Attenuated proliferation and transdifferentiation of prostatic stromal cells indicate suitability of phosphodiesterase type-5 inhibitors for prevention and treatment of benign prostatic hyperplasia

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Abbreviations list

BPH…benign prostatic hyperplasia

ED…erectile dysfunction

IGFBP3…insulin-like growth factor binding protein 3

IHC…immunohistochemistry

LUTS…lower urinary tract symtoms

NO…nitric oxide

NOX4…NAD(P)H oxidase 4

PCa…prostate carcinoma

PDGF…platelet derived growth factor

PDE…cyclic nucleotide phosphodiesterase

PDE5…PDE type 5

PKG…protein kinase G

PrEC…primary prostatic basal epithelial cells

PrSC… primary prostatic stromal fibroblasts

qPCR...quantitative PCR

SMA…smooth muscle cell actin

SMC…smooth muscle cell

SNP…sodium nitroprusside

Abstract

Benign prostatic hyperplasia (BPH) is characterized by tissue overgrowth and stromal reorganization primarily due to cellular proliferation and fibroblast-to-myofibroblast transdifferentiation. To evaluate the potential of PDE5 inhibitors like Tadalafil for prevention and treatment of BPH we analyzed the role of the NO/cGMP/PDE5 pathway for cellular proliferation and transforming growth factor beta 1 (TGFβ1)-induced fibroblast-to-myofibroblast transdifferentiation in primary prostate stromal cells (PrSC). Inhibition by Tadalafil of PDE5 which is mainly expressed in the stromal compartment of the prostate reduced proliferation of PrSCs 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 transdifferentiation. The inhibitory effect on transdifferentiation was also observed after siRNA-mediated PDE5 knockdown. As confirmed by the MEK1 inhibitor PD98059 this effect was mediated via MEK1 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 transdifferentiation processes.

Introduction

The cyclic nucleotide phosphodiesterases (PDEs) comprise a superfamily of phosphohydrolases that regulate cellular levels of the second messenger molecules cGMP and cAMP. PDE type 5 (PDE5) specifically hydrolyzes cGMP and is the major therapeutic target in 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 prostatic hyperplasia (BPH) and prostate cancer (PCa) frequently associated with erectile dysfunction (ED). Complete or partial loss of erectile function is a common side effect of clinically localized PCa treatment (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 transdifferentiation to myofibroblasts/smooth muscle cells (SMC) (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 PCa (10, 11). Furthermore, we and others previously demonstrated that TGFβ1 induces fibroblast-to-myofibroblast transdifferentiation of primary prostatic stromal fibroblasts (PrSCs) *in vitro* (12, 13) and exogenous administration of TGFβ1 is sufficient to induce myofibroblast differentiation *in vivo* (14).

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 since an anti-proliferative 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 the prostate are studied *in vitro* at a cellular level to elucidate the underlying molecular and cellular mechanisms of the described beneficial effects on BPH patients. These investigations are aimed to assess the mechanisms of PDE5 inhibition to prevent and treat BPH. Data demonstrate expression of PDE5 in the stromal compartment of the gland. Inhibition of PDE5 reduced proliferation and transdifferentiation 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 unless otherwise specified. Highly pure Tadalafil was kindly provided by ICOS Corporation (Eli Lilly and Company). The kinase inhibitors KT2358 and PD98059 were purchased from Calbiochem. Antibodies against PDE5 and p-ERK1/2 were purchased from Cell Signaling Technology. SMAD2/3 and p-SMAD antibodies were from Upstate, SMC-α-actin (SMA) and β-actin from Sigma-Aldrich, IGFBP3 from R&D Systems and α-tubulin from Santa Cruz Biotechnology. Mouse monoclonal anti-SMA for immunofluorescence was purchased from DakoCytomation.

Immunohistochemistry

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 anti-human PDE5 polyclonal, 1µg/ml, Cell signaling) at 4°C. Primary antiserum was detected after incubation with a biotinylated secondary antibody (biotinylated goat anti-rabbit IgG, Dako Cytomation) using HRP conjucated streptavidin (Dako Cytomation) and the FAST DAB Tablet Set (Sigma). Sections were counterstained with Meyer's Hemalum and mounted with Entellan (Merck). 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 8-well culture slides (Falcon BD Labware). After fixation in acteton/methanol (1:1)

and permeabilization with 0.2% Triton-X-100 cells were blocked with PBS containing 3% BSA for 45 min at room temperature (RT). Anti-SMA antibody (1 µg/mL) was applied for 2 hours at RT. After washing with PBS cells were incubated for 45 min with a secondary fluorochrome-labelled antibody (polyclonal goat anti-mouse TEXAS red, Invitrogen) and nuclei were counterstained for 30 min with DAPI (4',6-Diamidin-2'-phenylindol-dihydrochlorid, Molecular Probes). Cells were embedded in fluorescent mounting medium (DakoCytomation), viewed by the Zeiss Axiovert 200 microscope and images aquired by the Axiovision 4.7 software (Carl Zeiss Microscopy).

Cell lines and tissue culture

Human PrSC cultures and human prostatic basal epithelial cell (PrEC) cultures were established as described previously (22). PrSC were cultured in stromal cell growth medium (SCGM, Clonetics), PrEC on collagen I-coated plates in prostate epithelial cell growth medium (PrEGM, 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 days of culture. Therefore PrECs were treated with collagenase type-I and detached by trypsin/EDTA (Clonetics) and PrSCs were detached by trypsin (PAA Laboratories). Cells were stained with trypan blue staining solution and counted in a Fuchs-Rosenthal counting chamber.

