Expression of *VSX1* in Human Corneal Keratocytes during Differentiation into Myofibroblasts in Response to Wound Healing

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Purpose. To characterize the expression of the visual system homeobox gene (*VSX1*) in human corneal keratocytes both in vitro and in vivo.

Methods. The expression of *VSX1* was evaluated through semiquantitative RT-PCR, immunofluorescence and in situ hybridization both in corneas (either freshly obtained or wounded) and in collagenase/hyaluronidase–isolated keratocytes grown in the absence or presence of serum to promote keratocyte-to-myofibroblast differentiation.

Results. Quiescent or resting keratocytes normally residing in the corneal stroma or cultured in vitro in the absence of serum did not express VSX1. In wounded corneas or when cultured in the presence of serum to mimic wound-healing responses, keratocytes underwent fibroblastic transformation (with appearance of α-SMA and disappearance of CD-34 and keratocan signals) and started expressing VSX1.

Conclusions. The results show that VSX1 is expressed in vitro and in vivo during human corneal wound healing, a process in which differentiation of corneal keratocytes into myofibroblasts occurs. These data may help to elucidate the role of VSX1 in cornea physiology suggesting a potential involvement in cornea-related diseases such as keratoconus. (*Invest Ophthalmol Vis Sci.* 2006;47:5243–5250) DOI:10.1167/iovs.06-0185

Human *VSX1*[1,2] is a member of the Vsx1 group of vertebrate paired-like homeodomain transcription factors. These transcription factors are distinguished by the presence of the CVC domain, a highly conserved region of unknown function that lies C-terminal of the homeodomain. The vsx1 gene was first identified in goldfish,[3] and orthologues have since been identified in zebrafish,[4] chicken,[5] bovine,[1] and mouse.[6–7] In situ hybridization studies on these species showed that vsx1/VSX1 is expressed in the outer tier of the inner nuclear layer of the retina, suggesting that it plays a role in the development of retinal bipolar interneurons. In humans, VSX1 mRNA has been detected in the inner nuclear layer of the retina[1] and in embryonic craniofacial tissue.[1] In the mouse, Vsx1 expression was first detected by in situ hybridization (ISH) at postnatal day 5 and later was shown to be restricted to cone bipolar cells in adult mouse retina.[6,8]

Despite this role in adult and developing retina, human *VSX1* mutations were unexpectedly found to be associated with dominant posterior polymorphous corneal dystrophy (PPCD) and keratoconus diseases,[9–11] two conditions known to affect solely corneal tissues. In addition, a *VSX1* mutation leading to ocular and nonocular abnormalities including severe craniofacial malformations, central nervous system defects, and impairment of hearing functions was reported.[12] To explain a potential mechanism underlying these corneal disorders VSX1 gene expression was therefore investigated in normal and pathologic corneal tissues. Despite initial reports identifying VSX1 transcripts in adult corneas,[2] these finding were no longer confirmed in further studies in normal or keratoconus-affected corneas.[9,13,14] In contrast, Vsx1 expression was detected in the mouse cornea, even if at very low levels.[6,15]

In this article we describe the expression and localization of VSX1 in the stroma of human corneas, thus suggesting a potential involvement in corneal stromal dystrophies.

Materials and Methods

Isolation and Culture of Human Keratocytes and Corneal Epithelial Cells

Human keratocytes were obtained from fresh human corneal stroma using a collagenase/hyaluronidase digestion protocol. An 8-mm biopsy punch (Aesculap AG & Co. KG, Tuttlingen, Germany) was used to remove a central corneal button. After the corneal epithelium was removed, the corneal stroma was sliced into quarters and digested overnight with 2.0 mg/mL collagenase (Roche, Basel, Switzerland) and 0.5 mg/mL hyaluronidase (Worthington Biochemicals, Lakewood, NJ) in DMEM at 37°C. Isolated cells were washed in DMEM and cultured either in serum-free conditions for maintenance of keratocytes in culture or in DMEM supplemented with 10% fetal calf serum (FCS; Invitrogen-Gibco, Grand Island, NY) for differentiation into fibroblast and myofibroblast cultures. The cells were cultured on tissue culture–treated plastic at 4×10^4 cells/cm^2 (keratocytes) or 1×10^4 cells/cm^2 (fibroblasts and myofibroblasts) and fixed in 3% paraformaldehyde after 5 to 6 and 7 to 10 days, respectively. For experiments testing the role of cell density in myofibroblast differentiation, corneal fibroblasts were plated at low (5 cells/mm^2) and high density (500 cells/mm^2) on glass coverslips and fixed after 6 to 7 and 2 to 3 days, respectively.

