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SOX2 Regulates P63 and Stem/Progenitor Cell State in the Corneal Epithelium

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ABSTRACT

Mutations in key transcription factors SOX2 and P63 were linked with developmental defects and postnatal abnormalities such as corneal opacification, neovascularization, and blindness. The latter phenotypes suggest that SOX2 and P63 may be involved in corneal epithelial regeneration. Although P63 has been shown to be a key regulator of limbal stem cells, the expression pattern and function of SOX2 in the adult cornea remained unclear. Here, we show that SOX2 regulates P63 to control corneal epithelial stem/progenitor cell function. SOX2 and P63 were co-expressed in the stem/progenitor cell compartments of the murine cornea in vivo and in undifferentiated human limbal epithelial stem/progenitor cells in vitro. In line, a new consensus site that allows SOX2-mediated regulation of P63 enhancer was identified while repression of SOX2 reduced P63 expression, suggesting that SOX2 is upstream to P63. Importantly, knockdown of SOX2 significantly attenuated cell proliferation, long-term colony-forming potential of stem/progenitor cells, and induced robust cell differentiation. However, this effect was reverted by forced expression of P63, suggesting that SOX2 acts, at least in part, through P63. Finally, miR-450b was identified as a direct repressor of SOX2 that was required for SOX2/P63 downregulation and cell differentiation. Altogether, we propose that SOX2/P63 pathway is an essential regulator of corneal stem/progenitor cells while mutations in SOX2 or P63 may disrupt epithelial regeneration, leading to loss of corneal transparency and blindness. STEM CELLS *2019;37:417–429*

SIGNIFICANCE STATEMENT

The role of transcription factors in the regulation of limbal epithelial stem cell self-renewal and differentiation is poorly understood. This study demonstrates that SOX2 regulates and interacts with P63 and that both transcription factors control the stem/progenitor cell state. By contrast, downregulation of SOX2/P63 by miR-450b induces cell differentiation. Given that congenital point mutations in SOX2 or P63 were linked with multiple eye abnormities, a better understanding of this molecular network will shed light on disease mechanisms and may potentially be harnessed into novel therapeutic approaches to restore vision.

INTRODUCTION

The corneal epithelium is the outermost transparent tissue that serves as a barrier against external insults and undergoes continuous regeneration by stem cells. The unique compartmentalization of stem, progenitor, and differentiated cells to spatially segregated regions makes the cornea an excellent model for stem cell biology. The stem cells of the cornea reside in a ring-shaped zone in the corneal periphery, known as the limbus. Slow cycling cells were identified in the limbal epithelium [1] and indeed, lineage tracing experiments confirmed that the limbus is the main if not only source of long-term corneal regeneration [2, 3]. Limbal stem cells (LSCs) which were recently labeled by K15-GFP reporter transgene [4] give rise to progenitor cells that undergo centripetal movement toward the corneal center, and upon their transition to the supra basal layers of the corneal epithelium, they become post-mitotic, terminally differentiated, and shed off the eye. Uncontrolled LSC activity may lead to abnormal balance between proliferation and differentiation and give rise to tissue hyperplasia and cancer. Conversely, in case of irreversible loss or damage to LSCs, the cornea becomes neovascularized, its transparency is lost, leading to visual impairment and blindness [5–8]. LSC deficiency (LSCD) can be caused by various factors including eye burn, inflammation, and hereditary disease. Point mutations in *PAX6* [9–11] and *P63* [12, 13] lead to multiple eye abnormalities including LSCD. In line, P63 regulates corneal development [13] and epithelial stem cell maintenance [14, 15] while P63 expression was positively correlated with successful outcome of LSC therapy [16]. Mutations in *SOX2* were linked with anophthalmia (eye absence) in some patients [17–20], consistent with the critical role of SOX2 in early eye development [21, 22]. However, the expression and role of SOX2 in the adult stage cornea remained virtually unknown.

In the present study, we provide evidence that SOX2 is essential for corneal epithelial stem/progenitor cell state. SOX2 was co-expressed with and controlled P63, and in line, SOX2 prevented cell differentiation and was essential for colony-forming capacity and cell proliferation. Finally, miR-450b was identified as a direct repressor of SOX2 which was essential for the downregulation of SOX2/P63 pathway and the induction of cell differentiation.

MATERIALS AND METHODS

Cell Culture, Differentiation, Transfection, and Cloning

Cells were cultured at 37°C, 5% CO_2, and 20% O_2. Human limbal rings from cadaveric corneas were obtained post-mortem under the approval of the local ethical committee. Epithelium was separated from the underlying stroma following incubation with dispase (Gibco, Life Technologies, USA). Cells were grown in co-culture with mitomycinized growth-arrested J2-NIH3T3 cells (40×10^3 cells per square centimeter) in epithelial medium, as previously described [23]. For efficient transfection and for controlled calcium-induced differentiation, cells were switched to defined medium with supplements (SCMK001, Millipore, USA) containing 1% penicillin/streptomycin and low calcium (150 μM). For differentiation, 15×10^4 cells per square centimeter were seeded, grown to 80%–100% confluence, and then switched to high (1.2 mM) calcium for up to 1 week.

Cells were collected at indicated time points for different analyses. Clonogenicity test [23, 24], corneal epithelial differentiation of human embryonic stem cells [25–27], and neural differentiation of mouse embryonic stem cells [28] were carried out as previously reported. For cell proliferation assay, cells were transfected with indicated factors, and 48 hours later, cell proliferation was measured with alamar blue (Biorad) as detailed in the kit. For cell viability assay after transfection, trypan blue assay was performed (Sigma, UK, 93595). HEK293 cells were grown in high glucose- and L-glutamine-containing Dulbecco's modified Eagle's medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Invitrogen, Darmstadt, Germany). For transfections, cells were seeded on Collagen I (Sigma, USA, C8919)-coated dishes and on the next day co-transfected (Lipofectamine 3000 Invitrogen) with 40 ng of Renilla luciferase vector (pRL-CMV), pMIR-REPORT *SOX2*-3′ untranslated region (UTR) vector (400 ng, kind gift of K. S. Kosik [29]), and pre-miR mimic or control oligonucleotides (50 nM). Alternatively, cells were transfected with indicated transcription factor encoding plasmids (1 μg), P63 enhancer luciferase constructs (500 ng, kind gift of C. Missero), or specific endonuclease-digested silencing RNAs (esiRNAs; 50 nM). Luciferase activity was measured 48 hours after transfection using Dual-Luciferase Reporter Assay System (Promega, USA), and light emission was measured over 10 seconds. The efficiency of transfection was normalized to Renilla.

Cloning of the *MUT-SOX2-3′UTR* was done by ligation of a double stranded DNA fragment containing the entire length of *SOX2-3′UTR* that contains point mutations in all five predicted binding sites (for the sequence, see Supporting Information Fig. S4). For C38-C40-MUT-SOX2 plasmid, SOX2 binding site (atgaatgttcttgt) was mutated (to acacggtttcaagt) by polymerase chain reaction (PCR) using Fwd-5′gcctagtacttgaaaccgtgtctcccaaacacatcatttc3′ and Rev-5′aagacatagcttgcaggagaacatctggagc3′ primers.

All esiRNA reagents were from Sigma, USA including esiRNA-SOX2 (EHU184131), esiRNA-P63 (EHU122601), and control esiRNA against Enhanced Green Fluorescent Protein (EHUEGFP), Renilla Luciferase (EHURLUC), and SIC004. PM450a (AM17100) and PM450b (AM17100) mimic or AM450a (AM17000) and AM450b (AM17000) inhibitor and their control oligos (Ctl-PM–AM17120) and Ctl-AM (AM17012) were from Ambion, USA.

Western Blots, Immunoprecipitation, Immunostaining, and Flow Cytometry

Cells were washed with cold phosphate-buffered saline (PBS) twice, and lysates were obtained in RIPA buffer (Tris-HCl 10 mM, 10 mg/ml deoxycholate, 1% NP40, 1% SDS, 150 mM NaCl, and protease inhibitors cocktail [Roche, Mannheim, Germany]). Total protein was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Proteins were separated on 12% polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad) as reported [30–32]. The membranes were blocked with trisma base buffer supplemented with 0.1% tween 20 (TBST, Sigma, USA) containing 5% milk (Bio-Rad, USA) and probed with one of the following antibodies diluted in blocking solution: rabbit anti-SOX2 (1:1,000, Millipore, USA), mouse anti-P63 (1:500, 4A4 Santa Cruz Biotechnology, USA), mouse anti-K14 (1:1,000, Millipore, USA), mouse anti-K3 (1:1,000, Millipore, USA), goat anti-K12 (1:1,000, Santa Cruz Biotechnology, USA), and rabbit anti-ERK (1:3,500, Santa Cruz Biotechnology, USA) at 4°C, overnight, followed by three washes with TBST. Furthermore, the membranes were exposed to peroxidase-conjugated goat anti-mouse IgG or peroxidase-conjugated goat anti-rabbit IgG or peroxidase-conjugated donkey anti-goat IgG (all at 1:3,000) for 1 hour at room temperature and washed three times with TBST. Protein bands were visualized with EZ-ECL Enhanced Chemiluminescence Detection Kit (Biological Industries, Israel). Immunoprecipitation was performed as previously described [33] with 5 μg of rabbit anti-SOX2 and 5 μg of rabbit anti-myc.