For BrdU (bromodeoxyuridine) incorporation assays four thousand early passage PrSC were seeded in triplicates into individual wells of a 96-well plate (Nunc) 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 DMSO equivalent for 30 min prior to 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) according to manufacturer's instructions.

Transdifferentiation experiments

PrSC of passage 2–4 were incubated in RPMI 1640 (Clonetics) containing 1% charcoal treated fetal calf serum (FCS; Hyclone) 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 bFGF as control to maintain the fibroblast phenotype. Where indicated cells were pretreated with DMSO, KT2358, PD98059 or DMSO equivalent for 60 min and Tadalafil and/or sodium nitroprusside (SNP) for 30 min.

siRNA-mediated PDE5 knockdown

PrSC were seeded in 6 cm dishes and transfected with siRNA targeting *PDE5* (Invitrogen Cat. No. HSS112695) or scrambled control (Invitrogen Cat. No. 12935-300) using Lipofectamin™ 2000 (Invitrogen) according to manufacturer's instructions. 72 h post-transfection transdifferentiation experiments were started.

Quantitative Real-Time PCR

mRNA was extracted by the use of the TriFast™ Reagent (PeQLAB Biotechology). cDNA first strand synthesis was reverse transcribed from 2 µg total RNA preparation using Reverse Transcription System (Promega) 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).

153 ***Western blotting***

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

157

158 ***Statistics***

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

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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 primary prostatic basal epithelial (PrEC) and stromal cells (PrSC) 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 anti-proliferative 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 herein due to its higher specificity for PDE5 over other PDE isoenzymes and its prolonged half-life in plasma (17.5 h vs. ~4 h for Sildenafil and Vardenafil) (1). Tadalafil has high selectivity ratios vs. PDE5 for all PDE isoenzymes except PDE11A, which might be inhibited by high concentrations (23) and is expressed in the human prostate (24, 25). To rule out potential effects mediated via *PDE11A* we analyzed its gene expression by qPCR in the used cell types. In comparison to prostatic tissue extracts (where according to ct-values *PDE5* and *PDE11A* were expressed at similar levels) *PDE11A* expression was very low in both, PrECs ($6.7\pm1.2\times10^{-4}$ fold) and PrSCs ($4.1\pm0.6\times10^{-4}$ 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 immunohistochemistry (IHC). Consistently, in the stromal compartment strong staining was observed while 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 µM and 25 µM) on the proliferation of primary prostate cells was analyzed over a one-week 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 reduced 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, subsequent investigations were focused on PrSCs. The dose-dependence of the anti-proliferative 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 exhibiting highly significant effects ($P < 0.01$; Fig. 2C).

Anti-proliferative 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 mentioned above, 5 µM Tadalafil significantly inhibited PrSC proliferation (BrdU signal 83±5% of control treated cells; 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 anti-proliferative effects of PDE5 inhibition are mediated via elevation of cGMP and the subsequent activation of cGMP dependent PKG.

Since cGMP has been reported to activate the MEK/ERK pathway independently of PKG (26), PrSCs were also pretreated with the MEK1 inhibitor PD98059. However, preincubation with PD98059 did not influence the effect of Tadalafil on proliferation (86±4% vs. 83±5%; P=0.34; Fig. 2D), demonstrating that the growth inhibition by Tadalafil is not mediated via MEK/ERK.

Tadalafil suppresses TGFβ1-mediated fibroblast-to-myofibroblast transdifferentiation

Besides stromal expansion the main histological change in the BPH stroma is transdifferentiation of fibroblasts to myofibroblasts/SMCs. This transdifferentiation can be modeled in vitro by stimulating PrSC with TGFβ1 (27, 28) as indicated by induction of the transdifferentiation marker genes smooth muscle

actin gamma 2 (*SMA*) and insulin-like growth factor binding protein 3 (*IGFBP3*; (13)). The transdifferentiation model is briefly introduced in Fig. 3. Stimulation of PrSCs with TGFβ1 led to a 15.8±2.6 fold and 80.7±16.5 fold increase of mRNA levels of *SMA* and *IGFBP3*, respectively (Fig. 3A), which was verified on protein levels by western blot analysis (Fig. 3B). Effective transdifferentiation is also marked by typical changes in cell morphology from the thin and elongated phenotype of fibroblasts to the flattened phenotype of myofibroblasts with actin bundles that stain positive for SMA (Fig. 3C).

The effect of PDE5 inhibition on PrSC transdifferentiation was studied by stimulation with TGFβ1 after preincubation with 25 µM Tadalafil. TGFβ1-induced transdifferentiation was significantly attenuated by Tadalafil as determined by qPCR of the marker genes. *E*xpression 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 transdifferentiated cells.

