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Transforming Growth Factor- β Regulates the Growth of Valve Interstitial Cells *in Vitro*

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Although valve interstitial cell (VIC) growth is an essential feature of injured and diseased valves, the regulation of VIC growth is poorly understood. Transforming growth factor (TGF)- β promotes VIC proliferation in early-stage wound repair; thus, herein, we tested the hypothesis that TGF- β regulates VIC proliferation under normal nonwound conditions using low-density porcine VIC monolayers. Cell numbers were counted during a 10-day period, whereas proliferation and apoptosis were quantified by bromodeoxyuridine staining and TUNEL, respectively. The extent of retinoblastoma protein phosphorylation and expression of cyclin D1, CDK 4, and p27 were compared using Western blot analysis. Adhesion was quantified using a trypsin adhesion assay, and morphological change was demonstrated by immunofluorescence localization of α -smooth muscle actin and vinculin. TGF- β -treated VICs were rhomboid; significantly decreased in number, proliferation, and retinoblastoma protein phosphorylation; and concomitantly had decreased expression of cyclin D1/CDK4 and increased expression of p27. TGF- β -treated VICs adhered better to substratum and had more vinculin plaques and α -smooth muscle actin stress fibers than did controls. Thus, the regulation of VIC growth by TGF- β is context dependent. TGF- β prevents excessive heart valve growth under normal physiological conditions while it promotes cell proliferation in the early stages of repair, when increased VICs are required. (*Am J Pathol* 2011, 179:1746–1755; DOI: 10.1016/j.ajpath.2011.06.007)

Valve interstitial cells (VICs) are present in all three layers^{1,2} of the adult heart valve and are the most prevalent cell type present.^{3–5} They are situated underneath the surface endocardium¹ and embedded in an extracellular

matrix (ECM) that they secrete and actively remodel.⁵ VICs are the master cells within the valve because they regulate both physiological and pathological processes.^{2,6} In their quiescent state, VICs show characteristics of fibroblasts and are critical for the maintenance of normal valve structure and function.^{2,5} During development or in response to injury and disturbed hemodynamic flow, VICs become activated^{2,6,7} and take on features of myofibroblasts.^{2,5–7} Activated VICs are characterized by increased α -smooth muscle actin (α -SMA) expression, stress fiber formation, contractility, and proteolytic enzyme secretion.^{2,4,6–8} Like other myofibroblasts,⁹ VICs regulate wound repair, as demonstrated by the increased number and enhanced production of ECM components, both in diseased valves and in *in vitro* wound models.^{10,11} Thus, understanding the regulation of the biological and pathobiological features of VICs is essential for understanding the pathogenesis of heart valve diseases.

Quiescent VICs are activated by several cytokines and growth factors, one of them being transforming growth factor (TGF)- β ,^{2,12} a 25-kDa protein of the TGF- β superfamily.¹³ The TGF- β ligand binds and brings together TGF- β receptor I (TGF- β RI) and TGF- β RII Ser-Thr kinases.¹⁴ TGF- β RII phosphorylates TGF- β RI (also known as ALK5) and propagates the signal through phosphorylation of Smad proteins or via non-Smad mechanisms, such as mitogen-activated protein kinase, ρ -like Rho-GTPase, and phosphatidylinositol 3-kinase (PI3K)–AKT pathways.^{14,15} TGF- β regulates a wide range of cellular processes, including cell proliferation, apoptosis, differentiation, migration, and ECM remodeling.^{8,12,14,16} This multifunctionality allows TGF- β to participate in wound repair in multiple tissues and organs of the body.^{12,17} Overexpression of TGF- β is often observed in *in vivo*

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wound sites.¹² It is also present in several heart valve-related diseases (eg, calcific aortic stenosis,^{18,19} mitral valve prolapse,^{20,21} and Marfan syndrome).²² By using a well-characterized wound model,¹¹ we also reported an up-regulation of TGF- β at the *in vitro* wound edge. The exogenous addition of TGF- β further activates the VICs along the wound edge and stimulates their proliferation to enhance wound closure within the first 24 hours after wounding.⁸

Although the beneficial role of TGF- β in wound repair has been studied, its effects on cell growth remain controversial, owing to its context-dependent nature. Depending on TGF- β concentration,^{23,24} cell type of interest,²⁵ and degree of cell differentiation,²⁶ varying responses in proliferation and apoptosis have been reported. Because VIC growth *in vitro* is poorly understood, we investigated the regulation of VIC proliferation by TGF- β .

