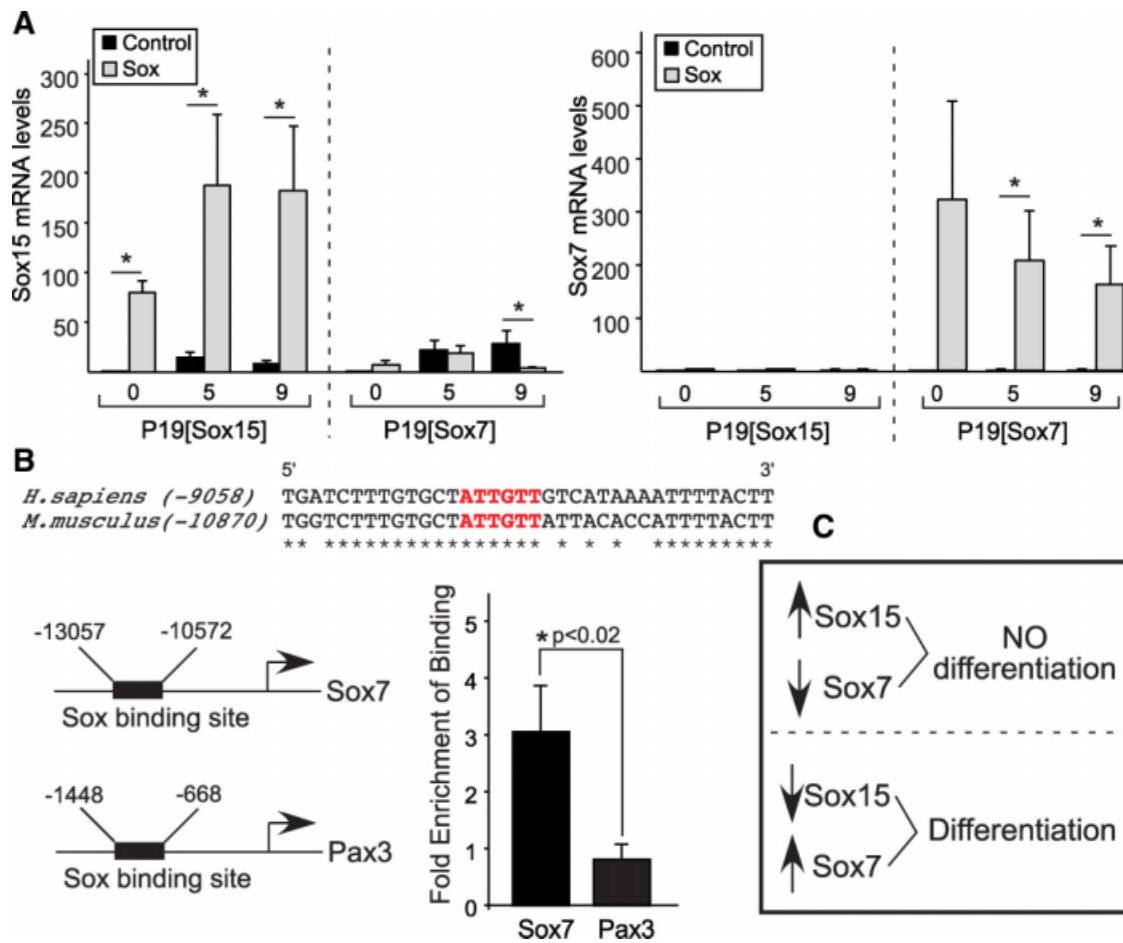
presence of Sox7. Sox7 was overexpressed an average of ( $162 \pm 73$ )-fold to ( $323 \pm 184$ )-fold over the time course of differentiation (Fig. 7A,  $n = 8$ ). A significant increase in the expression of Pax3/7, Meox1, and Foxc1, markers associated with muscle precursors, were observed in aggregated P19[Sox7] cells, compared with control cells (Fig. 6A). Unlike the phenotype observed for P19[Sox15] cells, Sox7 was able to enhance the expression of Myf5, MyoD, and myogenin mRNA as well as enhance MHC transcript expression (Fig. 6B). Using an anti-MHC antibody, we were also able to detect the presence of fully differentiated myocytes in P19[Sox7] cells, as compared to P19[control] cells in the absence of DMSO (Fig. 6C). Quantification of the number of MHC-positive cells in the total population revealed that Sox7 induced  $5\% \pm 1\%$  of total cells to differentiate into MHC-positive skeletal myocytes (Fig. 6D,  $n = 4$ ). Therefore, Sox7 was able to induce skeletal myogenesis in aggregated P19 cells and upregulate the expression of Pax3/7, Meox1, MRFs, and MHC.

