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Supplemental Information

TGFβ2 and TGFβ3 mediate appropriate context-dependent phenotype of rat valvular interstitial cells

Faye Wang, Cindy Zhang, Jae Kwagh, Brian Strassle, Jinqing Li, Minxue Huang, Yunling Song, Brenda Lehman, Richard Westhouse, Kamalavenkatesh Palanisamy, Vinay K. Holenarsipur, Robert Borzilleri, and Karen Augustine-Rauch

Supplemental Figures and Tables

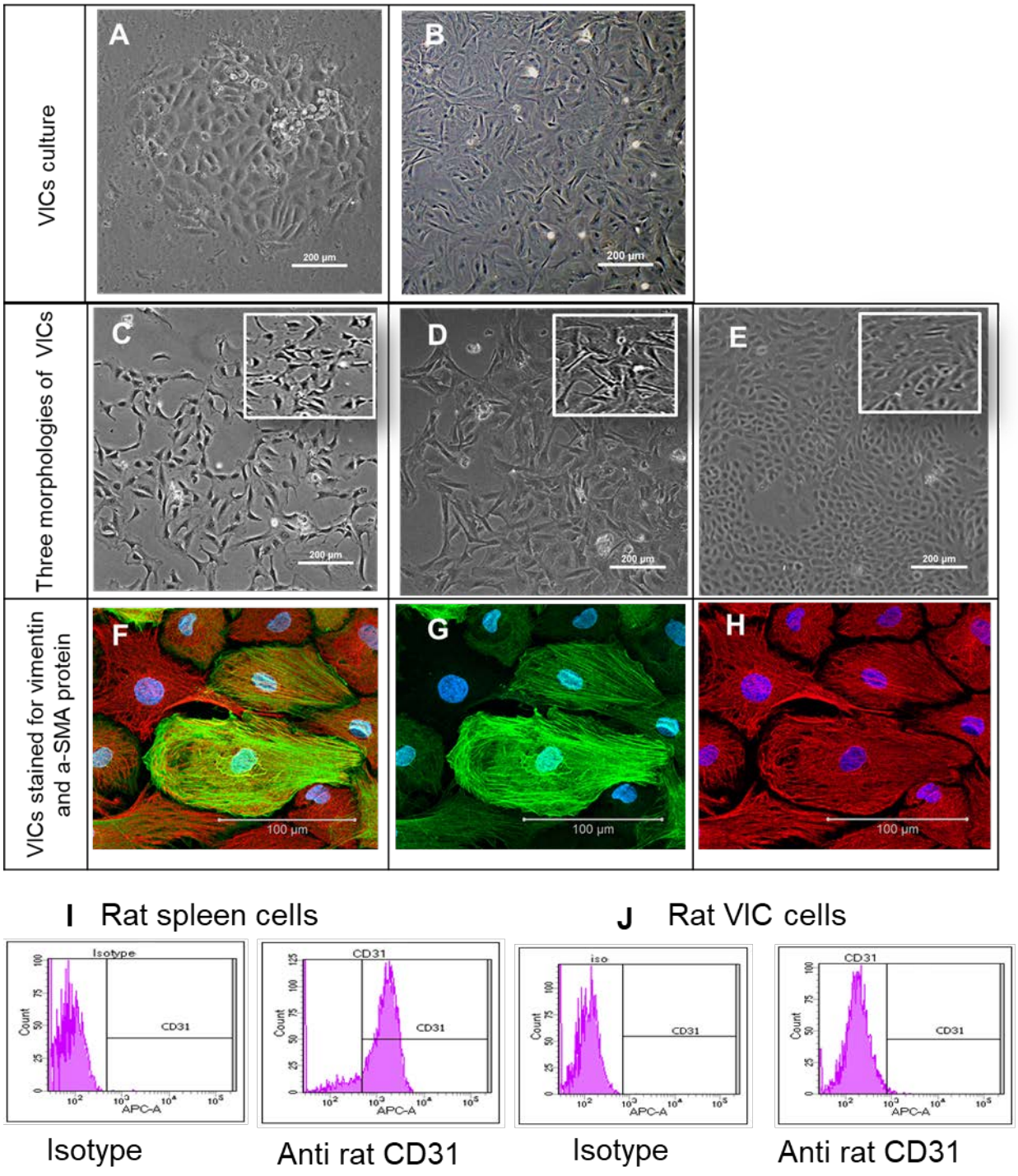

Figure S1. Rat Valvular Interstitial Cells (VICs): characterization of cultured cells and morphologies, Related to Results Section "Rat Valvular Interstitial Cells: Characterization of Cultured Cells and Morphologies."

(A) VICs exhibit multiple morphologies and grow by clonal expansion, where the respective morphology of the original cells is retained.

(B) Representative image of VIC cultured in a quiescent state induced by serum starvation (0.5% FBS) for 48 hours. In the quiescent state, primary rat VIC cultures present three morphologies:

(C) Condensed spindle-shaped (inset 2x original magnification).

(D) Flattened (inset 2x original magnification).

(E) Elongated, rounded morphology (inset 2x original magnification).

(F), (G) and (H) Immunofluorescent labeling for activated-VIC marker, α-SMA_{green} and a mesenchymal marker, $vimentin_{red}$, Nuclei labeled with $DAPI_{blue}$. Cells were positive for vimentin with activated VICs positive for vimentin and strongly positive for α-SMA.

(I) and (J) Flow cytometric evaluation of rat spleen cells (I) and VICs (J) labeled with isotype or with anti-CD-31 antibody. Lack of staining in VICs indicated no endothelial cell contamination.