For immunofluorescent staining, cells that were grown on glass coverslips were washed with PBS, fixed for 15 minutes in 4% paraformaldehyde (Sigma, USA) in PBS, and then permeabilized with 0.1% Triton X-100 (BioLab, Israel) in PBS for 10 minutes. Blocking was performed in saturation buffer (PBS with 3% bovine serum albumin [Biological Industries, USA], 3% donkey serum [Jackson, USA], and 0.1% Triton X-100) for at least 1 hour at room temperature in order to prevent unspecific antibody binding. Following these treatments, cells were incubated for 1 hour at room temperature with primary antibody mouse anti-SOX2 (1:100; Millipore, USA), rabbit anti-SOX2 (1:100; Abcam, UK), mouse anti-P63 (1:100; Santa Cruz Biotechnology, USA), rabbit anti-K14 (1:400; Covance, USA), rabbit anti-K12 (1:400; Abcam, UK), rabbit anti-K3 (1:400; Millipore, USA), rabbit anti-Ki67 (1:100; Santa Cruz Biotechnology, USA), mouse anti-TUJ1 (1:100; Covance, USA), and mouse anti-NESTIN (1:100; BD Pharmingen, USA) diluted in saturation buffer and washed three times with PBS. Next, cells were incubated for 1 hour at room temperature with secondary antibody (1:500) in saturation buffer, washed three times with PBS, and nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma, USA) and mounted (Thermo Scientific, USA). Secondary antibodies were Alexa Fluor-488 donkey anti-mouse IgG or Alexa Fluor-594 donkey anti-mouse IgG or Alexa Fluor-488 donkey anti-rabbit IgG or Alexa Fluor-594 donkey anti-rabbit IgG, diluted 1:500. Images were taken by Nikon Eclipse NI-E upright microscope, and quantification was performed using Nis-elements Analysis D software. Five to ten different fields were imaged and the average fluorescence intensity was calculated.

Quantitative Real-Time Polymerase Chain Reaction and TaqMan Assay for microRNAs

Cells were washed twice with PBS, and total RNA was isolated using TRI-Reagent (Sigma, USA) according to manufacturer's instructions. For mRNA, cDNA was prepared by reverse transcription-PCR (RT-PCR) using the high-capacity cDNA synthesis kit (Applied Biosystems, USA) using the following program: 1 hour at 37°C and 5 minutes at 95°C. Quantitative RT-PCR (qRT-PCR) was performed with KAPA SYBR FAST Universal kit (KAPA Biosystems, USA) using the appropriate specific primers (listed in Supporting Information Table S2) as follows: 3 minutes at 95°C, 40 cycles of 5 seconds at 95°C, 20 seconds at 60°C, and 10 seconds at 72°C. For TaqMan assays of microRNAs (miRNAs), 5 μl RNA (5 ng/μl) was subjected to RT-PCR using the reverse transcription kit and miRNA-specific primers (Applied Biosystems, USA) followed by qRT-PCR using TaqMan universal master mix and TaqMan miRNA-probes or U54 as control (Applied Biosystems). The relative amounts of each mRNA or miRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase or U54, respectively, and the relative expression of each reaction was calculated as a fold change relative to the control sample. Samples were cycled using Applied Biosystems StepOnePlus RT-PCR system.

Tissue Processing and Staining

Tissues were obtained from three to five mice and prepared for paraffin sections (5 μm). For immunofluorescent staining, tissues were dehydrated and then stained as detailed previously [2] using rabbit anti-SOX2 1:400, mouse anti-P63 1:100 (Santa Cruz Biotechnology, USA), goat anti-K12 1:400 (Santa Cruz Biotechnology, USA), rabbit anti-K15 1:500 (Abcam, UK; overnight at 4°C). Next, samples were washed (0.2% tween 20, 0.2% gelatine), incubated with secondary antibody (Alexa Fluor 488 and 594 [Invitrogen, USA]), and mounted as above. In situ hybridization for miR-450b was performed on whole embryos or on optimal cutting temperature (OCT) compound frozen sections as described previously [25].

Statistical Analysis

Data are presented as means ± SD. Normality was first evaluated using Shapiro-Wilk test. Then, *t* test or analysis of variance followed by Tukey's test were performed, as indicated in legends, to calculate *p* values. Differences were considered to be statistically significant from a *p* value below .05.

RESULTS

SOX2 Is Co-expressed with P63 by Stem/Progenitor Cells of the Corneal Epithelium

To characterize the expression pattern of SOX2 in vivo, we performed immunofluorescent staining on paraffin sections of the cornea of 2 months old mice. K15 and K12 antibodies were used to label the limbus and cornea, respectively (Fig. 1A). SOX2 was detected in the nucleus of stem/progenitor cells of the limbus, in committed progenitors throughout the basal layer of the corneal epithelium but not in supra basal differentiated epithelial cells and no signal was found in the corneal stroma (Fig. 1A, quantification in Supporting Information Fig. S1A, S1B). Interestingly, this expression pattern was very similar and overlapping with the expression pattern of P63, a well-known regulator of epithelial stem/progenitor cells. To further investigate the relevance of these findings to human, we established primary culture of human limbal epithelial stem/progenitor cells. Limbal rings were obtained from donated cadaveric cornea according to Helsinki ethical approval, the epithelial layer was separated from the underlying stroma and epithelial stem/progenitor cells were cultivated as detailed in Materials and Methods section. To study the expression and function of SOX2 during differentiation, limbal stem/progenitor cells were grown in low (150 μM) calcium levels to minimize spontaneous differentiation and then switched to high calcium levels (1.2 mM) to induce cell differentiation and stratification for the indicated time. Cells were harvested prior to differentiation induction (day 0) or following differentiation for 4–7 days. RT-PCR and Western blot analyses confirmed successful differentiation accompanied by a decrease in the expression of markers of stem/progenitor cells (P63 and K14) and an increase in K12 that marks terminally differentiated cells (Fig. 1B, 1C). Although SOX2 was detected in undifferentiated limbal epithelial stem/progenitor cells (day 0), its levels were significantly reduced upon early differentiation at the mRNA and protein levels (Fig. 1B, 1C). As compared to the expression in limbal stem/progenitor cells, very low or no signal was found in primary cultures of human limbal stromal cells or foreskin epidermal cells. Yet, significantly higher levels of SOX2 mRNA were documented in human pluripotent stem cells (Fig. 1D). To further corroborate these data and explore the cellular localization of SOX2 in human limbal epithelial stem/progenitor cells, we performed immunostaining. As shown in Figure 1E, 1F, SOX2 was detected in the nucleus of limbal stem/progenitor cells, although some perinuclear and cytoplasmic signal was sometimes evident. Finally, SOX2 was co-expressed with P63 (and K14) in undifferentiated cells, whereas it was not expressed by K12-positive differentiated cells (Fig. 1E, 1F). Altogether, these data indicate that SOX2 is co-expressed with P63 by stem/progenitor cells of the corneal epithelium.