#### Sox15 Binds the Genomic Region 5' to the Transcriptional Start Site of Sox7

Given that Sox7 expression is downregulated in the presence of a dominant-negative Sox15 mutant, and slightly upregu-

lated with the knock down of Sox15 (Fig. 5), we tested whether Sox7 expression is controlled by Sox15. Using Q-PCR, we measured the levels of Sox7 transcripts in P19[Sox15] cells, and the amount of Sox15 transcripts in P19[Sox7] cells. Expression of Sox15 in P19[Sox7] cells was significantly decreased compared with P19[control] cells, which is consistent with our observations that endogenous Sox15 levels were decreased during the differentiation process (Fig. 7A). While Sox15 was sufficient to induce Pax3/7, Meox1, and Foxc1 transcript levels from 10–500-fold over background levels (Fig. 2A, 2B), Sox15 did not significantly enhance Sox7 expression levels (Fig. 7A,  $n = 3-5$ ). Thus, Sox15 does not function as a positive activator of Sox7 gene expression in this system.

To characterize the role of Sox15 in regulating Sox7 expression, we used chromatin immunoprecipitation (ChIP) to determine if Sox15 occupies the chromatin upstream of the mouse Sox7 gene (Fig. 7B). Using MULAN (<http://mulan.d-code.org/>), we identified one conserved Sox binding site located within a conserved element approximately 11 kb upstream of the Sox7 transcriptional start site. Using an antibody against Sox15, a ( $3 \pm 1$ )-fold ( $n = 5$ ) enrichment in chromatin fragments corresponding to the Sox site located at 10,857 bp 5' of Sox7 was observed compared to immunoprecipitations performed using a goat IgG as a negative control.



**Figure 7.** Sox15 does not induce Sox7 expression. (A): Using quantitative polymerase chain reaction, we measured the relative levels of Sox15 and Sox7 in P19[Sox7] and P19[Sox15] cell lines differentiated in the absence of dimethyl sulfoxide. Error bars represent average  $\pm$  SEM. (B): In silico studies using MULAN identified one evolutionarily conserved SOX binding site upstream of the Sox7 transcriptional start site. ChIP was performed using an anti-Sox15 antibody to identify enriched Sox15 targets in P19[Sox15] cells on day 5 of differentiation. Sox15 appears to bind to conserved regions in the Sox7 gene but not to conserved regions in the Pax3 gene. Statistical analysis versus control at each time point was carried out using the Student's *t* test. (C): The relative levels of Sox15 and Sox7 correlate with the proliferation versus differentiation status of the P19 embryonal carcinoma cell.

Additionally, in silico studies revealed the presence of a conserved Sox binding site upstream of the Pax3 start site, which did not associate with Sox15, as determined by ChIP analysis (Fig. 7B). Thus, Sox15 appears to bind to a conserved element in the Sox7 gene, but not the Pax3 gene, in a population of differentiating P19 cells.

## DISCUSSION

We have shown that Sox15 was sufficient to upregulate the expression of markers of skeletal muscle precursors in P19 cells under non-muscle inducing conditions (Fig. 2). However, these cells, which lack Sox7 expression and have elevated/sustained levels of the myogenic inhibitors Id1 and Msx1 (Fig. 4), failed to progress past the myoblast stage and did not express MyoD, myogenin, or MHC (Fig. 3). In the presence of a dominant-negative Sox15, a reduction in Meox1, Pax3/7, and Foxc1 transcripts was observed as well as a decrease in MRF expression and overall myogenesis, as noted by the decline in the number of MHC-positive cells (Fig. 5A–5F). The knock down of endogenous Sox15 expression on the

other hand, resulted in a decrease of Pax3 and Meox1 only, suggesting that other factors may compensate for the lack of Sox15 and regulate muscle precursor cell fate and subsequent differentiation (Fig. 5G–5I). These results are consistent with the role of Sox15 in regulating satellite cell formation in mice [30]. Ectopic expression of Sox7 was sufficient to initiate and direct the entire myogenic program, giving rise to skeletal myocytes under non-muscle inducing conditions (Fig. 6). Finally, Sox15 and Sox7 did not upregulate each other's expression and Sox15 was recruited to a conserved site in the Sox7 but not Pax3 regulatory regions (Fig. 7).

