

## Phosphodiesterase Type 5 Inhibition Reverts Prostate Fibroblast-to-Myofibroblast *Trans*-Differentiation

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Phosphodiesterase type 5 (PDE5) inhibitors have been demonstrated to improve lower urinary tract symptoms secondary to benign prostatic hyperplasia (BPH). Because BPH is primarily driven by fibroblast-to-myofibroblast *trans*-differentiation, this study aimed to evaluate the potential of the PDE5 inhibitor vardenafil to inhibit and reverse *trans*-differentiation of primary human prostatic stromal cells (PrSC). Vardenafil, sodium nitroprusside, lentiviral-delivered short hairpin RNA-mediated *PDE5* knockdown, sodium orthovanadate, and inhibitors of MAPK kinase, protein kinase G, Ras homolog family member (Rho) A, RhoA/Rho kinase, phosphatidylinositol 3 kinase and protein kinase B (AKT) were applied to PrSC treated with basic fibroblast growth factor (fibroblasts) or TGF $\beta$ 1 (myofibroblasts) *in vitro*, in chicken chorioallantoic membrane xenografts *in vivo*, and to prostatic organoids *ex vivo*. Fibroblast-to-myofibroblast *trans*-differentiation was monitored by smooth muscle cell actin and IGF binding protein 3 mRNA and protein levels. Vardenafil significantly attenuated TGF $\beta$ 1-induced PrSC *trans*-differentiation *in vitro* and in chorioallantoic membrane xenografts. Enhancement of nitric oxide/cyclic guanosine monophosphate signaling by vardenafil, sodium nitroprusside, or *PDE5* knockdown reduced smooth muscle cell actin and IGF binding protein 3 mRNA and protein levels and restored fibroblast-like morphology in *trans*-differentiated myofibroblast. This reversal of *trans*-differentiation was not affected by MAPK kinase, protein kinase G, RhoA, or RhoA/Rho kinase inhibition, but vardenafil attenuated phospho-AKT levels in myofibroblasts. Consistently, phosphatidylinositol 3 kinase or AKT inhibition induced reversal of *trans*-differentiation, whereas the tyrosine phosphatase inhibitor sodium orthovanadate abrogated the effect of vardenafil. Treatment of prostatic organoids with vardenafil *ex vivo* reduced expression of myofibroblast markers, indicating reverse remodeling of stroma towards a desired higher fibroblast/myofibroblast ratio. Thus, enhancement of the nitric oxide/cyclic guanosine monophosphate signaling pathway by vardenafil attenuates and reverts fibroblast-to-myofibroblast *trans*-differentiation, hypothesizing that BPH patients might benefit from long-term therapy with PDE5 inhibitors. (*Endocrinology* 153: 5546–5555, 2012)

**B**enign prostatic hyperplasia (BPH) is characterized by progressive enlargement and reorganization of the stromal compartment of the gland, in particular increased extracellular matrix deposition and *trans*-differentiation of fibroblasts into myofibroblasts, the mitogenic secretome of which promotes proliferation, angiogenesis, and tumorigenesis (1–4). TGF $\beta$ 1 is considered to be a key inducer of pathogenic stromal reorganization, and we and

others have demonstrated that TGF $\beta$ 1 induces fibroblast-to-myofibroblast *trans*-differentiation (5–7).

BPH is commonly associated with bothersome lower urinary tract symptoms (LUTS). Inhibition of the phos-

Abbreviations: AKT, Protein kinase B; bFGF, basic fibroblast growth factor; BPH, benign prostatic hyperplasia; CAM, chorioallantoic membrane; cGMP, cyclic guanosine monophosphate; CNN1, calponin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMBS, hydroxymethylbilane synthase; IGFBP3, IGF binding protein 3; LUTS, lower urinary tract symptom; MEK, MAPK kinase; Na<sub>3</sub>VO<sub>4</sub>, sodium orthovanadate; NO, nitric oxide; NOX4, reduced nicotinamide adenine dinucleotide phosphate oxidase 4; PDE5, phosphodiesterase type 5; PI3K, phosphatidylinositol 3 kinase; PKG, protein kinase G; PrSC, prostatic stromal cell; qPCR, quantitative PCR; Rho, ras homolog family member; ROCK, RhoA/Rho kinase; ROS, reactive oxygen species; SCR, scrambled; shRNA, short hairpin RNA; SMA, smooth muscle cell actin; Smad, mothers against decapentaplegic homolog; SNP, sodium nitroprusside; SOD2, sodium dismutase 2.

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phodiesterase type 5 (PDE5), an enzyme expressed in the stromal compartment of the prostate (7) that metabolizes the second messenger cyclic guanosine monophosphate (cGMP), has been demonstrated to improve LUTS (for review see Refs. 8, 9). The precise mechanisms underlying these beneficial effects are unclear. However, it appears that PDE5 inhibition impacts several pathways involved in BPH/LUTS, including increased smooth muscle relaxation and reduced stromal cell proliferation (9, 10) via enhancing nitric oxide (NO)/cGMP signaling. We and others previously demonstrated antiproliferative effects of the PDE5 inhibitors tadalafil (7, 11) and vardenafil (11, 12) on prostatic stromal cells (PrSC). In addition to these effects, we recently reported that tadalafil attenuated fibroblast-to-myofibroblast *trans*-differentiation, a hallmark of stromal remodeling (7).

*In vivo* fibroblast-to-myofibroblast *trans*-differentiation occurs via a two-step process that is initiated by changes in mechanical tension of the extracellular matrix that are transmitted to the fibroblast cytoskeleton via Ras homolog family member (Rho) A/Rho kinase (ROCK) signaling (13). Consequently, fibroblasts adopt an activated phenotype and deposit new extracellular matrix components (14). Soluble factors and cytokines, in particular the extra domain-A splice variant of cellular fibronectin and TGF $\beta$ , especially TGF $\beta$ 1, play a key role in the differentiation to the  $\alpha$ -smooth muscle cell actin (SMA)-expressing myofibroblast phenotype (14). The effects of TGF $\beta$ 1 are mediated via mothers against decapentaplegic homolog (Smad) 2/3 activation and Smad-independent regulation of MAPK and phosphatidylinositol 3 kinase (PI3K) and protein kinase B (AKT) pathways (15–17). The RhoA/ROCK pathway seems to be the predominant pathway that regulates myofibroblast contraction (14) and has been shown to regulate the expression of  $\alpha$ -SMA in smooth muscle cells (18). Furthermore, RhoA has been shown to modulate TGF $\beta$ -induced smooth muscle cell differentiation via cross talk with Smad (19). The PDE5 inhibitors vardenafil and sildenafil have been shown to inhibit RhoA/ROCK in a NO/cGMP-dependent protein kinase G (PKG)-dependent manner in the bladder stroma and in vascular smooth muscle cells, respectively (20, 21). We previously demonstrated that PrSC fibroblast-to-myofibroblast *trans*-differentiation downstream of TGF $\beta$ 1 is driven by a prooxidant shift in redox homeostasis due to elevated production of reduced nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4)-derived reactive oxygen species (ROS) and concomitant decreases in antioxidant enzymes like sodium dismutase 2 (SOD2) (17).

In the present study, we evaluated the potential of the specific PDE5 inhibitor vardenafil that has been shown to significantly improve LUTS secondary to BPH (22), to

inhibit and moreover to revert stromal remodeling as characterized by fibroblast-to-myofibroblast *trans*-differentiation, and investigated the pathway underlying *trans*-differentiation reversal using inhibitors of MAPK kinase (MEK), PKG, RhoA, ROCK, PI3K, AKT, and the tyrosine phosphatase inhibitor sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>).