Corneal epithelial cells were cultivated on a feeder layer of lethally irradiated 3T3-J2 cells (a gift from Howard Green, Harvard Medical School, Boston, MA), as previously described.[16]

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TABLE 1. RT-PCR Primer Sequences

Primers	Sequence	Annealing Temperature (°C)	Product Length (bp)	GenBank Accession No.
Keratocan				
Sense	TATTCCTGGAAGGCAAGGTG	60	1249	NM_007035
Antisense	CATGTGACTGGCAAAAGCAT			
Lumican				
Sense	GGAAAACAATGCCCAGACTC	60	1258	NM_002345
Antisense	TGTTTACAAAACATTTCCCTCAGA			
Mimecan				
Sense	TCCAGTCACCTTGATTTTTGC	60	993	AF202167
Antisense	TTCCTTTACACATTGTTGAGACAGA			
VSX1				
Sense	5′ATTGACCTCTCCAGCTCTGC	60	201	NM_014588
Antisense	3′TGGACAATTTTTGTCTTTTGGA			
β-Actin				
Sense	5′GAGCGCAAGTACTCCGTGT	58	548	NM_001101
Antisense	3′ACGAAGGCTCATCATTCAAA			

Human Corneal Tissue Preparation

Human corneal tissues obtained from the Veneto Eye Bank Foundation (Mestre-Venezia, Italy) were collected at different times from 5 to 120 hours after death and evaluated through slit lamp examination immediately after retrieval. The corneas were managed in accordance with the guidelines in the Declaration of Helsinki for research involving human tissue. They were fixed in 3% paraformaldehyde (overnight at 4°C), embedded in optimal cutting temperature [OCT] compound, frozen, and sectioned (5- to 7-μm-thick slices). After hematoxylin staining, the corneas were divided into two groups: (1) normal corneas (typically fresh tissues), not showing epithelial defects, dehydration, edema, or inflammation, and (2) wounded corneas, characterized by central cornea defects and/or abrasions, usually due to loss of the epithelial integrity and/or to epithelial dehydration, focal breaks in Bowman's layer and stroma, degenerative pannus, disorganized collagen fibrils and opacity. In most cases, wounded corneas result from the incomplete closure of the eyelids after death.

RNA Extraction and Semiquantitative RT-PCR

Total RNA was extracted from tissues, cultured stromal and epithelial cells (RNeasy Micro kit; Qiagen, Valencia, CA). RT-PCR was then performed (OneStep RT-PCR kit; Qiagen). Primer sequences (synthesized by MWG Biotech, Ebersberg, Germany), annealing temperatures, and PCR product length are shown in Table 1. Reverse transcription reactions (45°C for 30 minutes, 95°C for 15 minutes) were followed by 40 PCR cycles (95°C for 10 seconds, 60°C for 1 minute, and 68°C for 2 minutes) and a final extension step (10 minutes at 68°C). VSX1, keratocan, mimecan, and lumican mRNA expression was analyzed by semiquantitative RT-PCR. β-Actin was used for normalization. cDNAs were synthesized starting from 0.5 to 1.5 μg of total RNA and PCR reactions performed by using 20, 24, 28, 32, 36, and 40 cycles. RT-PCR band intensity was quantified with commercial software (1D ver. 3.5; Eastman Kodak, Rochester, NY) and expressed as relative absorbance units (au).

In Situ Hybridization

Digoxigenin-labeled cRNAs were synthesized according to the manufacturer's instructions (DIG RNA Labeling kit; Roche), and hybridization was performed, also according to procedures recommended by the manufacturer (Roche). Primer pairs with Sp6/T7 promoter sequences (MWG Biotech) were used to obtain DNA templates for in vitro transcription. Digoxigenin was used for RNA probe labeling. Fluorescent antibodies (rhodamine-conjugated sheep anti-DIG Fab fragment; Santa Cruz Biotechnology, Santa Cruz, CA) were used for ISH in the cornea. A kit (BlueMap; Ventana Medical Systems, Tucson, AZ), was used for ISH in the retina.

Immunohistochemistry and Immunocytochemistry

Corneas fixed in 3% paraformaldehyde (overnight at 4°C) were embedded in OCT compound, frozen, and cut into 5- to 7-μm sections. Corneal sections and cells grown on tissue culture plastic or glass coverslips were analyzed by indirect immunofluorescence by using a VSX1 pAb (1:50; Santa Cruz Biotechnology), α-SMA mAb (1:100; Sigma-Aldrich), CD34 pAb (1:100; Santa Cruz Biotechnology), and CD-45 mAb (1:100; BD Biosciences, Chatsworth, CA) for 1 hour at 37°C. Rhodamine and FITC-conjugated secondary antibodies (1:100; Santa Cruz Biotechnology) were incubated for 1 hour at room temperature. Specimens were analyzed with a laser scanning microscope (LSM 510; Carl Zeiss Meditec, GmbH, Oberkochen, Germany), and image analysis was performed with fully automatic software (Axiovision version 4.4 software; Carl Zeiss Meditec, GmbH). For statistical analysis approximately 1000 cells per sample (three fields randomly chosen per slide) were analyzed. All data are expressed as the mean ± SD.

