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Transforming Growth Factor- β Regulates *in Vitro* Heart Valve Repair by Activated Valve Interstitial Cells

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The regulation of valve interstitial cell (VIC) function in response to tissue injury and valve disease is not well understood. Because transforming growth factor- β (TGF- β) has been implicated in tissue repair, we tested the hypothesis that TGF- β is a regulator of VIC activation and associated cell responses that occur during early repair processes. We used a well-characterized wound model that was created by mechanical denudation of a confluent VIC monolayer to study activation and repair 24 hours after wounding. VIC activation was demonstrated by immunofluorescent localization of α -smooth muscle actin (α -SMA), and α -SMA mRNA levels were quantified by real-time polymerase chain reaction. Proliferation and apoptosis were quantified by bromodeoxyuridine staining and terminal deoxynucleotidyl transferase dUTP nick end labeling, respectively. Repair was quantified by measuring VIC extension into the wound, and TGF- β expression was shown by immunofluorescent localization of intracellular TGF- β . Compared with nonwounded monolayers, VICs at the wound edge showed α -SMA staining, increased α -SMA mRNA content, elongation into the wound with stress fibers, proliferation, and apoptosis. VICs at the wound edge also showed increased TGF- β and pSmad2/3 staining with co-expression of α -SMA. Addition of TGF- β neutralizing antibody to the wound decreased VIC activation, α -SMA mRNA content, proliferation, apoptosis, wound closure rate, and stress fibers. Conversely, exogenous addition of TGF- β to the wound increased VIC activation, proliferation, wound closure rate, and stress fibers. Thus, wounding activates VICs, and TGF- β signaling modulates VIC response to injury. (*Am J Pathol* 2008, 173:1275–1285; DOI: 10.2353/ajpath.2008.080365)

Valve interstitial cells (VICs) are the predominant cell type in the heart valve.¹ Under normal circumstances, VICs are quiescent and maintain the structural integrity and function of the valve.^{2,3} In response to valve injury, VICs undergo phenotypic changes and become activated.^{4,5} α -Smooth muscle actin (α -SMA), a cytoskeletal isoform of actin not normally found in the quiescent VICs of normal heart valves, is a marker for activated VICs. Diseased heart valves show up-regulation of α -SMA staining in VICs.^{6–10} Activated VICs have features of myofibroblasts showing increased contraction, actin stress fibers, and other contractile proteins.^{3–5,11–13} Myofibroblast-type cells regulate wound repair in many organs¹⁴ and it is likely that activated VICs also regulate wound repair in the heart valve.¹⁵ Thus, understanding the regulation of VIC activation and the associated cellular responses that occur in early wound repair is critical to understanding the pathobiology of heart valve diseases.

Transforming growth factor (TGF)- β ,¹⁶ a 25-kDa protein that is a member of the TGF- β superfamily, is a well-studied regulator of extracellular matrix deposition in wound repair. It is secreted by numerous cell types¹⁷ including VICs with potent autocrine effects.^{18,19} It is known to promote differentiation of mesenchymal cells into myofibroblasts^{20,21} and to regulate multiple aspects of the myofibroblast phenotype through transcriptional activation of α -SMA, collagen,²² matrix metalloproteinases,²³ and other cytokines such as connective tissue growth factor²⁴ and basic fibroblast growth factor.^{25–27} TGF- β is present in mitral valve prolapse^{6,28} and calcific aortic stenosis.^{7,29,30} Heart valves of carcinoid syndrome patients show VIC activation and increased expression of TGF- β , which is associated with increased collagen deposition, changes in the organization of extracellular matrix components, and calcification.^{31,32}

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The regulation of the early stages of VIC wound repair are less well understood than the later stages of fibrosis and wound contracture. Because TGF- β has been implicated in several tissue repair conditions, we tested the hypothesis that TGF- β regulates VIC activation and associated cell functions that are implicated in early wound repair including VIC activation, extension of elongated stress fiber-rich VICs into the wound, proliferation, and apoptosis. We choose to use an *in vitro* model that has been extensively used to study endothelial, smooth muscle cell, and epithelial wound repair. Wounding is achieved by mechanical denudation of a confluent monolayer.^{1,33–36} We demonstrate that injury to a confluent VIC monolayer leads to TGF- β and VIC activation. VIC cultures treated with TGF- β neutralizing antibodies and exogenous TGF- β alter VIC activation and the associated cellular activities that occur in the early stages of wound repair. We examine changes in VIC proliferation and apoptosis, which are processes intrinsic to repair and remodeling that contribute directly to wound closure and show that TGF- β is required to maintain VIC activation and is a key regulator of wound repair by VICs.