Scale bar= 200μm

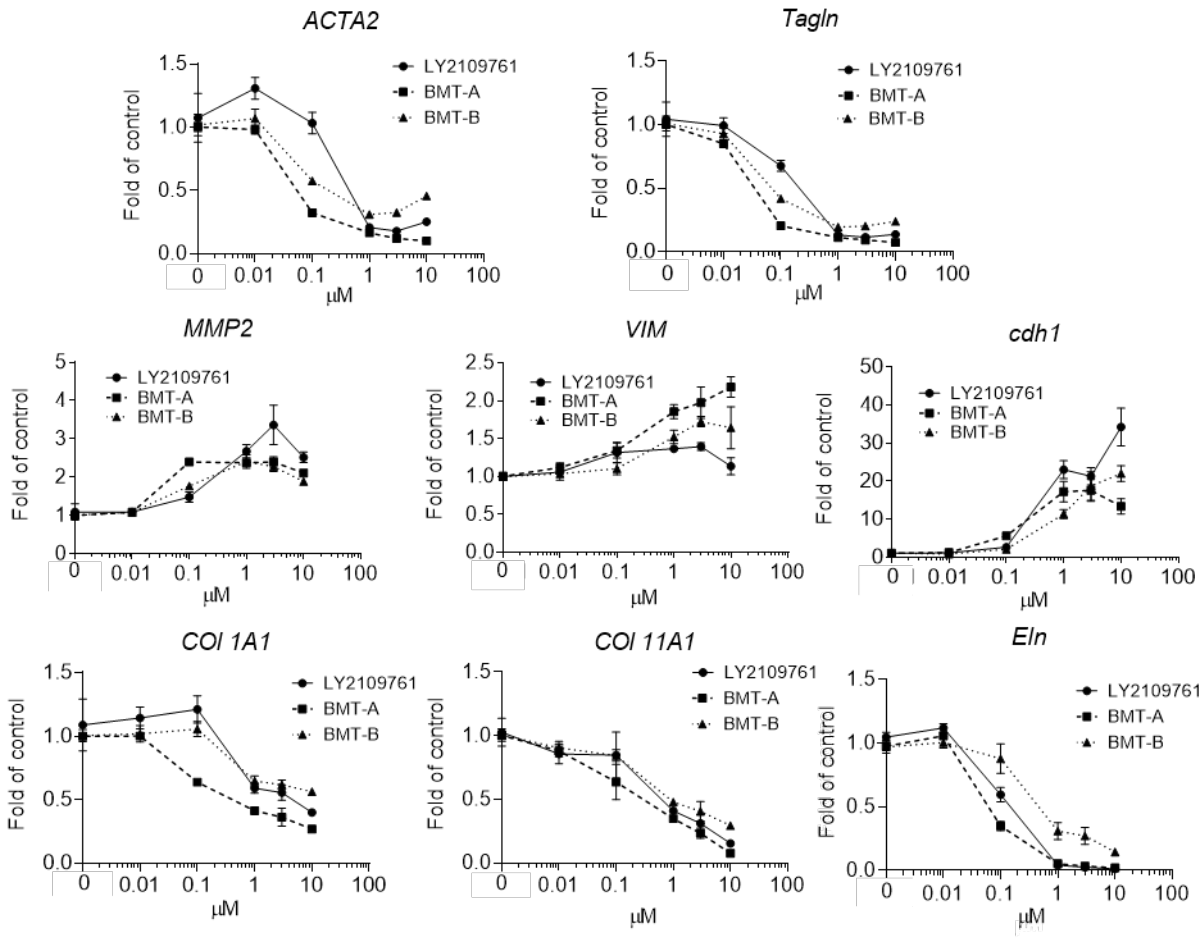

Figure S2. Early transcriptional alterations following TGFβRi treatment of VICS, Related to Table 4 and Figure 4.

Following 48 hour treatment, TGFβRi's caused decreased transcription of myofibroblast targets (ACTA2 and Tagln) but increased expression of EMT targets (MMP2 and vimentin) and epithelial marker, E-cadherin. There was also decreased transcription of ECM targets (COL 1A1, COL 11A1 and Eln).

For each target, at least 3 TGFβRi-treated VIC samples were analyzed with 2 technical replicates (>6 PCR tests) in calculating ±SEM.