RESULTS

Localization of VSX1 in the Human Eye

To evaluate the expression of VSX1 in the human eye, we analyzed sections from the retina and cornea by RNA ISH. As shown in Figure 1A, VSX1 transcripts were restricted to the inner nuclear layer (INL) of the human retina, thus confirming previously published data showing a similar pattern of VSX1 expression in goldfish, mouse, and bovine retinas.[1,4,6] No detectable staining was observed when human retinal sections were hybridized with a control sense riboprobe (Fig. 1B). Of note, when VSX1 expression was analyzed in human corneas, transcripts were observed only in tissues characterized by epithelial defects, dehydration, edema, or inflammation (known as wounded corneas; Fig. 1C), but not in freshly obtained normal corneas (data not shown). VSX1 mRNA was predominantly localized in stromal keratocytes with barely detectable signals observed in the basal layer of the corneal epithelium (Fig. 1C). These data were confirmed by semiquantitative RT-PCR on cultured cells grown in the presence of serum and showed that VSX1 was strongly detected in stromal cells, but little, if at all, in epithelial cells (Fig. 2A). VSX1 expression was not detected in RNAs extracted from both freshly isolated keratocytes and the whole normal stroma of

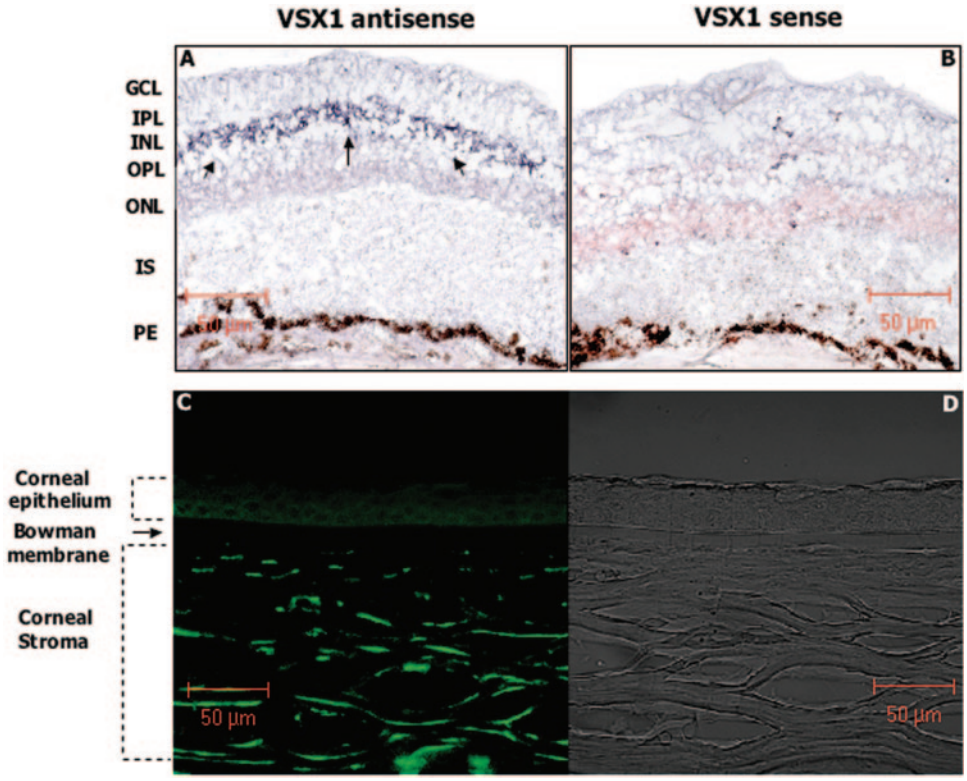

FIGURE 1. In situ localization of VSX1 mRNA in the adult human retina and cornea. (**A**) A continuous stripe of VSX1 expression was localized in the INL of the retina. (**C**) VSX1 is strongly expressed in the stromal keratocytes of wounded cornea. Barely detectable signals were observed in the basal layer of the corneal epithelium. (**B**, **D**) No staining was detected when human retina or cornea sections were hybridized with control sense riboprobes. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; IS: inner segment; PE: pigmented epithelium.

various corneal tissues ($n = 14$ corneas from human donors), as shown in Supplementary Figure S1, online at http://www.iovs.org/cgi/content/full/47/12/5243/DC1. In summary, VSX1 expression was found in the INL of the human retina and in the stroma of wounded corneas but was never detected in freshly isolated keratocytes or in intact corneas.

Expression of VSX1 in Fibroblasts, Myofibroblast, and Keratocytes

To explain the presence of VSX1 expression in the stroma of wounded, but not freshly obtained, normal corneas, in vitro cultures of stromal cells were set up mimicking conditions observed either in fresh corneas, in which resting or quiescent cells are present, or in wounded tissues, in which differentiation and wound-healing pathways are activated. It has long been reported that corneal stromal cells (also known as keratocytes) can be induced to alter their "in situ phenotype" by changing the culturing conditions. Keratocytes cultured in the presence of serum rapidly differentiate into fibroblasts and myofibroblasts, thus mimicking conditions observed in wound-healing responses. In the absence of serum, keratocytes maintain native biosynthetic and phenotypic appearances typical of resting or quiescent cells, as observed in freshly obtained normal corneas.[17,18] Furthermore, cultures of quiescent primary keratocytes have been reported to secrete proteoglycans (PGs) similar to those found in vivo, including the three major corneal keratan sulfate-containing PGs (KSPGs): lumican, keratocan, and mimecan.[19,20] On the contrary when corneal keratocytes are serially propagated in vitro or when serum is added (1) differentiation into fibroblasts and myofibroblasts is observed, (2) secretion of KSPGs is greatly reduced and (3) keratocan expression is definitely lost.[19]