Materials and Methods

VIC Growth Curve

VICs were harvested from porcine heart valve explants, as previously described.¹ VICs at passages 3 to 5 were plated in triplicate 35-mm tissue culture dishes (Falcon; BD Biosciences, San Jose, CA) at a density of 2000 cells/cm² and cultured in 2 mL of 5% fetal bovine serum containing media. Porcine TGF- β 1 (R&D Systems, Minneapolis, MN) was reconstituted in a vehicle solution of 4 mmol/L HCl and 0.1% bovine serum albumin. Media containing the vehicle solution or active porcine TGF- β 1 (0.1, 0.5, or 5 ng/mL) were first added to cultures 18 hours after plating and every 2 days thereafter. Cell counts were performed in triplicate during a 10-day period using the Countess Automated Cell Counter (Invitrogen, Eugene, OR) at 18, 42, and 66 hours after plating and then every 2 days.

In an attempt to rescue the TGF- β -mediated reduction in cell number, both a neutralizing antibody and a potent receptor inhibitor were used. TGF- β -neutralizing antibody (R&D Systems) was reconstituted in sterile PBS to a 10-mg/mL stock solution. At 18 hours after plating and every 2 days thereafter, media containing 15- μ g/mL neutralizing antibody was added to the culture. SB431542 is a potent inhibitor of TGF- β RI.^{27–29} Solid anhydrous SB431542 (Sigma, St Louis, MO) was dissolved in sterile dimethyl sulfoxide (DMSO) and prepared into a 10 mmol/L stock solution. A specific amount of the stock solution was added to the culture media to achieve desired further dilutions. In experiments involving SB431542 treatment alone, media containing DMSO or 1 to approximately 10 μ mol/L of SB431542 were added to the cell cultures, as previously described. For the experiments involving both SB431542 and TGF- β 1, VICs were pretreated with the inhibitor 2 hours before the addition of TGF- β 1. Cell counts were performed in triplicate during a 10-day period using a Coulter counter (Coulter Electronics Inc., Hialeah, FL) at the time points previously specified. To confirm the results of SB431542 inhibition,

SD208 (Tocris Bioscience, Bristol, UK) was used. SD208 is also a potent and selective inhibitor of TGF- β RI.³⁰ Preparation and treatment with SD208 follows the previously mentioned procedure for SB431542.

Immunofluorescent Staining

VICs were plated on 22 $\times$ 22-mm square glass coverslips (Fisher Scientific, Pittsburgh, PA) in 35-mm tissue culture dishes at a density of 2000 cells/cm² and cultured under the same conditions as previously described. Vehicle and TGF- β -treated monolayers were immunofluorescently stained 4 or 6 days after first addition of TGF- β . Briefly, VICs on glass coverslips were fixed with methanol-acetone (1:1) at 4°C (in the case of α -SMA) or 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) [in the case of vinculin and phosphorylated Smad (p-Smad)], rinsed three times with PBS for 5 minutes in tissue culture dishes, permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 3.5 minutes (in the case of α -SMA and p-Smad) or with 0.2% Triton X-100 in PBS for 5 minutes (in the case of vinculin), and rinsed again three times in PBS at 5-minute intervals. The coverslips were incubated with mouse anti- α -SMA (1:400; Sigma), mouse anti-vinculin (1:50; Sigma), and rabbit anti-p-Smad2/3 (Ser423/425) (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies at room temperature for 1 hour and were then washed three times with PBS at 5-minute intervals. Secondary antibodies were goat anti-mouse and goat anti-rabbit Alexa 488 (1:200; Molecular Probes, Invitrogen, Eugene, OR). Hoechst 33342 (1:2000; Lonza, Basel, Switzerland) was used to counterstain nuclei. After a 20-minute incubation with secondary antibody, coverslips were dipped in deionized water, mounted with Prolong Gold antifade reagent (Molecular Probes, Invitrogen), and stored at 4°C. Coverslips were examined using $\times$ 10 and $\times$ 60 objectives of an Olympus FluoView 1000 Laser Scanning Confocal Microscope (Olympus, Markham, ON, Canada) equipped with lasers providing exciting wavelengths of 405 and 488 nm, respectively. Serial optical sections were obtained at 0.1- μ m intervals for a total of 4.5 to 8.5 μ m. Images were captured at randomly selected regions using FV10-ASW 1.6 software (Olympus) to avoid saturation.