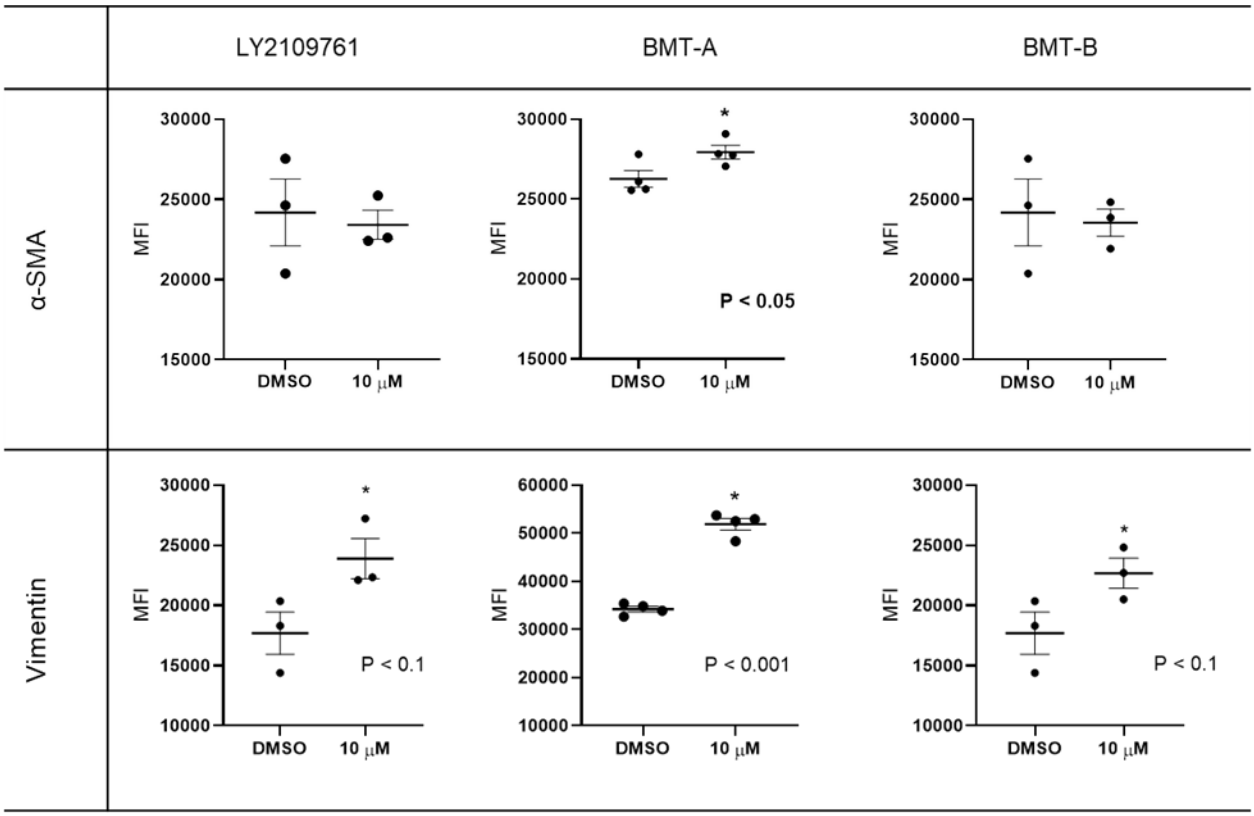

Figure S3. Evaluation of VIC expression of phenotypic markers following 48 hours TGFβRi treatment, Related to Figure 5.

VICs were stained with antibodies against α-SMA, vimentin, CD-31 and EpCAM to determine status of phenotype. α-SMA and vimentin are expressed in all VICs but vimentin expression is increased in VICs treated with TGFβRi's. VICs remained negative for staining with the endothelial marker, CD-31, or epithelial marker, EpCAM, following TGFβRi treatment (data not shown).

Error bars represent ± SEM.

Statistical comparisons employed one-way or two-way ANOVA followed by Dunnett's Test.