Semiquantitative RT-PCR was performed on stromal cells grown either in the presence or absence of serum. We observed that in the absence of serum, keratocytes retained the biosynthetic features resembling those of stromal cells in

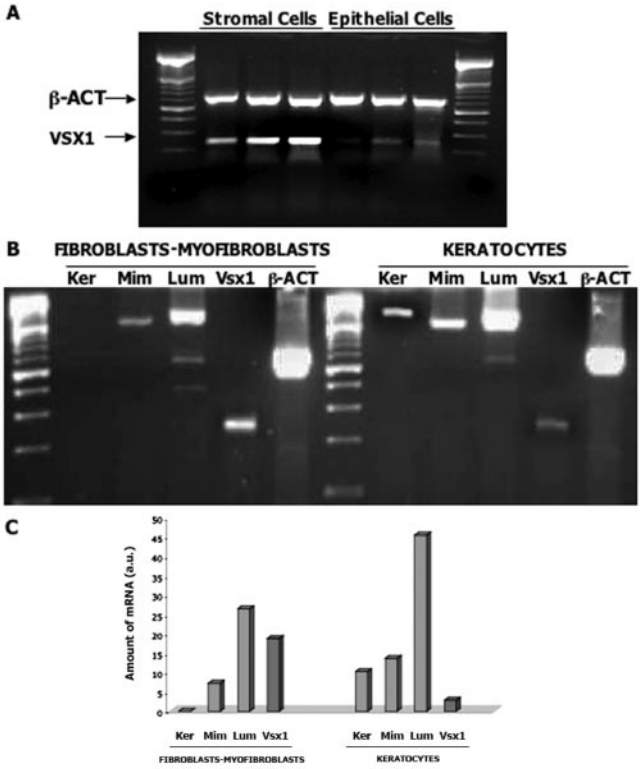

FIGURE 2. (**A**) Semiquantitative RT-PCR analysis from cultured stromal and epithelial cells. VSX1 was strongly detected in stromal cells but not in epithelial cells. (**B**) Semiquantitative RT-PCR performed on stromal cells grown either in the presence or absence of serum. VSX1 was expressed strongly in fibroblast and myofibroblast cultures, but only slightly in keratocytes. (**C**) In fibroblast and myofibroblast cultures, VSX1 expression was 8- to 10-fold higher than in keratocyte cultures, keratocan (Ker) was lost, and lumican (Lum) and mimecan (Mim) were highly reduced. β-act: β-actin.

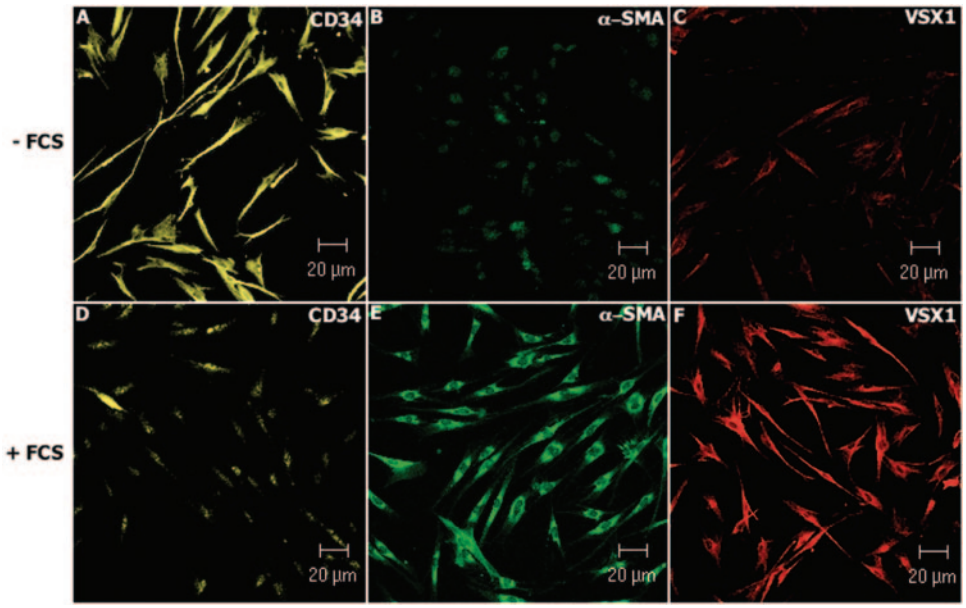

FIGURE 3. Immunostaining of VSX1, α-SMA, and CD-34 in keratocyte (serum-free cultures: −FCS) and fibroblasts and myofibroblast cultures (serum -containing cultures: +FCS). When cultured in the absence of serum keratocytes showed a dendritic morphology and expressed CD-34, but not α-SMA and VSX1 (**A–C**). Myofibroblasts showed positive α-SMA and VSX1 expression, but not CD-34 staining (**D–F**).

freshly obtained normal corneas (i.e., expression of major corneal KSPGs and keratocan). As expected VSX1 was barely expressed (Fig. 2B). In the presence of serum, KSPGs were still expressed, but keratocan was not, thus suggesting that keratocytes had differentiated into both activated keratocyte phenotypes (fibroblasts and myofibroblasts), after the change in cell culture conditions. In fibroblast and myofibroblast cultures, VSX1 expression was 8- to 10-fold higher than in keratocyte cultures (Fig. 2C), thus suggesting a likely involvement of VSX1 in differentiation and wound-healing pathways. These data were confirmed when stromal cells were cultured either in the presence or the absence of serum and expression of specific markers analyzed by immunofluorescence.