Materials and Methods

Cell Culture

Porcine hearts were obtained from a local abattoir, and explants were prepared from the distal third of the anterior leaflet of porcine mitral valves as previously described.³⁷ Briefly, the atrial and ventricular surfaces of the explants were scraped with a scalpel blade and rinsed with phosphate-buffered saline (PBS), pH 7.4, to remove valve endothelial cells. The explants were cut into 4 × 5-mm pieces, placed in 35-mm tissue culture dishes (Falcon; BD Biosciences, San Jose, CA), and grown in medium 199 (M-199) supplemented with 10% fetal bovine serum (FBS), and 2% penicillin, streptomycin, and Fungizone (Life Technologies Inc., Rockville, MD) in a humidified 95% air and 5% carbon dioxide atmosphere in an incubator at 37°C. VICs that grow out of the explants were detached with TrypLE Express (Invitrogen Corp., Carlsbad, CA) and subcultured. In all experiments, VICs were cultured in 10% or in 0.5% FBS M-199 with 2% penicillin, streptomycin, and Fungizone. VICs of passages two to four were used.

Concentration of TGF- β in Culture Media

Conditioned media was collected from wounded and control nonwounded monolayers (NWMs) cultured in parallel in 0.5% or 10% FBS containing media. VIC-conditioned media was collected at time 0 after wounding and at 24 hours after wounding and enzyme-linked immunosorbent assay (ELISA) for TGF- β 1 was performed. Porcine TGF- β 1 immunoassay kit (R&D Systems, Minneapolis, MN) was used to measure the concentration of active porcine TGF- β 1 in culture supernatants. Following the manufacturer's instructions, before ELISA, conditioned media were treated with 1 N HCl for 10 minutes

followed by 1.2 N NaOH/0.5 mol/L HEPES to activate TGF- β before performing the ELISA measurements. Measurements were performed in duplicate. Three independent experiments were performed. To determine the concentration of active TGF- β in conditioned media collected at 24 hours after wounding from cultures treated with TGF- β neutralizing antibody, Dynabeads Protein G and Dynal MPC-S Magnet (Invitrogen) were used to first precipitate complexes of TGF- β /TGF- β -neutralizing antibodies from media. Then the supernatants were assayed for TGF- β by ELISA.

Experimental Wounds

VICs were plated at a density of 1700 cells per well onto 18-mm diameter glass coverslips (Fisher Scientific, Pittsburgh, PA) in 12-well tissue culture dishes. VICs were then cultured in 1 ml of standard media per well for 6 to 8 days until a confluent monolayer was formed on top of the glass coverslip. This allowed the VICs to deposit their own matrix.³⁶ A 1-mm-wide flat blade was dragged across the monolayer in a linear manner completely removing a strip of VICs and associated extracellular matrix in the center of the monolayer to form a linear wound containing two straight edges. Approximately 10% of cells were removed from the monolayer to form the wound. The wounded monolayer was washed three times with serum-free M-199 media to remove cell debris. NWMs were cultured in parallel to wounded monolayers as controls. Throughout all of the experiments, the morphology of the monolayer in the wounded cultures away from the wound edge (WE) was similar to the monolayer in the nonwounded control cultures.

Treatment with TGF- β Neutralizing Antibody

A pan-TGF- β neutralizing antibody (R&D Systems) was reconstituted in sterile PBS to a concentration of 10 mg/ml. After wounding and washing of VIC monolayers, the cultures were incubated in 10% FBS containing media with 15 μ g/ml TGF- β neutralizing antibody. At 24 hours after wounding, the coverslips were washed in serum-free M-199 media and immunofluorescently stained for α -SMA. Apoptosis, proliferation, and extent of wound closure were also quantified. Three independent experiments were performed in triplicate coverslips.

Treatment with Exogenous TGF- β

In preliminary experiments, addition of exogenous TGF- β to cultures in 10% FBS-containing media led to focal areas of contraction with disruption of the confluent monolayer and retraction of the WE. By lowering the serum concentration, these effects disappeared and the monolayer was not perturbed. Therefore, for the experiments involving treatment with exogenous TGF- β , VICs were incubated in 0.5% FBS media that contains 0.1 ± 0.07 ng/ml of TGF- β as measured by ELISA. Porcine TGF- β 1 (R&D Systems) was reconstituted in a vehicle solution of 4 mmol/L HCl and 0.1% bovine serum albu-

min. Media containing vehicle or 0.5 ng/ml of active porcine TGF- β 1 was added to cultures after wounding and washing. This allowed us to add five times the concentration of TGF- β that was available for activation in the 0.5% FBS media. At 24 hours after wounding, the coverslips were washed in serum-free M-199 media and immunofluorescently stained for α -SMA. Apoptosis, proliferation, and extent of wound closure were also quantified. Three independent experiments were performed in triplicate coverslips.

