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Attenuated Proliferation and *Trans*-Differentiation of Prostatic Stromal Cells Indicate Suitability of Phosphodiesterase Type 5 Inhibitors for Prevention and Treatment of Benign Prostatic Hyperplasia

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Benign prostatic hyperplasia (BPH) is characterized by tissue overgrowth and stromal reorganization primarily due to cellular proliferation and fibroblast-to-myofibroblast *trans*-differentiation. To evaluate the potential of phosphodiesterase type 5 (PDE5) inhibitors like tadalafil for prevention and treatment of BPH, we analyzed the role of the nitric oxide/cyclic GMP (cGMP)/PDE5 pathway for cellular proliferation and TGFβ1-induced fibroblast-to-myofibroblast *trans*-differentiation in primary prostate stromal cells. Inhibition by tadalafil of PDE5, which is mainly expressed in the stromal compartment of the prostate, reduced proliferation of primary prostate stromal cells and to a lesser extent of primary prostate basal epithelial cells. Attenuated proliferation due to elevated intracellular cGMP levels was confirmed by inhibition of the cGMP-dependent protein kinase G by its inhibitor KT2358. Moreover, tadalafil strongly attenuated TGFβ1-induced fibroblast-to-myofibroblast *trans*-differentiation. The inhibitory effect on *trans*-differentiation was also observed after small interfering RNA-mediated PDE5 knockdown. As confirmed by the MAPK kinase 1 inhibitor PD98059, this effect was mediated via MAPK kinase 1 signaling. We conclude that BPH patients might benefit from adjuvant therapies with PDE5 inhibitors that inhibit stromal enlargement due to cell proliferation, as well as TGFβ1-induced *trans*-differentiation processes. **(*Endocrinology* 151: 0000–0000, 2010)**

The cyclic nucleotide phosphodiesterases (PDEs) comprise a superfamily of phosphohydrolases that regulate cellular levels of the second messenger molecules cyclic GMP (cGMP) and cAMP. PDE type 5 (PDE5) specifically hydrolyzes cGMP and is the major therapeutic target in erectile dysfunction (ED). Inhibition of PDE5 increases intracellular cGMP levels and thereby enhances nitric oxide (NO)/cGMP signaling. The resulting activation of the cGMP-dependent protein kinase G (PKG) and subsequent relaxation of penile vascular smooth muscle leads to erection (1). Besides treatment of ED, PDE5 inhibitors are also approved for the treatment of pulmonary hypertension, and there is evidence that chronic PDE5 inhibition improves heart rate recovery in patients with heart failure (2).

In the urogenital tract, PDE5 is expressed in the corpus cavernosum, prostate, bladder, vas deferens, epididymis, and testis (3). Highest protein levels were shown in the corpus cavernosum and in the prostate. The latter is affected by two age-related proliferative disorders, benign

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Abbreviations: bFGF, Basic fibroblast growth factor; BPH, benign prostatic hyperplasia; BrdU, bromodeoxyuridine; cGMP, cyclic GMP; DMSO, dimethylsulfoxide; ED, erectile dysfunction; HMBS, housekeeping gene porphobilinogen deaminase; IGFBP, IGF binding protein; IHC, immunohistochemistry; LUTS, lower urinary tract symptoms; NO, nitric oxide; PDE, phosphodiesterase; PDE5, PDE type 5; PDGF, platelet-derived growth factor; PKG, protein kinase G; PrEC, primary prostatic basal epithelial cell; PrSC, primary prostatic stromal cell; SCR, scrambled; siRNA, small interfering RNA; SMA, SMC-α-actin; SMAD, mothers against decapentaplegic homolog; SMC, smooth muscle cell; SNP, sodium nitroprusside; qPCR, quantitative PCR.

TABLE 1. Primer sequences

Gene symbol	UniGene ID	Primer sequences	Melting point (C)	PCR-product length (bp)
ACTG2 (*SMA*)	Hs.403989	Sense, 5-agaagagctatgagctgcca; antisense, 5-gctgtgatcttcctttctgcat	86	247
HMBS	Hs.82609	Sense, 5-ccaggacatcttggatcttgg; antisense, 5-atggtagcctgcatggtctc	88	213
IGFBP3	Hs.450230	Sense, 5-caagcgggagacgaatatg; antisense, 5-ttatccacacaccagcagaa	85	189
PDE5	Hs.587281	Sense, 5-caaaaccctggcctattcaa; antisense, 5-gcatctatgaacccaacttgc	80	163
PDE11A	Hs.570273	Sense, 5-tgaattgatgtccccaaagt; antisense, 5-gatcctggtaggcatcactg	82	158

prostatic hyperplasia (BPH) and prostate cancer frequently associated with ED (4).

BPH is rare in young men (present in 20% of men at age 40), but its prevalence increases with age to 70% at age 60 (5). Moreover, BPH is commonly associated with bothersome lower urinary tract symptoms (LUTS) with a lifetime risk for surgery of 25–30% (6, 7). It is characterized by progressive histological changes that arise initially in the stromal compartment, which becomes enlarged and altered in its cellular composition by fibroblast *trans*-differentiation to myofibroblasts/smooth muscle cells (SMCs) (5, 8, 9). The stromal reorganization is likely to be induced by elevated production of TGFβ1 as tissue and circulating TGFβ1 levels correlate with risk of BPH and prostate cancer (10, 11). Furthermore, we and others previously demonstrated that TGFβ1 induces fibroblast-to-myofibroblast *trans*-differentiation of primary prostatic stromal cells (PrSCs) *in vitro* (12, 13) and exogenous administration of TGFβ1 is sufficient to induce myofibroblast differentiation *in vivo* (14).