Similarly to observations in normal corneas, when cultured in the absence of serum keratocytes appear typically quiescent and flattened and express CD-34, a glycosylated transmembrane protein.[18] In this case VSX1 was not detectable (Figs. 3A, 3C).

In wounded corneas, keratocytes become activated, migrate to the wound site, start dividing, and express F-actin stress fibers containing the muscle protein α-smooth muscle actin (α-SMA) which leads to a myofibroblastic phenotype.[21] As we said, this can be easily mimicked in vitro by switching to serum-containing culture medium. As shown in Figure 3, keratocytes rapidly lost their dendritic morphology and acquired a fibroblastic morphology. In addition, expression of VSX1, α-SMA, but not CD-34 was observed (Fig. 3). To evaluate whether myofibroblasts express VSX1, double staining for both VSX1 and α-SMA was performed on cell cultures (Figs. 4A–F). All cells showing α-SMA in stress fibers expressed VSX1 as well. However, several cells showed VSX1, but not α-SMA expression, thus suggesting an earlier activation of VSX1 that it is likely to occur during keratocyte-to-fibroblast transition and to be maintained also in myofibroblasts. It has been reported that cultured fibroblasts differentiate into myofibroblasts through a cell-density–dependent mechanism.[22] To confirm that VSX1 is ubiquitously expressed in fibroblast/myofibroblast populations, cells were plated at low (5 cells/mm^2) or high density (500 cells/mm^2), conditions known to lead to enriched myofibroblast and fibroblast populations, respectively. As shown in Figure 4G, high (60%–70%) and comparable levels of VSX1 expression were observed when fibroblasts were plated at low (containing 55% myofibroblasts) or high (with approximately 12% myofibroblasts) densities. In summary, VSX1 is expressed in both activated keratocyte phenotypes, (fibroblasts and myofibroblasts) but not in normal quiescent keratocytes.

Modulation of VSX-1, α-SMA, CD34, and CD45 Expression in Human Corneas

The expression of keratocyte- and/or myofibroblast-specific markers and the involvement of VSX1 in wound-healing responses was further investigated in normal and wounded corneas. As expected, wounded corneas showed positive VSX1 staining in the corneal stroma, whereas no signal was observed in normal tissues (Fig. 5). When VSX1 expression was correlated with CD-34 (keratocyte-specific marker) and α-SMA (myofibroblast-specific marker) expression in normal or wounded corneas, we observed that VSX1 was strongly associated with upregulation of α-SMA and downregulation of CD-34. Normal corneas typically characterized by a quiescent keratocyte population showed high CD-34 (Fig. 6I) expression and barely detectable α-SMA (Fig. 6E) expression, but did not express VSX1 (Fig. 6A), which is consistent with the presence of a quiescent keratocyte population. In wounded corneas ($n = 3$) VSX1 expression was strictly correlated with that of α-SMA. Corneas exhibiting increasing values of α-SMA (Figs. 6E–H) showed intense VSX1 staining (Figs. 6A–D). Those wounded corneas showed the highest VSX1 and α-SMA expression (Figs. 6D, 6H) also showed no CD-34 expression (Fig. 6N), thus indicating that a transition from keratocytes to myofibroblasts had occurred. It is known that corneal injury leads to the recruitment of inflammatory cells in the wounded area.[23] To verify that the cells involved in the wound-healing process were resident stromal cells rather than inflammatory cells, we checked for expression of CD-45, a marker of bone-marrow–derived cells contained in the normal corneal stroma.[24,25] As shown in Figures 6O–R no significant modulation of CD-45 expression was observed in wounded corneas, thus suggesting that the VSX1-expressing cells were corneal fibroblasts or myofibroblasts and not inflammatory or immune cells.[26] Of interest, at higher magnifications (63×), VSX1 signal was clearly localized either in the nucleus or in the cytoplasm (Fig. 7), which confirms previous data showing that VSX1, like other homeoproteins, has the capacity for continuous shuttling between the cytoplasm and the nucleus.[27]

To confirm our findings, in a limited cohort of mice ($n = 4$), corneal wounding was achieved by means of alkali burns and