Our results are consistent with a model in which Sox15 may regulate the expression of Pax3/7, Meox1, and Foxc1, and therefore may function positively to enhance the specification of cells into the myogenic lineage (Fig. 2). A role for Sox15 upstream of Pax3/7, Meox1, and Foxc1, is supported by the early expression of Sox15 in the mouse blastocyst, although Sox15 expression in the paraxial mesoderm or early somite has not yet been examined [28]. The finding that only a portion of the stable P19[Sox15] cells expressed Pax3 and Meox1 protein suggests the requirement for another factor, which would be expressed in a subset of cells upon cellular aggregation. This observation is consistent with results for all

of the other stable P19 cell lines tested, including MyoD, Pax3,  $\beta$ -catenin, and Gli2 [9, 11, 26, 43]. It is also consistent with the idea that factors, including Sox15, may have opposing functions, such as maintaining the stem cell phenotype and enhancing differentiation, depending on the cellular context in which they are found. For example, Oct4 and Wnt3a can perform both functions, depending on their context [26, 58, 59]. It is likely that each transcription factor is influenced by the combination of factors, the signaling pathways, and the current chromatin structure available in each cell type.

In agreement with previous results in C2C12 myoblasts [32], we observed that P19 cells with high levels of Sox15 failed to progress past the precursor/myoblast stage (Fig. 3). Further, P19[Sox15] cell lines did not enhance Sox7 expression levels, and knock down of Sox15 slightly upregulated Sox7 (Fig. 7). It is possible that Sox15 is acting as a repressor of Sox7 expression under these circumstances. The loss of Sox7 in the P19[Sox15/EnR] cells (Fig. 5) is consistent with our finding that Sox15 may bind to the Sox7 regulatory sequences, shown by the ChIP assay (Fig. 7B), but it does not indicate whether Sox15 activates or represses Sox7 expression. Thus, cell lines expressing high levels of Sox15 and low levels of Sox7 fail to terminally differentiate, whereas cell lines expressing low levels of Sox15 and high levels of Sox7 exhibit normal myogenesis (Fig. 7C). Although we have established that Sox15 does not activate Sox7 expression, a more detailed examination of the Sox7 regulatory sequences and the role of Sox15 is required to confirm if Sox15 represses Sox7.

Our finding that Sox15 knock down results in the loss of Pax3 and Meox1 expression, but not overall myogenesis (Fig. 5G–5I), is consistent with the phenotype of mice lacking Sox15. These mice develop relatively normal muscle but lack efficient satellite cell formation [30] and efficient adult muscle regeneration, shown by a loss of MyoD but not Myf-5 expression [30, 33]. As Pax3 is an important determinant of satellite cell formation and MyoD regulation, our results in P19 cells are consistent with the Sox15<sup>-/-</sup> mouse phenotype [5, 13]. Interestingly, embryonic expression of Meox1 or Pax3 was not examined in Sox15<sup>-/-</sup> mice [33]. We would predict a downregulation of Pax3 and Meox1 expression in the developing myotome.

Gene expression analysis revealed that Wnt3a expression is elevated and sustained in P19[Sox15] cell lines (Fig. 4), and that these cells failed to progress through the myogenic pathway. Interestingly, a constitutively active  $\beta$ -catenin expressed in satellite cells caused an increase in the proportion of cells expressing Pax7, without a concomitant increase in MyoD [57]. These results suggest that  $\beta$ -catenin promotes self-renewal of muscle precursors, with fewer cells undergoing myogenesis. It is tempting to speculate that the elevated levels of Wnt3a observed in our system are contributing to the maintenance of muscle precursors at the expense of differentiation.

Our finding that Sox15 failed to induce myogenesis in P19[Sox15] cells aggregated in the absence of DMSO (Fig. 3) is consistent with previous findings showing that ectopic expression of Sox15 is not sufficient to initiate the myogenic program [33]. Stable expression of Sox15 in 3T3 fibroblasts did not result in positive MHC staining, or any detectable MyoD/Myf5 expression. In other studies, ectopic expression of Sox15 in C2C12 proliferating myoblasts antagonized differentiation and decreased MyoD expression, whereas a C-terminal truncation of Sox15 showed no adverse effects on myogenesis [32]. Finally, Sox15 expression decreased during myoblast differentiation. Therefore, in these studies, increased levels of Sox15 appeared inhibitory toward myoblast maintenance and differentiation [32].