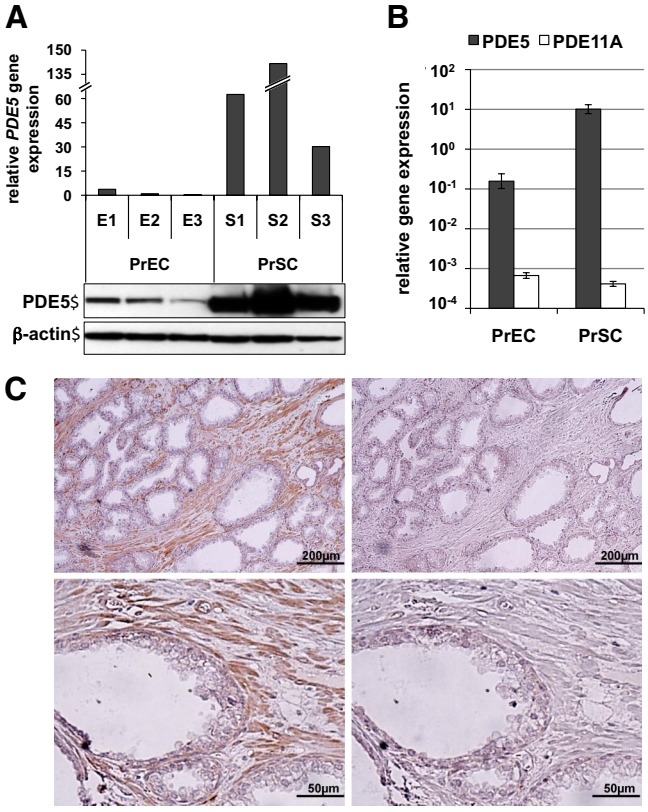

FIG. 1. PDE5 localizes predominantly to the prostatic stroma. A, *PDE5* mRNA levels were analyzed by qPCR and PDE5 protein levels by Western blot analysis in PrECs and PrSCs established from three independent donors (E1–E3, S1–S3). cDNA concentrations were normalized by the HMBS. β-Actin shown as loading control in Western blot analysis. B, PDE5 and PDE11A mRNA levels were analyzed by qPCR in PrECs (n = 3), PrSCs (n = 3), and normalized to prostate tissue specimens (n = 5; relative expression = 10^0). Note the logarithmic y-axis. C, IHC of PDE5 in normal prostate tissue (*top left*, *enlarged bottom left*). Signals could be specifically blocked by PDE5 blocking peptide (*top right*, *enlarged bottom right*).

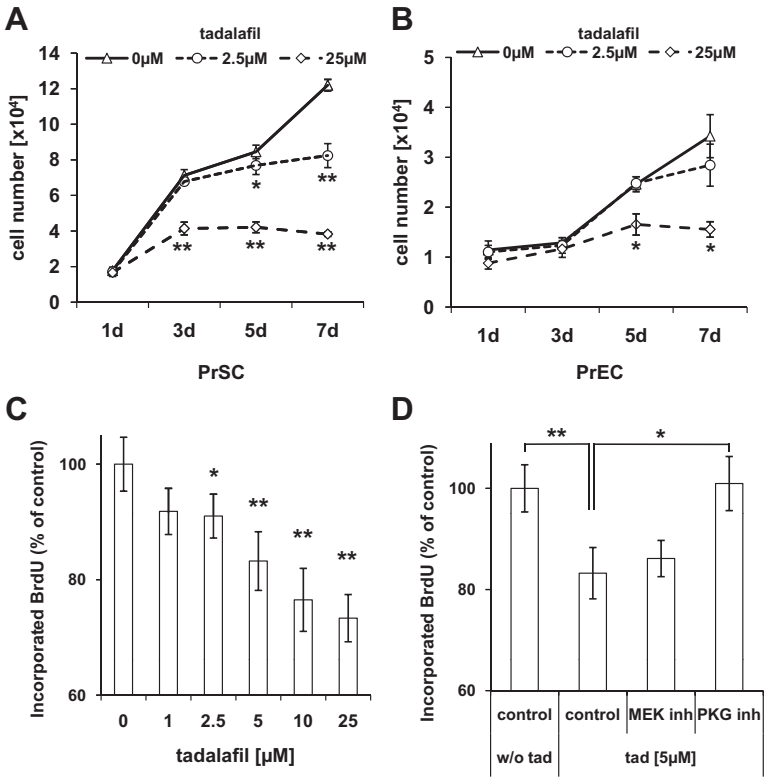

FIG. 2. Antiproliferative action of PDE5 inhibition on prostate cells. Cells seeded in triplicates were treated with the indicated concentration of inhibitor. Every other day, cell counts were obtained in a counting chamber after staining with trypan blue. A, Tadalafil significantly reduced proliferation of PrSCs at concentrations of 2.5 and 25 μM. B, Tadalafil at a concentration of 2.5 μM did not significantly alter proliferation of PrECs, whereas proliferation was reduced by 25 μM tadalafil. C, PrSCs were treated with the indicated concentrations of tadalafil and cell proliferation determined by BrdU incorporation after 72 h. Concentrations above 2.5 μM significantly reduced proliferation in a dose-dependent manner. D, The MAPK kinase 1 inhibitor PD98059 (20 μM) did not interfere with the antiproliferative effect of 5 μM tadalafil on PrSC. However, the protein kinase G (PKG) inhibitor KT2358 (200 nM) significantly attenuated the growth inhibitory effect of tadalafil, indicating that the antiproliferative effect of PDE5 inhibition is mediated via PKG. Data are expressed as mean ± SEM of at least three independent experiments. Statistical significance *vs.* controls was determined by paired Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). w/o tad, Control without tadalafil.

Beneficial effects of PDE5 inhibitors were observed on LUTS secondary to BPH in patients treated for ED (15, 16). The effect of PDE5 inhibition on the prostate is thought to be mainly caused by relaxation of smooth muscle lowering urethral pressure and thus affecting the dynamic component of the disease (17–19). However, the prostate size may also be affected, because an antiproliferative effect of PDE5 inhibitors on prostate stromal cells has been reported (20, 21). Elevated cGMP levels have been reported in prostate tissue after treatment with PDE5 inhibitors (17). It is thought that similar to the corpus cavernosum, the effects of PDE5 inhibition on the prostate arise via enhanced NO/cGMP signaling.

In the present study, the influences of PDE5 inhibition by the specific inhibitor tadalafil on prostate tissue remodeling are studied *in vitro* at a cellular level to elucidate the underlying molecular and cellular mechanisms of the described beneficial effects on BPH patients. Data demonstrate expression of PDE5 in the stromal compartment of the gland. Inhibition of PDE5 reduced proliferation and *trans*-differentiation of PrSC *in vitro*, suggesting effects on the static component of BPH *in vivo*. Our data indicate the potential clinical value of specific PDE5 inhibitors, such as tadalafil, in preventing and treating stromal enlargement and myofibroblast differentiation of stromal cells in BPH.