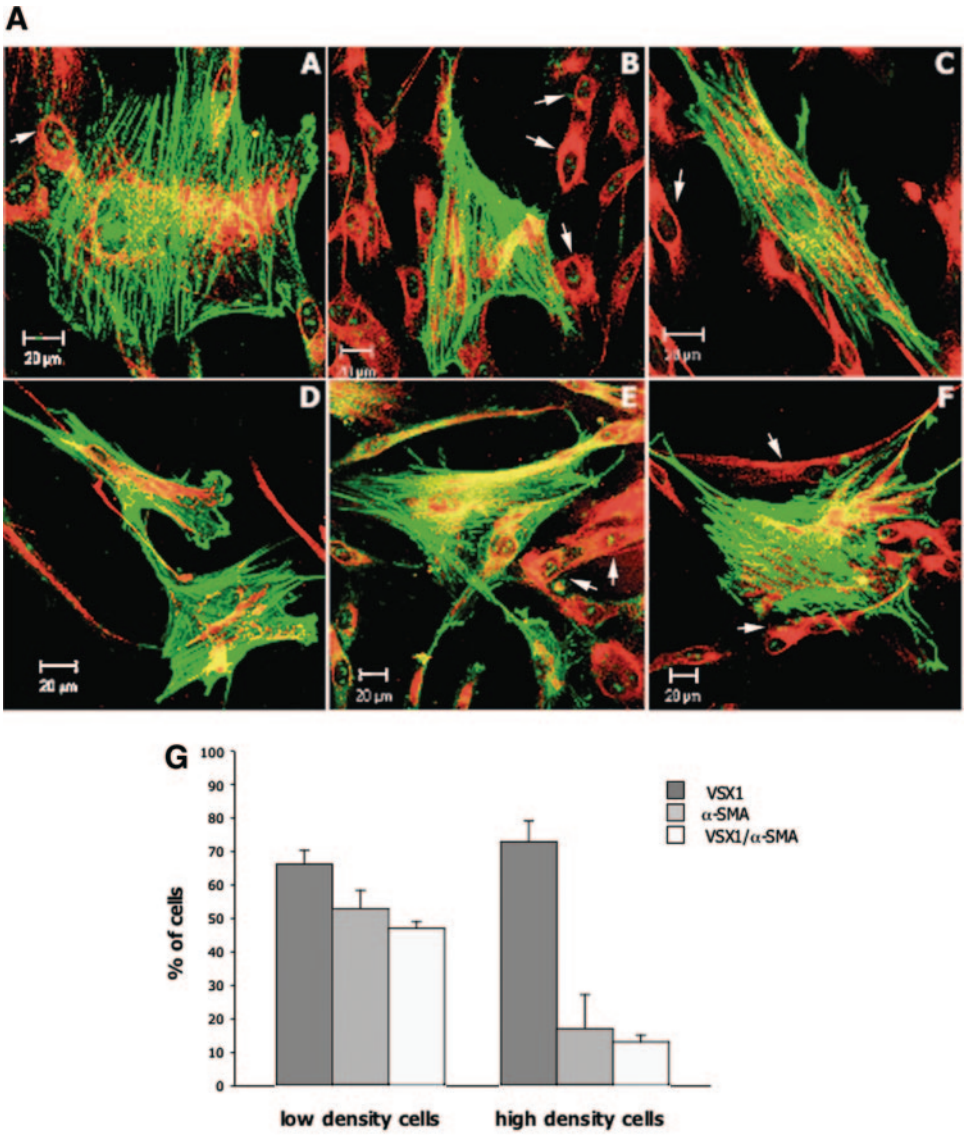

FIGURE 4. Double-immunostaining of cultured human corneal fibroblasts and myofibroblasts. (**A–F**) Corneal myofibroblasts showed positive labeling for VSX1 (*red*) and α-SMA in F-actin–containing stress fibers (*green*). (**A–F**) Note that fibroblasts showed positive staining for VSX1 but not for α-SMA (*arrows*). Quantification of fluorescence immunocytochemistry obtained by laser scanning confocal microscopy. The histogram shows the percentage of cells expressing VSX1 and α-SMA in cells cultured at low and high density and the percentage of cells in which VSX1 and α-SMA colocalized. Error bars, SD of the mean of triplicate samples within one experiment (**G**).

mechanical removal of the epithelium as previously described.[28] Whereas VSX1 was never detected in the untreated eyes, it was always observed in the wounded eye of each treated mouse, with patterns of expression similar to that described in human samples (Supplementary Fig. S2, online at http://www.iovs.org/cgi/content/full/47/12/5243/DC1).

DISCUSSION

Wound healing is a complex process requiring cellular and subcellular events, occurring under the influence of extracellular matrix proteins and growth factors and involving the coordinated interplay of several phases of proliferation, migration, matrix synthesis, and contraction. After stromal wounding, a multistep process is typically observed: (1) keratocytes start proliferating and migrate into the wounded area stimulated by the release of specific cytokines[29]; (2) keratocytes undergo fibroblastic transformation with the resultant expansion of the fibroblastic population by mitosis after approximately 48 to 72 hours[30]; (3) fibroblasts differentiate into myofibroblasts, which are characterized by the development of prominent actin filament bundles and the expression of the smooth-muscle-specific α isoform of actin (α-SMA).[21] Myofibroblasts play a critical role in wound repair by organizing into a unique network of interconnected cells that exert mechanical force through a smooth-muscle–like contractile mechanism, resulting in wound contraction and matrix organization.[31,32]