Similarly, in our experiments, Sox15 seemed to inhibit MyoD expression, since an increase in Pax3 expression did not result in a concomitant increase in MyoD expression (Figs. 2, 3), as would be predicted from the phenotype of P19[Pax3] cells [11]. Our results support a model in which Sox15 enhances Pax3 and Meox1 expression during muscle specification and then inhibits myoblast maintenance and/or differentiation if not subsequently downregulated. A dual positive/negative function for stem cell transcription factors is also observed during melanocyte stem cell differentiation. In this case, Pax3 acts early to activate expression of Mitf, while simultaneously inhibiting expression of melanin-synthesizing genes. These cells are thus committed, yet will not differentiate until the Pax3-mediated inhibition is relieved [60]. Similarly, upregulation of Id1 and Msx1 (Fig. 4), may contribute to the lack of differentiation in P19[Sox15] cells. In summary, the lack of upregulation of MyoD in this study may be due to the presence of inhibitors of differentiation, such as Id1 and Msx1, and/or the absence of an essential activator, such as Sox7.

As knock down of Sox15 resulted in a decrease of Meox1 and Pax3 expression (Fig. 5), it is possible that these two genes are immediate downstream targets of Sox15. Attempts to determine Pax3 and Meox1 promoter occupancy by Sox15 using chromatin immunoprecipitation (ChIP) were unsuccessful (Fig. 7, data not shown). In this case, Pax3 or Meox1 are likely indirect targets, although regions outside the 25 kb promoter region were not examined. It is possible that other members of the Sox family may be regulating Pax3 directly, given the conserved cis-acting site identified, and given that Sox15 is able to activate the Pax3 promoter (Fig. 5). Several other Sox factors are upregulated during P19 cell myogenesis, including Sox8, Sox11, and Sox17, which may bind Pax3/Meox1 directly. In contrast, Sox7 appears to be a direct target of Sox15 and expression analysis suggests that Sox15 is not a positive regulator of Sox7 expression. Further studies are required to elucidate the network of direct and indirect factors regulated by Sox15 and Sox7, and other family members, ideally using high throughput methods.

## SUMMARY

In summary, we have provided evidence that Sox15 and Sox7 may be regulators of early muscle precursor cell fate by functioning as upstream regulators of Pax3/7, Meox1, and Foxc1 expression. This regulation may enhance the specification of cells into the muscle lineage. Furthermore, Sox7, but not Sox15, regulates myoblast formation and differentiation by upregulating MRF and MHC expression. Thus, we propose differential roles for Sox15 and Sox7 during myogenesis. These findings could have implications for future stem cell therapy, which requires the ability to control differentiation versus proliferation and efficient satellite cell repopulation.

## ACKNOWLEDGMENTS

We wish to thank Dr. Ashraf Al-Madhoun, Jennifer Dawson, Virja Mehta, and Anastassia Voronova for critically reading the manuscript. We acknowledge the technical assistance of Sophie Boisvenue and Flavia Sendi-Mukasa. We are grateful to Dr. Alain Stintzi for his expertise and assistance with quantitative polymerase chain reaction analysis. We thank Dr. Valerie Wallace for helpful discussions. J.S. was supported by a NSERC

studentship as well as a Canadian Institute of Health Doctoral Research Award (Institute of Aging). I.S.S. was supported by a Canadian Institute of Aging Investigator Award. This work was supported by grant MOP-84458 (to I.S.S.) from the Canadian Institutes of Health Research.

### DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

### REFERENCES

- Cossu G, Borello U. Wnt signaling and the activation of myogenesis in mammals. *EMBO J* 1999;18:6867–6872.
- Currie PD, Ingham PW. The generation and interpretation of positional information within the vertebrate myotome. *Mech Dev* 1998;73:3–21.
- Tajbakhsh S, Cossu G. Establishing myogenic identity during somitogenesis. *Curr Opin Genet Dev* 1997;7:634–641.
- Arnold HH, Braun T. Genetics of muscle determination and development. *Curr Top Dev Biol* 2000;48:129–164.
- Relaix F, Montarras D, Zaffran S et al. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol* 2006;172:91–102.
- Laclef C, Hamard G, Demignon J et al. Altered myogenesis in Six1-deficient mice. *Development* 2003;130:2239–2252.
- Candia AF, Hu J, Crosby J et al. Mox-1 and Mox-2 define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. *Development* 1992;116:1123–1136.
- Mankoo BS, Skuntz S, Harrigan I et al. The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. *Development* 2003;130:4655–4664.
- Petropoulos H, Gianakopoulos PJ, Ridgeway AG et al. Disruption of Meox or Gli activity ablates skeletal myogenesis in P19 cells. *J Biol Chem* 2004;279:23874–23881.
- Maroto M, Reshef R, Munsterberg AE et al. Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. *Cell* 1997;89:139–148.
- Ridgeway AG, Skerjanc IS. Pax3 is essential for skeletal myogenesis and the expression of Six1 and Eya2. *J Biol Chem* 2001;276:19033–19039.
- Borycki AG, Mendham L, Emerson CP Jr. Control of somite patterning by Sonic hedgehog and its downstream signal response genes. *Development* 1998;125:777–790.
- Buckingham M, Relaix F. The role of Pax genes in the development of tissues and organs: Pax3 and Pax7 regulate muscle progenitor cell functions. *Annu Rev Cell Dev Biol* 2007;23:645–673.
- Goulding M, Lumsden A, Paquette AJ. Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. *Development* 1994;120:957–971.
- Williams BA, Ordahl CP. Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* 1994;120:785–796.
- Relaix F, Rocancourt D, Mansouri A et al. Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev* 2004;18:1088–1105.
- Reshef R, Maroto M, Lassar AB. Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. *Genes Dev* 1998;12:290–303.
- Skerjanc IS. Cardiac and skeletal muscle development in P19 embryonal carcinoma cells. *Trends Cardiovasc Med* 1999;9:139–143.
- Bajard L, Relaix F, Lagha M et al. A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. *Genes Dev* 2006;20:2450–2464.
- Relaix F, Rocancourt D, Mansouri A et al. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* 2005;435:948–953.
- Gros J, Manceau M, Thome V et al. A common somitic origin for embryonic muscle progenitors and satellite cells. *Nature* 2005;435:954–958.
- Candia AF, Wright CV. Differential localization of Mox-1 and Mox-2 proteins indicates distinct roles during development. *Int J Dev Biol* 1996;40:1179–1184.
- Capdevila J, Tabin C, Johnson RL. Control of dorsoventral somite patterning by Wnt-1 and beta-catenin. *Dev Biol* 1998;193:182–194.
- Wagner J, Schmidt C, Nikowits W Jr et al. Compartmentalization of the somite and myogenesis in chick embryos are influenced by wnt expression. *Dev Biol* 2000;228:86–94.
- Fan CM, Lee CS, Tessier-Lavigne M. A role for WNT proteins in induction of dermomyotome. *Dev Biol* 1997;191:160–165.
- Petropoulos H, Skerjanc IS. Beta-catenin is essential and sufficient for skeletal myogenesis in P19 cells. *J Biol Chem* 2002;277:15393–15399.
- Schmidt K, Glaser G, Wernig A et al. Sox8 is a specific marker for muscle satellite cells and inhibits myogenesis. *J Biol Chem* 2003;278:29769–29775.
- Yoshikawa T, Piao Y, Zhong J et al. High-throughput screen for genes predominantly expressed in the ICM of mouse blastocysts by whole mount in situ hybridization. *Gene Expr Patterns* 2006;6:213–224.
- Sarraj MA, Wilmore HP, McClive PJ et al. Sox15 is up regulated in the embryonic mouse testis. *Gene Expr Patterns* 2003;3:413–417.
- Meeson AP, Shi X, Alexander MS et al. Sox15 and Fhl3 transcriptionally coactivate Foxk1 and regulate myogenic progenitor cells. *EMBO J* 2007;26:1902–1912.
- Maruyama M, Ichisaka T, Nakagawa M et al. Differential roles for Sox15 and Sox2 in transcriptional control in mouse embryonic stem cells. *J Biol Chem* 2005;280:24371–24379.
- Beranger F, Mejean C, Moniot B et al. Muscle differentiation is antagonized by SOX15, a new member of the SOX protein family. *J Biol Chem* 2000;275:16103–16109.
- Lee HJ, Goring W, Ochs M et al. Sox15 is required for skeletal muscle regeneration. *Mol Cell Biol* 2004;24:8428–8436.
- Shiozawa M, Hiraoka Y, Komatsu N et al. Cloning and characterization of *Xenopus laevis* xSox7 cDNA. *Biochim Biophys Acta* 1996;1309:73–76.
- Takash W, Canizares J, Bonneaud N et al. SOX7 transcription factor: Sequence, chromosomal localisation, expression, transactivation and interference with Wnt signalling. *Nucleic Acids Res* 2001;29:4274–4283.
- Taniguchi K, Hiraoka Y, Ogawa M et al. Isolation and characterization of a mouse SRY-related cDNA, mSox7. *Biochim Biophys Acta* 1999;1445:225–231.
- Katoh M. Expression of human SOX7 in normal tissues and tumors. *Int J Mol Med* 2002;9:363–368.
- Zhang C, Basta T, Fawcett SR et al. SOX7 is an immediate-early target of VegT and regulates Nodal-related gene expression in *Xenopus*. *Dev Biol* 2005;278:526–541.
- Pendeville H, Winandy M, Manfroid I et al. Zebrafish Sox7 and Sox18 function together to control arterial-venous identity. *Dev Biol* 2008;317:405–416.
- Cermenati S, Moleri S, Cimbro S et al. Sox18 and Sox7 play redundant roles in vascular development. *Blood* 2008;111:2657–2666.
- Herpers R, van de Kamp E, Duckers HJ et al. Redundant roles for sox7 and sox18 in arteriovenous specification in zebrafish. *Circ Res* 2008;102:12–15.
- Zhang C, Basta T, Klymkowsky MW. SOX7 and SOX18 are essential for cardiogenesis in *Xenopus*. *Dev Dyn* 2005;234:878–891.
- Skerjanc IS, Slack RS, McBurney MW. Cellular aggregation enhances MyoD-directed skeletal myogenesis in embryonal carcinoma cells. *Mol Cell Biol* 1994;14:8451–8459.
- Ridgeway AG, Petropoulos H, Wilton S et al. Wnt signaling regulates the function of MyoD and myogenin. *J Biol Chem* 2000;275:32398–32405.
- Skerjanc IS, Petropoulos H, Ridgeway AG et al. Myocyte enhancer factor 2C and Nkx2-5 up-regulate each other's expression and initiate cardiomyogenesis in P19 cells. *J Biol Chem* 1998;273:34904–34910.
- Natoli TA, Ellsworth MK, Wu C et al. Positive and negative DNA sequence elements are required to establish the pattern of Pax3 expression. *Development* 1997;124:617–626.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning, A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press, 1989.
- Wilton S, Skerjanc H. Factors in serum regulate muscle development in P19 cells. *In Vitro Cell Dev Biol Anim* 1999;35:175–177.
- Li C, Hung Wong W. Model-based analysis of oligonucleotide arrays: Model validation, design issues and standard error application. *Genome Biol* 2001;2:RESEARCH0032.
- Yu JY, DeRuiter SL, Turner DL. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci USA* 2002;99:6047–6052.
- Thompson-Jaeger S, Raghov R. Exogenous expression of Msx1 renders myoblasts refractory to differentiation into myotubes and elicits enhanced biosynthesis of four unique mRNAs. *Mol Cell Biochem* 2000;208:63–69.