Immunofluorescent Staining

Nonwounded and wounded monolayers were immunofluorescently stained at 24 hours after wounding. VICs on glass coverslips were fixed with 4% paraformaldehyde, rinsed three times in PBS, incubated with 0.1% Triton X-100 in PBS for 5 minutes, and rinsed again three times in PBS at 5-minute intervals. The coverslips were incubated with mouse anti- α -SMA (1:500; Sigma, Oakville, Canada), rabbit anti-TGF- β (against TGF- β 1, -2, -3), anti-phosphorylated Smad2/3 (Ser423/425) (1:100 and 1:50, respectively; Santa Cruz Biotechnology, Santa Cruz, CA), anti-TGF- β 3 (1:100; Abcam, Cambridge, MA), anti-cleaved caspase-3 (1:50; Cell Signaling, Danvers, MA), and mouse anti-BrdU (1:100, Cell Signaling) primary antibodies at room temperature for 1 hour. The coverslips were then washed three times with PBS at 5-minute intervals. Secondary antibodies were goat anti-mouse Alexa 488 or 568 and goat anti-rabbit Alexa 488 or 568 (1:200; Molecular Probes, Invitrogen, Eugene, OR). Hoechst 33342 (1:500; Lonza, Basel, Switzerland) or propidium iodide (1:5000, Sigma) were used to counterstain nuclei. After secondary antibody, coverslips were dipped in deionized water and mounted with Prolong Gold antifade reagent (Molecular Probes, Invitrogen) allowing the samples to cure overnight. Equivalent amounts of mouse IgG and rabbit IgG protein (Jackson ImmunoResearch, West Grove, PA) were used for negative control. Coverslips were examined using the $\times 20$, $\times 40$, and $\times 60$ objective of a Nikon C1 scanning confocal laser-imaging system (Nikon Canada, Mississauga, Canada) fitted with an argon-krypton mixed-gas laser with excitation wavelengths of 488 and 568 nm. Serial optical sections were taken at 0.5- μ m intervals for a total of 5 to 8 μ m depending on cell or monolayer thickness. Volume rendering of optical sections were compiled using the EZ-C1 software (Nikon Canada). Images were captured at randomly selected regions within the NWM or at the WE using the FV10-ASW 1.6 software (Olympus, Markham, Canada) to avoid saturation.

Quantification of α -SMA Stress Fibers

VICs at the WE showing α -SMA stress fiber staining were quantified at 24 hours after wounding. Random counting of VICs was performed using the $\times 40$ objective of the Nikon C1 confocal microscope starting five fields away from the edge of the coverslip and was continued linearly counting cells in every other field of view for 30 fields

along the WE. A total of 40 to 50 cells per field were counted. Both WEs were counted for a total of 60 fields per wound. Thirty random fields in the center of the coverslip were counted as controls in the nonwounded confluent monolayer.

Quantification of Proliferation

Bromodeoxyuridine labeling reagent (Sigma) was added in 1:500 dilution to VIC cultures at 22 hours after wounding. At 24 hours after wounding, the coverslips were washed in serum-free M-199 media, fixed in ethanol:acetic acid:H₂O (90:5:5) for 20 minutes, incubated with denaturing solution (Calbiochem, La Jolla, CA) for 1 hour and immunofluorescently stained. VIC nuclei were counterstained with propidium iodide (1:5000, Sigma). Both the total number and the number of proliferating VICs at the WE and in the NWM were counted randomly as described above for the counting of VICs with α -SMA stress fibers. Proliferation was calculated as a percentage of the labeled cells out of the total number of VICs.

Quantification of Apoptosis

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed using the TACS TdT-fluorescein *in situ* apoptosis detection kit (R&D Systems) to identify apoptotic VICs. VICs were also immunofluorescently stained for cleaved caspase-3. VICs were counterstained with propidium iodide. The total number of VICs and the number of VICs undergoing apoptosis in the NWM and at the WE were counted as described above for the counting of VICs with α -SMA stress fibers. The apoptotic index was calculated as a percentage of the labeled cells out of the total number of VICs.

Quantification of Wound Closure

Confocal images of wounded coverslips stained for α -SMA were captured using the $\times 20$ objective. Fifteen to twenty fields were captured from every other field along the WE. Confocal z-stacks were volume rendered using the FV10-ASW 1.6 software. Measurements were made using the NIS-Elements software (Nikon Instruments Inc., Melville, NY) of the distance between the original nonstained multilayered WE and the stained front row of cells at the WE at 24 hours after wounding.

RNA Extraction and Isolation

All reagents used for RNA isolation were purchased in the PicoPure RNA Isolation Kit (Arcturus Bioscience Inc., Mountain View, CA). Wounded and nonwounded VICs cultured in 10% FBS standard media with or without TGF- β neutralizing antibody treatment were washed three times with serum-free M-199 media. VICs were then observed under a phase contrast microscope at $\times 100$ magnification and a 27G $\frac{1}{2}$ PrecisionGlide needle

(Becton-Dickinson, Mountain View, CA) was bent and used to capture VICs under the phase contrast microscope in the NWM or at the WE. Needle points containing VICs of interest were then immersed immediately in extraction buffer to obtain cell extract. Approximately 300 to 500 cells were captured from the NWM or WE.