Increase of NO signaling enhances suppressive effects of Tadalafil on fibroblast transdifferentiation

In the normal prostate nitric oxide synthases (NOS) are mainly expressed in the epithelial compartment (29). Since fibroblasts have low NOS expression levels NO/cGMP signaling in the stroma is mainly stimulated by NO synthesized from neurons. Thus in a pure PrSC culture NO levels are presumably low. As PDE5 inhibition enhances the NO/cGMP signaling the relative high concentration of Tadalafil needed to significantly attenuate transdifferentiation might be attributed to a low cGMP synthesis rate. Thus the soluble NO donor sodium nitroprusside (SNP) was used to enhance the NO/cGMP pathway. SNP dose dependently attenuated the induction of *SMA* (10 µM SNP: 90±6% of control, *P*=0.12; 100 µM SNP: 68±7% of control, *P*=0.02; Fig. 4A) and *IGFBP3* (10 µM SNP: 94±5% of control, *P*=0.17; 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 at a concentration of 25 µM synergistically enhanced the effect of SNP on the transcription of the transdifferentiation markers (Fig. 4A and B). These findings were also confirmed at the protein level by western blotting (Fig. 4C 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 if Tadalafil and SNP directly interfere in TGFβ1 signaling, SMAD2 and ERK1/2 phosphorylation was analyzed by western blots using phospho-specific antibodies. PDE5 protein levels were not significantly influenced by TGFβ1, Tadalafil or SNP (Fig. 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 transdifferentiation via the MEK/ERK pathway

As for the anti-proliferative effects of PDE5 inhibition the attenuation of PrSC transdifferentiation 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 transdifferentiation as monitored by marker gene expression of *SMA* and *IGFBP3* (Fig. 5B). Therefore we tested implication of the MEK/ERK pathway since as mentioned above 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 transdifferentiation 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 while KT2358 did not influence protein expression (Fig. 5C and D). Taken together these findings indicate that the attenuation of TGFβ1-induced transdifferentiation by NO/cGMP is mediated via the MEK1 pathway and not via activation of PKG.

RNAi mediated knockdown of PDE5 attenuates fibroblast-to-myofibroblast transdifferentiation

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 transdifferentiation 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 to cells treated with scrambled siRNA (Fig. 6A). Additionally, the induction of transdifferentiation markers upon TGFβ1 stimulation was significantly reduced by *PDE5* knockdown (*SMA*: 48±8% of SCR control, *P*=0.01; *IGFBP3*: 62±19% of SCR control, *P*=0.03; Fig. 6B). Transdifferentiation of PrSC had lower efficiency in *PDE5* knockdown cells as monitored by SMA and IGFBP3 protein levels (Fig 6C and D). Taken together siRNA-mediated knockdown of *PDE5* mimicked the attenuation of fibroblast-to-myofibroblast transdifferentiation achieved with Tadalafil, indicating that the effect of Tadalafil was derived from a specific inhibition of PDE5.

Discussion

Given the recently reported beneficial effects of PDE5 inhibitors on lower urinary tract symptoms secondary to BPH (15, 16) this study aimed to investigate the influence of PDE5 inhibition on the prostate at a cellular level. We investigated the expression 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 lower PDE5 expression we found a less pronounced anti-mitogenic effect of PDE5 inhibition on prostatic basal epithelial cells. Given that PDE11A, which might be inhibited by Tadalafil, was not significantly expressed in the used PrSCs and PrECs, these effects were ascribed to inhibition of PDE5. However, since PDE11A has been localized in the glandular epithelium (24, 25) and *PDE11A* mRNA levels were similar to *PDE5* levels in prostatic tissue extracts, the anti-proliferative effects of Tadalafil on epithelial cells might be enhanced *in vivo* by additional inhibition of PDE11A activity. An anti-mitogenic 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). Anti-proliferative effects on prostate stromal cells have also been reported for the PDE5 inhibitors Vardenafil (20) and Zaprinast (21). Vardenafil enhanced the anti-proliferative effects of the NO/cGMP pathway activator SNP and BAY 41-8543 (a stimulator of soluble guanylyl cyclase) of prostatic SMCs, while prostatic fibroblasts were not investigated (30).

PDE5 inhibition leads to elevated cGMP levels with increased levels of cyclic nucleotides associated with anti-proliferative effects (36). Several signaling pathways downstream of cGMP have been implicated in the anti-mitotic activity of PDE5 inhibition. Sildenafil and organic nitrates reduce 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) while 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 FCS-stimulated proliferation via PKG (21). Consistently, the anti-mitotic effect of Tadalafil was blocked when PrSC were preincubated with the PKG inhibitor KT2358, while inhibition of MEK1 had no influence. Our findings suggest that in PrSCs the anti-proliferative activity of PDE5 inhibition is mediated via elevated cGMP levels and downstream activation of PKG independently of the MEK/ERK pathway.

BPH is characterized by an initial stromal proliferation and increased myofibroblast/SMC to fibroblast ratio caused by transdifferentiation. TGFβ1 has been shown to induce fibroblast differentiation into myofibroblast/SMCs in the human prostate, which is considered to be the major mechanism *in vivo* (12, 27, 38, 39). In the present study we investigated the effect of PDE5 inhibition on transdifferentiation 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 transdifferentiation but elevating the endogenous cGMP synthesis by the soluble NO donor SNP increased 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 anti-proliferative effect the attenuation of transdifferentiation by Tadalafil/SNP was unaffected by the PKG

inhibitor KT2358. However, the MEK inhibitor PD98059 significantly abrogated the cGMP mediated transdifferentiation block. Thus, increased cGMP levels caused by Tadalafil/SNP treatment attenuate TGFβ1-induced transdifferentiation downstream via a PKG independent MEK/ERK pathway. This pathway might include activation of p21Ras by cGMP potentially mediated via guanine nucleotide exchange factors like CNRasGEF as suggested by Oliveira et. al (2003).