SOX2 Regulates P63 Enhancer and P63 Expression

As SOX2 and P63 were co-expressed in stem/progenitor cells, we hypothesized that they may coregulate each other. To this end, we examined the possibility that SOX2 may regulate putative promoter/enhancer regions of P63 and vice versa using in silico analysis that predicts transcription factor binding sites (MatInspector, GenomatixSuite v3.10). Conserved P63

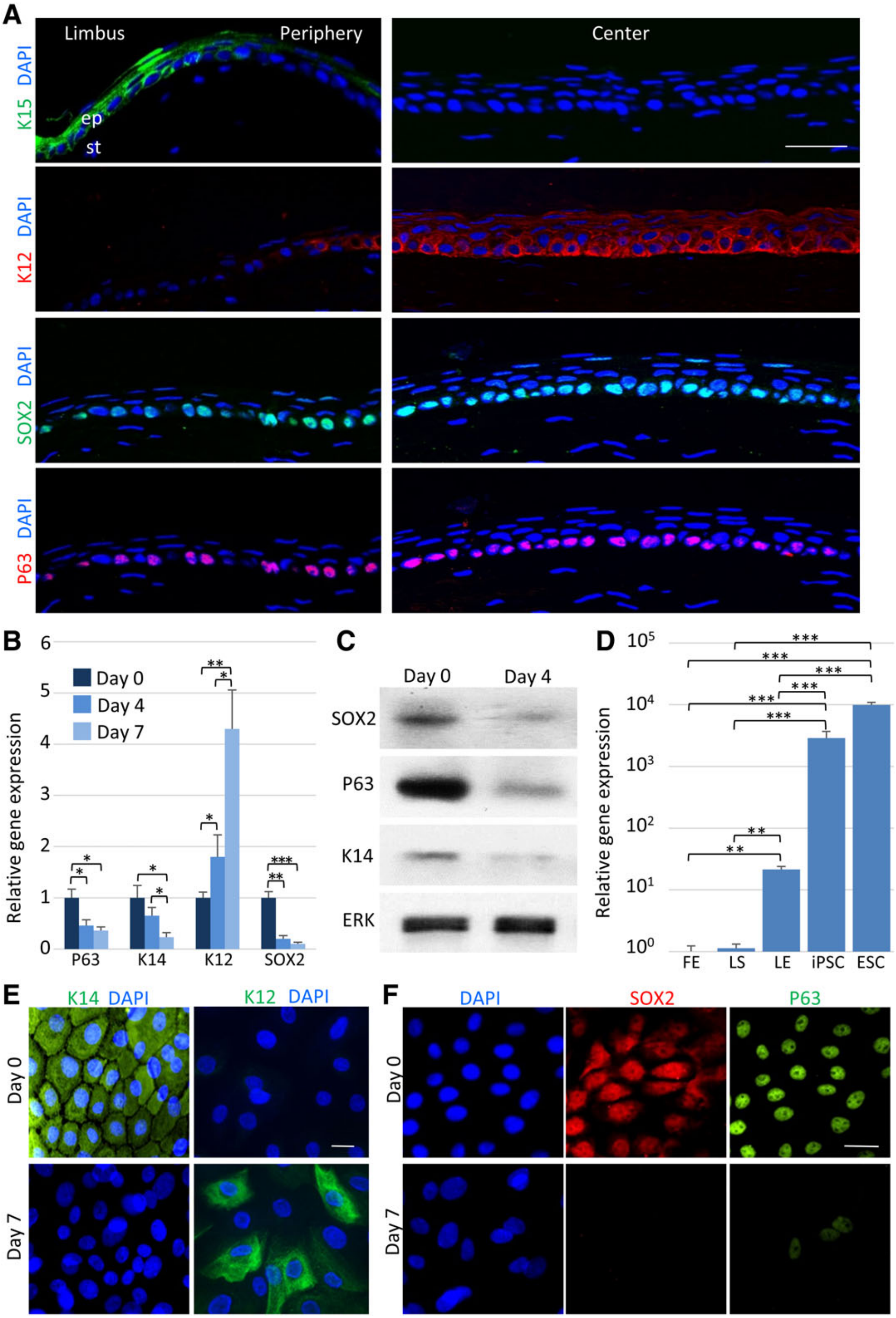

Figure 1. SOX2 is co-expressed with P63 in stem and progenitor cells of the corneal epithelium in vivo and in vitro. **(A):** Immunofluorescence staining of the indicated proteins was performed on paraffin sections of the adult mouse cornea. The regions of the limbus, peripheral cornea, and corneal center are shown. **(B, C, E, F):** Primary human limbal epithelial cells were differentiated for the indicated times, and the expression of the indicated marker was tested by quantitative real-time polymerase chain reaction (qPCR) **(B)** or Western blot analysis **(C)** or immunostaining **(E, F)**. ERK served as loading control in **(C)**. **(D):** A comparative qPCR analysis of SOX2 in the following human cells: primary FE, LS, LE, iPSC, and ESCs. **(B, D):** Data were normalized to housekeeping gene and is presented (mean ± SD, $n = 3$) as fold increase compared to control sample. Statistical analysis was performed by one-way analysis of variance followed by Tukey's test (*, $p < .05$; **, $p < .01$; ***, $p < .001$). **(A, E, F):** Nuclei were detected by DAPI counter staining. Scale bars are 25 μm **(A)** and 12 μm **(E, F)**. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ep, epithelium; ESC, embryonic stem cell; FE, foreskin epidermal cells; iPSC, induced pluripotent stem cell; LE, limbal epithelium stem/progenitor cells; LS, limbal stromal cells; st, stroma.

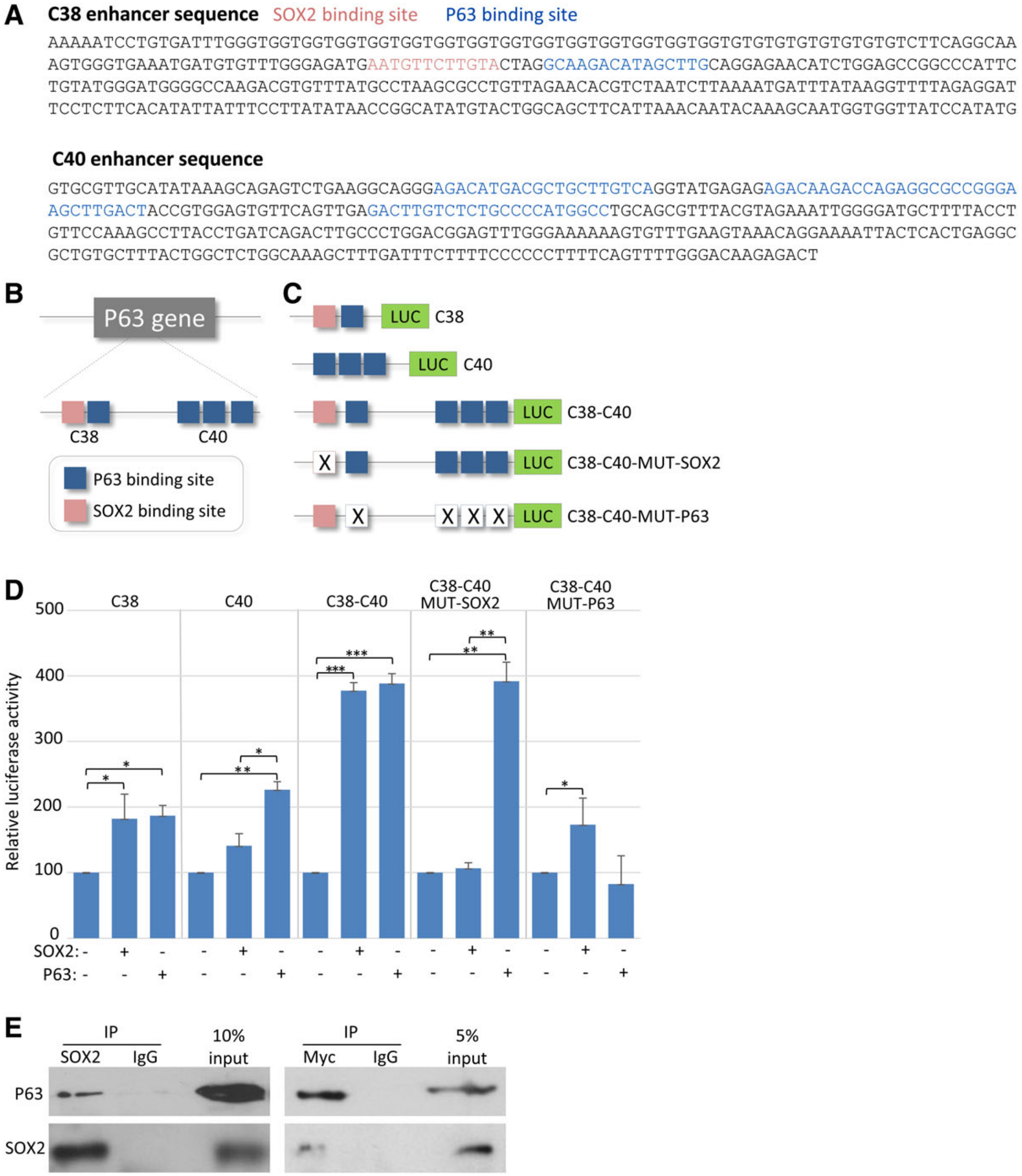

Figure 2. SOX2 can activate *P63* enhancer and interact with P63 protein. **(A, B):** The sequence **(A)** and location **(B)** of C38 and C40 enhancers within *P63* gene. Consensus binding sites of SOX2 and P63 are highlighted in pink and blue, respectively. **(C):** Schematic representation of luciferase construct containing C38, C40, C38-C40, and C38-C40-mutated constructs lacking the indicated P63 or SOX2 binding sites. **(D):** HEK293 cells were co-transfected with the indicated luciferase construct and with SOX2 or P63 or control empty plasmid (−), as indicated. Luciferase activity represents the relative read that was normalized to Renilla and presented as fold increase compared to control sample (mean ± SD, $n = 3$). Statistical significance was assessed by one-way analysis of variance followed by Tukey's test (*, $p < .05$; **, $p < .01$; ***, $p < .001$). **(E):** HEK293 cells were co-transfected with plasmids encoding for SOX2 and myc-tagged P63. Lysates were collected 2 days later and IP was performed with the indicated antibodies or with nonspecific antibodies (IgG) as control. Expression in lysates is shown as control (input). Abbreviation: IP, immunoprecipitation.