RNA isolation protocols specified in the PicoPure RNA isolation kit (Arcturus Bioscience Inc.) were followed. Briefly, RNA purification columns were preconditioned with 250 μ l of conditioning buffer for 5 minutes at room temperature followed by centrifugation at $16,000 \times g$ for 1 minute. Cell extracts are mixed with 50 μ l of 70% ethanol by pipetting up and down. The cell extract and ethanol mixtures were applied to the preconditioned purification columns and centrifuged for 2 minutes at $100 \times g$, followed immediately by a centrifugation at $16,000 \times g$ for 30 seconds. One hundred μ l of wash buffer 1 was applied to each purification column followed by $8000 \times g$ centrifugation for 1 minute. One hundred μ l of wash buffer 2 was applied to each purification column followed by $8000 \times g$ centrifugation for 1 minute. RNA was eluted with 10 μ l of elution buffer by first incubating the elution buffer on the purification column for 1 minute at room temperature then centrifuging at $1000 \times g$ for 1 minute followed by at $16,000 \times g$ for 1 minute.

First-Strand cDNA Synthesis

First strand cDNA synthesis from RNA isolated from VICs was completed using the SuperScriptIII reverse transcriptase kit (Invitrogen). Briefly, 2 μ l of random decamers (Ambion, Austin, TX), 2 μ l of 10 mmol/L dNTP mix (Invitrogen), and 9 μ l of RNA were mixed and incubated for 5 minutes at 65°C followed by incubation on ice for 1 minute. Four μ l of 5 $\times$ first-strand buffer, 1 μ l of 0.1 mol/L dithiothreitol, 1 μ l of RNaseOUT (40 U/ μ l), and 1 μ l of Superscript III reverse transcriptase (2000 U) were then added to each reaction mixture, making a total reaction volume of 20 μ l per sample. Each reaction mixture was incubated at 25°C for 5 minutes, 50°C for 50 minutes, and 70°C for 15 minutes.

Real-Time Polymerase Chain Reaction (PCR)

Real-time PCR primers (ACGT Inc., Wheeling, IL) were designed using Primer Express (Applied Biosystems, Foster City, CA) and span across an intron whenever possible. Primer sequences were: HPRT-F 5'-AACGGCT-TGCTCGAGATGTG-3'; HPRT-R 5'-TCCAGCAGGTCAG-CAAAGAA-3'; β -actin-F 5'-TGTCCACCTTCCAGCAGATG-T-3'; β -actin-R 5'-TGCAACTAACAGTCCGCCTAGA-3'; α -SMA-F 5'-TCTGTAAGGCCGGCTTTGC-3'; α -SMA-R 5'-TGTCCTCCATCCACCATCA-3'. For each real-time PCR reaction, 12.5 μ l of Power SYBR Green PCR Master Mix, 1.5 μ l of 10 μ mol/L forward and reverse primers mixture, 10 μ l of first-strand cDNA, and 1 μ l of H₂O were mixed for a total reaction volume of 25 μ l. Real-time PCR reactions were performed using a Prism 7900 HT sequence detection system (Applied Biosystems). Thermal cycling conditions were designed as follows: 50°C for 2 minutes,

initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. A final dissociation stage was added at 95°C for 15 seconds followed by 60°C for 15 seconds. Fluorescent measurements of SYBR green incorporation were recorded during each annealing step. Dissociation curve analyses were performed to confirm specificity of the SYBR green signals in each experiment. Relative quantifications were performed using the comparative standard curve method (Sequence Detection Systems Software 2.0, Applied Biosystems). α -SMA values were divided by HPRT and β -actin values independently for standardization and the resulting values were averaged. The relative α -SMA mRNA content for VICs in a nontreated NWM was assigned a value of 1, and the α -SMA mRNA content in VICs at the nontreated WEs, treated WEs, and treated NWM were expressed as folds increase from that of the nontreated NWM.

Statistical Analysis

A value of $P < 0.05$ was considered significant. Student's *t*-test was used to compare control and treated groups. One-way analysis of variance followed by Bonferroni posttest was used to reflect multiple comparisons. These statistical analyses were performed using GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA).

Results

Wounding Activates VICs and Leads to Increased Intracellular TGF- β Expression and TGF- β Signaling

After wounding, VICs participate in wound repair by closing the wound from both sides of the wound forming two advancing linear WEs. By 4 days after wounding, ~90% of the wound is filled in with VICs, and wound closure occurs in 6 days. The wounded area is not identifiable by phase contrast microscopy by 10 days. At 24 hours after wounding, all VICs at the WE show significantly increased α -SMA staining (Figure 1B) compared to the control NWM where α -SMA is absent (Figure 1A). In the monolayer away from the WE, α -SMA staining is also absent. Wounded cultures were stained at 24, 48, 72, 96 hours, 6 days, and 10 days, and α -SMA staining at the WE begins to disappear at 6 days and was absent at 10 days (data not shown).