## Materials and Methods

### Reagents

Reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Human recombinant TGF $\beta$ 1 was from R&D Systems (Minneapolis, MN). Kinase inhibitors and concentrations employed were as follows: MEK inhibitor PD98059 (50  $\mu$ M; Calbiochem, San Diego, CA); PKG inhibitor KT5823 (1  $\mu$ M; Calbiochem); RhoA inhibitor C3 exoenzyme (1  $\mu$ g/ml; Calbiochem); ROCK inhibitor Y27632 (5  $\mu$ M); PI3K inhibitor LY-294002 (20  $\mu$ M; Calbiochem); and AKT inhibitor triciribine (20  $\mu$ M). Antibodies were obtained as follows: AKT, phospho-AKT, phospho-p44/42 MAPK (ERK1/2), PDE5 (Cell Signaling Technology, Beverly, MA);  $\alpha$ -tubulin, vimentin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); IGF binding protein 3 (IGFBP3) (R&D Systems);  $\alpha$ -SMA (Sigma, St. Louis, MO);  $\alpha$ -SMA for immunohistochemistry (IHC) (Dako, Glostrup, Denmark); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, MA); and horseradish peroxidase-conjugated secondary antibodies (antimouse from Promega Corp., Madison, WI; antirabbit from Dianova, Hamburg, Germany; and antigoat from Dako). Tissue culture grade vardenafil was kindly provided by Peter Sandner (Bayer HealthCare, Wuppertal, Germany).

### Culture of primary cells and prostatic organoids, *trans*-differentiation, and lentiviral-mediated PDE5 knockdown

Human primary PrSC were established as described previously (23) and cultured in stromal cell growth medium (Quantum 333; PAA Laboratories, Pasching, Austria).

Fibroblast-to-myofibroblast *trans*-differentiation was induced by 1 ng/ml TGF $\beta$ 1, whereas control cells were incubated with 1 ng/ml basic fibroblast growth factor (bFGF) in RPMI 1640 (PAA Laboratories) supplemented with 1% charcoal-treated bovine calf serum (HyClone Laboratories, Logan, UT) and antibiotics for 72 h as described (7). For *trans*-differentiation reversal studies, cells were subsequently stimulated for additional 72 h with bFGF, TGF $\beta$ 1, or TGF $\beta$ 1 with vardenafil, sodium nitroprusside (SNP), and/or kinase inhibitors and Na<sub>3</sub>VO<sub>4</sub>.

For PDE5 knockdown, 72-h *trans*-differentiated PrSC were transduced with lentiviral particles at multiplicity of infection 8 and analyzed 6 d after transfection (media were replaced by fresh medium containing bFGF or TGF $\beta$ 1 after 72 h). Production of lentiviral particles was carried out according to the manufacturer's protocol (Addgene, Cambridge, MA) as described previously (24). The target sequence (5-gacagcttgatcttctgcaatt) was located within the coding region of PDE5, scrambled short hairpin RNA (shRNA) vector (Addgene plasmid 1864) was used as control.

Human prostatic organoids were established from radical prostatectomy from tissue-wedges from the ventral prostate

showing no histological signs of malignancy. These explants were minced into organoids of approximately 1 mm<sup>3</sup> and seeded in 24-well plates in serum-free RPMI 1640 containing antibiotics. After 24 h and subsequently every other day, medium was renewed by serum-free medium supplemented with 50 μM vardenafil or mock control. Organoids were subjected to RNA isolation after 7 d of vardenafil treatment.

### Preparation of onplants and ex ovo chick chorioallantoic membrane (CAM) assay

Onplants were prepared as described previously (25). Briefly, native, nonpepsinized, type I rat-tail collagen (BD Biosciences, San Jose, CA) was neutralized with 0.1 M NaOH solution and mixed with 10× DMEM (Life Technologies, Inc., Carlsbad, CA) on ice. Then, 3 × 10<sup>5</sup> lentiviral transduced or normal PrSC were added to 30 μl of this solution and dropped on a 3 × 4 mm NITEX nylon mesh (Sefar, Inc., Depew, NY). After incubation at 37 C for 45 min, onplants were submerged in RPMI 1640 medium and cultured at 37 C in a humidified atmosphere containing 5% CO<sub>2</sub> for 3 d. Subsequently, onplants were applied to the chicken CAM.

The CAM assay was performed as described elsewhere (25) with slight modifications. Fertilized white leghorn chicken eggs (SPF eggs; Charles River, Germantown, MD) were incubated at 37 C with 80% humidity for 3 d. Subsequently, eggs were opened and transferred to plastic weighing boats. *Ex ovo* cultures were covered with a square Petri dish and incubated at 37 C and 80% humidity for 5 d. Then, PrSC collagen-onplants containing vardenafil (1.7 ng/μl) or distilled water as control were applied to CAM (four equal onplants/CAM) and incubated for 5 d. For histological analysis, onplants were excised from the CAM, fixed in 4% paraformaldehyde solution, and processed for paraffin sectioning.

### IHC, immunofluorescence, Western blot analysis, and quantitative real-time PCR

IHC, immunofluorescence, and Western blot analysis were performed as described previously (6, 7).

mRNA extraction, cDNA synthesis and quantitative PCR (qPCR) were performed as described elsewhere (7, 17). Primer sequences are given in Table 1. For PrSC experiments, cDNA concentrations were normalized by the internal standard hydroxymethylbilane synthase (*HMBS*); for prostatic organoids,

cDNA was normalized to *HMBS* and eukaryotic translation elongation factor 1α1 (*EEF1A1*).

### Statistical analysis

Results are expressed as mean values ± SEM. Statistical differences between treatments were calculated by paired Student's *t* test and considered significant when *P* < 0.05 (\*, *P* < 0.05; \*\*, *P* < 0.01).

## Results

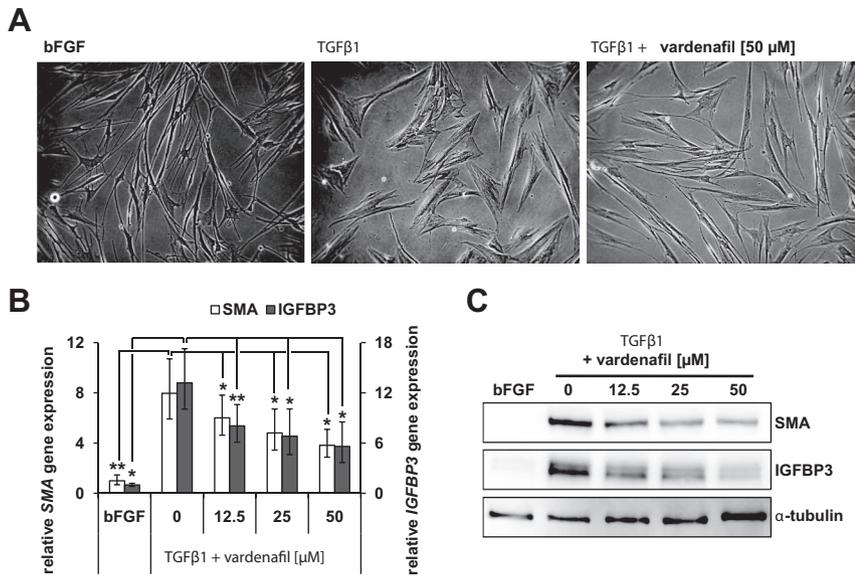
### Vardenafil inhibits prostatic fibroblast-to-myofibroblast *trans*-differentiation

To investigate the potential of vardenafil to inhibit fibroblast-to-myofibroblast *trans*-differentiation *in vitro*, PrSC were differentiated with TGFβ1. Effective *trans*-differentiation is characterized by typical changes in cell morphology from the thin and elongated phenotype of fibroblasts to the flattened phenotype of myofibroblasts. Treatment with vardenafil maintained PrSC in a fibroblast-like phenotype in the presence of TGFβ1, indicating inhibition of fibroblast-to-myofibroblast *trans*-differentiation (Fig. 1A). At molecular level, *trans*-differentiation can be monitored by the induction of marker genes like SMA and IGFBP3. Although stimulation with TGFβ1 significantly enhanced mRNA levels of both markers, treatment with vardenafil significantly attenuated the potential of TGFβ1 to induce *trans*-differentiation (Fig. 1B), which was verified at the protein level by Western blot analysis (Fig. 1C).