consensus binding sites were not identified in *SOX2* promoter/enhancer regions. Interestingly, SOX2 consensus binding site was identified in C38 (but not C40), an evolutionarily conserved cis-regulatory enhancer [34]. The C38-C40 modules, which reside in an intron of *P63* gene, were shown to act in synergism and were bound and controlled by P63 transcription factor via multiple binding sites that facilitate self-regulatory mechanism [34, 35] (Fig. 2A, 2B). To test the impact of SOX2 on *P63* enhancer, HEK293 cells were co-transfected with SOX2 or P63 or empty plasmid (as control), together with luciferase expression vector in which luciferase gene was cloned downstream to C38 or C40 enhancer sequences alone or in tandem

(C38-C40; as illustrated in Fig. 2C). Importantly, overexpression of SOX2 significantly enhanced the luciferase activity of C38 construct but had only mild and nonsignificant effect on C40, which lacks SOX2 binding site (Fig. 2D). The effect of SOX2 on C38-C40 was even stronger compared to the induction of C38, despite the fact that C40 alone was low or insensitive to SOX2. This implies that the enhanced effect of SOX2 on C38-C40 may involve cis-interaction between elements on C38 and C40. Next, we performed site-directed mutagenesis to disrupt the binding site of SOX2. Indeed, the mutated construct (C38-C40-MUT-SOX2) was insensitive to SOX2 transfection. Altogether, this set of experiments strongly suggests that SOX2 activates *P63* enhancer through the newly identified evolutionary conserved binding site.

To gain further insights on potential cis-interactions, we first tested the effect of P63 on these constructs. As expected from a previous report [34], P63 transfection enhanced the activity of C38 or C40 enhancers and caused an even stronger effect on the combined construct (C38-C40), whereas the mutated construct that lacks all P63 binding sites (C38-C40-P63-MUT) was insensitive to P63 transfection (Fig. 2D). Interestingly, mutations in P63 binding sites resulted in attenuated response to SOX2 effect. The latter observation together with the proximity of SOX2 and P63 binding sites in C38 enhancer suggest that these transcription factors may directly interact. To test this possibility, we overexpressed SOX2 and P63 conjugated to a myc tag (myc-P63) and performed co-immunoprecipitation assay. As shown in Figure 2E, a significant band of P63 was detected following SOX2 pull-down, whereas SOX2 was detected following immunoprecipitation of myc-P63. P63 and SOX2 were detected in lysates (input), whereas no signal was found following immunoprecipitation using nonspecific IgG antibodies that were used as negative control. Altogether, these data and the co-ocalization of SOX2 and P63 suggest that SOX2 can regulate C38-C40 enhancers and may control the transcription of P63 mRNA.

SOX2 Is Essential to Maintain Stem/Progenitor Cell State

To test whether SOX2 controls *p63* gene in limbal stem/progenitor cells, we performed knockdown experiments using esiRNA against SOX2 (siSOX2) to induce specific and efficient gene silencing, and nonspecific sequences served as control (siCtl). As shown in Figure 3A and 3B, siSOX2 significantly inhibited SOX2 mRNA and protein. Importantly, SOX2 repression resulted in a significant reduction of P63 mRNA and protein, indicating that SOX2 is upstream to P63. Moreover, SOX2 repression resulted in a reduction in the expression of stem/progenitor markers ABCG2 and K14 and an increase in differentiation markers K3 and K12 with no significant impact on the expression of epidermal marker, K10 (Supporting Information Fig. S1C). Similarly, transfection of esiRNA against P63 induced cell differentiation (Supporting Information Fig. S1D, S1E, S1F), suggesting a common pathway for SOX2 and P63 in regulating stemness and differentiation. To further assess the involvement of SOX2 in the differentiation process, primary limbal stem/progenitor cells were transfected and then subjected to calcium-induced differentiation for 4 days. SOX2 inhibition resulted in reduced expression of ABCG2 and K14 (Fig. 3C) and a dramatic increase in the differentiation markers (Fig. 3D). Moreover, siSOX2 reduced the expression of K14 and induced the expression of K3 at the protein level, as evident by Western blot (Fig. 3E) and flow cytometry (Fig. 3F) analyses. In line, SOX2 repression was correlated with typical changes in cell morphology that occurred during differentiation, for example, enlarged cell body, loss of hexagonal organized pattern, and appearance of disorganized colonies (Fig. 3G).

A well-known hallmark of LSCs is their ability to form long-term proliferative large clones known as holoclones [14, 36]. P63 is known to be important for stemness and its repression was shown to drastically affect colony-forming capacity of LSCs. To further explore the role of SOX2 in regulating stem/progenitor cells, undifferentiated limbal stem/progenitor cells were transfected with siSOX2 or control esiRNA, seeded at clonal density, and then allowed to expand for 2–3 weeks (as detailed in Materials and Methods section). Notably, colony-forming efficiency was drastically affected by SOX2 depletion (Fig. 4A). Quantification revealed that the number of colonies significantly decreased (Fig. 4B) while the size of the remaining colonies was profoundly smaller following SOX2 repression (Fig. 4C), suggesting that SOX2 is essential for the long-term proliferative capacity of LSCs. A comparable robust effect was also observed upon P63 depletion (Fig. 4D, 4E, 4F), in line with a previous report [14]. Finally, knockdown of SOX2 attenuated by ~25% the prevalence of Ki67$^+$ proliferative cells (Fig. 4G, 4H) and led to ~40% decrease in cell growth, as shown by the alamar blue viability assay (Fig. 4I). Altogether, these data indicate that SOX2 is required for cell proliferation while its inhibition enhances cell differentiation.

Finally, we performed rescue experiment in which limbal cells were co-transfected with siSOX2 with or without P63. While siSOX2 reduced clonogenicity, forced expression of P63 restored the colony formation (Fig. 5A, 5B; Supporting Information Fig. S2B) with no significant difference in cell death (Fig. 5C), and the expression of K14 and ABCG2 was significantly restored (Fig. 5D). Given that siRNA was efficiently delivered to the vast majority of the cells (Supporting Information Fig. S2A) and that restored colonies showed low SOX2 and high P63 (Supporting Information Fig. S2B), these data suggest that P63 acts downstream to SOX2. Taken together, these data suggest that SOX2 maintains stem/progenitor cell state at least in part via the regulation of P63 expression.

The microRNA Cluster *MIR450* Can Repress SOX2

Forced SOX2 knockdown in vitro had strong impact on cell differentiation (Figs. 3, 4). The in vivo observation that SOX2 is drastically downregulated upon the transition of cells from the basal corneal epithelial layer to supra basal layers suggests that an active and efficient mechanism mediates SOX2 degradation in supra basal differentiated cells. Such mechanism may be mediated by miRNAs. Intriguingly, TargetScan algorithm (http://www.targetscan.org/) predicted multiple potential binding sites for members of *MIR450* cluster (miR-450a and miR-450b) in *SOX2*-3′UTR (Fig. 6A, 6B). According to miRBase databases (http://www.mirbase.org/index.shtml), *MIR450* is a cluster of miRNAs composed of six miRNA encoding genes (Fig. 6C). Within this cluster, four miRNA genes (two copies of miR-450a and single copies of miR-450b and miR-542) are at close proximity to each other, while they are separated by 4,891 bases from two additional miRNAs (miR-503, miR-424; illustrated in Fig. 6C). This suggests that these two groups may be regulated by separate promoter/enhancer elements. To test this possibility, we chose to examine the expression profile of these miRNA genes in the course of embryonic stem cell differentiation as