Quantification of Apoptosis

TUNEL was performed using the TACS TdT-fluorescein *in situ* apoptosis detection kit (R&D Systems) to identify apoptotic VICs. Total nuclei were counterstained with Hoechst. A positive and a negative control were used for comparison.

Quantification of Proliferation

Bromodeoxyuridine (BrdU) labeling reagent (Amersham Sciences, Buckinghamshire, UK) was added in 1:1000 dilution to vehicle and TGF- β -treated cultures 2 and 4 days after first treatment at the time of media change and incubated for 6 hours. The coverslips were then washed in PBS, fixed in ethanol-acetic acid (95:5) for 20 minutes

at 4°C in a coplin jar, and denatured with 2N HCl for 30 minutes in an incubator at 37°C. Incubation for 1 hour with mouse anti-BrdU (1:1000; Sigma) primary antibodies at 37°C was followed by secondary antibody, as previously described. VIC nuclei were counterstained with propidium iodide (1:1000; Sigma). Images were captured using the $\times 20$ objective of a Nikon TE300 (Nikon, Melville, NY) inverted fluorescence microscope, starting two fields away from the left top corner, taking every other field until the whole coverslip was covered. A total of 15 fields were captured for each coverslip, with approximately 200 to 500 cells and 30 to 100 cells per fields for vehicle and TGF- β -treated monolayers, respectively. The total number of nuclei was counted using Simple PCI 6.5 (Hamamatsu, Shizuoka Prefecture, Japan), and the number of BrdU-stained nuclei was counted manually. Proliferation was quantitated as the percentage of labeled cells in the total number of cells counted.

Trypsin Adhesion Assay

By using a previously characterized trypsin adhesion assay³¹ with slight modification, the degree of adhesiveness to the substratum was compared. Briefly, vehicle or TGF- β -treated VICs were incubated in 1.0 mL of 1:10 diluted trypsin-EDTA (Invitrogen) for 0.5 or 1.0 minutes, followed by immediate neutralization with 0.5 mL of media containing 5% fetal bovine serum. The number of VICs that were detached during the incubation period was counted using a Coulter counter. The remaining VICs in the monolayer were washed three times with PBS, detached using full trypsin-EDTA, and counted. The percentages of vehicle or TGF- β -treated VICs detached during the incubation period were compared.

Western Blot Analyses

To harvest enough cells for protein extraction, TGF- β - or SB431542-treated VICs and their respective controls were cultured in 100-mm tissue culture dishes (Falcon) until they reached confluency. Whole cell protein extracts were obtained by lysing the VICs in ice-cold radioimmunoprecipitation assay buffer (50 mmol/L Tris, pH7.4; 150 mmol/L NaCl; 1% Triton; 0.25% Na-deoxycholate; and 1 mmol/L EDTA) supplemented with protease inhibitor (Roche Applied Science, Indianapolis, IN), 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium fluoride (Sigma). Nuclear and cytosolic protein extracts were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL), supplemented with protease inhibitor (Roche Applied Science), 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium fluoride, following the extraction protocols specified by the kit. The protein concentration was determined by Bradford analysis, and 10 to 40 μ g of protein per lane was resolved by SDS-PAGE. Transfer and blotting were performed on an iBlot gel transfer device (Invitrogen) and a SNAP i.d. protein detection system (Millipore, Danvers, MA), following the instructions specified. The blots were reacted with

rabbit anti-phosphorylated retinoblastoma protein (pRb) (Ser807/811) (1:200), mouse anti- α -SMA (1:5000), anti-cyclin D1 (1:100), anti-CDK4 (1:200), and anti-p27 (1:100) (Santa Cruz Biotechnology). Mouse anti- β -tubulin (1:5000; Sigma) and anti-histone H1 (1:5000; Millipore) antibodies were used to confirm equal protein loading in the whole cell/cytosolic and nuclear portion, respectively. Goat anti-mouse and goat anti-rabbit secondary antibodies (horseradish peroxidase conjugated), at a dilution of 1:5000, were used. The immune complexes were detected using Luminata Western HRP Substrates (Millipore).