In the past three decades, the CAM assay developed to an accepted and reliable *in vivo* model to replace animal experiments for testing different substances and chemotherapeutics (26–29). To study *trans*-differentiation in an *in vivo* environment, a PrSC CAM xenograft model was established. Due to the growth factor-rich environment in the developing embryo, PrSC in the onplant *trans*-differ-

**TABLE 1.** Primer sequences

Gene	Primer sequences	
	Sense	Antisense
<i>ACTA2</i> ( <i>SMA</i> )	5-tacaatgagcttctgtgttgc	5-cgtccagaggcatagagaga
<i>CNN1</i>	5-ggtgaacgtgggagtgagt	5-ggtccagaggctgggtctgt
<i>EEF1A1</i>	5-cacacggctcacattgca	5-cacgaacagcaaaagcgacc
<i>HMBS</i>	5-ccaggacatcttgatctgg	5-atggtagcctgcatgggtctc
<i>IGF1</i>	5-ggaggctggagatgtattgc	5-gatgtgtccttggccaacct
<i>IGFBP3</i>	5-caagcgggagacgaatag	5-ttatccacacaccagcagaa
<i>NOX4</i>	5-tggcaagagaaacagacctga	5-tgggtccacaacagaaaaca
<i>PDE5</i>	5-aaaaccctggcctattcaa	5-gcatctatgaaccaacttgc
<i>PLN</i>	5-acagctgccaaggctaacta	5-gcttttgacgtgcttgttga
<i>SOD2</i>	5-tccactgcaaggaaacaacag	5-tcttgctgggatcattaggg



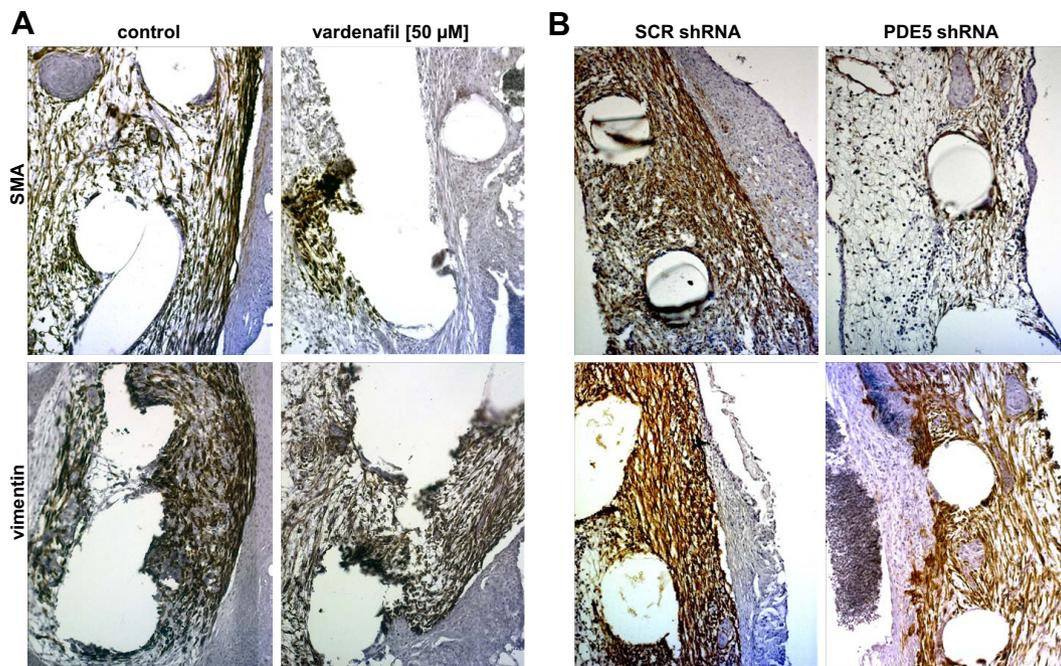
**FIG. 1.** Vardenafil attenuates fibroblast-to-myofibroblast *trans*-differentiation *in vitro*. PrSC were incubated with bFGF, TGFβ1, or TGFβ1 + vardenafil as indicated for 72 h before phase contrast microscopy (A), qPCR (B), or Western blotting (C) for the *trans*-differentiation markers SMA and IGFBP3. Note the thin, elongated, and light-refractive phenotype of bFGF-treated PrSC (fibroblasts) in comparison with the flattened and less light-refractive morphology of TGFβ1-differentiated PrSC (myofibroblasts). A and C, Images are representative of at least three independent experiments using different donors. B, Values represent mean ± SEM. Significance is indicated (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ;  $n = 5$ ). C, GAPDH served as loading control.

entiated into myofibroblasts *in vivo*, as determined by SMA expression. However, SMA staining was reduced in onplants treated with vardenafil, indicating that PDE5 inhibition attenuated fibroblast-to-myofibroblast *trans*-dif-

ferentiation *in vivo* (Fig. 2A). To confirm that this was indeed mediated via inhibition of PDE5, specific lentiviral-delivered shRNA was employed. Consistently, PDE5 shRNA attenuated SMA expression in PrSC compared with control scrambled (SCR) shRNA (Fig. 2B). The presence of human stromal cells in the onplants was verified by IHC for human vimentin (Fig. 2).

**PDE5 inhibition by vardenafil reverses fibroblast-to-myofibroblast *trans*-differentiation**

We previously reported that *trans*-differentiated PrSC overcome TGFβ1-induced growth arrest by mitogenic stimulation but maintained expression of SMA and calponin-1 (CNN1), indicating a stable conversion to a myofibroblast phenotype (6). To determine whether PDE5 inhibition restored the fibroblast phenotype to predifferentiated cells, PrSC were *trans*-differentiated for 72 h and subsequently treated with different concentrations of vardenafil in the presence of TGFβ1 for additional 72 h, whereas control cells were maintained with either bFGF (fibroblast) or TGFβ1 (myofibroblast) (Fig. 3A). Vard-



**FIG. 2.** PDE5 inhibition attenuated fibroblast-to-myofibroblast *trans*-differentiation *in vivo*. IHC of chicken CAM PrSC xenograft onplants. Treatment with vardenafil (A) and infection with PDE5 shRNA lentivirus (B) reduced SMA expression as compared with untreated (A) and SCR shRNA control (B) PrSC, respectively. The presence of human stromal cells in the onplants was verified by IHC for human vimentin.

enafil-treated cells adopted a fibroblast-like morphology and stained less intense for SMA in immunofluorescence, indicating that PDE5 inhibition induced a reversal of *trans*-differentiation (Fig. 3B). Consistently, vardenafil reduced the mRNA levels of the myofibroblast markers SMA and IGFBP3 in a dose-dependent manner that was significant at 50  $\mu\text{M}$  of the inhibitor (Fig 3C). This was verified at the protein level by Western blot analysis (Fig. 3D).