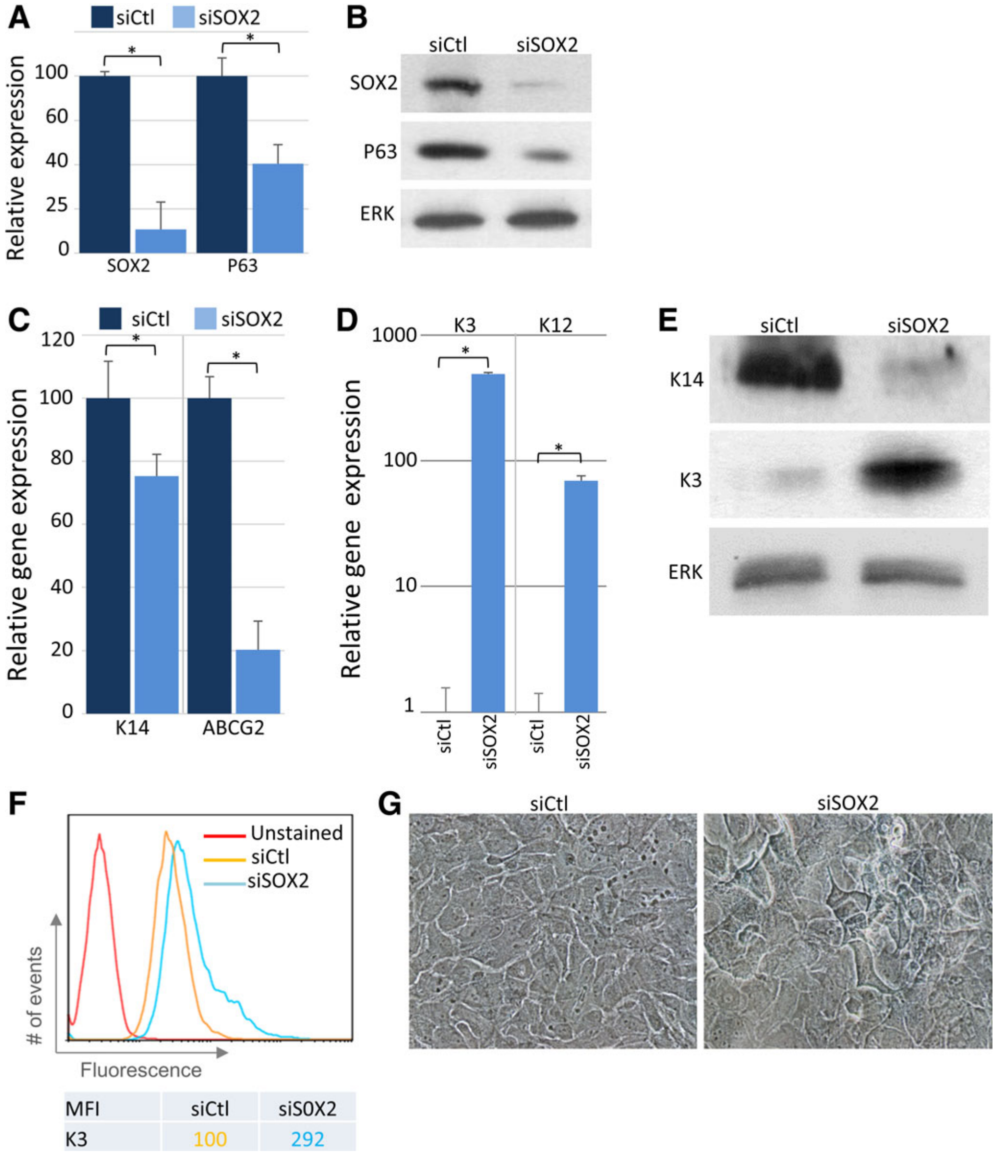

Figure 3. SOX2 prevents cell differentiation. Primary limbal cells were transfected with siSOX2 or control esiRNA, and 72 hours later, the expression of SOX2 and P63 was examined by real-time polymerase chain reaction (PCR) **(A)** or Western blot analysis **(B)**. ERK served as loading control. Quantitative real-time PCR analysis of the indicated markers of stem/progenitor cells **(C)** or markers of differentiated cells **(D)**, or cells were lysed and subjected to Western blot analysis of the indicated markers **(E)** (ERK served as loading control) or immunostaining of K3 was followed by flow cytometry analysis **(F)**. **(G):** The morphological changes upon siSOX2 repression are shown by bright field microscopy (×20 objective). **(A, C, D):** Data represent mean ± SD, $n = 3$ and statistical significance was assessed by t test (*, $p < .05$). Abbreviations: esiRNA, endonuclease-digested silencing RNA; siSOX2, esiRNA against SOX2; siCtl, control esiRNA.

large changes in gene expression during differentiation are known to take place in this process. Human embryonic stem cells were differentiated into corneal epithelial-like cells as previously reported [25, 27] and cells were collected at the indicated time points for analysis. Indeed, each group displayed a different expression profile (Fig. 6D), suggesting that the two groups of genes represent two separately regulated clusters.

Notably, miR-450a and miR-450b are well conserved in mammals, and their seed sequence differs by a single nucleotide at position 8, suggesting they may have both overlapping and separate sets of target genes (Supporting Information Table S1). We thus focused on miR-450b that had four predicted binding sites on SOX2-3′UTR. SOX2 is expressed in early eye development [21, 22], in the adult cornea (Fig. 1), and in neural cells [37]. To explore the expression of miR-450b in this context, we performed whole mount in situ hybridization of mouse embryos at different developmental stages. A low but significant signal for miR-450b was documented in the developing lens of 10-day-old embryos (E10.5), and this signal dramatically peaked by E11.5 (Fig. 6E). Strikingly, this de novo expression coincided with a significant reduction in SOX2 protein shown by immunofluorescent staining of tissue sections (Fig. 6F). In the adult murine cornea, miR-450b was low or not detected in the limbus but was specifically expressed in the cornea of 2-month-old mice (Fig. 6G). Closer inspection of the expression of miR-450b signal on tissue sections has shown that miR-450b was low in corneal basal layer (black arrow, Fig. 6H), whereas it was highly expressed by differentiated supra basal layers (white arrow, Fig. 6H). Thus, miR-450b