The intensities of protein bands were quantified and compared using Image J 1.43 (US NIH, Bethesda, MA). The densitometric data were normalized such that the group that has a higher expression level is set at 100.

Statistical Analysis

The Student's *t*-test was used to compare vehicle with TGF- β 1-treated groups. *P* < 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA).

Results

TGF- β Treatment Significantly Inhibits the Increase in VIC Number over Time

Staining for the p-Smad2/3 complex, a key complex involved in TGF- β signaling, is used to confirm activation of the pathway. At both 4 and 6 days after treatment, TGF- β -treated VICs (Figure 1, B and D) showed significantly more prominent p-Smad nuclear localization (Figure 1E) compared with the control, in which nuclei are only faintly stained (Figure 1, A and C).

Under phase-contrast microscopy, the vehicle and TGF- β -treated VICs initially showed no observable difference in cell density or morphological features (Figure 2, A–D). After 4 days, treatment with TGF- β resulted in a significant reduction in VIC density (Figure 2, E–J). Although vehicle-treated VICs became superconfluent 8 days after treatment, TGF- β -treated VICs failed to reach confluency, even when they were cultured for up to 12 days. Instead, prolonged treatment caused VICs to cluster and form nodules. The growth of TGF- β -treated VICs leveled off after 4 days of treatment, whereas that of the vehicle-treated cells remained in the log phase. The inhibitory effect is concentration dependent, as shown in Figure 3A. Maximal inhibition was observed with 0.5 ng/mL TGF- β treatment because a higher concentration (5 ng/mL) resulted in the same extent of inhibition (data not shown).

TGF- β -Mediated Inhibition of VIC Proliferation Is Prevented by Inhibitors to TGF- β

As confirmed by intracellular labeling of the p-Smad2/3 complex (data not shown), TGF- β signaling via the Smad path-

Figure 1. p-Smad in nuclei. Immunofluorescent confocal micrographs of vehicle (A and C) and 5 ng/mL TGF- β -treated (B and D) subconfluent VIC monolayers 4 days (A and B) and 6 days (C and D) after first TGF- β addition are shown. VICs are stained for p-Smad2/3 (Ser423/425) (green; nuclei, red). There was more prominent p-Smad nuclear localization in treated VICs. Scale bar = 10 μ m (D). Original magnification, $\times 600$. **E:** Comparison of the percentages of p-Smad-incorporated nuclei in vehicle and TGF- β -treated monolayers after 4 and 6 days. * $P < 0.0001$ for statistical significance between groups. $n = 9$.

way was effectively blocked by the inhibitor, SB431542. Because SB431542 functions by disrupting the kinase activity of TGF- β RI, we incubated the cells with the inhibitor for 2 hours before adding any exogenous TGF- β in an effort to block any of its downstream signaling. The inhibitory effect of TGF- β was abolished in the presence of the inhibitor (Figure 3B).

We found that treatment with SB431542 alone without TGF- β resulted in decreased cell growth (Figure 3C), and this growth-inhibitory effect was dose dependent. Maximal inhibition was observed at a concentration of 10 μ mol/L. Another potent inhibitor of TGF- β receptor, SD208, also inhibited VIC growth (data not shown). Using a neutralizing antibody to TGF- β did not significantly affect VIC number (Figure 3D).

TGF- β Treatment Decreases Proliferation but Has No Effect on Apoptosis

Replicating VICs incorporate BrdU into their newly synthesized DNA during a 6-hour incubation period with the base analog. TGF- β -treated VICs showed significantly fewer BrdU-stained nuclei (Figure 4, A–F). The percentages of BrdU-incorporated nuclei were counted, and the proliferation of TGF- β -treated VICs at 2 days (Figure 4G) and 4 days (Figure 4H) after initial TGF- β treatment was $9.4\% \pm 0.4\%$ and $16.1\% \pm 0.7\%$ lower than the control, respectively. Notably, even though the differences in cell number became significant after 4 days of treatment, proliferation rates started to differ at as early as 2 days after treatment. Because there was no significant difference in BrdU incorporation in VICs treated with 0.5 or 5 ng/mL of TGF- β , a treatment concentration of 0.5 ng/mL was used in all experiments conducted thereafter. In TGF- β - or vehicle-treated VICs, no apoptotic nuclei were observed (Figure 5, C and D). Apoptosis was also not observed in untreated confluent monolayers (data not shown).