**Enhancement of NO/cGMP signaling reverses fibroblast-to-myofibroblast *trans*-differentiation**

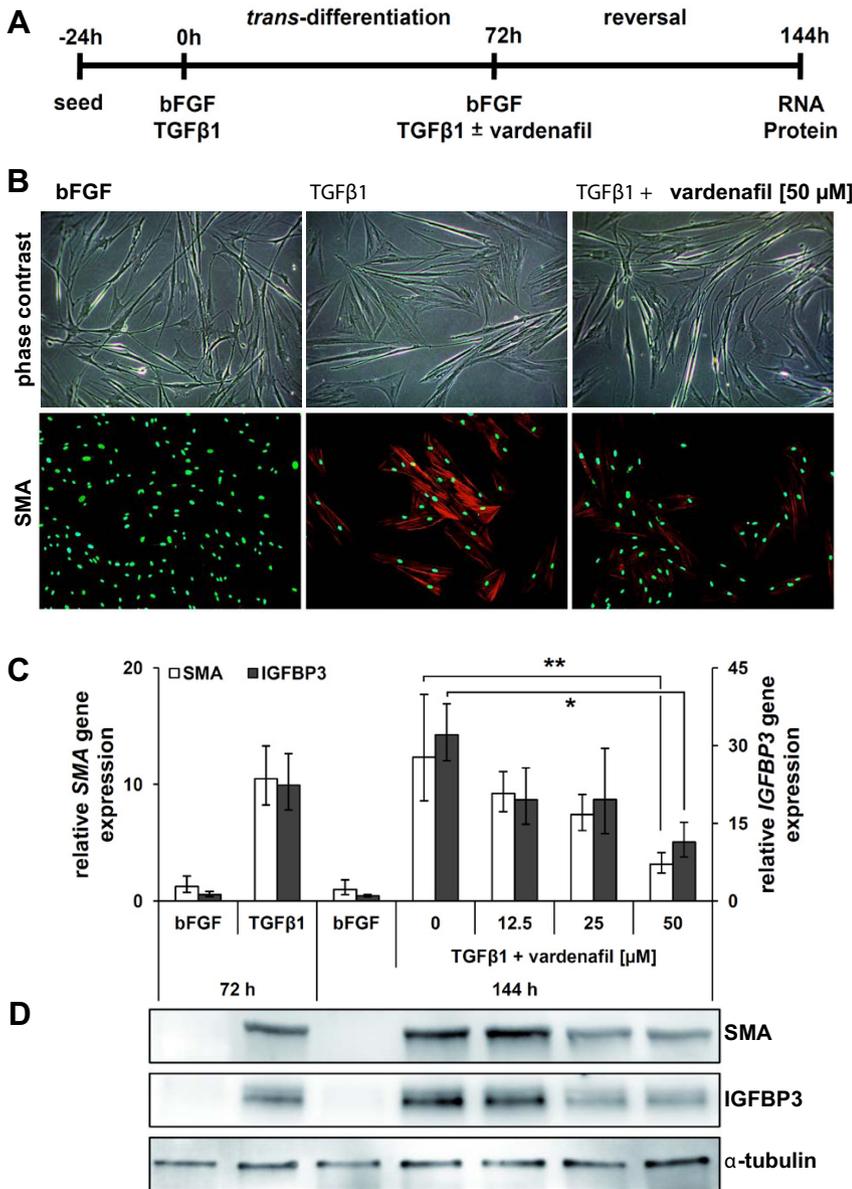
To confirm that the observed partial reversal of *trans*-differentiation upon vardenafil treatment was mediated via elevated cGMP levels, the soluble NO donor SNP was used. As with vardenafil, SNP dose dependently reduced SMA and IGFBP3 levels of *in vitro trans*-differentiated myofibroblasts (Fig. 4, A and B).

Although vardenafil is highly specific for PDE5 (30),

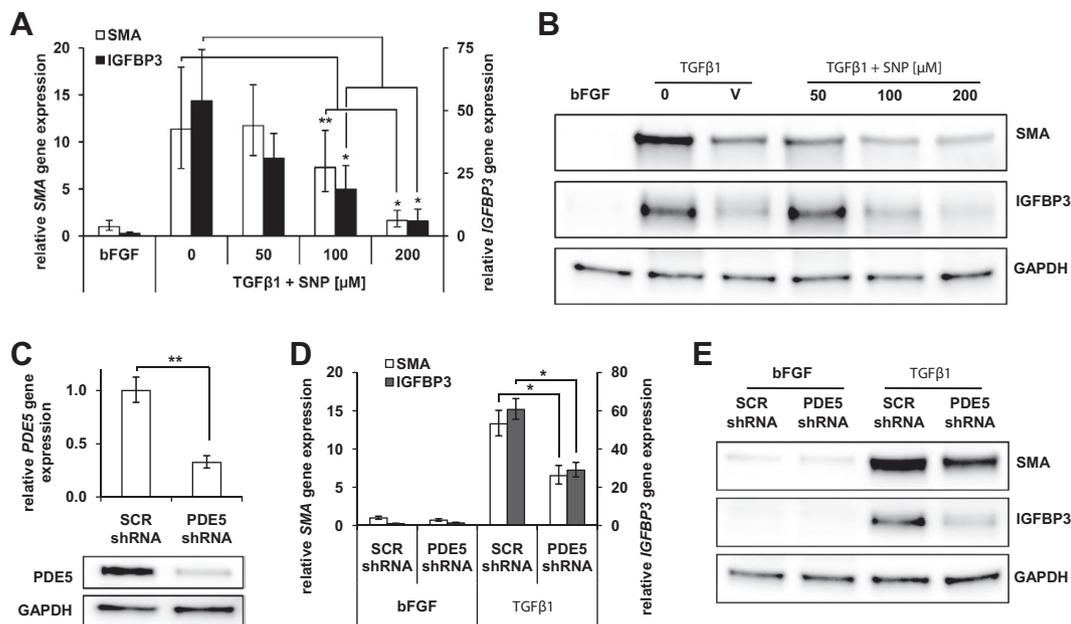
this does not exclude potential interactions with other molecules. To verify that the reversal of fibroblast-to-myofibroblast *trans*-differentiation via vardenafil was by direct inhibition of PDE5, we analyzed the effect of specific lentiviral-delivered shRNA. PDE5 shRNA significantly reduced *PDE5* mRNA and protein levels compared with cells treated with SCR shRNA (Fig. 4C). Additionally, *PDE5* knock-down significantly reduced mRNA levels of the myofibroblast markers in *trans*-differentiated PrSC in the presence of TGF $\beta$ 1 (Fig. 4D), which was verified at the protein level (Fig. 4E), indicating that the effect of vardenafil was derived from a specific inhibition of PDE5.

**PDE5 inhibition reduces myofibroblast marker levels in *ex vivo*-treated prostatic organoids and restores SOD2 levels *ex vivo* and *in vitro***

Next, we addressed the question whether reversal of *trans*-differentiation by vardenafil is limited to freshly *in vitro*-differentiated myofibroblast or can be applied to *in vivo*-generated myofibroblasts in a three-dimensional tissue. Thus, prostatic organoids from prostatectomy tissue were cultured in serum-free medium and treated with vardenafil for 7 d *ex vivo*. Subsequently, mRNA levels of myofibroblast-related genes were compared with control-treated organoids. A panel of markers significantly regulated during TGF $\beta$ 1-induced *trans*-differentiation of PrSC *in vitro* was employed (Fig. 5A). *SMA*, *CNN1*, and *PLN* that were up-regulated during *trans*-differentia-



**FIG. 3.** Vardenafil partially reverses fibroblast-to-myofibroblast *trans*-differentiation *in vitro*. PrSC were incubated with bFGF, TGF $\beta$ 1, or TGF $\beta$ 1 + vardenafil as indicated (A) for 144 h before phase contrast microscopy and immunofluorescence staining for SMA (B), qPCR (C), or Western blotting (D) for the *trans*-differentiation markers SMA and IGFBP3. Note the thin, elongated, and light-refractive phenotype of bFGF-treated PrSC (fibroblasts) in comparison with the flattened and less light-refractive morphology of TGF $\beta$ 1-differentiated PrSC (myofibroblasts). B and D, Images are representative of at least three independent experiments using different donors. C, Values represent mean  $\pm$  SEM. Significance is indicated (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ;  $n = 5$ ). D,  $\alpha$ -Tubulin served as loading control.