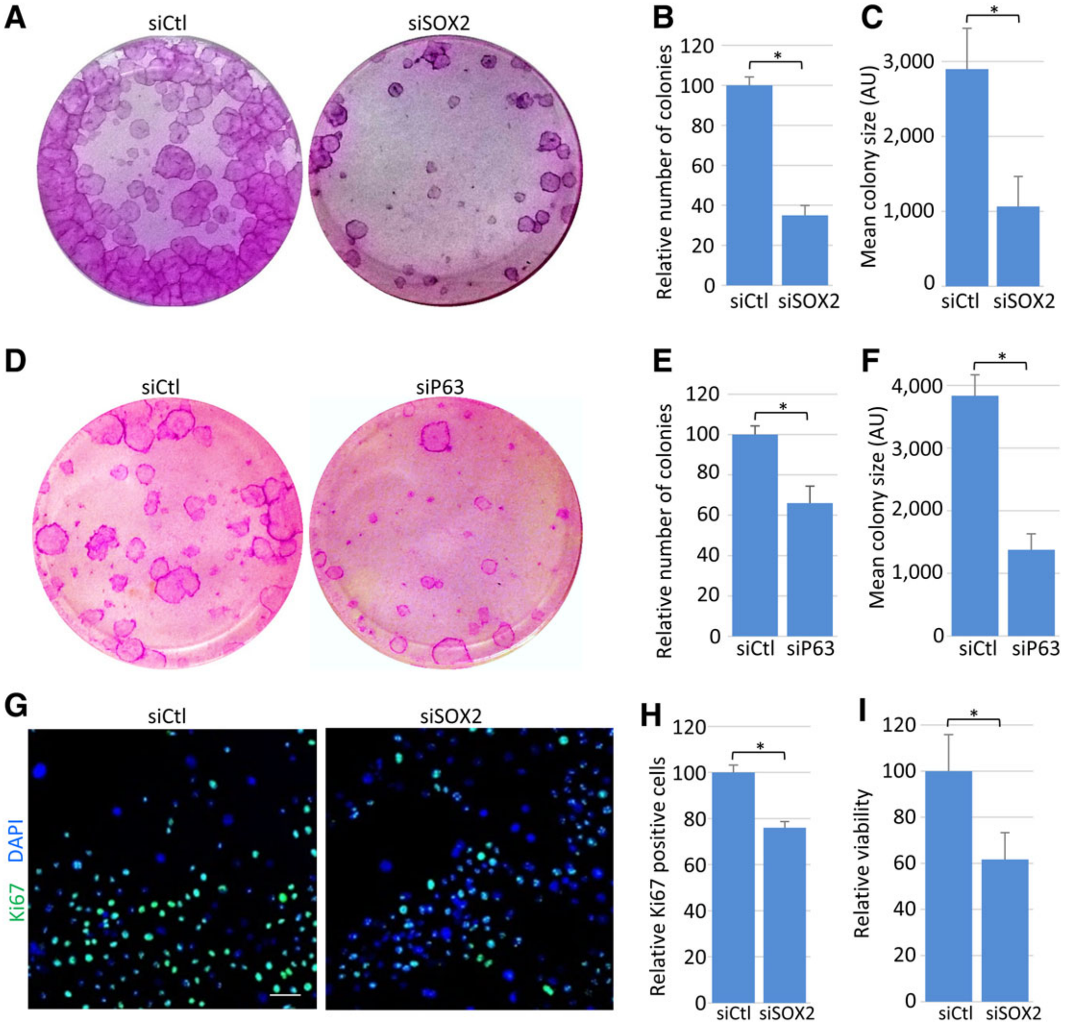

Figure 4. SOX2 regulates long-term colony-forming efficiency and cell proliferation. Limbal cells were transfected with siSOX2 or siP63 or siCtl and 48 hours later, subjected to clonogenicity test as detailed in Materials and Methods section. Colonies were visualized by Rhodamin staining 3 weeks later **(A, D)**, and quantification of the number of colonies relative to control **(B, E)** and the average size of colonies **(C, F)** was performed by Nis-Element software as detailed in Materials and Methods section. **(G–I):** Limbal stem/progenitor cells were transfected with siSOX2 or siCtl and 72 hours later, cells were immunostained for the proliferative marker Ki67 **(G)**, and quantification (by Nis-Element software) of the relative number of Ki67-positive cells is shown **(H)**. Transfectents were grown for 72 hours and then subjected to alamar blue viability test **(I)**. **(B, C, E, F, H, I):** Data represent mean ± SD, n = 3. Significance assessed by t test (*, $p < .05$). Nuclei were counterstained with DAPI and scale bars are 30 μm. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; siSOX2, endonuclease-digested silencing RNA against SOX2; siP63, endonuclease-digested silencing RNA against P63; siCtl, control endonuclease-digested silencing RNA.

displays spatially inversed expression pattern with SOX2 in the adult cornea (compare Fig. 6H with SOX2 pattern in Fig. 1A). Similarly, a moderate inverse correlation between SOX2 and miR-450b was found in the course of in vitro differentiation and maturation of embryonic stem cells into specific neurons (Supporting Information Fig. S3). These data suggest that miR-450b may serve as a SOX2 repressor.

To test whether miR-450b can bind and repress SOX2 through interactions with *SOX2-3′UTR*, we performed a luciferase assay in 293HEK cells. The 3′UTR region of *SOX2* which was cloned downstream to a luciferase gene (*SOX2-3′UTR*) and co-transfected with synthetic oligonucleotide mimic of pre-miR-450a (PM450a), pre-miR-450b (PM450b), or both, or scrambled mimic that served as control (Ctl). Twenty-four hours later, cells were lysed and luciferase activity was measured as detailed in Materials and Methods section. As shown in Figure 7A, both miRNAs inhibited luciferase activity when co-transfected with *SOX2-3′UTR* luciferase plasmid. However, a more significant decrease in the luciferase activity was observed in the presence of miR-450b, in line with the fact that it contains four binding sites in *SOX2-3′UTR.* Additionally, enhanced repression was observed when both miR-450a and miR-450b were co-transfected, suggesting that they might act in synergism. To test whether this effect depends on the binding to the predicted sites in *SOX2-3′UTR*, we performed site-directed mutagenesis. All five binding sites for miR-450a and miR-450b were disrupted by point mutations in a mutated construct (*MUT-SOX2-3′UTR*) that was generated (Supporting Information Fig. S4). Evidently, the luciferase activity of the mutated construct was insensitive to miR-450b, suggesting that miR-450b binds to these predicted sites to repress SOX2 (Fig. 7A).

To further test this possibility and its relevance to human, we tested the expression of SOX2 and miR-450b during differentiation of human limbal stem/progenitor cells. As shown in

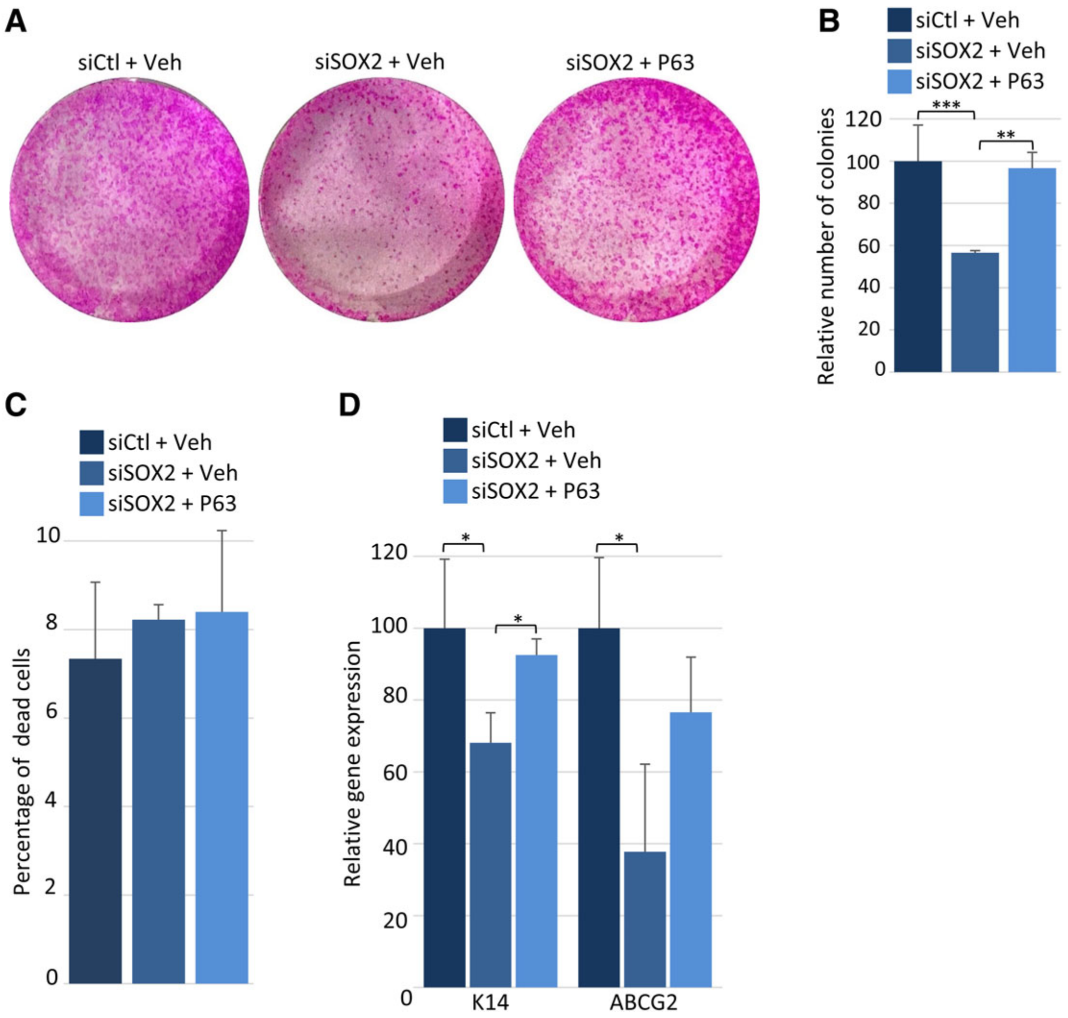

Figure 5. P63 rescues stemness in SOX2 knockdown cells. Limbal cells were co-transfected with siSOX2 or siCtl and P63 expression plasmid or empty plasmid (veh). Seventy-two hours later, cells were subjected to clonogenicity test **(A)**, and number of the colonies were quantified by Nis-Element software as detailed in Materials and Methods section **(B)**. **(C):** Cells were taken for trypan blue assay to quantify dead cells after transfection of indicated factors. Quantitative real-time polymerase chain reaction analysis of the indicated markers of stem/progenitor cells were performed **(D)**. **(B–D):** Data represents mean ± SD, $n = 3$. Statistical significance was assessed by one-way analysis of varaince followed by Tukey's test (*, $p < .05$; **, $p < .01$; ***, $p < .001$). Abbreviations: siSOX2, endonuclease-digested silencing RNA against SOX2; siCtl, control endonuclease-digested silencing RNA.