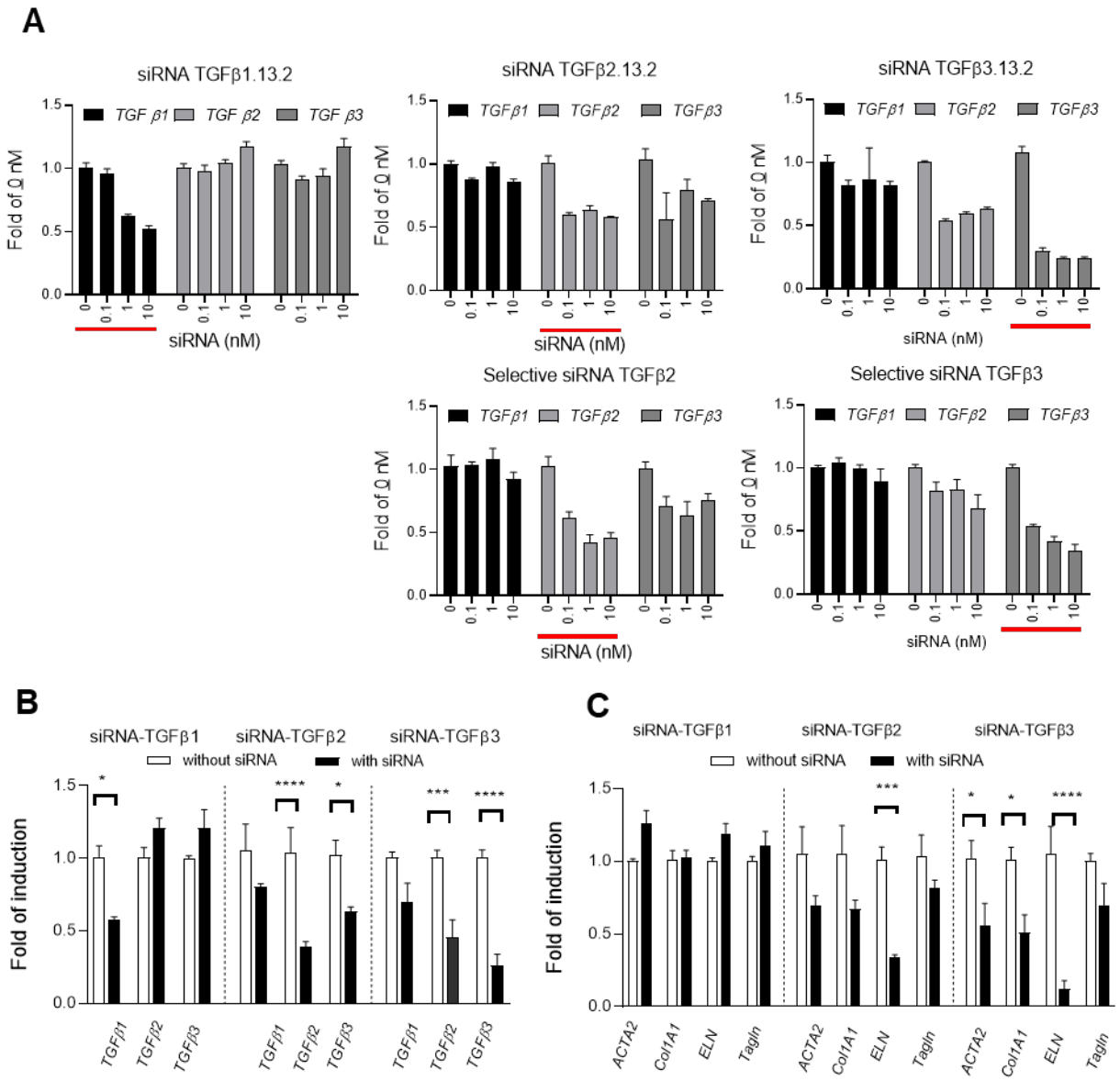

Figure S4. Small interfering RNA knock down of respective TGFβs cause differential alterations of myofibroblast and collagen target expression, Related to Figure 6.

(A) Small interfering RNA (siRNA) constructs from multiple sources were evaluated for potency and selectivity of TGFβ ligand mRNA reduction at 16 hours post transfection. A total of 3-4 siRNA constructs for each ligand were evaluated with representative data for 1 or 2 constructs designed against each ligand are presented. All siRNA constructs designed for TGFβ2 or TGFβ3 decreased both TGFβ2 and TGFβ3 mRNA levels. Underlined bars represent the TGFβ ligand targeted by the respective siRNA construct.

(B) Silent RNA (10nM) knockdown of TGFβ ligands and effect on myofibroblast and collagen target transcription at 16 hours post siRNA transfection in rat VICs.

Represented data are the average of 3 experiments, ± SEM.

Statistical comparisons employed one-way or two-way ANOVA followed by Dunnett's Test. The p values are expressed as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$; **** $p \leq 0.0001$

Table S1. TaqMan assays used in study, Related to Transparent Methods: RT-PCR.

Gene symbol	Gene name	Thermofisher Scientific Gene ID
Acta2	Alpha smooth muscle actin	Rn01759928_g1
Cdh1	E-cadherin	Rn00580109_m1
Col1a1	Collagen type I, alpha 1	Rn01463848_m1
Col11a1	Collagen, type XI, alpha 1	Rn01523309_m1
ELN	Elastin	Rn01499782_m1
MMP2	Matrix metallopeptidase 2	Rn01538170_m1
Tagln	Transgelin	Rn01642285_g1
TGFβRI	Transforming growth factor, beta receptor I	Rn00562811_m1
TGFβRII	Transforming growth factor, beta receptor II	Rn00579682_m1
TGFβRIII	Transforming growth factor, beta receptor III	Rn00568482_m1
TGFβ1	Transforming growth factor, beta 1	Rn00572010_m1
TGFβ2	Transforming growth factor, beta 2	Rn00676060_m1
TGFβ3	Transforming growth factor, beta 3	Rn00565937_m1
VIM	Vimentin	Rn00667825_m1
Hprt1	Hypoxanthine phosphoribosyl transferase 1	Rn01527840_m1

Table S2. Antibodies used in immunohistochemical characterization (IHC), flow cytometry (Flow Cyt) and TGFβ neutralizing antibody studies (N), Related to associated sections in the Transparent Methods.

Antibody	Assay	Host species	Species reactivity	Catalogue number and supplier	Dilution
Anti-α-SMA	IHC	mouse	mouse, human, cat. etc.	ab184675, Abcam	1:100
Anti Vimentin	IHC	rabbit	mouse, rat, human	ab154207, Abcam	1:500
IgG2a	IHC	mouse	mouse, rat, human	ma5-18169, Invitrogen	1:200
IgG	IHC	rabbit		ab208568, Abcam	1:500
E-cadherin Antibody (DECMA-1)	IHC	mouse	rat, human, dog	sc-59778, Santa Cruz	1:50
Anti-mouse IgG (H+L), Alexa Fluor 488	IHC	donkey	highly cross-absorbed	A32766, Invitrogen	1:666
Anti-Rabbit IgG (H+L), Alexa Fluor 594	IHC	donkey	highly cross-absorbed	A32754, Invitrogen	1:666
Anti-SMA	Flow Cyt	rabbit	rat	ab223921, Abcam	1:200
Anti-Vimentin	Flow Cyt	mouse	rat, horse, chicken, cow, cat, dog, human, pig	ab195877, Abcam	1:500
Anti-CD31	Flow Cyt/IHC	mouse	rat	50-0310-82, Invitrogen	0.25µg/test
IgG	Flow Cyt/IHC	rabbit		ab232814	1:200
IgG1	Flow Cyt/IHC	mouse		ab170190	1:500
IgG1	Flow Cyt/IHC	mouse		50-4714-82, Invitrogen	0.25µg
Anti-EpCAM	Flow Cyt/IHC	mouse	rat	ab187276, abcam	1:50
Goat Anti-Mouse IgG H&L Alexa Fluor® 488	Flow Cyt/IHC	goat	mouse	ab150117, abcam	2 µg/ml
TGF beta-1,2,3 Monoclonal Antibody (1D11)	N	mouse	bovine, chicken, human, mouse	MA5-23795, ThermoFisher	1-10ug/ml
TGF-beta 1 Antibody (141322)	N	mouse	human, rat, mouse	MAB2401, Novus Biologicals	1-10ug/ml
TGF-beta 2 Antibody (771213)	N	rat	mouse	MAB7346, Novus Biologicals	1-10ug/ml
TGF beta3 Antibody	N	goat	mouse, human	AF-243, Novus Biologicals	1-10ug/ml

Table S3. IC_{50} (μM) of Akt/mTOR phosphoprotein after 48 hours treatment with TGFβRi, Related to Table 2.