Materials and Methods

Reagents

All reagents were purchased from Sigma-Aldrich (Vienna, Austria) unless otherwise specified. Highly pure tadalafil was kindly provided by ICOS Corp. (Eli Lilly and Co., Indianapolis, IN). The kinase inhibitors KT2358 and PD98059 were purchased from Calbiochem (Merck Biochemicals, Darmstadt, Germany). Antibodies against PDE5 and p-ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA). Mothers against decapentaplegic homolog (SMAD)2/3 and p-SMAD antibodies were from Upstate (Bedford, MA), SMC-α-actin (SMA) and β-actin from Sigma-Aldrich, IGF binding protein (IGFBP)3 from R&D Systems (Minneapolis, MN), and α-tubulin from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-SMA for immunofluorescence was purchased from Dako Cytomation (Vienna, Austria).

Immunohistochemistry (IHC)

Paraffin-embedded tissue sections were deparaffinized and hydrated in xylene and graded alcohol series. Thereafter, antigen retrieval was performed by microwave treatment in citrate buffer (10 mM, pH 6.0), and endogenous peroxidase activity was blocked with 3% H_2O_2/methanol. Sections were incubated in blocking solution containing 10% bovine calf serum (Dako Cytomation) for 45 min and then stained overnight with a 1:100 dilution of primary antiserum (rabbit antihuman PDE5 polyclonal, 1 μg/ml; Cell Signaling Technology) at 4 C. Primary antiserum was detected after incubation with a biotinylated secondary antibody (biotinylated goat antirabbit IgG; Dako Cytomation) using horseradish peroxidase conjugated streptavidin (Dako Cytomation) and the FAST DAB Tablet Set (Sigma-Aldrich). Sections were counterstained with Meyer's Hemalum and mounted with Entellan (Merck Biochemicals). Specificity controls of the PDE5 polyclonal antibody were performed by blocking experiments with an excess of PDE5 blocking peptide (50 μg/ml; Cell Signaling Technology).

Immunofluorescence

Cells were plated on eight-well culture slides (Falcon BD Labware, Erembodegem, Belgium). After fixation in acteton/methanol (1:1) and permeabilization with 0.2% Triton X-100,

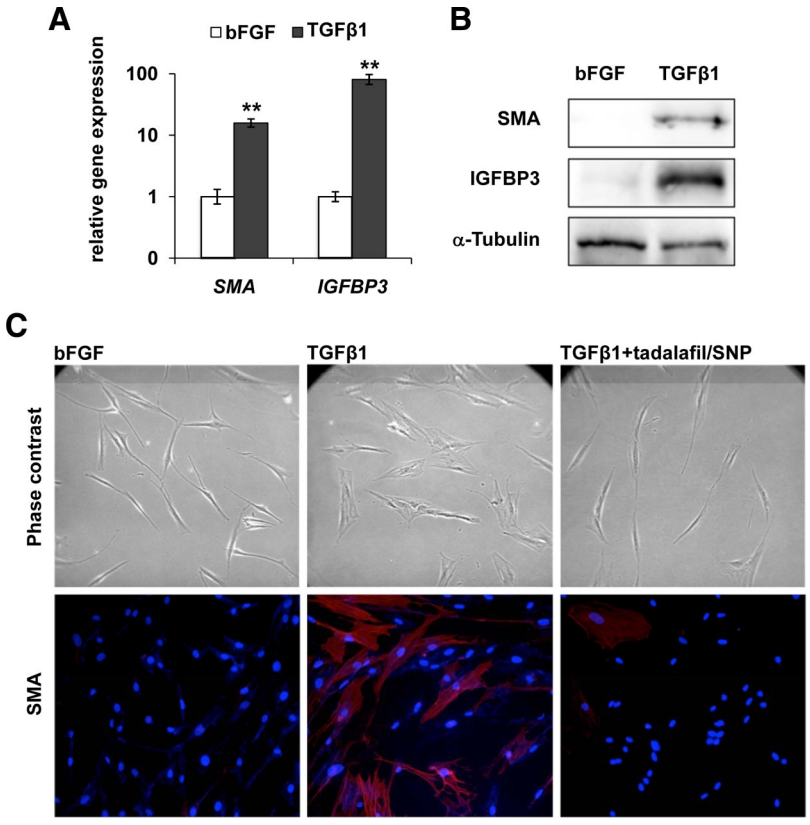

FIG. 3. TGFβ1 induced fibroblast-to-myofibroblast *trans*-differentiation. PrSCs were stimulated with 1 ng/ml TGFβ1 to induce *trans*-differentiation and with 1 ng/ml bFGF (control), respectively. A, mRNA levels of the *trans*-differentiation markers *SMA* and *IGFBP3* were analyzed by qPCR. cDNA concentrations were normalized by the HMBS. TGFβ1 led to a significant increase of *SMA* (15.8 ± 2.6 fold) and *IGFBP3* (80.7 ± 16.5 fold) mRNA levels after 24 h. Data are expressed as mean ± SEM of independent experiments. Statistical significance *vs.* controls was determined by paired Student's *t* test (**, $P < 0.01$). B, SMA and IGFBP3 protein levels were analyzed by Western blot analysis from total cell lysates taken 72 h after stimulation. α-Tubulin served as loading control. C, Phase contrast microscopy (*upper panel*) of PrSCs stimulated with bFGF or TGFβ1 for 72 h. Note the thin, elongated, and light refractive phenotype of fibroblasts (bFGF-treated PrSCs) in comparison with the flattened and less light refractive morphology of myofibroblasts (TGFβ1-*trans*-differentiated PrSCs). Pretreatment with 25 μM tadalafil and 100 μM SNP inhibited the morphological changes induced by TGFβ1. PrSCs were stimulated as in C before immunofluorescent staining of SMA (*red; lower panel*). TGFβ1-treated PrSCs stain positive for the myofibroblast marker SMA. Nuclei were counterstained with 4′,6-diamidin-2′-phenylindol-dihydrochlorid.

cells were blocked with PBS containing 3% BSA for 45 min at room temperature. Anti-SMA antibody (1 μg/ml) was applied for 2 h at room temperature. After washing with PBS, cells were incubated for 45 min with a secondary fluorochrome-labeled antibody (polyclonal goat antimouse TEXAS red; Invitrogen, Lofer, Austria), and nuclei were counterstained for 30 min with 4′,6-diamidin-2′-phenylindol-dihydrochlorid (Molecular Probes, Eugene, OR). Cells were embedded in fluorescent mounting medium (Dako Cytomation), viewed by the Zeiss Axiovert 200 microscope, and images acquired by the Axiovision 4.7 software (Carl Zeiss Microscopy, Oberkochen, Germany).