Figure 7B, as SOX2 decreased during differentiation, the levels of miR-450a and miR-450b significantly increased. As miR-450b had multiple binding sites (Fig. 6A, 6B) and showed enhanced repression (Fig. 7A), we transfected human limbal stem/progenitor cells with miR-450b mimic (PM) or control pre-miR sequence (Ctl), and transfection efficiency was validated by RT-PCR analysis (Supporting Information Fig. S5). A significant repression of SOX2 and P63 (Fig. 7C) further supported the hypothesis that miR-450b is a direct repressor of SOX2. Similar to the effect of siSOX2, transfection with PM resulted in a decrease in the stem/progenitor marker K14, cell proliferation (Fig. 7C, 7D), reduced clonogenic potential (Fig. 7E, 7F), and an increase in the differentiation marker K3 (Fig. 7C). Finally, miR-450b antagonist had an opposite effect on cell differentiation (Fig. 7G), proliferation (Fig. 7H), and clonogenic capacity (Fig. 7I, 7J). Taken together, we conclude that SOX2 regulates P63 and maintains the stem/progenitor cell state, whereas miR-450b represses SOX2 and induces cell differentiation at least in part, by directly targeting SOX2 and consequently by affecting P63 pathway.

DISCUSSION

It is likely that some of SOX2 functions are common in different cellular contexts, including regulation of stem cell self-renewal, asymmetric cell division, and chromatin remodeling. In neurons, it was proposed that SOX2 regulates stemness [37] and cooperates with other proteins to prevent the activity of polycomb repressive complex 2 [38, 39]. In this study, we show that SOX2 is expressed by stem/progenitor cells of the corneal epithelium and support their state. We propose that at least partly, SOX2 mediates its functions through the control of P63 expression. A newly identified SOX2 consensus binding site was found in C38, a putative enhancer of P63 gene [34]. We therefore propose that through the binding to this specific site, SOX2 regulates the expression of P63. Additionally, few observations from this study support the hypothesis that SOX2 and P63 cooperate through physical interaction: (i) the close proximity of P63 and SOX2 binding sites on C38, (ii) disruption of P63 sites by mutagenesis also affected SOX2-mediated enhancer activation, and

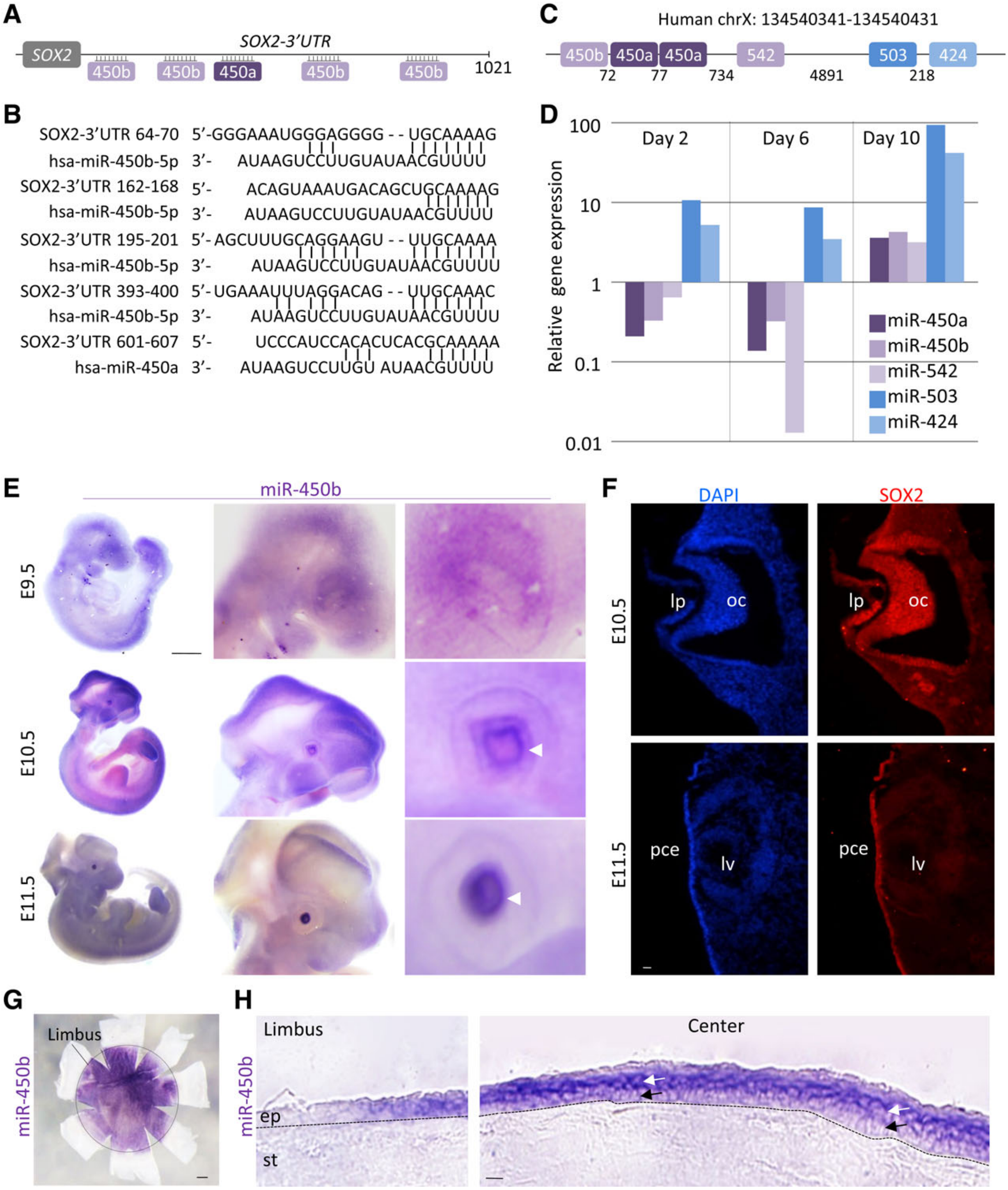

Figure 6. Evidence for *MIR450* cluster as a potential repressor of SOX2. **(A):** Schematic representation of the 3′UTR of human *SOX2* and predicted binding site of miR-450a and miR-450b identified by TargetScan. **(B):** Sequence and complementation of the predicted binding site. **(C):** Illustration of the human *MIR450* cluster (defined by miRBase) that includes six miRNAs genes. **(D):** Human embryonic stem cells were seeded on collagen IV-coated dishes in the present of corneal fibroblast conditional media to induce corneal epithelial differentiation for the indicated time. Relative expression of the indicated miRNAs is shown and data represent the normalized expression as fold change in expression relative to undifferentiated cells. **(E):** Wholemount in situ hybridization for miR-450b on mouse embryos of the indicated embryonic day. Increased magnifications are shown from left to right, and lens is annotated by white arrowheads. **(F):** Immunofluorescence staining of SOX2 on mouse head sections at E10.5 and E11.5. Nuclei were counterstained with DAPI. **(G, H):** In situ hybridization of miR-450b on whole cornea **(G)** or sections of cornea **(H)** of 2-month-old mice. Scale bars are 250 µm **(E, G)** and 25 µm **(F, H)**. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; lp, lens pit; lv, lens vesicle; oc, optic cup; pce, presumptive corneal epithelium; UTR, untranslated region.