TGFβRi	AKT	GSK3B	IRSI	p70S6K
LY2107961	0.48±0.13	0.58±0.31	0.92±0.01	0.41±0.1
BMT- A	0.19±0.07	0.24±0.12	0.34±0.33	0.18±0.07
BMT- B	0.97±0.41	0.61±0.39	1.68±0.61	2.2±2.01

Average of 2 experiments; ±SEM.

*Table S*4. Flow cytometry analysis of apoptosis and necrosis of VICs at 48 hours following BMT-B treatment, Related to Table 3 and Figure 3.

μM	% of Dead cells	% of Apoptosis
0	4.4	1.4
0.01	3.0	0.6
0.1	3.9	1.1
1	3.7	1.3
10	2.6	0.7

Table S5. Average of IC_{50}/EC_{50} (μM) of target transcriptional expression at 48 hours treatment with TGFβR inhibitors, Related to Table 4 and Figure S2.

Gene	Category	Ly2109761	BMT- A	BMT- B
TGFβ1 (IC_{50})	TGFβ ligands	0.1613	0.12	0.43
TGFβ2 (IC_{50})	TGFβ ligands	0.1314	0.13	0.24
TGFβ3 (IC_{50})	TGFβ ligands	1.041	0.79	≥ 10
TGFβRI (IC_{50})	TGFβ receptors	0.1723	0.09	0.47
TGFβRII (EC_{50})	TGFβ receptors	0.42 ± 0.02	0.94 ± 0.53	0.56± 0.18
TGFβRIII (EC_{50})	TGFβ receptors	0.49 ± 0.045	0.58 ± 0.24	0.61± 0.22
ACTA2 (IC_{50})	Myofibroblast Marker	0.15 ± 0.06	0.14 ± 0.02	0.87 ± 0.67
Tagln (IC_{50})	Myofibroblast Marker	0.12 ± 0.04	0.40 ± 0.33	0.17 ± 0.11
Cdh1 (EC_{50})	Epithelial Marker	0.41	0.14	1.18
VIM (EC_{50})	Mesenchymal Marker	0.017	0.33	0.4
MMP2 (EC_{50})	MMP2 EMT Marker	0.48 ± 0.36	0.07 ± 0.05	0.05
COL1A1 (IC_{50})	ECM	0.27 ± 0.14	0.09 ± 0.03	0.31 ± 0.15
COL11A1 (IC_{50})	ECM	0.25 ± 0.12	0.40 ± 0.22	0.53 ± 0.11
ELN (IC_{50})	ECM	0.15 ± 0.06	0.07 ± 0.02	0.71 ± 0.52

For each target, at least 3 TGFβRi- treated VIC samples were analyzed with 2 technical replicates ($\geq$6 PCR tests) in calculating EC_{50}/IC_{50}, respectively. In cases that included multiple experiments, the EC_{50}/IC_{50} values are presented with $\pm$SEM.

Transparent Methods

Rat VIC isolation. All animal procedures were approved by the Institutional Animal Care and Use Committee of Bristol-Myers Squibb Company and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Male or female Crl:CD® (Sprague-Dawley) rats (Charles River Laboratories, Kingston, NY) between 8-13 weeks were used in the surgical harvest of heart valves to provide VICs for culture. Approximately 1-2 rats were used to support sufficient VICs for culture, where passages 3-8 were used to support in vitro experiments. Tissue harvest and VIC cultures were re-established after passage 8 to support additional in vitro experiments. Rat valves (mitral, tricuspid, pulmonary and aortic) were isolated according to previous methods (Liu et al., 2015, Walker et al., 2004) with some modification. Briefly, heart valves were isolated under sterile conditions, rinsed in cold phosphate buffered saline (PBS) and placed on ice, then centrifuged followed by two digestions. The initial digestion was to remove endothelial cells with 1 ml of digested collagenase solution (MillporeSigma, C5894) at 600 units/ml in 1X Trypsin inhibitor, 0.1% BSA, 100 units/ml penicillin/ 100 µg/ml streptomycin (P/S, ThermoFisher Scientific, 15140122) and media 199 (ThermoFisher Scientific, 11150-067). Samples were incubated 37^{o} for 5 minutes, followed by pipetting 5 times, then the media without valves was removed as much as possible. The second digestion was performed to disassociate the VICs from the collagen network using 10ml of media containing collagenase at 42.5 units/ml at 37^{o}C for 60 minutes with shaking at 50 times/minute. After washing with PBS twice with centrifugation, cells were placed in four wells of a 24 well plate in 10% (V:V) fetal bovine serum (FBS, ThermoFisher Scientific, 10082147), P/S with media 199 (complete medium). After 7-10 days, when cells reached confluence, cells were transferred to a 6-well plate. All additional cell passages came from the original 24 and 6-well plates and were harvested by cell scraping. VICs were maintained in complete media that contained one third of previous passage media as conditioned media. For cell passages used for experiments, detached cells were cultured in a collagen I (MillporeSigma,125-50) coated T-75 flask for 5-7 days with media change every 2-3 days and cells were removed by incubation for 3 minutes at 37^{o}C with Trypsin-EDTA (0.05%) (ThermoFisher Scientific, 25300054), and plated in 24, or 96 well plates.