TGF- β staining using the anti-TGF- β (against TGF- β 1, -2, -3) is increased in VICs at the WE (Figure 1D) at 24 hours after wounding compared to VICs in the NWM (Figure 1C). TGF- β staining is frequently concentrated in the perinuclear area (Figure 1D). Using anti-TGF- β 3 staining did not show the presence of TGF- β 3 in the VIC cultures (data not shown), and TGF- β 2 is not present in mesenchymal cells.³⁸ TGF- β 1 concentration in the non-conditioned 10% FBS-containing media is 1.2 ± 0.07 ng/ml measured by ELISA. VIC-conditioned media col-

Figure 1. Immunofluorescent confocal photomicrographs of VIC NWMs (**A**, **C**, **E**, and **G**) and wounded VIC monolayers at the WE (**B**, **D**, **F**, and **H**) at 24 hours after wounding. VICs are stained for α -SMA (**A** and **B**, green; nuclei, blue), TGF- β (**C** and **D**), phosphorylated Smad2/3 (**E** and **F**), and are double stained (**G** and **H**) for α -SMA (red) and TGF- β (green). α -SMA, TGF- β , and phosphorylated Smad2/3 are expressed at low levels in the NWM and are increased at the WE. α -SMA and TGF- β are co-expressed at the WE and not in the NWM. Note that phosphorylated Smad2/3 complexes are localized to the nucleus of VICs. **Arrow** indicates direction of wound closure. Original magnifications, $\times 600$.

lected at 24 hours after wounding contains 1.5 ± 0.06 ng/ml of TGF- β 1.

Staining for the phosphorylated Smad2/3 complex, key transcription factors forming a signaling complex in the TGF- β signaling pathway, is increased at the WE at 24 hours after wounding (Figure 1F) whereas the NWM was faintly stained (Figure 1E). At the WE, phosphorylated Smad2/3 complexes are concentrated in the nuclear region with some perinuclear staining. VICs at the WE co-stain for α -SMA and TGF- β at 24 hours after wounding

(Figure 1H). This α -SMA and TGF- β co-expression is not observed in VICs in the NWM (Figure 1G).

TGF- β Neutralizing Antibody Reduces VIC Wound Repair through Decreasing Activation, α -SMA mRNA Content, Proliferation, Apoptosis, Wound Closure, and Formation of α -SMA Stress Fibers in VICs at the WE

At 24 hours after wounding, α -SMA staining is significantly increased at the WE compared to the NWM (Figure 1, A and B). When $15 \mu\text{g/ml}$ of TGF- β neutralizing antibody is applied to nonwounded and wounded VIC cultures reducing the available TGF- β from 1.2 ± 0.07 ng/ml to 0.85 ± 0.05 ng/ml through ELISA measurements, α -SMA staining intensity is significantly reduced at the WE (Figure 2, A and B).

Quantification of relative α -SMA mRNA abundance in VICs in the NWM and at the WE showed that α -SMA mRNA is 2.06 ± 0.43 times as abundant in VICs at the WE than in VICs in the NWM (Figure 2C). When VIC cultures are treated with $15 \mu\text{g/ml}$ of TGF- β neutralizing antibody, no significant change in α -SMA mRNA is observed in the NWM. However, α -SMA mRNA content in VICs at the WE is reduced 6.90 ± 0.25 -fold in $15 \mu\text{g/ml}$ of TGF- β neutralizing antibody-treated cultures compared to non-treated cultures (Figure 2C).

At 24 hours after wounding, VIC proliferation as quantified by bromodeoxyuridine staining is $35.07 \pm 2.04\%$ higher at the WE than VIC proliferation within the NWM (Figure 3A). After $15 \mu\text{g/ml}$ of TGF- β neutralizing antibody treatment, VIC proliferation at the WE is reduced by $22.75 \pm 3.73\%$ in treated cultures compared to non-treated cultures at 24 hours after wounding (Figure 3A). TGF- β neutralizing antibody treatment did not show an effect on VIC proliferation in the NWM compared to the nontreated NWM.

At 24 hours after wounding, $6.15 \pm 0.34\%$ of the VIC population at the WE undergo apoptosis as determined by TUNEL (Figure 3B). When VIC cultures are treated with $15 \mu\text{g/ml}$ of TGF- β neutralizing antibody, VIC apoptosis index at the WE is decreased to $1.93 \pm 0.12\%$, a 69% decrease relative to nontreated cultures at 24 hours after wounding (Figure 3B). VICs in the NWM show no apoptosis both in nontreated and treated cultures (data not shown). Staining for cleaved caspase-3 show an index of $3.34 \pm 0.20\%$ at 24 hours after wounding, and treatment with neutralizing antibody show a significantly decreased index of $0.45 \pm 0.05\%$.

When wounded VIC cultures are treated with $15 \mu\text{g/ml}$ of TGF- β neutralizing antibody, wound closure is decreased by $106.40 \pm 5.36 \mu\text{m}$ at 24 hours after wounding (Figure 4A). The percentage of VICs showing α -SMA stress fibers at the WE is reduced by $19.91 \pm 1.10\%$ at 24 hours after wounding when wounded VIC cultures are treated with $15 \mu\text{g/ml}$ of TGF- β neutralizing antibody (Figure 4B).