**FIG. 4.** Enhancement of NO/cGMP signaling reverses fibroblast-to-myofibroblast *trans*-differentiation *in vitro*. PrSC were *trans*-differentiated with TGF $\beta$ 1 for 72 h and subsequently stimulated with SNP or subjected to lentiviral transduction in the presence of TGF $\beta$ 1 before qPCR or Western blotting for the *trans*-differentiation markers SMA and IGFBP3. Control cells were maintained in medium containing bFGF. SNP dose dependently attenuated SMA and IGFBP3 mRNA levels after 72 h (A) and reduced myofibroblast marker protein levels to a similar extent than treatment with 50  $\mu$ M vardenafil (V) (B). Lentiviral-delivered PDE5 shRNA significantly reduced PDE5 mRNA and protein levels compared with SCR control shRNA as determined at d 6 after transduction (C) and reduces SMA and IGFBP3 mRNA (D) and protein levels in *trans*-differentiated myofibroblasts in the presence of TGF $\beta$ 1 (E). A, C, and D, Values represent mean  $\pm$  SEM. Significance is indicated (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ;  $n = 5$ ). B, C, and E, GAPDH served as loading control, images are representative of at least three independent experiments using different donors.

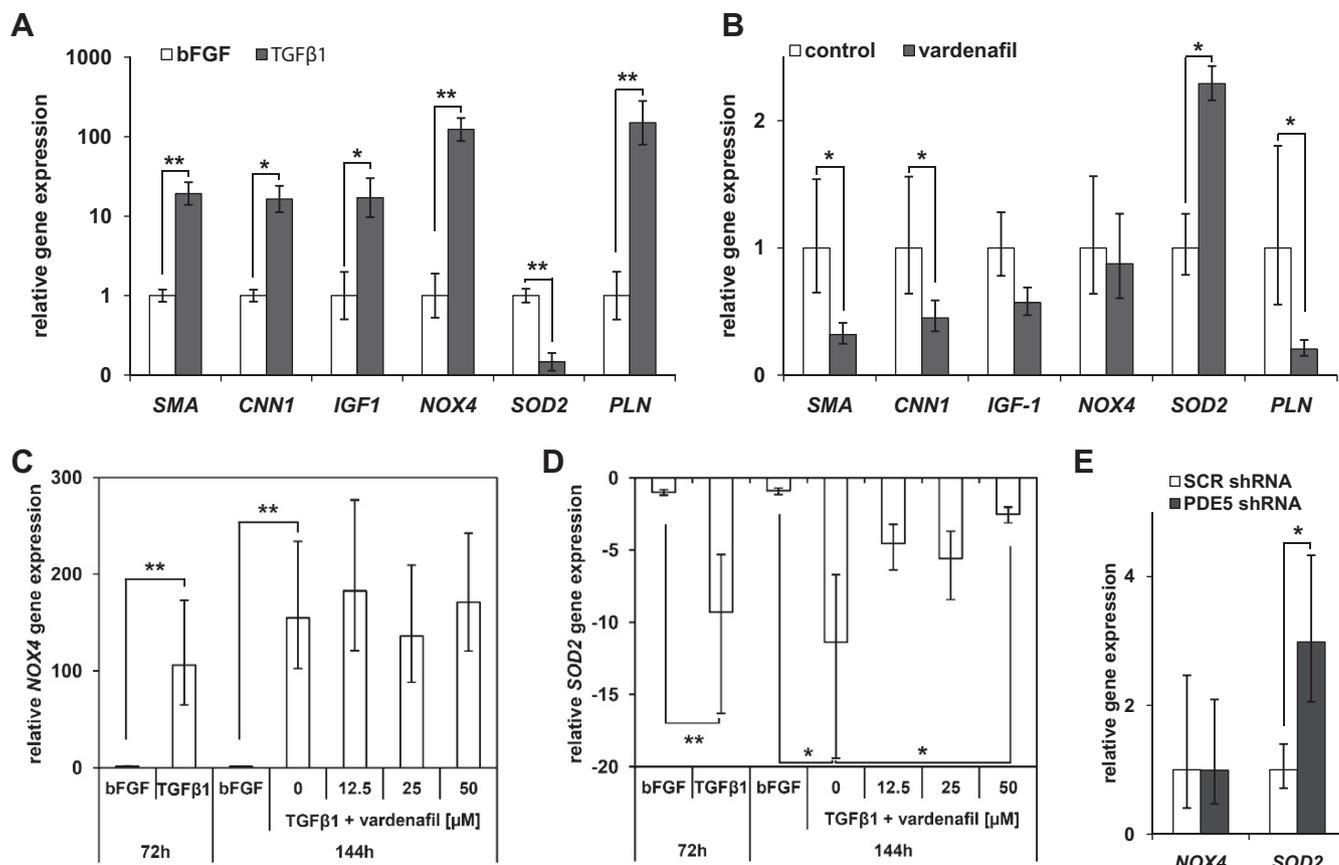
tion were found significantly reduced, whereas *SOD2* that was down-regulated during *trans*-differentiation was found significantly induced in vardenafil-treated organoids (Fig. 5B). The TGF $\beta$ 1-induced genes *IGF1* and *NOX4* were not significantly regulated upon vardenafil treatment of organoids, although there was a trend for *IGF1* to be reduced ( $P = 0.15$ ). These data indicate remodeling of the stroma toward a higher fibroblast/myofibroblast ratio by PDE5 inhibition in prostatic organoids and thus a reversed *trans*-differentiation of *in vivo*-generated myofibroblasts to a fibroblast-like phenotype.

PrSC myofibroblast *trans*-differentiation is driven by a prooxidant shift in redox homeostasis due to induction of ROS-producing NOX4 and concomitant decrease in ROS-scavenging enzymes (17). The findings obtained in prostatic organoids indicate that reversal of *trans*-differentiation upon PDE5 inhibition does not affect NOX4 levels but restores expression of antioxidant enzymes like *SOD2* to counteract the prooxidant shift. To further substantiate this finding, *NOX4* and *SOD2* mRNA levels were analyzed during vardenafil-induced reversal of *trans*-differentiation in *in vitro*-predifferentiated cells treated according to Fig. 3A. Indeed, *NOX4* levels were unaffected by PDE5 inhibition (Fig. 5C), whereas *SOD2* levels were restored to almost control (bFGF) levels upon treatment with 50  $\mu$ M vardenafil (Fig. 5D). To verify that reg-

ulation of *SOD2* expression was specific for PDE5 inhibition, the effect of lentiviral shRNA on *NOX4* and *SOD2* expression was investigated in PrSC. Consistently, *PDE5* knockdown by PDE5 shRNA significantly induced *SOD2* expression but did not affect *NOX4* levels in PrSC compared with SCR shRNA-treated cells (Fig. 5E).

### Reversal of myofibroblast *trans*-differentiation is mediated via inactivation of PI3K/AKT signaling

The signaling pathway underlying vardenafil-induced reversal of *trans*-differentiation was investigated using specific kinase inhibitors. We previously reported that PDE5 inhibition reduced proliferation of PrSC via PKG and attenuated *trans*-differentiation via the MEK pathway (7). However, neither the PKG inhibitor KT5823 nor the MEK inhibitor PD98059 abrogated the potential of vardenafil to reverse *trans*-differentiation (Fig. 6A). PDE5 inhibition has been demonstrated to inhibit RhoA/ROCK downstream of NO/cGMP/PKG (20, 21). To investigate whether vardenafil-induced reversal of *trans*-differentiation was mediated via inhibition of RhoA signaling, the effect of RhoA inhibition by C3 exoenzyme and ROCK inhibition by Y27632 on myofibroblast markers was tested. Likewise the PKG inhibitor, neither C3 exoenzyme nor Y27632 significantly affected *IGFBP3* and *SMA* mRNA levels in myofibroblasts and vardenafil-treated