As Tadalafil attenuates both proliferation and differentiation of PrSCs the question remains in what state the cells are transferred upon PDE5 inhibition. Potential mechanisms involved could be apoptosis, senescence or quiescence. However, we observed no increased apoptosis or senescence in our PrSCs. Therefore it is likely that the cells enter a quiescence state due to the elevated cGMP levels. This is in agreement with the previous finding that prolonged NO treatment shifted vascular smooth muscle cells to a quiescent state (41).

The conclusions drawn from this study are summarized in Figure 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 transdifferentiation 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 transdifferentiation processes.

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Figure Legends

Figure 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 primary prostate epithelial (PrEC) and stromal cells (PrSC) established from 3 independent donors. cDNA concentration was normalized against the house keeping gene porphobilinogen deaminase (HMBS). β-actin shown as loading control in western blot (B) *PDE5* and *PDE11A* mRNA levels were analyzed by qPCR in PrECs (n=3), PrSCs (n=3) and prostate tissue specimens (n=5).. Note the logarithmic y-axis. (C) Immunohistochemistry 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).

Figure 2. Anti-proliferative 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 obtainted in a counting chamber after staining with trypan blue. (A) Tadalafil significantly reduced proliferation of PrSCs at a concentration 2.5 and 25 µM. of 25 µM and proliferation of PrSC at (B) Tadalafil at a concentration of 2.5 µM did not significantly alter proliferation of PrECs, while 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 MEK1 inhibitior PD98059 (20 µM) did not interfere with the anti-proliferative effect of 5 µM Tadalafil on PrSC. However, the PKG inhibitor KT2358 (200 nM) significantly attenuated the growth inhibitory effect of Tadalafil, indicating that the anti-proliferative 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 (* $P < 0.05$; ** $P < 0.01$).

Figure 3. TGFβ1 induced fibroblast-to-myofibroblast transdifferentiation. PrSCs were stimulated with 1 ng/ml TGFβ1 to induce transdifferentiation and with 1 ng/ml bFGF (control), respectively. (A) mRNA levels of the transdifferentiation markers *SMA* and *IGFBP3* were analyzed by qPCR. cDNA concentration was normalized against the house keeping gene porphobilinogen deaminase (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 of PrSCs stimulated with bFGF or TGFβ1 for 72 h. Note the thin, elongated and light refractive phenotype of bFGF-treated PrSCs (fibroblasts) in comparison to the flattened and less light refractive morphology of TGFβ1- transdifferentiated PrSCs (myofibroblasts). Pretreatment with 25 μM Tadalafil and 100 μM SNP attenuated the morphological changes induced by TGFβ1. (D) PrSCs were stimulated as in (C) before immunofluorescent staining of SMA (red). TGFβ1 treated PrSCs stain positive for SMA. Nuclei were counterstainen with DAPI.

Figure 4. PDE5 inhibition attenuates PrSC fibroblast-to-myofibroblast transdifferentiation. PrSCs were preincubated with 25 μM of Tadalafil and increasing concentrations of SNP before stimulation with 1 ng/ml bFGF (control) or 1 ng/ml TGFβ1 to induce transdifferentiation. Tadalafil significantly attenuated the induction of transdifferentiation 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% transdifferentiation) was determined by paired Student's t-test (* $P < 0.05$; ** $P < 0.01$) (C) Total protein extracts from three different PrSC cultures treated as in (A, B) were pooled and subjected to western blotting with the indicated antibodies. α-tubulin served as loading control. (D) Densitometric analysis of (C). Values represent mean±SEM from three independent experiment using different donors.