Figure 2. Immunofluorescent confocal photomicrographs of wounded VIC monolayers at the WE stained for α -SMA (green) in TGF- β neutralizing antibody-treated or nontreated cultures at 24 hours after wounding. **A:** VICs show prominent α -SMA staining at the nontreated WE. **B:** Treatment with TGF- β neutralizing antibody show decreased α -SMA staining intensity. **Arrow** indicates direction of wound closure. **C:** Real-time PCR quantification of α -SMA mRNA in VICs at the WE and in the NWM in TGF- β neutralizing antibody-treated and nontreated cultures at 24 hours after wounding. An increase in α -SMA mRNA content is observed at the WE compared to NWM. In TGF- β neutralizing antibody-treated cultures, decreased α -SMA mRNA content is observed at the WE compared to nontreated cultures. No difference in α -SMA mRNA content is observed in the NWM between treated and nontreatment cultures. ** and *** denotes significance between the indicated groups. $P < 0.05$; $n = 6$. Original magnifications, $\times 600$.

Exogenous TGF- β Enhances VIC Wound Repair through Increasing Activation, Proliferation, Wound Repair, and Formation of Stress Fibers in VICs at the WE

Treatment of VIC cultures with 0.5 ng/ml of exogenous TGF- β in 0.5% FBS-containing media at the time of wounding leads to increased staining intensity of α -SMA in VICs at the WE of treated cultures (Figure 5B) compared to vehicle (Figure 5A) at 24 hours after wounding. No α -SMA staining in the NWM is observed in treated cultures and vehicle (data not shown). At 24 hours after wounding, VIC proliferation at the WE is $7.55 \pm 0.90\%$ higher than in the NWM in 0.5% FBS-containing media (Figure 6). In cultures treated with 0.5 ng/ml of exogenous TGF- β in 0.5% FBS-containing media, proliferation at the WE is increased by $6.67 \pm 1.56\%$ compared with that at the WE of vehicle. No difference in proliferation is observed between treated cultures and vehicle in the NWM. No difference in VIC apoptosis is observed at the WE of 0.5 ng/ml exogenous TGF- β -treated cultures compared to vehicle at 24 hours after wounding. Treatment of wounded VIC cultures with 0.5 ng/ml of exogenous TGF- β

in 0.5% FBS-containing media leads to increased wound closure by $83.50 \pm 5.31 \mu\text{m}$ or 35% at 24 hours after wounding (Figure 7A). Furthermore, the number of VICs showing α -SMA stress fibers is increased by $31.16 \pm 2.38\%$ in exogenous TGF- β -treated cultures compared to vehicle at 24 hours after wounding (Figure 7B).

Discussion

This is the first heart valve study to investigate the regulatory role of TGF- β on several VIC functions that occur during the early stages of wound repair. We show that at 24 hours after wounding, VICs at the WE become activated, form α -SMA stress fibers, proliferate, undergo apoptosis, and demonstrate TGF- β up-regulation and signaling (Figure 8). We also show that these cell functions are regulated by changes in TGF- β concentration in the media. Our mechanical wound model does have similarities to *in vivo* tissue repair because VIC activation, cell proliferation and migration, and apoptosis do occur in our model. However in this current study, VICs are not exposed to inflammation, hemodynamic and mechanical forces, and surface microthrombosis that is observed to

Figure 3. **A:** Percentage of VIC proliferation at the WE in TGF- β neutralizing antibody-treated and nontreated cultures as determined by BrdU staining at 24 hours after wounding. VIC proliferation is higher at the WE compared to the NWM. TGF- β neutralizing antibody treatment leads to a decrease in proliferation of VICs at the WE compared to nontreated cultures. No effect on proliferation is observed in the NWM in treated compared to nontreated cultures. ** and *** denote significant differences between groups as indicated. $P < 0.05$; $n = 6$. **B:** Apoptosis index of VICs at the WE in TGF- β neutralizing antibody-treated and nontreated cultures determined by TUNEL at 24 hours after wounding. TGF- β neutralizing antibody-treated cultures show lower apoptosis index of VICs at the WE compared to nontreated cultures. ***Significant differences of groups as indicated. $P < 0.05$; $n = 7$.

Figure 4. A: Measure of extent of VIC wound closure in TGF- β neutralizing antibody-treated and nontreated cultures at 24 hours after wounding. Treatment of wounded VIC cultures with TGF- β neutralizing antibody reduces extent of wound closure. ***Significant differences between groups as indicated. $P < 0.05$; $n = 6$. **B:** Percentage of VICs showing α -SMA stress fibers at the WE in TGF- β neutralizing antibody-treated and nontreated cultures at 24 hours after wounding. Treatment with TGF- β neutralizing antibody decreases the number of VICs at the WE showing α -SMA stress fibers. ***Significant differences between groups as indicated. $P < 0.05$; $n = 6$.

occur at the site of *in vivo* valve injury and disease. These factors can be added to this type of model in future experiments as has been done successfully in experimental vascular studies.^{39,40}

Our observations of α -SMA expression and stress fiber formation in VICs at the WE indicate that wounding in our model activates the myofibroblast phenotype of quiescent VICs. Myofibroblasts are central in wound healing in many types of tissue and are defined biochemically by α -SMA expression and morphologically by contractile structures represented by stress fibers.^{14,41} Stress fibers are responsible for maintaining an isometric tension applied to the extracellular matrix through focal adhesion sites and occur in mesenchymal,^{42,43} epithelial,^{44,45} and endothelial cells, especially during the early stages of repair.^{46,47} Myofibroblast α -SMA expression and its assembly into stress fibers are important in cell migration, force generation, and wound contracture both *in vitro* and *in vivo*.^{48,49} In our model it is likely the increased stress fibers at the WE is associated with elongation and migration of the VICs into the wound.