**FIG. 5.** Vardenafil reduces myofibroblast marker expression in prostatic organoids *ex vivo* and restores *SOD2* expression. A, PrSC ( $n = 3$ ) were incubated with bFGF (fibroblasts) or TGF $\beta$ 1 (myofibroblasts) for 72 h before qPCR of *trans*-differentiation regulated genes. B, Prostatic organoids were adapted to serum-free medium and subsequently treated with 50  $\mu$ M vardenafil or mock control for 6 d before qPCR of *trans*-differentiation-regulated genes. C and D, PrSC were incubated with bFGF, TGF $\beta$ 1, or TGF $\beta$ 1 + vardenafil as indicated before qPCR analysis of *NOX4* (C) and *SOD2* (D). E, Lentiviral-delivered PDE5 shRNA significantly induced *SOD2* mRNA levels in PrSC compared with SCR control shRNA as determined at 6 d after transduction. Values represent mean  $\pm$  SEM. Significance is indicated (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

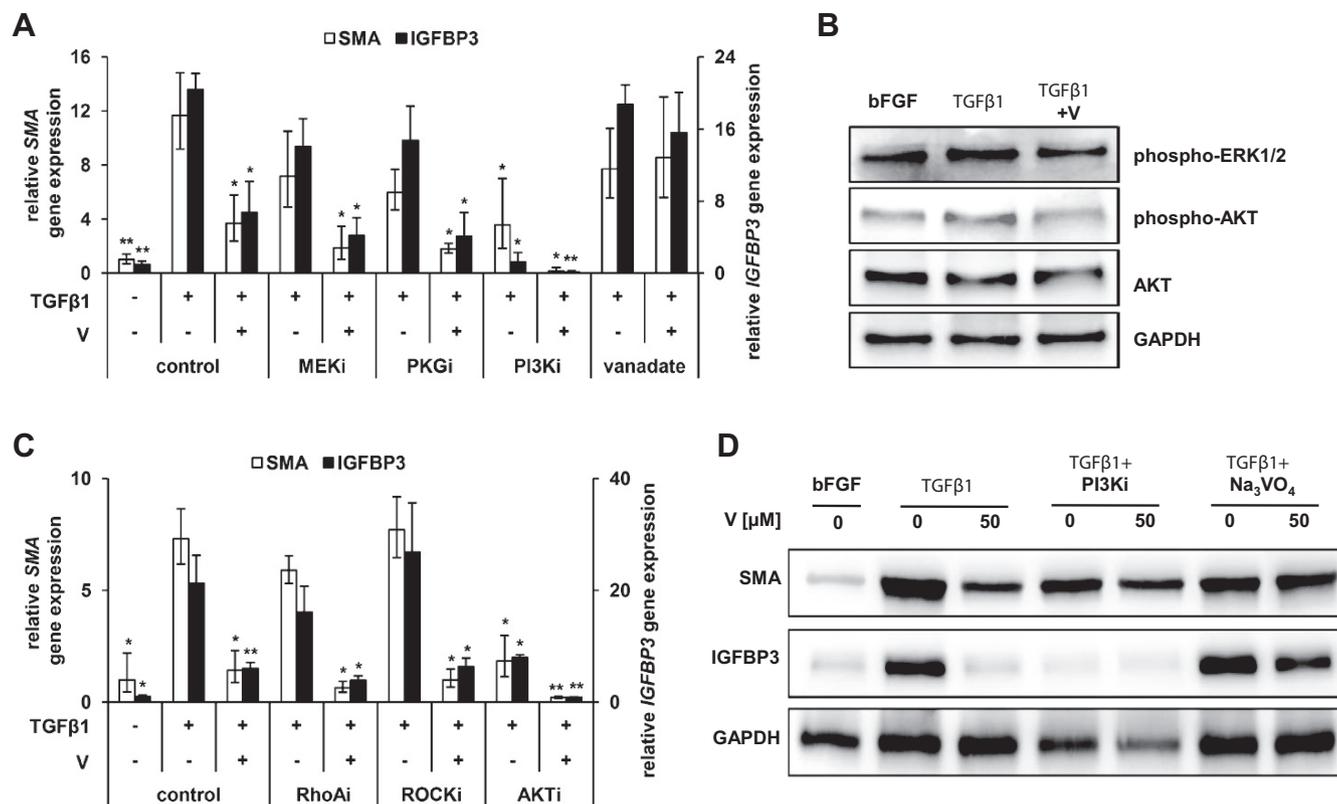
myofibroblasts (Fig. 6C). In agreement with the finding that vardenafil-induced reversal of myofibroblast *trans*-differentiation was unaffected by MEK inhibition, short-term treatment of *trans*-differentiated PrSC with vardenafil did not enhance phospho-ERK1/2 levels but attenuated phospho-AKT levels (Fig. 6B), indicating that inactivation of PI3K/AKT signaling might underlie reversal of *trans*-differentiation. Consistently, the PI3K inhibitor LY-294002 significantly reduced SMA and IGFBP3 mRNA and protein levels (Fig. 6, A and D). Because LY-294002 besides PI3K also inhibits other kinases, such as glycogen synthase kinase-3 (31), we additionally used the highly selective AKT inhibitor triciribine. Likewise PI3K inhibition, triciribine significantly reduced SMA and IGFBP3 levels synergistically with vardenafil, indicating that attenuation of PI3K/AKT signaling is causative for PDE5 inhibition-induced reversal of myofibroblast *trans*-differentiation (Fig. 6C). Consistently, the broad-spectrum phosphatase inhibitor  $\text{Na}_3\text{VO}_4$  that acts as a phosphate analog and inhibits tyrosine phosphatases and other enzymes, including alkaline phosphatases and ATPases, ab-

rogated the potential of vardenafil to reverse *trans*-differentiation (Fig. 6, A and D). Taken together, these findings indicate that PDE5 inhibition-induced reversal of fibroblast-to-myofibroblast *trans*-differentiation is mediated via inactivation of PI3K/AKT signaling potentially via phosphatases that inactivate AKT.

## Discussion

Stromal remodeling via fibroblast-to-myofibroblast *trans*-differentiation promotes development and progression of BPH. Based on the reported beneficial effects of PDE5 inhibitors on LUTS secondary to BPH (8, 9), we investigated the potential of the PDE5 inhibitor vardenafil to inhibit and revert fibroblast-to-myofibroblast *trans*-differentiation of PrSC and demonstrate here that vardenafil not only attenuated but also reversed *trans*-differentiation.

Besides inhibition of *trans*-differentiation *in vitro*, vardenafil and shRNA-mediated PDE5 knockdown inhibited *trans*-differentiation in an *in vivo* CAM xenograft



**FIG. 6.** Vardenafil-induced reversal of fibroblast-to-myofibroblast *trans*-differentiation is mediated via attenuation of PI3K/AKT signaling. PrSC were *trans*-differentiated with TGFβ1 for 72 h and subsequently incubated with the indicated inhibitors in the presence of TGFβ1 before qPCR or Western blotting. Control cells were maintained in medium containing bFGF (TGFβ1–). **A**, Effect of MEK inhibition (MEKi) (50 μM PD98059), PKG inhibition (PKGi) (1 μM KT5853), PI3K inhibition (PI3Ki) (20 μM LY-294002), and 50 μM Na<sub>3</sub>VO<sub>4</sub> (vanadate) alone or in combination with 50 μM vardenafil (V) on the myofibroblast markers *SMA* and *IGFBP3* gene expression after 72 h of stimulation (n = 4). **B**, *Trans*-differentiated PrSC were stimulated with 50 μM vardenafil in the presence of TGFβ1 for 2 h before Western blotting for the indicated antibodies. **C**, Effect of RhoA inhibition (RhoAi) (1 μg/ml exoenzyme C3), ROCK inhibition (ROCKi) (5 μM Y27632), and AKT inhibition (AKTi) (20 μM triciribine) alone or in combination with 50 μM vardenafil (V) on the myofibroblast markers *SMA* and *IGFBP3* gene expression after 72 h of stimulation (n = 3). **D**, PrSC were treated as in **A** before Western blotting for the myofibroblast markers *SMA* and *IGFBP3*. **A** and **C**, Values represent mean ± SEM. Significance vs. TGFβ1 control is indicated (\*, P < 0.05; \*\*, P < 0.01). **B** and **D**, GAPDH served as loading control, images are representative of at least three independent experiments using different donors.