Cell lines and tissue culture

Human PrSC cultures and human primary prostatic basal epithelial cell (PrEC) cultures were established as described previously (22). PrSCs were cultured in stromal cell growth medium (Clonetics, Lonza, Verviers, Belgium), PrEC on collagen I-coated plates in prostate epithelial cell growth medium (Clonetics). All experiments were performed with cells from at least three individual donors.

Cell proliferation assays

Early passage PrSC and PrEC were seeded at a density of 20,000 cells/well in 24-well plates (Falcon BD Labware) in triplicates in 400 μl culture medium. After adhesion, cells were stimulated with the indicated concentrations of tadalafil, and cell numbers were determined after 1, 3, 5, and 7 d of culture. Therefore, PrECs were treated with collagenase type-I and detached by trypsin/EDTA (Clonetics), and PrSCs were detached by trypsin (PAA Laboratories, Pasching, Austria). Cells were stained with trypan blue staining solution and counted in a Fuchs-Rosenthal counting chamber.

For bromodeoxyuridine (BrdU) incorporation assays, 4000 early passage PrSC were seeded in triplicates into individual wells of a 96-well plate (Nunc, Roskilde, Denmark) in 100 μl culture medium and left to adhere overnight. Thereafter, fresh medium was supplemented with tadalafil at the indicated concentrations. For kinase inhibitor experiments, cells were preincubated with 200 nM KT2358, 20 μM PD98059, or dimethylsulfoxide (DMSO) equivalent for 30 min before addition of tadalafil. Media was replaced every 24 h. Proliferation rate after 72 h was analyzed by a BrdU cell proliferation ELISA (Roche Applied Science, Vienna, Austria) according to manufacturer's instructions.

Trans-differentiation experiments

PrSCs of passage 2–4 were incubated in RPMI 1640 (Clonetics) containing 1% charcoal treated fetal calf serum (HyClone, South Logan, UT) and 1% penicillin/streptomycin·L-glutamine (PAA Laboratories). Subsequently, cells were stimulated with either 1 ng/ml human recombinant TGFβ1 (R&D Systems) or 1 ng/ml human basic fibroblast growth factor (bFGF) as control to maintain the fibroblast phenotype. Where indicated, cells were pretreated with KT2358, PD98059, or DMSO (vehicle) equivalent for 60 min and tadalafil and/or sodium nitroprusside (SNP) for 30 min.

Small interfering RNA (siRNA)-mediated PDE5 knockdown

PrSCs were seeded in 6-cm dishes and transfected with siRNA targeting *PDE5* (catalog no. HSS112695; Invitrogen) or scrambled control (catalog no. 12935-300; Invitrogen) using Lipofectamin 2000 (Invitrogen) according to manufacturer's instructions. Seventy-two hours after transfection, *trans*-differentiation experiments were started.

Quantitative real-time PCR

mRNA was extracted by the use of the TriFast Reagent (PeQLAB Biotechnology, Erlangen, Germany). cDNA first

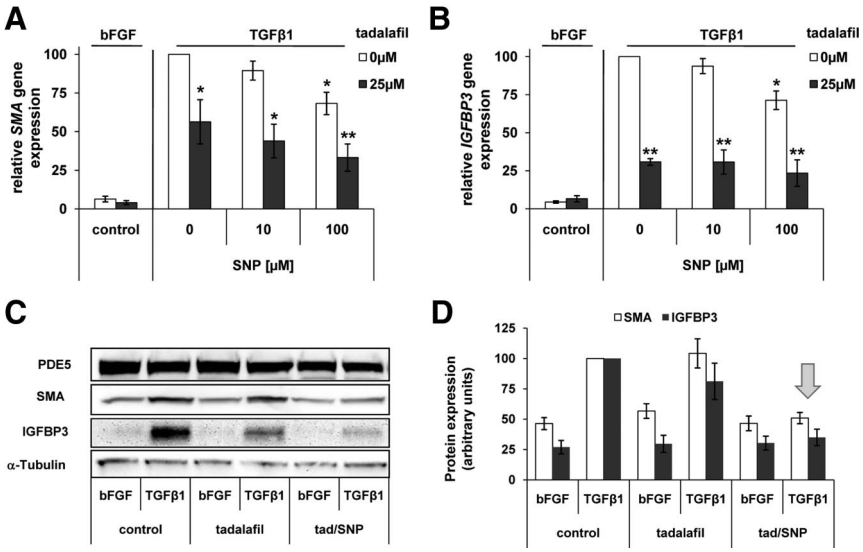

FIG. 4. PDE5 inhibition attenuates PrSC fibroblast-to-myofibroblast *trans*-differentiation. PrSCs were preincubated with 25 μM of tadalafil and increasing concentrations of SNP before stimulation with 1 ng/ml TGFβ1 to induce *trans*-differentiation or 1 ng/ml bFGF (control). Tadalafil significantly attenuated the induction of *trans*-differentiation markers *SMA* (A) and *IGFBP3* (B) as determined by qPCR after 24 h of stimulation. SNP dose dependently enhanced the effect of tadalafil. Statistical significance *vs.* TGFβ1 treatment (100% *trans*-differentiation) was determined by paired Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). Total protein extracts from three different PrSC cultures treated as in A and B were subjected to Western blot analysis with the indicated antibodies (C) and densitometrically analyzed (D). Pretreatment with tadalafil and 100 μM SNP attenuated the induction of the *trans*-differentiation markers at the protein level (*gray arrow*). α-Tubulin served as loading control. Values represent mean ± SEM from three different donors.

strand synthesis was reverse transcribed from 2 μg of total RNA preparation using Reverse Transcription System (Promega, Madison, WI) and oligo dT15 and random hexamer primers. Quantitative PCR (qPCR) was performed by the FastStart DNA Master SYBR Green I kit and the Light Cycler 480 System (Roche Applied Science) according to manufacturer's instructions. Specificity of PCR products was confirmed by melting curve analysis. Primer sequences are given in Table 1. cDNA concentrations were normalized by the housekeeping gene porphobilinogen deaminase (HMBS).

Western blot analysis

Total cell extracts were prepared and analyzed by Western blotting as described previously (22). Primary antibodies were used at dilutions of 1:1000 (PDE5, p-ERK1/2, p-SMAD, SMAD2/3, and IGFBP3) or 1:5000 (SMA, α-tubulin, and β-actin).