TGF- β Treatment Leads to Hypophosphorylation of pRb via Altered Expression of Cell Cycle Proteins

Alteration in the pRb phosphorylation status on specific residues (Ser807/811) is a critical regulatory step in G₁ to S phase transition. The expression of phosphorylated pRb (Ser807/811) in both the cytoplasmic and nuclear compartments was significantly down-regulated by TGF- β treatment (Figure 6A). Such an effect was reversed when the VICs were treated with SB431542 (Figure 6B).

The kinase complex responsible for the phosphorylation of pRb at Ser807/811 is the cyclin D1–CDK4 complex.³² TGF- β treatment significantly decreased the expressions of both cyclin D1 and CDK4 (Figure 6C). At the same time, an inhibitor of the kinase complex, p27, was markedly up-regulated (Figure 6C). The differences in expression levels between groups are determined to be statistically significant (Figure 6, D–F).

TGF- β Treatment Alters VIC Morphological Features and Increases VIC Adhesion to the Substratum

TGF- β -treated VICs were rhomboid, as opposed to spindle shaped. They were flattened out on the substratum and larger at the same magnification. They also possessed more cellular protrusions. This change in VIC morphological features was reflected in the α -SMA expression level (Figure 7, E and F) and localization at 4 days (Figure 7, A and B) and 6 days (Figure 7, C and D) after treatment. TGF- β -treated VICs showed more prominent and denser stress fiber when compared with the control.

Initially, in preparation for counting TGF- β -treated VICs, more incubation time in trypsin was required to fully

Figure 2. Phase-contrast micrographs of vehicle (A, C, E, G, and I) and 5 ng/mL TGF- β -treated (B, D, F, H, and J) VICs taken 1 day (A and B), 2 days (C and D), 4 days (E and F), 6 days (G and H), and 8 days (I and J) after initial treatment with TGF- β , showing the change over time in cell density and morphological features. Most treated cells appeared rhomboid, whereas the vehicle cells remained spindle shaped. Scale bar = 100 μ m (J). Original magnification, $\times 100$.

detach from the substratum. The trypsin adhesion assay, which is designed to compare the degree of adhesion, revealed that TGF- β treatment resulted in significantly fewer VICs becoming detached during the 0.5- or 1.0-minute incubation period in diluted trypsin (Figure 8C). More explicitly, after 4 days of TGF- β treatment, VICs showed approximately eightfold stronger adhesion to the

substratum. TGF- β treatment also led to increased intensity of staining for vinculin, a surrogate for focal adhesion complex. As shown in Figure 8, A and B, TGF- β -treated VICs showed significantly more prominent vinculin plaques around the cell periphery when compared with the control, in which plaques were absent.

Discussion

To our knowledge, this is the first *in vitro* study to investigate the role of TGF- β on VIC growth, independent of injury. We demonstrate that, in a subconfluent monolayer, exogenous TGF- β decreases VIC proliferation by down-regulation of cyclin D1 and CDK4, resulting in reduced phosphorylation of pRb and cell cycle arrest in the G₁ phase. Our findings show that VICs are unique in being inhibited by TGF- β , unlike other fibroblasts in other tissues. Thus, for translational valve regeneration and tissue engineering studies, it is advisable to use VICs and not other fibroblast-type cells because the latter do not necessarily reflect the phenotype of VICs. Our *in vitro* findings and those of others will need to be validated *in situ*, where factors such as physical forces, inflammation, and physiological matrix are present.

TGF- β is a highly versatile molecule that mediates a wide range of biological processes in several systems, and its cellular responses are known to be cell-type dependent.¹⁶ In the vascular system, TGF- β inhibits the proliferation of endothelial cells while it stimulates that of the adjacent smooth muscle cells,^{33,34} despite their close proximity. Conversely, it has a pro-apoptotic effect on endothelial cells while being anti-apoptotic in smooth muscle cells.³⁵ Overall, TGF- β is recognized as a potent growth inhibitor in cells of epithelial origin^{36–41} and a growth stimulator in fibroblast-type cells.^{23,25,42} Although VICs are fibroblast-type cells, the anti-proliferative response in these fibroblasts could be partially explained by the fact that they are derived from surface endothelial cells that have undergone epithelial-to-mesenchymal transformation during embryonic development.² It is possible that VICs from adult heart valves still possess characteristics of valve endothelial cells. In carefully considering our data, we suggest that, instead of categorizing the proliferative responses on the basis of cell type, studies should focus on the profile of activation of different downstream pathways and determine their individual contributions to the net results observed.