TGFβR Inhibitors. Three TGFβRi's with potent TGFβRI or -R1/-R2 selectivity were evaluated in this study to assess target/class mediated alterations of VIC biology: LY2109761, BMT-A and BMT-B. LY2109761 is an orally active TGFβRI/II dual kinase inhibitor (I, K_i = 38 nmol/L; II, K_i = 300 nmol/L *in vitro* kinase assay) (Melisi et al., 2008), purchased from Selleckchem, cat. # S2704. BMT-A and BMT-B, are TGFβRI inhibitors of the azaindole series, with BMT-A also having inhibitory activity on TGFβRII, were synthesized by Bristol-Myers Squibb (Table 1) (Zhang et al., 2018). The structure of BMT-A and BMT-B can be found as Example 14B and 47, respectively (Fink et al 2017, US Patent 09708316). All compounds were formulated in dimethyl sulfoxide (DMSO, MillporeSigma, D2438) as 20mM stock solutions.

Rat In Vivo Toxicology Studies: All animal experiments were approved by the Institutional Animal Care and Use Committee of Bristol-Myers Squibb Company and were conducted in accordance with the Guide for

the Care and Use of Laboratory Animals. Male or female Sprague-Dawley rats (Newsted et al.,2019) of approximately 8-10 weeks age were treated daily by oral gavage with the respective TGFβRi for 4 days, with necropsy on Day 14 or 15. In the case of LY2109761, rats were dosed for 4 days or 14 days with necropsy on day 4 or day 15, and confirmed previous reports that doses that caused valvulopathy were not histologically apparent by Day 4 but was obvious by 2 weeks with either 4 days or 14 days of dosing (Herbertz et al., 2015, Maratera, 2009). Based upon finding that 4 days of dosing with LY2109761 was sufficient to induce valvulopathy that was histologically apparent by 2 weeks post initial dose, BMT-A and BMT-B were subsequently evaluated following a dose schedule of 4 consecutive days followed by a 10 day dose holiday prior to necropsy and tissue collection. On day 1-2 plasma was collected along a time course for toxicokinetic evaluation. Hearts were collected and fixed in 10% neutral buffered formalin, paraffin embedded and step-sectioning was performed to thoroughly evaluate integrity all heart valves. Valves were stained with hematoxylin and eosin or trichrome for histopathological assessment. At least 5 rats were included in each treatment group.

VIC Experimental Designs. Rat VICs were plated and cultured with complete media (without conditioned media) for 2-3 days to achieve $\leq$90% of coverage in either 96 or 24 well plates. The VICs were then serum starved at least 24 hours in M199 with 0.5% of FBS prior to treatments with the TGFβRi's (Gu and Masters, 2010). With exception of the SMAD3 phosphorylation studies, all experiments were undertaken as follows: after treatment with a concentration range (0.01, 0.1, 1, 3, 10 μM) of TGFβRi's or 0.05% DMSO in serum starved media, cells were evaluated for various biological endpoints at 48 hours post treatment. For the p-SMAD3 phosphorylation studies, cells were lysed and processed for p-SMAD3 AlphaLISA measurements at 1 hour or 48 hours post TGFβRi treatment. When stimulating with human TGFβ (hTGFβ, VWR, 47743-604), 0.1ng/ml of hTGFβ in 0.1% BSA/PBS was added 30 minutes prior cell collection for p-SMAD3 measurement, with exception of the time course study of hTGFβ induced p-SMAD3 assay. To evaluate p-SMAD3 induction following hTGFβ stimulation, hTGFβ concentrations were tested in a concentration range of 0.001 -10 ng/ml.

Flow cytometry assessment: Approximately 1×10^6 cells in 1 mL PBS were labeled with 1 μl of the reconstituted fluorescent reactive dye to 1 mL of the cell suspension for 30 minutes with protection from light. Live and dead cells were distinguished by using a viability dye, LIVE/DEAD™ Fixable Near-IR dye (ThermoFisher Scientific, L34976). The fluorescence of Near-IR dye with 633 nm excitation and 780 nm emission was detected through APC-Cy7 channel.

Cell apoptosis was evaluated by using CellEvent™ Caspase-3/7 Green Detection Reagent (Invitrogen, C10423). Cells were incubated with 100 μl of 5 μM CellEvent™ Caspase-3/7 Green Detection Reagent in PBS with 5% fetal bovine serum for 30 minutes at 37°C. Apoptotic cells with activated caspase-3/7 show bright green nuclei was detected through FITC channel.

Cultured VIC cells treated with BMS-A, BMS-B, or LY2109761 for 48h were collected for flow cytometric phenotyping. The cells were stained with Anti- rat CD31-efluo660 (Invitrogen, cat. 50-0310-82), alpha smooth muscle actin-Allophycocyanin (abcam, ab223921), vimentin-Alexa Fluor® 488 (abcam, ab195877) and Anti-CD31 antibodies. (Table S2).

All flow cytometry experiments were run by using BD FACSCanto II flow cytometer with Diva software.

VIC proliferation, ATP and oxidative stress assessment. At 48 hours following treatment, VICs were evaluated for proliferation by CyQUANT® Direct Cell Proliferation Assay (ThermoFisher Scientific, C35011). VICs were cultured for 60 minutes with 2X CyQUANT test reagent, and fluorescence was read at wavelengths of 480/535 nm using an EnVision plate reader (Perkin-Elmer, Waltham, MA). ATP measurement was undertaken using CytoTox™ Homogeneous Membrane Integrity Assay (Promega , G7890) and chemoluminescent signal was read by the EnVision plate reader after a 15 minute incubation with CellTiter-Glo® reagent. Oxidative Stress was evaluated using the ROS-Glow™ H_2O_2 Assay (Promega, G8821). To this end, VICs were cultured with H_2O_2 at the last 6 hours of the experiment, following 48 hours of compound treatment. Following the 6 hour H_2O_2 treatment, the VICs were then incubated for 20 minutes with ROS-Glo™ Detection Solution. Chemiluminescence was measured using an EnVision plate reader.