Statistics

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

Results

PDE5 is predominantly expressed in the stromal compartment of the prostate

To identify the potential target cells of PDE5 inhibitors in the prostate, the expression of *PDE5* in human prostate PrECs and PrSCs was analyzed by qPCR. Expression of *PDE5* was significantly 65 ± 19-fold higher in PrSCs compared with PrECs, a finding confirmed at the protein level in cell lysates (Fig. 1A). Given the reported antiproliferative effects of PDE5 inhibitors, we evaluated the impact on primary prostate cell proliferation. Of the three PDE5 inhibitors approved for the treatment of ED, tadalafil was used here due to its high specificity for PDE5 over other PDE isoenzymes and its prolonged half-life in plasma (17.5 *vs.* ~4 h for sildenafil and vardenafil) (1). Tadalafil has high selectivity ratios *vs.* PDE5 for all PDE isoenzymes except PDE11A (selectivity ratio of $IC_{50} = 5$) (23), which is expressed in the human prostate predominantly in the epithelium (24, 25). To rule out potential effects mediated via *PDE11A*, we analyzed its gene expression by qPCR in both PrECs and PrSCs. In comparison with prostatic tissue extracts, *PDE11A* expression was very low in both PrECs ($6.7 \pm 1.2 \times 10^{-4}$ fold) and PrSCs ($4.1 \pm 0.6 \times 10^{-4}$ fold), respectively (Fig. 1B).

To verify whether the PDE5 expression pattern observed *in vitro* reflects that *in vivo*, prostate tissue sections were stained for PDE5 by IHC. Consistently, in the stromal compartment, strong staining was observed, whereas in the epithelial compartment, no PDE5 specific immunoreactivity was detectable (Fig. 1C). Signals could be specifically blocked by PDE5 blocking peptide. Collectively, these results demonstrate that in the human prostate, PDE5 is predominantly expressed in the fibromuscular stromal compartment.

Tadalafil reduces PrSC proliferation in a dose-dependent manner

The effect of PDE5 inhibition by tadalafil (2.5 and 25 μM) on the proliferation of primary prostate cells was analyzed over a 1-wk period. In agreement with the high endogenous PDE5 levels, tadalafil had a pronounced effect on proliferation of PrSCs; 2.5 μM tadalafil was sufficient to significantly reduce proliferation of PrSCs (Fig. 2A) but not PrECs (Fig. 2B). Proliferation of PrECs was significantly reduced only at the higher concentration of tadalafil (25 μM), reflecting the low PDE5 expression (Fig. 1).

These data further demonstrate that the stroma is the main target of PDE5 inhibition in the prostate. Thus, sub-

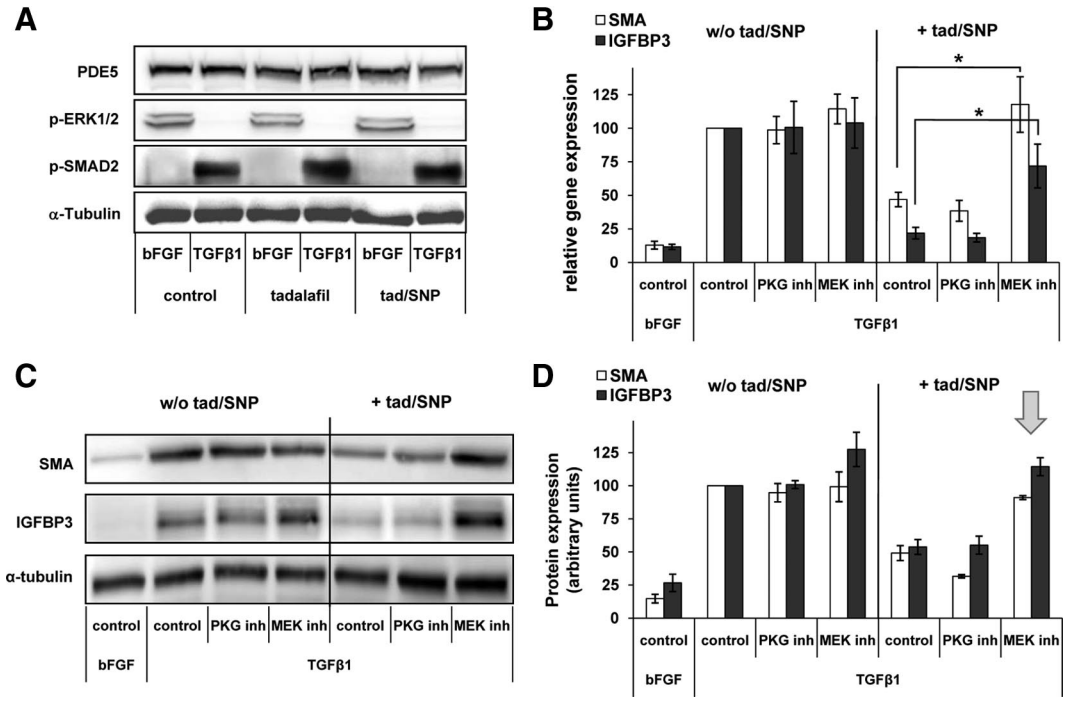

FIG. 5. Tadalafil/SNP do not interfere with TGFβ signaling but activate MEK/ERK signaling. A, Total protein extracts were prepared of PrSCs from three independent donors after 1 h of bFGF or TGFβ1 stimulation. Extracts were pooled and 30 μG protein analyzed by Western blot analysis. Pretreatment of cells with 25 μM tadalafil and 100 μM SNP did not affect p-ERK1/2 dephosphorylation and p-SMAD2 phosphorylation. α-Tubulin served as loading control. B, PrSCs were incubated with 25 μM tadalafil and 100 μM SNP after control (DMSO), PKG inhibitor (1 μM KT2358), or MEK1 inhibitor (100 μM PD98059) treatment and stimulated with bFGF or TGFβ1. PD98059, but not KT2358, reversed the blocking effect of tadalafil/SNP on fibroblast-to-myofibroblast *trans*-differentiation as determined by qPCR of the *trans*-differentiation markers *SMA* and *IGFBP3* after 24 h. Statistical significance was determined by paired Student's *t* test (*, $P < 0.05$). C, Total protein extracts from three different PrSC cultures treated for 72 h as in A and B were subjected to Western blot analysis with the indicated antibodies and (D) densitometrically analyzed. The PKG inhibitor KT2358 did not reverse the reduction of SMA and IGFBP3 proteins by tadalfil/SNP. The MEK1 inhibitor PD98059 reversed the blocking effect of tadalafil/SNP on SMA and IGFBP3 protein levels (*gray arrow*). α-Tubulin served as loading control. Values represent mean ± SEM from three different donors.

sequent investigations were focused on PrSCs. The dose dependence of the antiproliferative effect was analyzed by BrdU incorporation assays. Increasing levels of tadalafil (1–25 μM) attenuated proliferation of PrSC in a dose-dependent manner with concentrations above 5 μM showing highly significant effects ($P < 0.01$) (Fig. 2C).