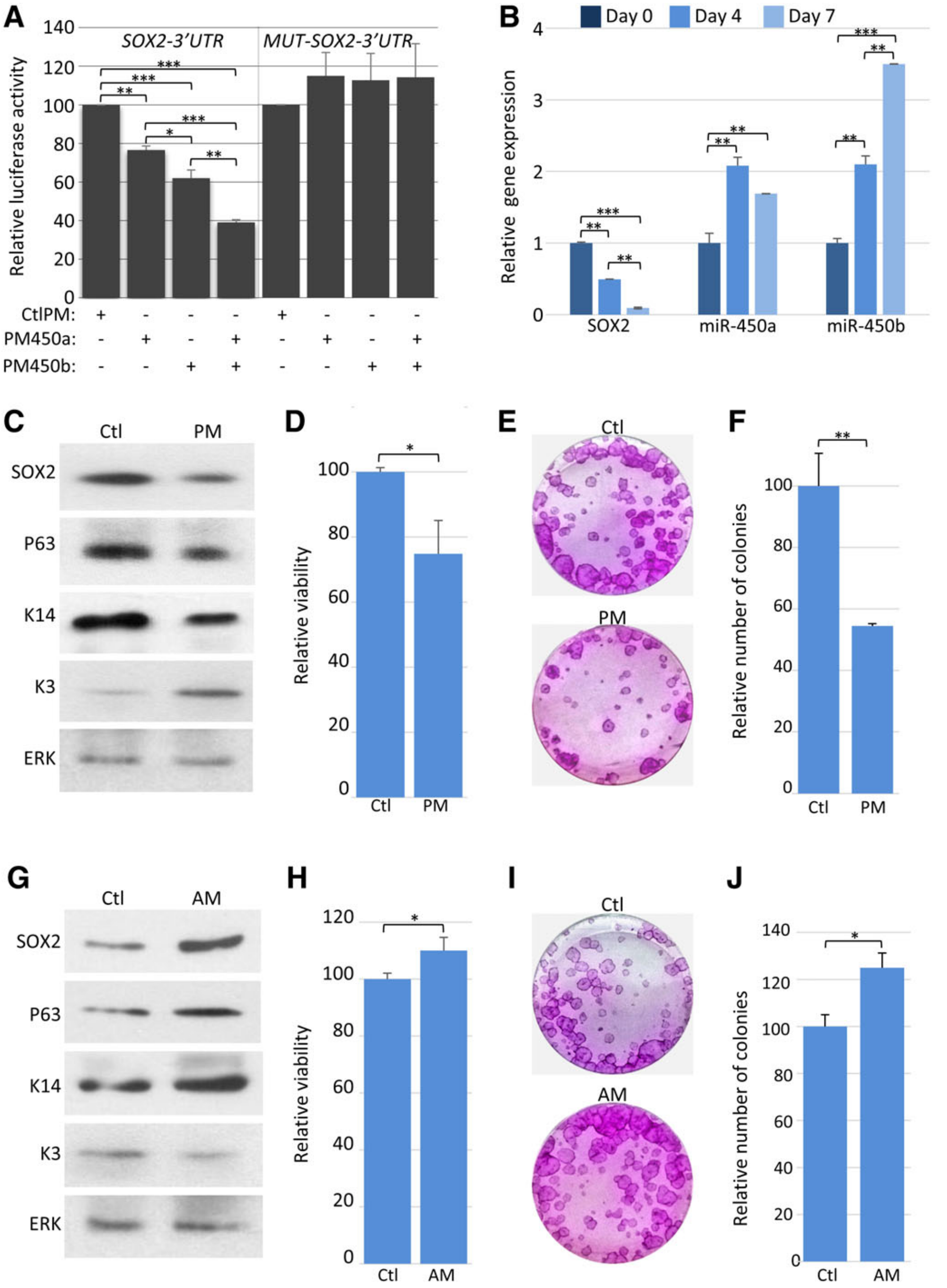

Figure 7. miR-450b represses SOX2 and induces differentiation of limbal epithelial stem/progenitor cells. **(A):** 293HEK cells were co-transfected with *SOX2-3′UTR* luciferase plasmid or with a mutated plasmid with disrupted miR-450a, b binding sites (*Mut-SOX2-3′UTR*, see Fig. S4), and with pre-miR-450a (PM450a) or pre-miR-450b-5p (PM450b) or both or control (CtlPM), as indicated. Data represent the normalized luciferase activity relative to control sample. **(B):** Primary human limbal stem/progenitor cells were induced to differentiate for the indicated time and the expression of the indicated genes was examined by quantitative polymerase chain reaction. **(C–J):** Primary human limbal stem/progenitor cells were transfected with PM or AM or Ctl and then subjected to differentiation for 4 days and Western blot analysis of the indicated genes **(C, G)**, or transfectants were allowed to grow for 72 hours and then cell viability was tested by alamar blue assay **(D, H)**, or transfectants were subjected to clonogenicity test and colonies were revealed by rhodamine staining **(E, I)** and quantified **(F, J)** by Nis-Element software. Data represent mean ± SD, $n = 3$. **(A, B):** Statistical significance was assessed by one-way analysis of variance followed by Tukey's test and **(D, F, H, J)** *t* test (*, $p < .05$; **, $p < .01$; ***, $p < .001$). Abbreviations: AM, pre-miR-450b mimic antagonist; Ctl, control pre-miR sequence; PM, pre-miR-450b mimic

(iii) these two proteins could co-immunoprecipitate and display overlapping functions. Altogether, it is tempting to hypothesize that SOX2 and P63 interact in vivo at least in the context of C38-C40-mediated activation of P63 transcription and potentially in additional genomic loci to control the regulation of stem/progenitor cell state and prevent cell differentiation. In line with this model, a recent study reported the co-occupancy of SOX2 and P63 in genomic loci and their cooperation in the regulation of gene expression in squamous cell carcinoma [40, 41].

Like other members of the SOX family, SOX2 possesses low DNA-binding affinity [42]. Therefore, SOX2 interactions with cofactors are essential to propagate its function. It would be therefore interesting to characterize SOX2 genomic binding sites and explore its interactions with potential coregulatory factors such as P63 and PAX6. Such cooperation is expected to control the corneal epithelial differentiation program, corneal avascularity, and/or corneal cell identity. In contrast to P63 that seemingly plays an overlapping role in the cornea and epidermis [13–15, 43], SOX2 was not detected in epidermal cells [44] (Fig. 1D). This expression pattern seems to be similar to that of PAX6 that could induce transdifferentiation of epidermal cells into corneal epithelial-like cells [45–47]. In fact, SOX2 and PAX6 have already been shown to coordinate key event in early lens placode development [21]. Altogether, it is possible that some of SOX2-mediated functions reported here are driven by SOX2 interactions with P63 and/or PAX6.

Our experiments suggest that SOX2 plays a role as a guardian of stem/progenitor cell state. Knockdown of SOX2 dramatically reduced the clonogenic potential of primary limbal stem/progenitor cells, induced a reduction in stem/progenitor cell markers, and attenuated cell proliferation. Notably, SOX2 was found to be expressed not only by stem/progenitor cells located in the limbus but also by corneal committed progenitor cells (i.e., basal corneal epithelial cells). In fact, it is likely that SOX2 prevents the terminal differentiation of corneal committed progenitors. SOX2 was detected in basal corneal progenitor cells but absent from terminally differentiated cells in vivo, while SOX2 inhibition by siRNA or by miR-450b induced significant cell differentiation in vitro. Thus, these data strongly suggest that SOX2 is essential for preventing terminal differentiation.

The rapid loss of SOX2 signal upon detachment from the basal cell layer implies for active mechanisms to remove residual SOX2. We propose that this mechanism is mediated by miR-450b, which is a direct repressor of SOX2 that induces cell differentiation. The 3′UTR of SOX2 contains multiple binding sites for miR-450b. In line, miR-450b reduced SOX2, P63, clonogenicity, and cell proliferation and induced cell differentiation. These results of miR-450b transfection were similar to the effects of SOX2 siRNA, suggesting that by directly repressing SOX2, miR-450b induces differentiation of corneal epithelial cells. Yet, like other miRNAs, miR-450b may have multiple target genes other than SOX2. In fact, PAX6 is a targeted by miR-450b and this regulation was shown to be important for corneal epithelial lineage commitment of embryonic stem cells [25]. Collectively, given that miR-450b targets these key transcription factors, it seems that miR-450b may be an important miRNA that rewards further investigation in vivo and in pathology. SOX2 and PAX6 share common roles in eye development and in the maintenance of neural SC self-renewal. However, very little is known regarding the regulation of their expression. The remarkable number of five binding sites in *SOX2 3′UTR*, the efficient repression in vitro, and the clear reciprocal expression in lens development in vivo suggest that miR-450b is a key regulator of SOX2. Knockout of SOX2 in human and mice led to an early failure in eye development and anophthalmia, whereas point mutation in one allele of SOX2 is linked with mental retardation and multiple eye defects [17–20]. Therefore, incorrect dosage of SOX2 because of miR-450b deficiency or its hyper activation is expected to result in eye and neural abnormalities. Thus, it would be interesting to examine the regulation of SOX2 by miR-450b in vivo and its potential association with corneal and/or neural hereditary diseases.

CONCLUSION

Altogether, we propose that SOX2 controls P63 and that both transcription factors are essential regulators of stem cell progenitor cell states in the corneal epithelium. In line with this model, mutations in these genes were linked with congenital eye pathologies that involve corneal abnormalities. It will be of importance to further characterize the interactions between SOX2 and P63, and their signaling network in different cellular compartments in the corneal epithelium in health and disease. A better understanding of the molecular network that is controlled by these key transcription factors will shed light on LSC self-renewal pathways and will potentially be harnessed into novel therapeutic approaches for corneal pathologies.

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AUTHOR CONTRIBUTIONS

S.B.: conceptual/design, data analysis and interpretation, prepared the figures, manuscript writing; L.S., E.N., D.D. and A.A.: conceptual/design, data analysis, prepared the figures; M.K. and B.T.: provided materials, data analysis and interpretation; P.H., L.P., C.L., D.A., and R.S.F.: conceptual/design, data analysis and interpretation, and manuscript writing. All authors approved the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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