In this work, we have analyzed VSX1 expression and its involvements in wound-healing processes, both in vitro culture conditions mimicking this process (presence of serum) and in vivo in human wounded corneas.[17,18] The results suggest a strong correlation between VSX1 expression and wound-healing responses. VSX1 is absent in freshly obtained normal corneas where keratocytes maintain more native biosynthetic and phenotypic appearances typically observed in quiescent cells. Similar results are observed in vitro when stromal keratocytes are cultured in the absence of serum. Differently, VSX1 is abundantly expressed in wounded corneas and in cells cultured in the presence of serum. Our data suggest that VSX1 is expressed only when keratocytes undergo fibroblastic transformation (i.e., in wounded corneas or when cells are cultured in the presence of serum). In these cases we observe that keratocytes assume fibroblast phenotypes and express myofibroblast-specific markers (α-SMA, but not keratocan and CD-34). This finding is consistent with previously published works showing that when keratocytes differentiate into myofibro-

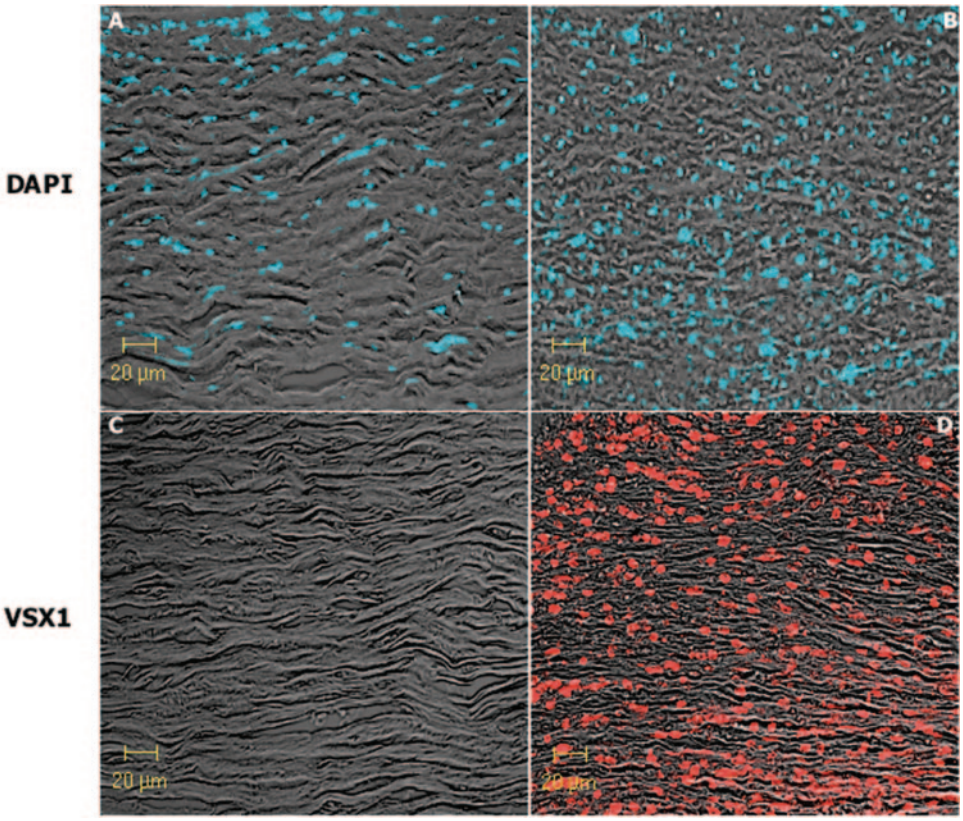

FIGURE 5. DAPI and VSX1 staining in human normal and wounded corneas. Note that an increased amount of stromal cells can be observed in the wounded stroma compared with the normal tissue (**A**, **B**). Wounded cornea showed positive VSX1 staining in the corneal stroma, whereas no signal was observed in normal tissues (**C**, **D**).