Mitochondria function measurement. An extracellular flux assay was used to assess mitochondrial toxicity in VICs by determining the oxygen consumption rate (OCR) utilizing the XF^e96 flux analyzer (Agilent), as described by (Brand and Nicholls, 2011). Briefly, rat VICs were plated onto XF^e96 plates (Agilent), cultured, and test compounds were applied as described in VIC Experimental Designs. The cells were washed twice in un-buffered DMEM assay medium: XF Base Medium Minimal DMEM (Agilent Technologies, 103680), supplemented with 10mM glucose, 1mM pyruvate, 2mM L-glutamine (medium pH7.4, 37°C). Cells were then incubated in 180µl assay media in a CO_2 free incubator at 37°C for 60min. The XF^e 96 microplate cartridges were loaded with 20 µl of dosing solution. Three measurements of OCR were taken and a mitochondrial stress test was performed by consecutive addition of the ATP synthase inhibitor, oligomycin (1 µM), the uncoupler, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 0.5 µM) and finally the ETC inhibitors rotenone (1µM) plus antimycin A (1µM) (Rot/AA). Five functional parameters including ATP production, maximal respiration, spare capacity, proton leak, and non-mitochondrial respiration, were calculated to determine potential mitochondrial toxicity caused by tested compounds.

RT-PCR. VICs were treated with TGFβRi's for 48 hours in triplicate in a 96-well plate then lysed according to TaqMan® Gene Expression Cells-to-CT™ Kit (ThermoFisher Scientific, AM1728); 10µl of lysis was used for reverse transcription at 37° for 1 hour, then 2µl of RT product was used in the real time PCR reaction. TaqMan primer assay (ThermoFisher Scientific, 4426961) sets (Table S1) were used to

characterize the VIC's response to TGFβRi's. PCR amplification was conducted using a ViiA7 system from ThermoFisher Scientific.

Phospho-protein measurement. VICs were cultured in 96 or 24 well plates and treated with TGFβRi's at a concentration range of 0.01, 0.1, 1, 3, 10 μM or 0.05% DMSO at 37°C for 60 minutes or 48 hours, according to the experimental design. Plates were washed with ice-cold PBS, then 1X MILLIPLEX®MAP lysis buffer (Millipore, 43-040) with complete proteinase inhibitor (Roche, 11 836 153 001), phosphate cocktail II (Sigma, P5726) and phosphate cocktail III (Sigma P0044) were added. The lysate was gently rocked for 10 minutes at 4°C, then filtrated with a filter plate (EMD, MAHVH 4510) and centrifuged at 2000g, 4°C for 5 minutes. p-SMAD3 was detected using an AlphaLISA® *SureFire Ultra* HV p-SMAD3 (Ser423/425) test kit (PerkinElmer, ALSU-PSM3-A-HV). p-SMAD signal was measured using an EnVision plate reader, PerkinElmer, 2105. p-SMAD2, p-AKT and p-ERK were measured with Luminex technology - MILLIPLEX MAP TGFβ Signaling Pathway, Magnetic Bead 6-Plex-Cell Signaling Multiplex Assay (Millipore, 48-614MAG) and Akt/mTOR phosphoprotein were tested with the same method (Millipore, 48-611MAG).

TGFβ ligand Measurement: TGFβ ligand Measurement: TGF-β1, 2, and 3 levels in rat serum and cell culture media were detected by using Bio-Rad Luminex kit (Bio-Plex Pro TGF-ß 3-plex Panel, 171W4001M). The detailed experimental protocol is described in Bio-Rad's Bio-Plex Pro™ TGF-β Assays Instruction Manual, *https://www.bio-rad.com/en-us/sku/171w4001m-bio-plex-pro-tgf-beta-3-plex-assay?ID=171w4001m*. Briefly, animal serum samples or filtered cell culture media were diluted either 16X or 4X with sample diluent or cell culture media and acidified with 1N HCl and neutralized with 1N NaOH/0.5 M HEPES to release TGF- β from latent complex. The samples were then incubated with magnetic beads with internal color-code multiple fluorescent dyes, detection antibody, and streptavidin-PE. A Bio-Plex® 200 Systems and Bio-Plex Manager™ software were used for sample acquisition and data analysis. The concentration of analyte bund to each bead is proportional to the MFI of reporter signal, and the result was presented as median fluorescence intensity (MFI) as well as concentration (pg/ml). A standard curve of TGF-β1, 2 and 3 were run with the samples in each assay.

VIC Cell Migration: VICs were seeded at $2.5X10^5$/well in 6-well plates. At confluence, media was switched to 0.5%FBS/M199 media for 24 hours. At Scratch Test was performed where a scratch was made in the middle of the well with a 200μl pipette tip (Liang et al., 2007), then respective TGFβRi's (10μM) in 0.5%FBS/M199 media was added to the wells. Three independent wells were photographed at 0, 6, 24 and 48 hours post-scratch using a Zeiss phase contrast microscope, where photographs were taken at the same marked location each time. The TIF files were analyzed using HALO image analysis software (INDICA LABS, NM, USA). The annotation of the scratched open area was performed for 0 hour image, and then the annotation was copied and pasted for its sequential 6, 24, and 48 hour. The measurement of

the cell density was performed by using the Area Quantification v1.0 algorithm. Thus, the area of the cells was measured and the percentage of cell density were calculated and compared to the Time 0 (T_0) density.