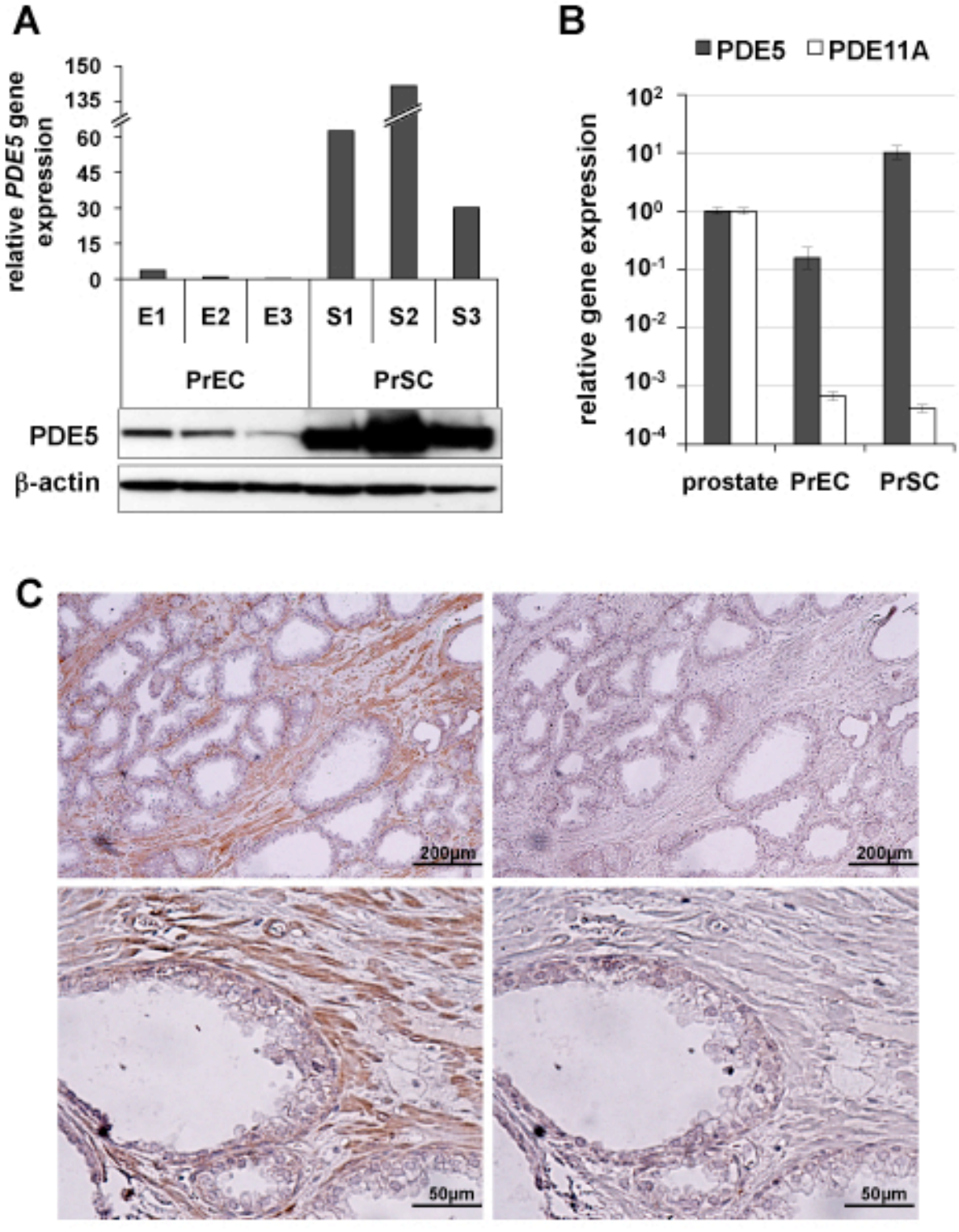
Figure 5. Tadalafil/SNP do not interfere in TGFβ signaling but activate MEK/ERK signaling. (A) Total protein extracts were prepared of PrSCs from three independent donors after 1 h of bFGF or TGFβ1 stimulation. Extracts were pooled and 30 µg protein analyzed by western blot. Pretreatment of cells with 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 effect of Tadalafil/SNP as determined by qPCR of the transdifferentiation markers *SMA* and *IGFBP3* after 24 h. Statistical significance was determined by paired Student's t-test (* $P < 0.05$). **(C)** Total protein extracts were prepared from three different PrSC cultures after 72 h, pooled and 30 mg protein analyzed by western blot. The PKG inhibitor KT2358 did not influence the reduction of SMA and IGFBP3 by Tadalfil/SNP. The MEK1 inhibitor PD98059 blocked the effect Tadalafil/SNP on SMA and IGFBP3 protein levels. α-tubulin served as loading control. (D) Densitometric analysis of (C). Values represent mean±SEM from three independent experiment using different donors.

Figure 6. siRNA-mediated knockdown of PDE5 mimicks the effects of Tadalafil on transdifferentiation. PrSCs were transfected either with scrambled (SCR) or PDE5 specific siRNA and stimulated with bFGF or TGFβ1 72 h post-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 transdifferentiation. 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 were pooled and 30 µg protein analyzed by western blot with the antibodies indicated. β-actin served as loading control. (D) Densitometric analysis of (C). Values represent mean±SEM from three independent experiment using different donors.

Figure 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 protein kinase 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 transdifferentiation independently of PKG activation and thereby reduces the BPH related increase of the myofibroblast ratio. Since attenuation of transdifferentiation in part is blocked the MEK inhibitor PD98059, these effects are mediated via MEK1 signaling. Taken together both distinct pathways activated by Tadalafil reduce cellular changes in the stroma associated with development and progression of BPH, thus indicating potential therapeutic use of PDE5 inhibiton to prevent and treat the disease.