Antiproliferative effects of tadalafil are mediated via cGMP and PKG

Elevating cGMP levels by PDE5 inhibition is supposed to enhance NO/cGMP signaling resulting in PKG activation. To investigate the downstream signaling pathway of cGMP leading to growth inhibition, PrSCs were stimulated with tadalafil after preincubation with the PKG inhibitor KT2358. As shown above, tadalafil at a concentration of 5 μM significantly inhibited PrSC proliferation ($P = 0.002$). Pretreatment with the PKG inhibitor blocked the effect of Tadalfil (101 ± 5% of control-treated cells −tadalafil; $P = 0.009$ *vs.* control +tadalafil) (Fig. 2D). Thus, the antiproliferative effects of PDE5 inhibition are mediated via elevation of cGMP and the subsequent activation of cGMP-dependent PKG.

Because cGMP has been reported to activate the MEK/ERK pathway independently of PKG (26), PrSCs were also pretreated with the MEK1 inhibitor PD98059 that did not abrogate the effect of tadalafil on proliferation (Fig. 2D), demonstrating that the growth inhibition by tadalafil is not mediated via MEK/ERK.

Tadalafil suppresses TGFβ1-mediated fibroblast-to-myofibroblast *trans*-differentiation

Besides stromal expansion, the main histological change in the BPH stroma is *trans*-differentiation of fibroblasts to myofibroblasts/SMCs. This *trans*-differentiation can be modeled *in vitro* by stimulating PrSC with TGFβ1 (27, 28) as indicated by induction of the *trans*-differentiation marker genes smooth muscle actin γ2 (*SMA*) and *IGFBP3* (13). Stimulation of PrSCs with TGFβ1 led to a 15.8 ± 2.6- and 80.7 ± 16.5-fold increase of mRNA levels of *SMA* and *IGFBP3*, respectively (Fig. 3A), which was verified at the protein level by Western blot analyses (Fig. 3B). Effective *trans*-differentiation is also characterized by typical changes in cell morphology from the thin and elongated phenotype of fibroblasts to

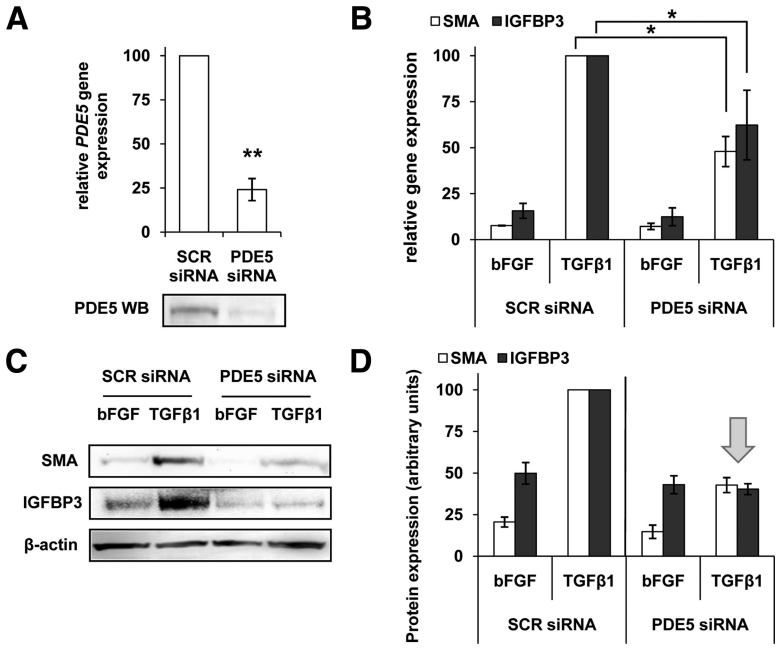

FIG. 6. Small interfering RNA-mediated knockdown of PDE5 mimics the effects of tadalafil on *trans*-differentiation. PrSCs were transfected either with SCR or PDE5-specific siRNA and stimulated with bFGF (control) or TGFβ1 (*trans*-differentiation) 72 h after transfection. A, PDE5-specific siRNA efficiently reduced PDE5 expression on mRNA and protein levels after 72 h as determined by qPCR and Western blot analysis, respectively. B, qPCR analysis after 24 h of bFGF or TGFβ1 stimulation of the genes indicated. PDE5 knockdown (PDE5 siRNA) significantly attenuated the TGFβ1-induced *trans*-differentiation. Results are expressed as mean ± SEM. Statistical significance was determined by paired Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). C, Total protein extracts were prepared of PrSCs from three independent donors after 72 h of bFGF or TGFβ1 stimulation, subjected to Western blot analysis with the indicated antibodies and (D) densitometrically analyzed. PDE5 knockdown attenuated the induction of the *trans*-differentiation markers at the protein level (*gray arrow*). β-Actin served as loading control. Values represent mean ± SEM from three different donors.

the flattened phenotype of myofibroblasts with actin bundles that stain positive for SMA (Fig. 3C).

The effect of PDE5 inhibition on PrSC *trans*-differentiation was studied by stimulation with TGFβ1 after preincubation with 25 μM tadalafil. TGFβ1-induced fibroblast-to-myofibroblast *trans*-differentiation was significantly attenuated by tadalafil as determined by qPCR of the marker genes. Expression of *SMA* was reduced to 56 ± 14% ($P = 0.046$) (Fig. 4A) and that of *IGFBP3* to 31 ± 2% ($P = 0.0005$) (Fig. 4B) of control *trans*-differentiated cells.