*ELISA Measurement of Collagen and Elastin in VIC lysates: V*ICs were cultured in 24 well plates. Following 48 hours of treatment with TGFβRi's, cells were lysed with 1X MILLIPLEX®MAP lysis buffer with complete proteinase inhibitor, phosphate cocktail II and phosphate cocktail III and 50ul of lysate was tested by ELISA with rat Col type1 (MyBioSource.com., MBS262647), rat Col type III (cat No., MyBioSource.com., MBS260802), rat Col typeV (MyBioSource.com., MBS2603034), rat Elastin (MyBioSource.com., MBS2503127). Manufacture procedures were followed for each respective ELISA measurement.
Cellular Impedance measurement: After background readings of Agilent E-Plate (Aligent, 300601110) were taken with 50ul of complete media, 15,000 cells/100 µL were seeded into the E-Plate. The plate was then transferred to the Aligent real time cell analyzer (RTCA) station, which was located in a cell culture incubator, and cells were cultured for 24 hours at 37^{O}C, followed by another 24 hours with 0.5%FBS/Media199, continued by treatment with TGFβRi's in a 0.01 to 10µM concentration range, in 0.5%FBS/Media199. Cell attachment, spreading, and proliferation were assessed as impedance recordings, which were monitored every 2 hrs starting after cell seeding through 5 days. Measured cellular impedance (CI) from cells in each individual well on the E-Plate were automatically converted to CI values by the RTCA software. CI value from TGFβRi's added at 72 hrs and continued to be monitored for an additional 48 hours were exported to Excel for further analysis.

Immunocytochemistry and Imaging. VIC morphology was observed by light microscopy during the culture period and assessed for cell morphology and confluence. For immunocytochemistry, 0.5 X 10^4/well cell suspension was added on BioCoat™ Collagen I 4 wells chamber slides, (VWR, 734-0206) and incubated in 5% CO_2 at 37^{o}C for 24-48 hours. After serum starving and treatment with TGFβRi's for 48 hours, slides were directly fixed with ice cold 100% methanol for 5 minutes, permeabilized with 0.1% Triton X-100 in 1 X PBS for 5 minutes and then blocked for 1 hour with 3% CD1 mouse plasma in PBST (PBS + 0.1% Tween 20) at room temperature. Primary antibodies against vimentin, α-SMA, α-CD31, α- E cadherin and α-Epcam in PBST with 1% of BSA were applied for one hour at room temperature in the dark. Following a wash sequence, secondary antibodies were applied for 1 hour at room temperature in the dark. After another wash sequence, slides were mounted using ProLong® Gold Antifade Mountant with DAPI (ThermoFisher Scientific, USA). Images were collected using a 20x objective on an LSM710 confocal microscope (Carl Zeiss, Thornwood, NY). Using the tiling/stitching software feature and an automated stage, a high-resolution image that covered the majority of the well was created. Details of the antibodies used for immunohistochemistry are summarized in (Table S2).

Silent RNA knockdown of TGFβ ligands. Three constructs were evaluated for transfection efficiency, target knockdown efficiency and selectivity, and cytotoxicity. The original set of evaluated constructs were from Integrated DNA Technologies: rn.Ri.TGFβ1.13, rn.Ri. TGFβ2.13, and rn.Ri.TGFβ3.13, three

constructs of each, named 13.1; 13.2 and 13.3. Due to the finding that all siRNAs for TGFβ2 and TGFβ3 caused knockdown of both TGFβ2 and TGFβ3 gene expression, Ambion® Silencer® Select Pre-designed siRNA (select TGFβ2 and select TGFβ3) were evaluated (Figure S4A). Based on acceptable transfection efficiency, selectivity in target knockdown and lack of cytotoxicity, siRNA TGFβ2 (ID s135786) and siRNA TGFβ3 (ID s130564) from ThermoScientifics and rn.Ri.TGFβ1.13.2 of Integrated DNA technologies were selected for this study and used with the X-tremeGENE™ siRNA (Sigma Aldrich, cat# 4476093001). 0.5%FBS in M199 media as the dilution solution.

TGFβ ligand neutralization studies: After VICs were starved for 24 hour, pan TGF beta Monoclonal Antibody (1D11) (ThermoFisher Scientific, MA5-23795), TGF-beta 1 Antibody (141322) (NOVUS biologicals, MAB2401), TGF-beta 2 Antibody (771213) (NOVUS biologicals, MAB7346) and/or TGF beta3 (NOVUS biologicals, AF-243) (Table S2) were added at 1,3,10 ug/ml with 1XPBS as control in 0.5%FBS/M199 media for 48 hours. Cells were measured for p-SMAD3 inhibition by alphalisa and gene expression by real time PCR by a set of TaqMan primers (Table S1). Cell media was collected and measured for TGFb ligand levels using Luminex.

Statistical analysis and EC_{50}/IC_{50} calculations. Data are presented in all tables and figures as mean ± standard deviation or standard error of the mean. The data were analyzed for significant changes with respect to the vehicle or DMSO vs. treatment using GraphPad Prism 8.0.2 (La Jolla, CA) with statistical significance defined at least $p \leq 0.05$. Statistical comparisons employed one-way or two-way ANOVA followed by Dunnett's Test. The data calculated for EC_{50} and IC_{50} was transformed and nonlinear regression fit using same program. Unless otherwise noted, the p values are expressed as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$; **** $p \leq 0.0001$

Supplemental References

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