Table 1. Primer sequences

Gene Symbol	Unigene ID	Primer sequences	Melting point (°C)	PCR-product length (bp)
ACTG2 (SMA)	Hs.403989	sense: 5-agaagagctatgagctgcca; anti-sense: 5-gctgtgatctccttctgcat	86	247
HMBS	Hs.82609	sense: 5-ccaggacatcttggatctgg; anti-sense: 5-atggtagcctgcatggtctc	88	213
IGFBP3	Hs.450230	sense: 5-caagcgggagacgaatatg; anti-sense: 5-ttatccacacaccagcagaa	85	189
PDE5	Hs.587281	sense: 5-caaaaccctggcctattcaa; anti-sense: 5-gcatctatgaacccaacttgc	80	163
PDE11A	Hs.570273	Sense: 5-tgaattgatgtccccaaagt; anti-sense: 5-gatcctggtaggcatcactg	82	158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60
45
30
15
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E2
E3
S1
S2
S3
PrEC
PrSC
PDE5
β-actin
B
PDE5
PDE11A
relative gene expression
10^2
10^1
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10^-2
10^-3
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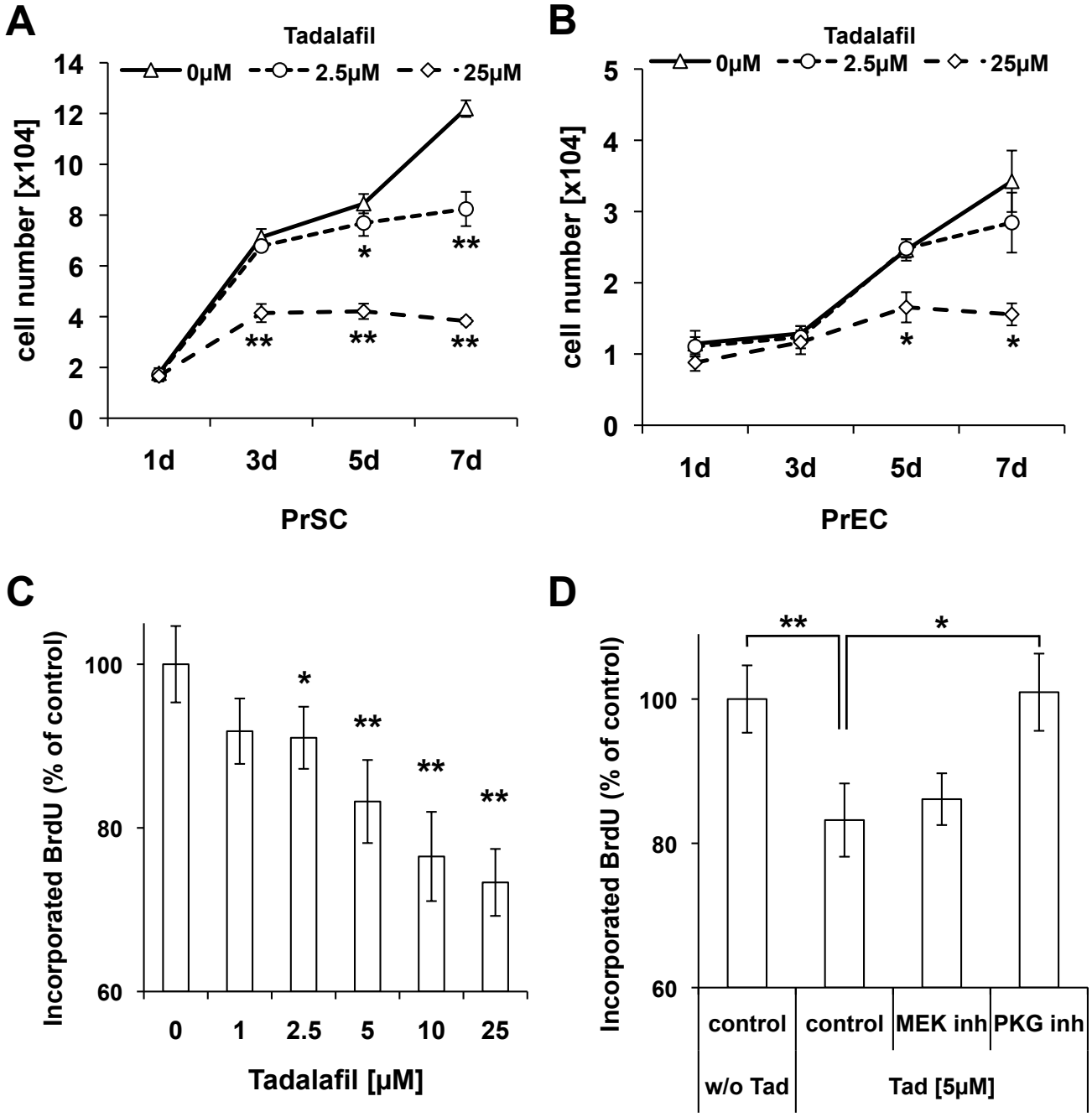
prostate
PrEC
PrSC
C
200μm
200μm
50μm
50μm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6
4
2
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1d
3d
5d
7d
PrSC
B
Tadalafil
0μM
2.5μM
25μM
cell number [x104]
5
4
3
2
1
0
1d
3d
5d
7d
PrEC
C
Incorporated BrdU (% of control)
100
80
60
0
1
2.5
5
10
25
Tadalafil [μM]
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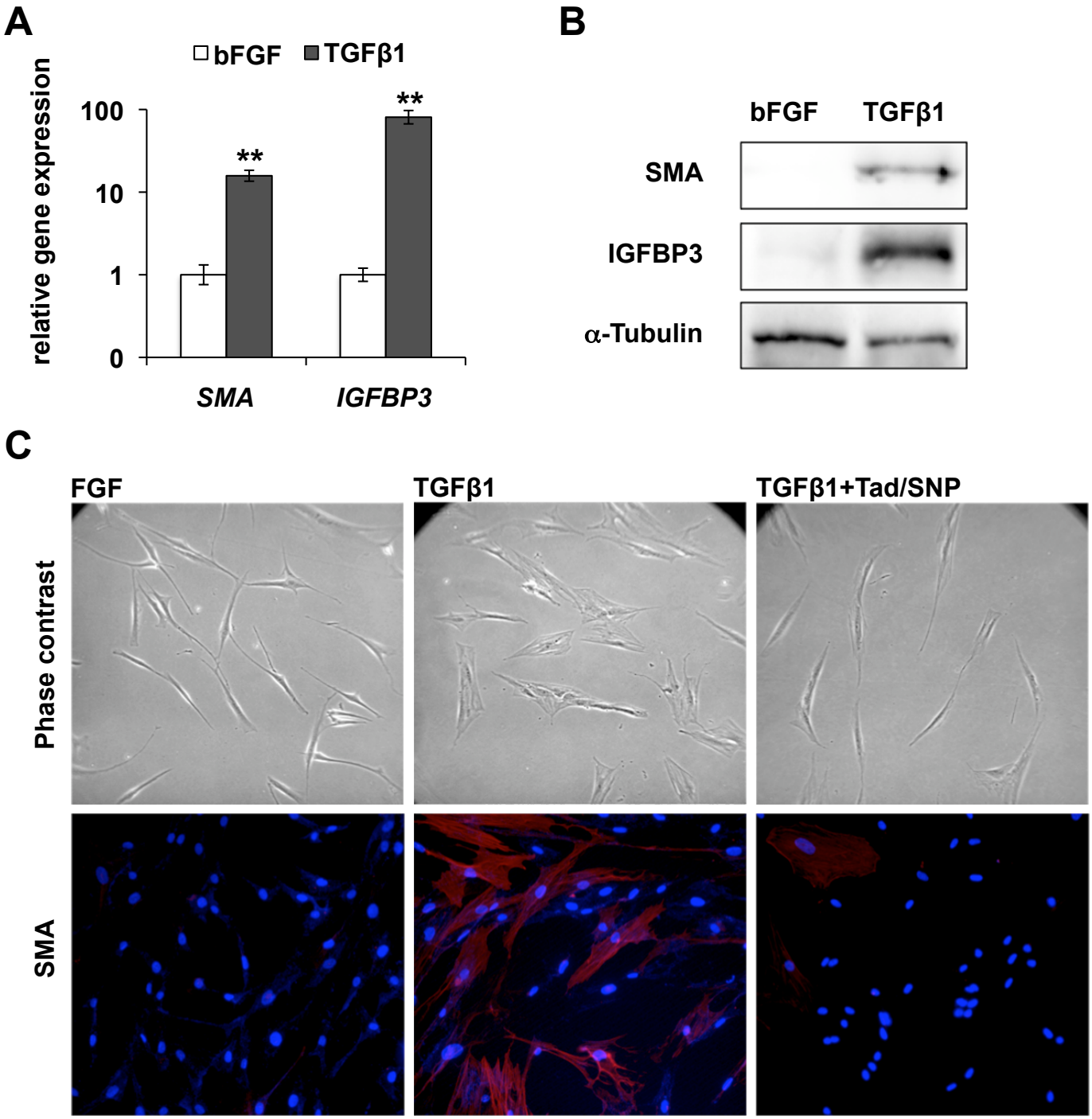
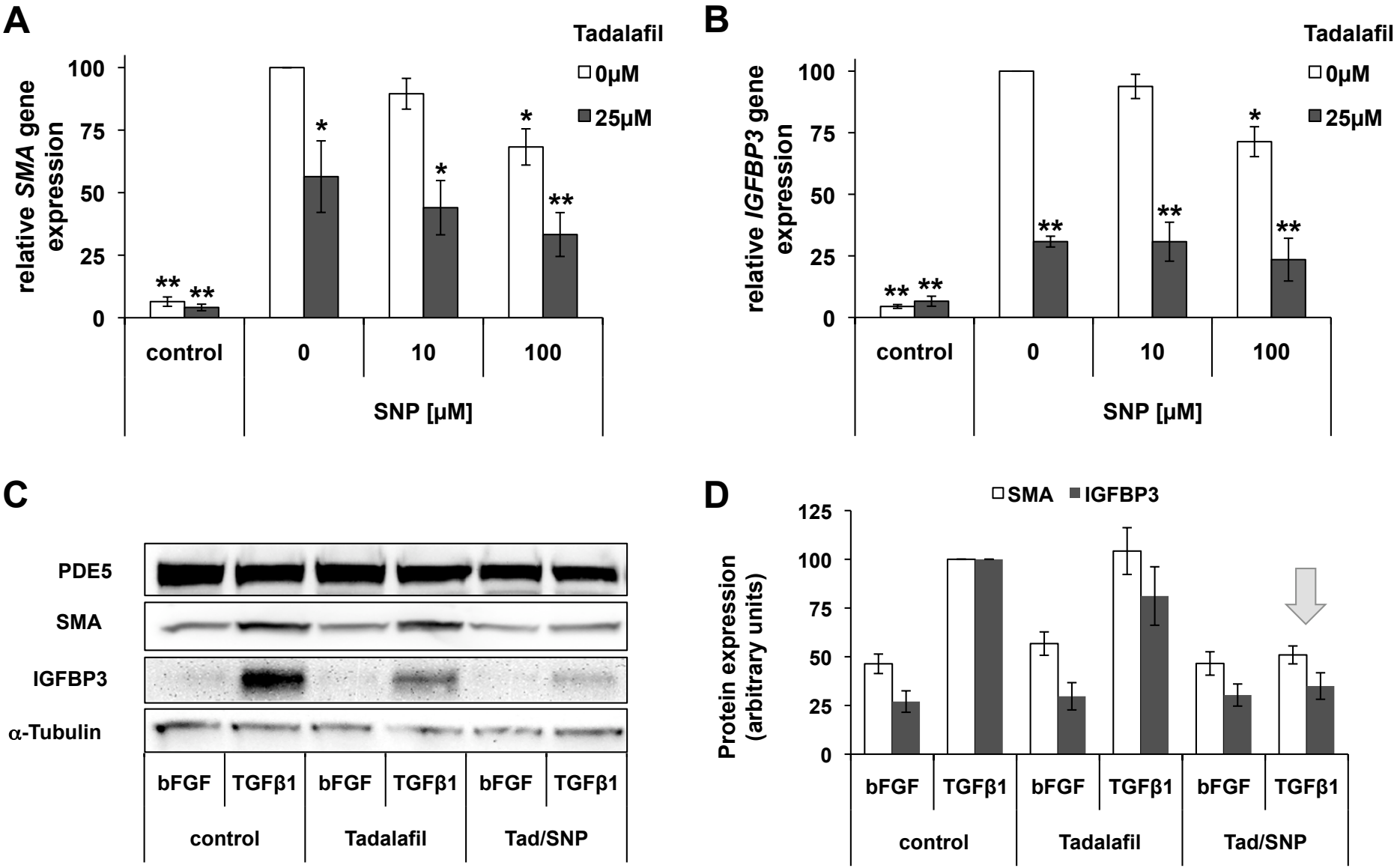
100
80
60
control
control
MEK inh
PKG inh
w/o Tad
Tad [5μM]