model. These results further substantiate our previous findings using PDE5 inhibition by tadalafil and small interfering RNA-mediated PDE5 knockdown *in vitro* (7), indicating that enhancement of the NO/cGMP signaling pathway attenuates PrSC *trans*-differentiation. These data are in line with previous studies implicating the NO/cGMP pathway in fibroblast-to-myofibroblast conversion in other tissues. In fibroblast cultures from human Peyronie's disease plaques sildenafil and the cGMP analog 8-bromo-cGMP reduced SMA levels (32) and sildenafil in combination with the soluble guanylyl cyclase activator BAY58–2667 inhibited human lung fibroblast-to-myofibroblast conversion (33). 8-Bromo-cGMP inhibited TGFβ1-induced *trans*-differentiation of cardiac fibroblasts isolated from wild-type mice (34). Similarly, the soluble guanylyl cyclase stimulator BAY41–2272 elevated intracellular cGMP levels and inhibited myofibroblast conversion in cultured cardiac fibroblasts and reduced the number of myofibroblasts in car-

diac fibrosis in rats with hypertension induced by suprarenal aortic constriction (35).

We demonstrate that enhancement of the NO/cGMP pathway by SNP, vardenafil, or PDE5 knockdown does not only inhibit fibroblast-to-myofibroblast conversion but moreover restores the fibroblast phenotype in *trans*-differentiated PrSC. To our knowledge, this the first study reporting a reversal of stromal remodeling as characterized by fibroblast-to-myofibroblast *trans*-differentiation in the prostate. A reversal of corneal myofibroblasts by FGF/heparin has been reported previously (36), and vardenafil treatment reduced myofibroblast numbers and total size of preformed TGFβ1-induced Peyronie's disease plaques in a rat model (37).

In a three-dimensional coculture models of porcine skin fibrosis, SOD significantly lowered the levels of TGFβ1 and SMA, indicating reversal of myofibroblasts into normal fibroblasts (38). Moreover, the ROS scavenger N-ace-

tylcysteine significantly decreases SMA and type I collagen levels in fibroblasts isolated from patients with idiopathic pulmonary fibrosis (39). We previously demonstrated that PrSC *trans*-differentiation is associated with a sustained prooxidant shift driven by induction of the ROS-producing enzyme *NOX4* and a concomitant down-regulation of several ROS-scavenging enzymes, including *SOD2* (17). Consistently, *trans*-differentiation was inhibited by SOD supplementation (17). Interestingly, in the present study, vardenafil treatment of prostatic organoids and predifferentiated myofibroblasts or *PDE5* knockdown in PrSC did not affect *NOX4* levels but restored *SOD2* expression (Fig. 5). Thus, PDE5 inhibition appears to enhance the antioxidative potential of PrSC thereby counteracting the TGF $\beta$ 1-induced sustained prooxidant shift.

Unlike inhibition of *trans*-differentiation that is mediated via the MEK pathway (7), reversal was neither affected by MEK inhibition nor by PKG, RhoA, or ROCK inhibition. In contrast, vardenafil attenuated phospho-AKT levels in *trans*-differentiated myofibroblasts, and PI3K or AKT inhibition induced reversal of the phenotype. Regulation of AKT signaling by cGMP has been described in guanylyl cyclase C (that generates cGMP) knockout mice, where AKT signaling was elevated in intestinal cells and could be repressed by oral cGMP supplementation (40). Moreover, phosphatase inhibition abrogated vardenafil-induced reversal of *trans*-differentiation, indicating that reversal due to enhanced NO/cGMP signaling is mediated via activation of phosphatases that inactivate protein kinases like AKT.

Enhancement of the NO/cGMP pathway partially but not completely reversed *trans*-differentiation in our model system. However, one has to keep in mind that this partially reversal occurred in the continued presence of the *trans*-differentiation-inducing TGF $\beta$ 1 stimulus within 72 h reflecting the *in vivo* situation. Vardenafil treatment in the absence of TGF $\beta$ 1 completely restores *trans*-differentiation markers to the levels of bFGF-treated fibroblasts (data not shown).

In conclusion, enhancement of the NO/cGMP signaling pathway by PDE5 inhibitors like vardenafil attenuate and revert fibroblast-to-myofibroblast *trans*-differentiation, a central process underlying stromal remodeling in BPH. Reversibility of *trans*-differentiation suggests that prostate fibroblasts and myofibroblasts are not terminally differentiated cell types but rather alternative and convertible phenotypes. Our findings indicate that BPH patients might benefit from long-term therapy with PDE5 inhibitors that attenuate and revert stromal remodeling. PDE5 inhibition seems to affect BPH/LUTS via several mechanism. Although increased smooth muscle cell relaxation mediates a readily relieve of LUTS, reduced stromal cell

proliferation and attenuation of myofibroblast formation might stop the progression of BPH, and reversal of myofibroblast *trans*-differentiation provides the potential regression of disease upon long-term therapy.

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## References

1. Ao M, Franco OE, Park D, Raman D, Williams K, Hayward SW 2007 Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium. *Cancer Res* 67:4244–4253
2. Yang F, Tuxhorn JA, Ressler SJ, McAlhany SJ, Dang TD, Rowley DR 2005 Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res* 65:8887–8895
3. Tuxhorn JA, McAlhany SJ, Yang F, Dang TD, Rowley DR 2002 Inhibition of transforming growth factor- $\beta$  activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. *Cancer Res* 62:6021–6025
4. Verona EV, Elkhouloun AG, Yang J, Bandyopadhyay A, Yeh IT, Sun LZ 2007 Transforming growth factor- $\beta$  signaling in prostate stromal cells supports prostate carcinoma growth by up-regulating stromal genes related to tissue remodeling. *Cancer Res* 67:5737–5746
5. Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR 2002 Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. *Clin Cancer Res* 8:2912–2923
6. Untergasser G, Gander R, Lilg C, Lepperdinger G, Plas E, Berger P 2005 Profiling molecular targets of TGF- $\beta$ 1 in prostate fibroblast-to-myofibroblast transdifferentiation. *Mech Ageing Dev* 126: 59–69
7. Zenzmaier C, Sampson N, Pernkopf D, Plas E, Untergasser G, Berger P 2010 Attenuated proliferation and *trans*-differentiation of prostatic stromal cells indicate suitability of phosphodiesterase type 5 inhibitors for prevention and treatment of benign prostatic hyperplasia. *Endocrinology* 151:3975–3984
8. Martinez-Salamanca JI, Carballido J, Eardley I, Giuliano F, Gratzke C, Rosen R, Salonia A, Stief C 2011 Phosphodiesterase type 5 inhibitors in the management of non-neurogenic male lower urinary tract symptoms: critical analysis of current evidence. *Eur Urol* 60: 527–535
9. Gacci M, Eardley I, Giuliano F, Hatzichristou D, Kaplan SA, Maggi M, McVary KT, Mirone V, Porst H, Roehrborn CG 2011 Critical analysis of the relationship between sexual dysfunctions and lower urinary tract symptoms due to benign prostatic hyperplasia. *Eur Urol* 60:809–825
10. Andersson KE, de Groat WC, McVary KT, Lue TF, Maggi M, Roehrborn CG, Wyndaele JJ, Melby T, Viktrup L 2011 Tadalafil for the