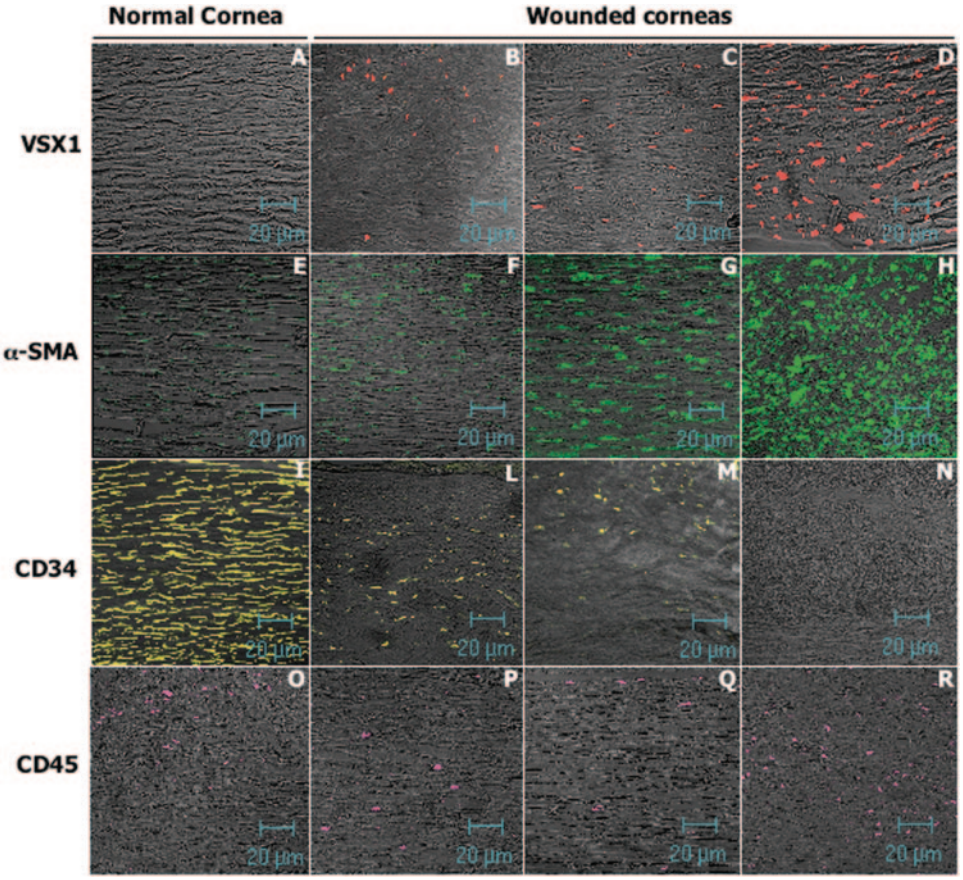

FIGURE 6. Immunodetection of VSX1, α-SMA, CD-34, and CD-45 in human normal and wounded corneas. VSX1 expression was strongly associated with upregulation of α-SMA and downregulation of CD-34. Normal cornea did not express VSX1 and showed barely detectable α-SMA and high CD-34 expression (**A**, **E**, **I**). Wounded corneas exhibited increasing values of α-SMA (**F**–**H**), showed high VSX1 staining (**B**–**D**) but no CD-34 expression (**L**–**N**). No significant modulation of CD-45 was observed (**O**–**R**).

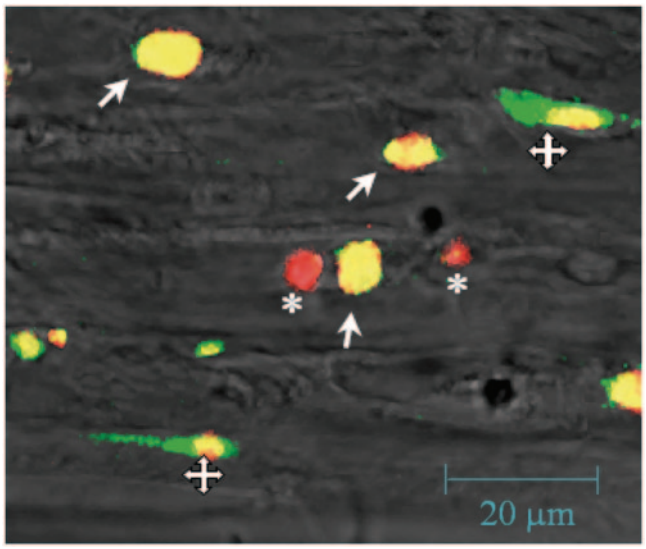

FIGURE 7. Double staining of DAPI (*red*) and VSX1 (*green*) in human wounded cornea. *Yellow* (*arrows*): merged image of the two stainings. VSX1 was clearly localized either in the nucleus (↑) or in the cytoplasm (+). Cells with DAPI but not VSX1 staining are *red* (✶). Magnification, ×63.

blasts, specific markers, such as keratocan and CD-34 are lost, and the cells loose their keratocyte-like phenotypic and biosynthetic characteristics.[17,18] The differentiation of keratocytes into myofibroblasts and the expression/disappearance of specific markers is reported to be a hallmark of wound-healing processes. In all these cases, we found VSX1 to be strongly expressed. These findings also explain previous difficulties encountered in evaluating and localizing VSX1 expression in freshly obtained normal corneas and reinforce our hypothesis suggesting a potential role for VSX1 in wound-healing processes. The potential involvement of VSX1 in pathways leading from keratocytes to myofibroblasts may confirm the role of this factor in differentiation processes. Similarly, Chow et al.[8] showed that VSX1 is required for the late differentiation and function of cone bipolar cells.

Recently, mutations in the VSX1 gene were found in patients affected by various corneal dystrophies such as keratoconus[10] and posterior polymorphous corneal dystrophy.[9] Keratoconus is a frequent (1: 2000) corneal dystrophy characterized by progressive conical protrusion of the cornea and noninflammatory central stroma.[33] Our findings showing that VSX1 is expressed also in the corneal stroma may elucidate the pathophysiological origin of genetic disorders such as keratoconus and suggest potential therapeutic treatments.

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