Increase of NO signaling enhances suppressive effects of tadalafil on fibroblast *trans*-differentiation

In the normal prostate, NO synthases are mainly expressed in the epithelial compartment (29). Because fibroblasts have low NO synthase expression levels, NO/cGMP signaling in the stroma is mainly stimulated by NO synthesized from neurons. Thus, the soluble NO donor SNP was used to enhance the NO/cGMP pathway in PrSCs. SNP dose dependently attenuated the induction of *SMA* (100 μM SNP, 68 ± 7% of control; $P = 0.02$) (Fig. 4A) and *IGFBP3* (100 μM SNP, 71 ± 6% of control; $P = 0.02$) (Fig. 4B) by TGFβ1, indicating that this attenuation is mediated via increased cGMP levels. Additional blocking of cGMP hydrolysis by tadalafil synergistically enhanced the effect of SNP on the transcription of the *trans*-differentiation markers (Fig. 4, A and B). These findings were also confirmed at the protein level by Western blot analysis (Fig. 4, C and D). Total PDE5 protein levels were not affected by any treatment.

Tadalafil does not influence early TGFβ1 signaling intermediates

Stimulation of PrSC with TGFβ1 leads to phosphorylation of the immediate signaling intermediate SMAD2. Additionally, upon TGFβ1 stimulation, ERK1/2 is dephosphorylated within 1 h. To evaluate whether tadalafil and SNP directly interfere with TGFβ1 signaling, SMAD2 and ERK1/2 phosphorylation was analyzed by Western blot analyses using phospho-specific antibodies. PDE5 protein levels were not significantly regulated by TGFβ1, tadalafil, or SNP (Figs. 4A and 5A). Our results revealed no alterations in the rapid TGFβ1 response upon treatment with tadalafil and/or SNP (Fig. 5A). Thus, PDE5 inhibition and stimulation of cGMP synthesis did not directly block initial steps of TGFβ1 signaling.

Tadalafil attenuates *trans*-differentiation via the MEK/ERK pathway

As for the antiproliferative effects of PDE5 inhibition, the attenuation of PrSC *trans*-differentiation is presumably mediated via elevated cGMP levels, resulting in PKG activation. Hence, the signaling pathway downstream of cGMP was again investigated by preincubation with the PKG inhibitor KT2358. However, inhibition of PKG with 1 μM KT2358 did not affect tadalafil/SNP-induced repression of *trans*-differentiation as monitored by marker gene expression of *SMA* and *IGFBP3* (Fig. 5B). Therefore, the importance of the MEK/ERK pathway was investigated, because cGMP has been reported to activate the MEK/ERK pathway independently of PKG (26). Indeed, preincubation with the MEK1 inhibitor PD98059 restored the potential of TGFβ1 to induce *trans*-differentiation markers [*SMA*, 118 ± 21% *vs.* 47 ± 5% ($P = 0.04$); *IGFBP3*, 72 ± 16% *vs.* 22 ± 4% ($P = 0.04$)] (Fig. 5B).

Consistently, the MEK1 inhibitor PD98059 blocked the effects of tadalafil/SNP on IGFBP3 and SMA protein levels, whereas KT2358 did not influence protein expres-

sion (Fig. 5, C and D). Taken together, these findings indicate that the attenuation of TGFβ1-induced *trans*-differentiation by NO/cGMP is mediated via the MEK1 pathway and not via activation of PKG.

RNAi-mediated knockdown of PDE5 attenuates fibroblast-to-myofibroblast *trans*-differentiation

Although tadalafil is highly specific for PDE5, this does not exclude potential interactions with other molecules. To verify that the attenuation of fibroblast-to-myofibroblast *trans*-differentiation via tadalafil was by direct inhibition of PDE5, we analyzed the effect of siRNA-mediated *PDE5* knockdown. PDE5 siRNA significantly reduced *PDE5* mRNA and protein levels compared with cells treated with scrambled siRNA (Fig. 6A). Additionally, the induction of *trans*-differentiation markers upon TGFβ1 stimulation was significantly reduced by *PDE5* knockdown [*SMA*, 48 ± 8% of scrambled (SCR) control ($P = 0.01$); *IGFBP3*, 62 ± 19% of SCR control ($P = 0.03$)] (Fig. 6B). *Trans*-differentiation of PrSC had lower efficiency in *PDE5* knockdown cells as monitored by SMA and IGFBP3 protein levels (Fig. 6, C and D). Taken together, siRNA-mediated knockdown of *PDE5* mimicked the attenuation of fibroblast-to-myofibroblast *trans*-differentiation achieved with tadalafil, indicating that the effect of tadalafil was derived from a specific inhibition of PDE5.

Discussion

BPH is characterized by an initial stromal proliferation and increased myofibroblast/SMC to fibroblast ratio caused by *trans*-differentiation. Given the recently reported beneficial effects of PDE5 inhibitors on LUTS secondary to BPH (15, 16), this study aimed to investigate the molecular mechanism of PDE5 inhibition on the prostate at a cellular level and elucidated that the PDE5 inhibitor tadalafil specifically inhibited prostatic fibroblast proliferation and *trans*-differentiation.

We investigated the expression pattern of PDE5 in the human prostate and demonstrated that PDE5 was mainly present in the stromal compartment of the gland but absent from epithelium. These findings are consistent with a recent study (30). PDE5 has also been reported in stromal cells of other human organs, including the corpus cavernosum, bladder, lung, and retina (3, 30–32). Moreover, lung fibroblasts expressed PDE5 *in vitro* (33).

In the present study, we demonstrated that specific PDE5 inhibition by tadalafil reduced cellular proliferation of prostate-derived fibroblasts in a dose-dependent manner. Moreover, in accordance to the much lower PDE5 expression, we found a less pronounced antimitogenic effect of PDE5 inhibition on prostatic basal epithelial cells. Given that PDE11A, which might be inhibited by tadalafil, was not significantly expressed in both cell types, PrSCs and PrECs, these effects were ascribed to inhibition of PDE5. However, due to the 50-fold lower PDE5 expression in PrECs and the fact that antiproliferative effects were only significant at a concentration of 25 μM tadalafil, possible additional off-target effects, *e.g.* interaction with enzymes other than PDE5 and PDE11A, cannot be completely ruled out and remains to be investigated in future studies.

An antimitogenic effect of PDE5 inhibition was first reported in bovine artery SMCs (34), where sildenafil was found to inhibit platelet-derived growth factor (PDGF)-stimulated proliferation. These findings were confirmed in human pulmonary artery SMCs (32, 35). Antiproliferative effects on prostate stromal cells have also been reported for the PDE5 inhibitors vardenafil (20) and zaprinast (21). Vardenafil enhanced the antiproliferative effects of the NO/cGMP pathway activator SNP and BAY 41-8543 (a stimulator of soluble guanylyl cyclase) of prostatic SMCs, whereas prostatic fibroblasts were not investigated (30).