- treatment of lower urinary tract symptoms secondary to benign prostatic hyperplasia: pathophysiology and mechanism(s) of action. *Neurourol Urodyn* 30:292–301
11. Tinel H, Stelte-Ludwig B, Hütter J, Sandner P 2006 Pre-clinical evidence for the use of phosphodiesterase-5 inhibitors for treating benign prostatic hyperplasia and lower urinary tract symptoms. *BJU Int* 98:1259–1263
  12. Fibbi B, Morelli A, Vignozzi L, Filippi S, Chavalmane A, De Vita G, Marini M, Gacci M, Vannelli GB, Sandner P, Maggi M 2010 Characterization of phosphodiesterase type 5 expression and functional activity in the human male lower urinary tract. *J Sex Med* 7:59–69
  13. Amano M, Nakayama M, Kaibuchi K 2010 Rho-kinase/ROCK: a key regulator of the cytoskeleton and cell polarity. *Cytoskeleton* 67:545–554
  14. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA 2002 Myofibroblasts and mechano-regulation of connective tissue remodeling. *Nat Rev Mol Cell Biol* 3:349–363
  15. Biernacka A, Dobaczewski M, Frangogiannis NG 2011 TGF- $\beta$  signaling in fibrosis. *Growth Factors* 29:196–202
  16. Hinz B, Phan SH, Thannickal VJ, Prunotto M, Desmoulière A, Varga J, De Wever O, Mareel M, Gabbiani G 2012 Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *Am J Pathol* 180:1340–1355
  17. Sampson N, Koziel R, Zenzmaier C, Bubendorf L, Plas E, Jansen-Dürr P, Berger P 2011 ROS signaling by NOX4 drives fibroblast-to-myofibroblast differentiation in the diseased prostatic stroma. *Mol Endocrinol* 25:503–515
  18. Mack CP, Somlyo AV, Hautmann M, Somlyo AP, Owens GK 2001 Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization. *J Biol Chem* 276:341–347
  19. Chen S, Crawford M, Day RM, Briones VR, Leader JE, Jose PA, Lechleider RJ 2006 RhoA modulates Smad signaling during transforming growth factor- $\beta$ -induced smooth muscle differentiation. *J Biol Chem* 281:1765–1770
  20. Morelli A, Filippi S, Sandner P, Fibbi B, Chavalmane AK, Silvestrini E, Sarchielli E, Vignozzi L, Gacci M, Carini M, Vannelli GB, Maggi M 2009 Vardenafil modulates bladder contractility through cGMP-mediated inhibition of RhoA/Rho kinase signaling pathway in spontaneously hypertensive rats. *J Sex Med* 6:1594–1608
  21. Guilluy C, Sauzeau V, Rolli-Derkinderen M, Guérin P, Sagan C, Pacaud P, Loirand G 2005 Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension. *Br J Pharmacol* 146:1010–1018
  22. Stief CG, Porst H, Neuser D, Beneke M, Ulbrich E 2008 A randomised, placebo-controlled study to assess the efficacy of twice-daily vardenafil in the treatment of lower urinary tract symptoms secondary to benign prostatic hyperplasia. *Eur Urol* 53:1236–1244
  23. Zenzmaier C, Untergasser G, Hermann M, Dirnhofer S, Sampson N, Berger P 2008 Dysregulation of Dkk-3 expression in benign and malignant prostatic tissue. *Prostate* 68:540–547
  24. Zenzmaier C, Hermann M, Hengster P, Berger P 2012 Dickkopf-3 maintains the PANC-1 human pancreatic tumor cells in a dedifferentiated state. *Int J Oncol* 40:40–46
  25. Deryugina EI, Quigley JP 2008 Chapter 2. Chick embryo chorioallantoic membrane models to quantify angiogenesis induced by inflammatory and tumor cells or purified effector molecules. *Meth ods Enzymol* 444:21–41
  26. Saw CL, Heng PW, Liew CV 2008 Chick chorioallantoic membrane as an in situ biological membrane for pharmaceutical formulation development: a review. *Drug Dev Ind Pharm* 34:1168–1177
  27. Taizi M, Deutsch VR, Leitner A, Ohana A, Goldstein RS 2006 A novel and rapid in vivo system for testing therapeutics on human leukemias. *Exp Hematol* 34:1698–1708
  28. Kunzi-Rapp K, Genze F, Küfer R, Reich E, Hautmann RE, Gschwend JE 2001 Chorioallantoic membrane assay: vascularized 3-dimensional cell culture system for human prostate cancer cells as an animal substitute model. *J Urol* 166:1502–1507
  29. Armstrong PB, Quigley JP, Sidebottom E 1982 Transepithelial invasion and intramesenchymal infiltration of the chick embryo chorioallantois by tumor cell lines. *Cancer Res* 42:1826–1837
  30. Montorsi F, Salonia A, Briganti A, Barbieri L, Zanni G, Suardi N, Cestari A, Montorsi P, Rigatti P 2005 Vardenafil for the treatment of erectile dysfunction: a critical review of the literature based on personal clinical experience. *Eur Urol* 47:612–621
  31. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P 2007 The selectivity of protein kinase inhibitors: a further update. *Biochem J* 408:297–315
  32. Valente EG, Vernet D, Ferrini MG, Qian A, Rajfer J, Gonzalez-Cadavid NF 2003 L-arginine and phosphodiesterase (PDE) inhibitors counteract fibrosis in the Peyronie's fibrotic plaque and related fibroblast cultures. *Nitric Oxide* 9:229–244
  33. Dunkern TR, Feurstein D, Rossi GA, Sabatini F, Hatzelmann A 2007 Inhibition of TGF- $\beta$  induced lung fibroblast to myofibroblast conversion by phosphodiesterase inhibiting drugs and activators of soluble guanylyl cyclase. *Eur J Pharmacol* 572:12–22
  34. Li P, Wang D, Lucas J, Oparil S, Xing D, Cao X, Novak L, Renfrow MB, Chen YF 2008 Atrial natriuretic peptide inhibits transforming growth factor  $\beta$ -induced Smad signaling and myofibroblast transformation in mouse cardiac fibroblasts. *Circ Res* 102:185–192
  35. Masuyama H, Tsuruda T, Sekita Y, Hatakeyama K, Imamura T, Kato J, Asada Y, Stasch JP, Kitamura K 2009 Pressure-independent effects of pharmacological stimulation of soluble guanylate cyclase on fibrosis in pressure-overloaded rat heart. *Hypertens Res* 32:597–603
  36. Maltseva O, Folger P, Zekaria D, Petridou S, Masur SK 2001 Fibroblast growth factor reversal of the corneal myofibroblast phenotype. *Invest Ophthalmol Vis Sci* 42:2490–2495
  37. Ferrini MG, Kovancec I, Nolzco G, Rajfer J, Gonzalez-Cadavid NF 2006 Effects of long-term vardenafil treatment on the development of fibrotic plaques in a rat model of Peyronie's disease. *BJU Int* 97:625–633
  38. Vozenin-Brotans MC, Sivan V, Gault N, Renard C, Geffrotin C, Delanian S, Lefaix JL, Martin M 2001 Antifibrotic action of Cu/Zn SOD is mediated by TGF- $\beta$ 1 repression and phenotypic reversion of myofibroblasts. *Free Radic Biol Med* 30:30–42
  39. Bocchino M, Agnese S, Fagone E, Svegliati S, Grieco D, Vancheri C, Gabrielli A, Sanduzzi A, Avvedimento EV 2010 Reactive oxygen species are required for maintenance and differentiation of primary lung fibroblasts in idiopathic pulmonary fibrosis. *PLoS One* 5:e14003
  40. Lin JE, Li P, Snook AE, Schulz S, Dasgupta A, Hyslop TM, Gibbons AV, Marszlowicz G, Pitari GM, Waldman SA 2010 The hormone receptor GUCY2C suppresses intestinal tumor formation by inhibiting AKT signaling. *Gastroenterology* 138:241–254