TGF- β arrests the cell cycle in the G₁ phase, as demonstrated by decreased activity of thymidine kinase, a marker of cells entering the S phase.⁴³ One key regulator of G₁ to S phase transition is pRb, a growth suppressor that becomes functionally inactive when phosphorylated on specific residues.^{43–45} To confirm pRb under-phosphorylation as a result of TGF- β stimulation, we examined the amount of phosphorylation on specific residues known to be involved in cell cycle regulation. The Ser807/811 site is phosphorylated by cyclin D1–CDK4 complexes,^{32,46} and its phosphorylation status is examined in our study. We show that, in TGF- β -treated VICs, phosphorylated pRb (Ser807/811) is reduced by as much as

Figure 3. Growth curves of control and TGF- β -treated VICs. **A:** VIC growth in 0.1 or 0.5 ng/mL TGF- β and corresponding vehicle-treated subconfluent monolayers. TGF- β -treated VICs showed significantly delayed growth after 4 days of treatment. The growth-inhibitory effect is concentration dependent because 0.5 ng/mL treatment resulted in a significantly greater extent of inhibition than 0.1 ng/mL treatment. (Only the statistical significance between the two treatment groups is shown.) **B:** VIC growth in two independent groups. In the control group, DMSO was added to the culture 2 hours before the addition of TGF- β vehicle solution (4 mmol/L HCl and 0.1% bovine serum albumin). In the treatment group, 2 μ mol/L SB431542 was added to the culture 2 hours before the addition of 5 ng/mL TGF- β . The TGF- β -mediated growth inhibition is completely abolished in the presence of SB431542, and the two growth curves overlap. **C:** VIC growth in DMSO and 1 μ mol/L SB431542 treated subconfluent monolayers. The inhibitor alone inhibits VIC growth. **D:** VIC growth in vehicle- and TGF- β -neutralizing antibody (Ab)-treated monolayers. No significant change in cell number was observed. In all experiments, cell counts were performed in triplicate. Error bars denote SEM. Statistical significance between groups: * P < 0.05, ** P < 0.01, and *** P < 0.001.

10-fold. As expected, there are concomitant decreases in the expression of both cyclin D1 and CDK4. P27 is an inhibitor of the kinase complex,^{47–50} and its expression is markedly up-regulated. Collectively, our data suggest

that the control VICs are able to enter the S phase because of the presence of phosphorylated and inactive pRb, which releases the transcription factor E2F to stimulate the production of DNA replication proteins.^{49,51,52} In

Figure 4. BrdU incorporation in control and TGF- β -treated VICs. A comparison of the proportion of proliferating cells in vehicle (**A**, **C**, and **E**) and 5 ng/mL TGF- β -treated (**B**, **D**, and **F**) VICs 2 days (**A** and **B**), 3 days (**C** and **D**), and 4 days (**E** and **F**) after first TGF- β addition is shown in immunofluorescent micrographs of the nuclei of BrdU-incorporated VICs. Total nuclei are counterstained in red, with BrdU-stained nuclei shown in green. TGF- β significantly decreases the proportion of proliferating cells in the treated monolayers (**B**, **D**, and **F**). Scale bar = 10 μ m (**F**). Original magnification, $\times$ 200. The percentages of proliferating cells in vehicle and 5 ng/mL or vehicle and 0.5 ng/mL TGF- β -treated subconfluent monolayers 2 days (**G**) and 4 days (**H**) after first TGF- β addition are compared. TGF- β at both concentrations tested significantly inhibits VIC proliferation at both time points. There is no significant difference in the percentages of proliferating cells treated with 5 and 0.5 ng/mL TGF- β . * P < 0.001 denotes significant differences between groups. n = 15.