We observe that α -SMA mRNA and protein are up-regulated concurrently with increased TGF- β in VICs at the WE. We also find that TGF- β neutralization reduces α -SMA protein, mRNA, and stress fibers whereas exogenous addition of TGF- β increases α -SMA protein, mRNA, and stress fibers. These findings are consistent with data showing that in tissue fibroblasts, TGF- β directly regulates α -SMA expression and plays a pivotal role in wound repair.^{20,50} Our observation of α -SMA

mRNA reduction when TGF- β is neutralized may be explained by the findings in fibroblasts that TGF- β stimulates α -SMA gene expression through the TGF- β control element at -42 to -61 from the transcriptional start site of the α -SMA promoter.^{51,52} In fibroblasts, BTEB2, GSKF, SP1, and other Kruppel-like factors are reported to bind and regulate α -SMA promoter activity.^{53,54} Sp1/3 and Smad3 proteins have also been shown to stimulate α -SMA enhancer activity in fibroblasts.⁵¹ Furthermore, our findings are consistent with a recent report showing that TGF- β induces α -SMA protein and stress fiber expression in porcine aortic VICs cultured at low-cell density on collagen substrates.¹³

TGF- β is produced by VICs in our model. We show intracellular TGF- β in VICs at the WE to be located in the perinuclear region, consistent with the known synthesis and secretion pathways of TGF- β through the endoplasmic reticulum and Golgi networks.^{17,55} Our ELISA measurements further support VICs as a source of TGF- β because we find that the concentration of TGF- β is increased in VIC-conditioned media collected at 24 hours after wounding compared to conditioned media collected at 0 hours after wounding.

Figure 5. Immunofluorescent confocal photomicrographs of wounded VIC monolayers at the WE stained for α -SMA (green; nuclei, blue) at 24 hours after wounding. VICs in vehicle cultures (**A**) show α -SMA staining at the WE and VICs in exogenous TGF- β -treated cultures (**B**) show increased α -SMA staining intensity compared to those at the WE of vehicle. **Arrow** indicates direction of wound closure. Original magnifications, $\times 600$.

Figure 6. Percentage of VIC proliferation at the WE in exogenous TGF- β -treated or vehicle cultures determined by BrdU staining at 24 hours after wounding. VIC proliferation is increased at the WE compared to the NWM in vehicle. Exogenous TGF- β treatment leads to a further increase in proliferation of VICs at the WE compared to vehicle. No effect on proliferation is observed in the NWM of treated cultures compared to vehicle. * and ** denote significant differences between groups as indicated. $P < 0.05$; $n = 6$.

Figure 7. A: Measure of extent of VIC wound closure in exogenous TGF- β -treated or vehicle cultures at 24 hours after wounding. Treatment of wounded VIC cultures with exogenous TGF- β leads to an increase in the extent of wound closure compared to vehicle. **Significant differences between groups as indicated. $P < 0.05$; $n = 6$. **B:** Percentage of VICs showing α -SMA stress fibers at the WE at 24 hours after wounding. Exogenous TGF- β treatment increases the number of VICs at the WE with α -SMA stress fibers compared to vehicle. ***Significant differences between groups as indicated. $P < 0.05$; $n = 6$.

TGF- β isoforms (TGF- β 1, -2, and -3), activin, nodals, bone morphogenetic proteins, growth and differentiation factor, and Mullerian inhibitory substance are all members of the TGF- β superfamily that all contain common sequence and structural features including a unique positioning of seven cysteine residues resulting in the formation of a core cysteine knot.⁵⁶ TGF- β 1 is the predominant isoform of TGF- β exerting biological effects in the adult human and pig. TGF- β 2 is not expressed in mesenchymal cells,³⁸ and we do not detect TGF- β 3 in our VIC cultures. Tethering of the secreted latent TGF- β complex through LTBP to matrix proteins such as fibronectin and heparin is an important cellular mechanism to tightly regulate TGF- β bioavailability, spatial gradients, and activation in the cell microenvironment.^{19,57–60} Direct fibronectin-TGF- β interactions or indirect fibronectin-TGF- β interactions through heparin activate porcine aortic VICs.¹⁹ TGF- β 1 activation has also been shown to be integrin-mediated *in vivo*.⁶¹ We previously report the up-regulation of fibronectin, changes in α v β 6 integrin, and prominent fibrillar adhesion assembly by VICs at the WE.³⁶ The tethering of latent TGF- β complexes to fi-

bronectin is necessary to allow α v β 6 integrin-mediated TGF- β activation.^{58,59,62}

One possibility in our model is that latent TGF- β becomes concentrated at the WE because of increased secretion by activated VICs and increased fibronectin. It is likely that our mechanical disruption of VIC-VIC and VIC-matrix interactions by mechanical wounding leads to activation of TGF- β by unknown mechanisms. Two possible mechanisms are that integrin activation and rearrangement at the WE and/or proteolytic enzymes, such as plasmin,⁶³ thrombin,⁶⁴ and matrix metalloproteinases,⁶⁵ which may be secreted by VICs at the WE activates TGF- β . This would result in active TGF- β concentrated specifically at the WE, which then signals to regulate VIC wound repair.