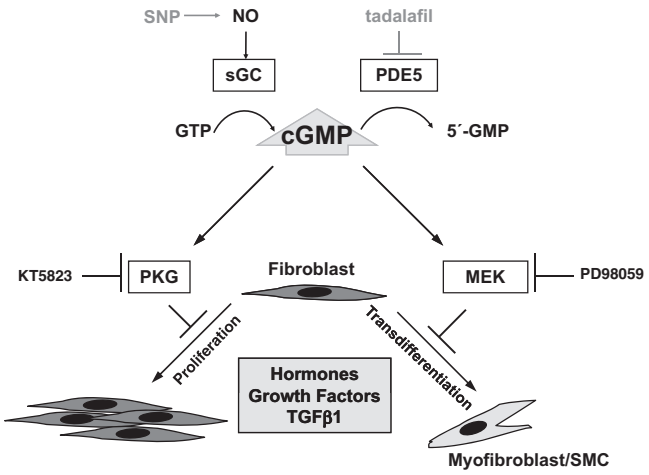

FIG. 7. Proposed pathways of PDE5 inhibition in the prostatic stroma. Age-related changes in local hormone and growth factor levels lead to enlargement and increased myofibroblast to fibroblast ratios in the stromal compartment. These changes lead to the development of BPH and related LUTS. PDE5 inhibition (by tadalafil) and/or NO donors (SNP) increase intracellular cGMP levels. Activation of the cGMP-dependent PKG reduces the proliferation rate of prostate fibroblasts, as demonstrated with the PKG inhibitor KT5823, thus reducing the rate of stromal enlargement. Additionally, elevated cGMP levels attenuate fibroblast-to-myofibroblast *trans*-differentiation independently of PKG activation and thereby reduces the BPH related increase of the myofibroblast ratio. Because attenuation of *trans*-differentiation in part is blocked the MEK inhibitor PD98059, these effects are mediated via MEK1 signaling. Taken together, the two distinct pathways activated by tadalafil attenuate cellular changes in the stroma associated with development and progression of BPH, thus indicating potential therapeutic use of PDE5 inhibition to prevent and treat the disease.

PDE5 inhibition leads to elevated cGMP levels with increased levels of cyclic nucleotides associated with antiproliferative effects (36). Several signaling pathways downstream of cGMP have been implicated in the antimitotic activity of PDE5 inhibition. Sildenafil and organic nitrates reduced PDGF-stimulated proliferation of bovine vascular SMCs by activating PKA but not PKG (34). In contrast, PDGF-stimulated proliferation of porcine pulmonary artery SMCs was inhibited by sildenafil via PKG and downstream degradation of ERK1/2 phosphorylation (37), whereas activation of the MEK/ERK pathway was reported as downstream response of cGMP in rabbit aortic endothelial cells (26). In human prostate stromal cells, zaprinast inhibited fetal calf serum-stimulated proliferation via PKG (21). Consistently, the antimitotic effect of tadalafil was blocked when PrSC were preincubated with the PKG inhibitor KT2358, whereas inhibition of MEK1 had no influence. Our findings suggest that in PrSCs, the antiproliferative activity of PDE5 inhibition is mediated via elevated cGMP levels and downstream activation of PKG independently of the MEK/ERK pathway.

TGFβ1 has been shown to induce fibroblast *trans*-differentiation into myofibroblast/SMCs in the human prostate, which is considered to be the major mechanism in BPH (12, 27, 38, 39). In the present study, we investigated the effect of PDE5 inhibition on *trans*-differentiation of PrSC and observed that tadalafil dose dependently attenuated the potential of TGFβ1 to induce expression of myofibroblast markers. This effect could be mimicked by siRNA-mediated knockdown of *PDE5*. Interestingly, PDE5 inhibition by sildenafil was not sufficient to block TGFβ1-induced lung fibroblast-to-myofibroblast differentiation monitored by SMA protein levels but required additional activation of soluble guanylyl cyclase (33). In contrast, tadalafil on its own was sufficient to attenuate PrSC *trans*-differentiation, but elevating the endogenous cGMP synthesis by the soluble NO donor SNP increased the suppressive effect of PDE5 inhibition.

PDE5 inhibition by tadalafil was not compensated by increased PDE5 expression in PrSC, resembling results obtained in cultures of human penile cells (40). In contrast to lung fibroblasts, stimulation with TGFβ1 did not lead to a reduced PDE5 expression (33).

The signaling pathway downstream of cGMP was again investigated by the use of PKG and MEK inhibitors. Early TGFβ1 response was unaffected by PDE5 inhibition and/or stimulation of soluble guanylyl cyclase, excluding direct interference with the TGFβ1 signaling cascade. Unlike the antiproliferative effect, the attenuation of *trans*-differentiation by tadalafil/SNP was unaffected by the PKG inhibitor KT2358. However, the MEK inhibitor PD98059 significantly abrogated the cGMP mediated *trans*-differentiation block. Thus, increased cGMP levels caused by tadalafil/SNP treatment attenuate TGFβ1-induced *trans*-differentiation downstream via a PKG-independent MEK/ERK pathway. This pathway might include activation of p21Ras by cGMP potentially mediated via guanine nucleotide exchange factors (GEFs) like cyclic nucleotide rasGEF (CNrasGEF) as suggested by Oliveira *et al*. (26).

The conclusions drawn from this study are summarized in Fig. 7. Age-related changes in local hormone and growth factor levels lead to enlargement and increased myofibroblast to fibroblast ratios in the stromal compartment. Elevated cGMP levels due to PDE5 inhibition and/or NO donors reduce proliferation of fibroblasts at least in part via PKG and reduced TGFβ1-induced *trans*-differentiation of PrSC via MEK1 signaling. Therefore, additionally to the effect of PDE5 inhibition on the dynamic component of BPH caused by relaxation of smooth muscle (17–19), PDE5 inhibition at a cellular level affects both hallmarks of the static component of the disease. Thus, we conclude that BPH patients might benefit from PDE5 inhibitors that inhibit stromal cell proliferation, as well as TGFβ1-mediated *trans*-differentiation processes.

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