Figure 5. A–D: Immunofluorescent micrographs of the nuclei of apoptotic VICs 6 days after first TGF- β treatment. Total nuclei are counterstained in red, with apoptotic nuclei shown in green. The positive control (**A**), with the addition of nuclease, shows a significant level of apoptosis, whereas the negative control (**B**), with the elimination of TdT enzyme, shows no sign of apoptosis; this is similar in the vehicle (**C**) and 5 ng/mL TGF- β -treated VIC monolayers. Scale bar = 10 μ m. Original magnification, $\times 200$.

contrast, TGF- β R-phosphorylated Smad complexes translocate to the nucleus and associate with other transcription factors to directly modulate the promoter activity of CDK inhibitors.^{53,54} By associating with the cyclin D1–CDK4 complexes and suppressing their kinase activity, these inhibitors are able to sequester the complexes in an inactive state. As a result of the combination effect of inhibitors and the direct TGF- β -mediated cyclin D1–CDK4 down-regulation, treated VICs have mostly under-phosphorylated active tumor suppressor proteins that contribute to the growth retardation. TGF- β 's effect on phosphorylation is effectively reversed by a selective and potent inhibitor of TGF- β RI, SB431542. By acting as an ATP mimetic, it disrupts the receptor kinase activity and abolishes TGF- β -mediated cellular responses in many cell types.^{28,55} Intriguingly, although this inhibitor is able to abolish the growth-suppressive effect of exogenous TGF- β , on its own, it decreases cell number without affecting cell survival. One likely explanation, as noted later, is that the various non-Smad pathways activated by TGF- β may function independently to oppose the Smad-mediated effects; in addition, the net cellular response

depends on the extent of activation or suppression of each individual pathway. Inhibition of the entire TGF- β signaling cascade rescues the Smad-mediated decrease in pRb phosphorylation. However, at the same time, suppression of the potentially proliferative non-Smad pathways counteracts this effect, resulting in the observed counterintuitive growth inhibition by SB431542. Another less likely explanation is that SB431542 does relieve the G₁ to S block. However, the inhibition itself may cause the arrest of the cell cycle at a different check point further downstream.

The outcome of TGF- β action depends on cell type and local environment. This concept is best illustrated in the cancer literature. Depending on the combination effects of multiple factors in the tumor microenvironment (eg, the presence of other growth factors), immune cell population dynamics, and ECM composition, TGF- β could either promote or suppress cancer progression and metastasis^{53,56,57} by altering the proliferative responses in the same cell line. In a previous study,⁸ we observed that TGF- β expression is up-regulated on mechanical wounding and that, in response to injury, exog-

Figure 6. The expression levels of phosphorylated pRb (Ser 807/811) in the cytoplasm and nucleus of vehicle and 0.5 ng/mL TGF- β -treated VICs (**A**) and vehicle and 5 μ M/L SB431542-treated VICs (**B**) are determined using Western blot analyses. Cell extracts were prepared 6 days after first TGF- β treatment. Extracts containing 20 μ g of protein were separated on an 8% polyacrylamide gel. **C:** Expression of whole cell CDK4, cyclin D1, and nuclear p27 in vehicle and 0.5 ng/mL TGF- β -treated VICs. Cell extracts were prepared 6 days after first TGF- β treatment. Extracts containing 40 μ g of protein were separated on a 12.5% polyacrylamide gel. Phosphorylated pRb, CDK4, cyclin D1, p27, and their corresponding loading controls (tubulin and histone) were detected by immunoblotting. **D–F:** The levels of expression are determined by densitometry and compared. Statistical significance between groups: * $P < 0.01$, ** $P < 0.001$, and *** $P < 0.0001$.

Figure 7. α -SMA expression in control and TGF- β -treated VICs. Immunofluorescent confocal micrographs of vehicle and 5 ng/mL TGF- β -treated subconfluent VIC monolayers 4 days (**A** and **B**) and 6 days (**C** and **D**) after first TGF- β addition are shown. VICs are stained for α -SMA, which is expressed at a much higher level and organized into stress fibers in the treated group (green; nuclei, blue). Scale bar = 10 μ m (**D**). Original magnification, $\times 600$. **E:** Expression of whole cell α -SMA in vehicle and 0.5 ng/mL TGF- β -treated VICs. Cell extracts were prepared 6 days after first TGF- β treatment. Extracts containing 10 μ g of protein were separated on an 8% polyacrylamide gel. α -SMA and tubulin loading control were detected by immunoblotting. **F:** The levels of expression in control and treated VICs are determined by densitometry and compared. * $P < 0.001$ denotes statistical significance between groups.