The most well-characterized TGF- β signaling mechanism is through the Smad2 and Smad3 proteins that are phosphorylated and activated by the type I receptor, and shuttle to the nucleus through Smad4.^{66,67} Our observations that phosphorylated Smad2 and Smad3 are up-regulated and accumulate in the nucleus of VICs at the WE suggest that this important TGF- β signaling pathway regulates VIC wound repair at least in part. The cellular responses to TGF- β signaling via Smad2 and Smad3 can be variable depending on cell type. TGF- β causes epithelial cells to undergo growth arrest and apoptosis, whereas in fibroblasts, it induces activation, proliferation, and differentiation.^{68–70} Other cellular processes regulated by TGF- β in fibroblasts include cell growth, apoptosis, migration, extracellular matrix production, and degradation.^{66–72} These findings are consistent with our current observations that VICs undergo activation, proliferation, apoptosis, and our previous observations of VIC migration and matrix deposition at the WE.³⁶

We show that there is increased VIC proliferation at the WE. Our findings are consistent with previous observations in a different model in which TGF- β promotes proliferation in sheep aortic VICs at low cell densities cultured on collagen substrates.¹⁸ The signaling mechanism of TGF- β in promoting proliferation has been under study for a number of cell types.^{25,73,74} In addition to the well-characterized Smad pathways in promoting proliferation, non-Smad TGF- β signaling pathways are recently found to be also important in regulating fibroblasts proliferation in which TGF- β signals through the phosphoinositide-3 kinase (PI3K) pathway leading to phosphorylation of Akt

Figure 8. Summary of the response of VICs to TGF- β in early stages of wound repair. VICs are quiescent when residing in a confluent monolayer. After wounding, VICs at the WE express α -SMA, a marker of VIC activation. Activated VICs require TGF- β to maintain activation through promoting α -SMA mRNA production and protein expression. Activated VICs at the WE undergo proliferation and apoptosis, as well as form α -SMA stress fibers, leading to wound closure and repair. Neutralization of TGF- β leads to reduced VIC activation, α -SMA mRNA production, proliferation, apoptosis, α -SMA stress fiber formation, and wound closure, decreasing VIC wound repair. Addition of exogenous TGF- β increases proliferation, stress fiber formation, and wound closures, enhancing VIC wound repair.

and PAK2 activation.^{75–79} These signaling mechanisms are still under elucidation and they may occur in VICs, which are myofibroblast-like in nature. TGF- β also promotes fibroblast proliferation via transcriptionally stimulating platelet-derived growth factor and basic fibroblast growth factor-2.⁸⁰ This finding in fibroblasts is consistent with our previous report of the stimulatory effect of basic fibroblast growth factor-2 on VIC wound repair.³⁴

The presence of VIC apoptosis at the WE indicates that cell turnover is another important aspect of valve wound repair. In the healing of a dermal wound, hypertrophic scarring occurs if apoptosis is dysregulated.⁴¹ In the same manner, if activated VICs did not undergo apoptosis, excessive extracellular matrix deposition and fibrosis would likely occur leading to valve scarring and dysfunction. Apoptosis is mediated by the caspase family of proteases that are controlled through the environment-mediated extrinsic pathway and/or the mitochondria-mediated intrinsic pathway. Caspases-8, -9, and -10 are initiator caspases that cleave and activate effector caspases-3, -6, and -7.⁸¹ The regulation of initiator caspases is dependent on the balance of pro-apoptosis and anti-apoptosis cues, and apoptosis is sometimes reversible through inhibition of initiator caspases. However, once apoptosis progresses to the cleavage of effector caspases, irreversible cell death results as effector caspases fragment DNA, disrupt cytoskeleton, and lead to metabolic breakdown of the cell.⁸² Thus, we identify VICs in the irreversible and terminal stages of apoptosis at the WE by using both TUNEL to detect DNA fragmentation and cleaved caspase-3 staining to detect caspase-3 activation. TGF- β is known to promote apoptosis through a number of signaling pathways. In a number of carcinomas, TGF- β pro-apoptotic responses by Smad-3 is followed by caspase-8 activation via the Fas receptor⁸³ or by activation of PI3K-Akt signaling.⁸⁴ Apoptosis may also be regulated by TGF- β through its effect on the balance of pro-apoptosis BH3-only proteins and anti-apoptosis Bcl-2 family members.^{85,86}

In conclusion, we demonstrate that wounding activates VICs that repair the wound. Wound repair is regulated by TGF- β by modulating α -SMA protein expression, mRNA expression, and stress fiber formation. TGF- β further regulates proliferation, apoptosis, and wound closure, which are critical processes in repair.

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