- 52 Lee H, Habas R, Abate-Shen C. MSX1 cooperates with histone H1b for inhibition of transcription and myogenesis. *Science* 2004;304:1675–1678.
- 53 Miller KA, Barrow J, Collinson JM et al. A highly conserved Wnt-dependent TCF4 binding site within the proximal enhancer of the anti-myogenic Msx1 gene supports expression within Pax3-expressing limb bud muscle precursor cells. *Dev Biol* 2007;311:665–678.
- 54 Bendall AJ, Ding J, Hu G et al. Msx1 antagonizes the myogenic activity of Pax3 in migrating limb muscle precursors. *Development* 1999;126:4965–4976.
- 55 Melnikova IN, Bounpheng M, Schatteman GC et al. Differential biological activities of mammalian Id proteins in muscle cells. *Exp Cell Res* 1999;247:94–104.
- 56 Vinals F, Ventura F. Myogenin protein stability is decreased by BMP-2 through a mechanism implicating Id1. *J Biol Chem* 2004;279:45766–45772.
- 57 Perez-Ruiz A, Ono Y, Gnocchi VF et al. beta-Catenin promotes self-renewal of skeletal-muscle satellite cells. *J Cell Sci* 2008;121:1373–1382.
- 58 Pan G, Thomson JA. Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Res* 2007;17:42–49.
- 59 Zeineddine D, Papadimou E, Chebli K et al. Oct-3/4 dose dependently regulates specification of embryonic stem cells toward a cardiac lineage and early heart development. *Dev Cell* 2006;11:535–546.
- 60 Lang D, Lu MM, Huang L et al. Pax3 functions at a nodal point in melanocyte stem cell differentiation. *Nature* 2005;433:884–887.



See [www.StemCells.com](http://www.StemCells.com) for supporting information available online.