enous TGF- β promotes VIC proliferation while it inhibits apoptosis in the early stage of wound repair. In contrast to what was reported at the wound edge, this current study shows that TGF- β decreases proliferation in low-density monolayers, independent of injury. The culture conditions used in this present study and the previously published wound model study are the same, suggesting that the differences observed are not a result of an *in vitro* artifact. Instead, one possibility is that mechanical wounding is an external stimulus that alters the signaling profile of quiescent VICs, leading to different cellular responses. Under both conditions and in essentially all cell

types, the Smad signaling pathway is activated in the presence of TGF- β . In other words, the same Smad molecules are up-regulated in all cellular and molecular contexts. Therefore, it is unlikely that Smad alone could mediate the full spectrum of TGF- β -related responses observed. In fact, TGF- β has activated many Smad-independent pathways, such as the mitogen-activated protein kinase, ρ -like Rho-GTPase, and PI3K-AKT pathways.¹⁵ One possibility is that one of these pathways may function independent of Smad and elicit opposing downstream effects. However, their signaling mechanisms and direct impacts on cell proliferation remain to be eluci-

Figure 8. VIC adhesion in control and TGF- β -treated VICs. Immunofluorescent confocal micrographs of vehicle (**A**) and 5 ng/mL TGF- β -treated (**B**) subconfluent VIC monolayers 4 days after first TGF- β addition are shown. VICs are stained for vinculin (green; nuclei, blue), which is expressed at a higher level and organized into vinculin plaques in the treated group. Scale bar = 10 μ m (**B**). Original magnification, $\times 600$. **C:** Results from the trypsin adhesion assay showing proportions of cells detached during the given incubation period. TGF- β -treated VICs showed significantly greater attachment to the substratum. Three independent trials were performed. Error bars denote SEM. Statistical significance between groups: * $P < 0.01$, ** $P < 0.001$.

dated. In one study,⁵⁸ it was suggested that the activation of the PI3K pathway is only observed in fibroblast cell lines in response to TGF- β stimulation, but not in epithelial cells, resulting in growth promotion in fibroblasts but inhibition in epithelial cells. One hypothesis is that VICs process different external stimuli and modulate the levels of the anti-proliferative Smad signaling and the pro-proliferative PI3K signaling to achieve the most desirable outcome. The net result of this active modulation is either VIC proliferation or wound closure in response to injury or growth inhibition under physiological conditions to prevent overgrowth and disruption of the valve structure. To design *in vitro* models that best replicate the *in vivo* conditions, every potential external stimulus and interaction should be considered. However, for the study of molecules with complex signaling cascades, such as TGF- β , it is more reasonable to start from the most basic system with few confounding variables and gradually build up the network of interactions. In this study of VIC growth regulation, we show that TGF- β has growth-inhibitory effects on intact monolayers. However, when an external stimulus, such as mechanical wounding, is applied to this system, VICs respond to the same molecule differently, likely by modulating the levels of activation of various downstream signaling pathways. Therefore, selectively determining the impact of each stimulus on the signaling profile is important in understanding the context-dependent effect of TGF- β under various conditions.

We also show that TGF- β addition leads to increased formation of α -SMA stress fibers and vinculin plaques, which may be a secondary effect of reduced growth or a condition that limits proliferation. In addition to a reduction in cell number, 0.5 ng/mL of TGF- β is sufficient to cause a significant increase in adhesion and a morphological change from spindle to rhomboid shaped after 4 days of treatment. Although TGF- β directly regulates cell cycle progression through several well-characterized mechanisms,^{37,39,40,59} decreased VIC proliferation can equally be attributed to increased cell-ECM adhesion and cytoskeletal rearrangement.⁶⁰ Consistent with our previous study⁶¹ of single VICs, the rhomboid cells that arise from TGF- β treatment show stronger attachments. They also possess abundant α -SMA stress fibers that, in turn, correlate with increased adherence.⁶² As a result, treated VICs require more time to physically round up from the substratum and proliferate. It is critical to recognize the bidirectionality of cell-matrix signaling. In addition to various ECM remodeling processes coordinated by signals transmitted or factors released from the cell, there is also matrix-to-cell signaling. In general, cell morphological features, cytoskeleton-matrix force transmission, adhesion, and matrix stiffness are all interrelated, and they act in concert to control the growth and differentiation of cells.^{63–65}

In summary, our two major findings are that TGF- β regulation of VIC growth is context dependent and that TGF- β inhibits VIC proliferation, similar to effects on epithelial, but not fibroblast-type, cells.

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