A
bFGF
TGFβ1
relative gene expression
100
10
1
0
**
**
SMA
IGFBP3
B
bFGF
TGFβ1
SMA
IGFBP3
α-Tubulin
C
FGF
TGFβ1
TGFβ1+Tad/SNP
Phase contrast
SMA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10
100
SNP [μM]
B
relative IGFBP3 gene expression
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0μM
25μM
control
0
10
100
SNP [μM]
C
PDE5
SMA
IGFBP3
α-Tubulin
bFGF
TGFβ1
control
Tadalafil
Tad/SNP
D
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IGFBP3
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75
50
25
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bFGF
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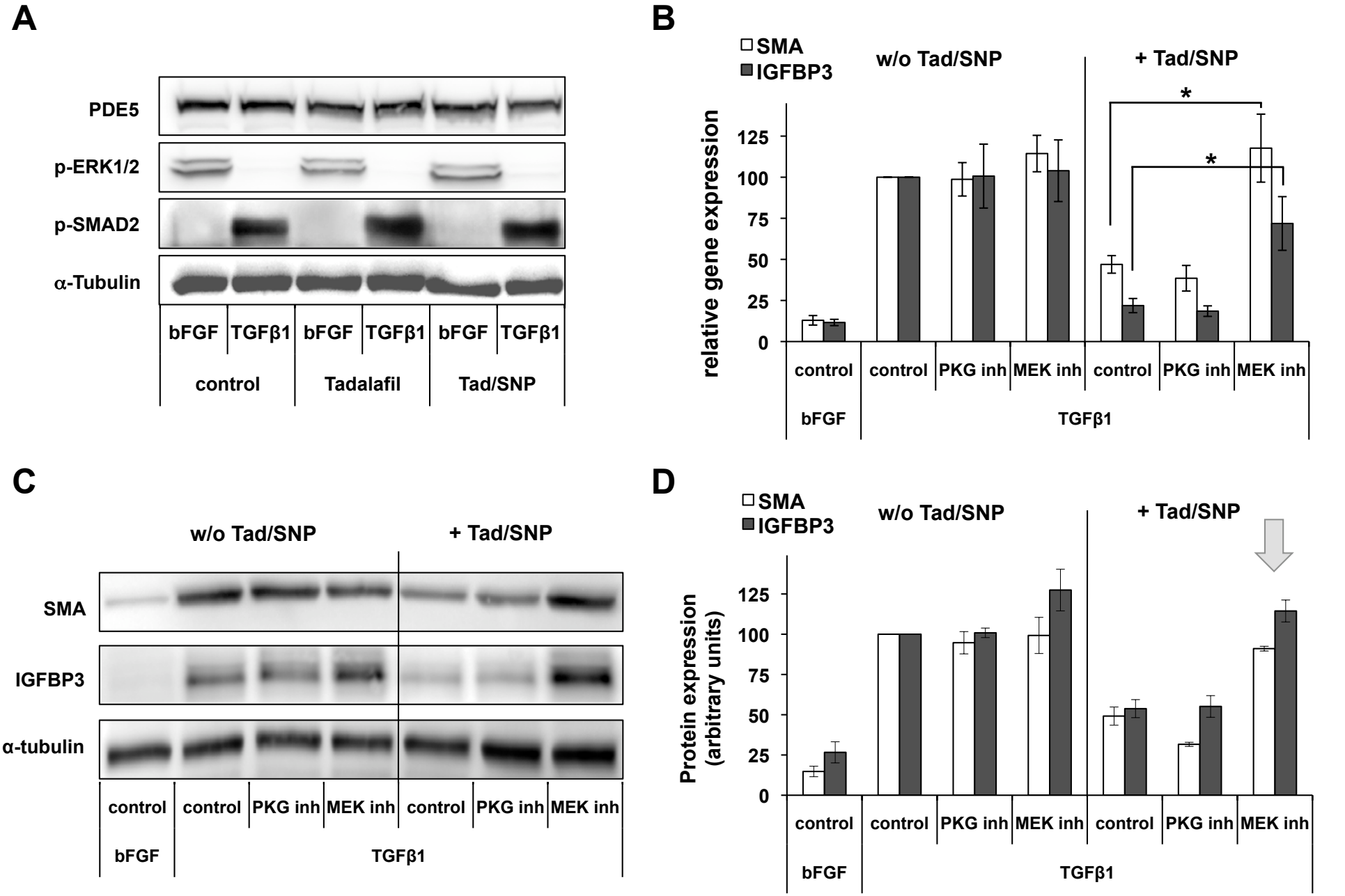
control
Tadalafil
Tad/SNP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bFGF
TGFβ1
bFGF
TGFβ1
bFGF
TGFβ1
control
Tadalafil
Tad/SNP
B
SMA
IGFBP3
w/o Tad/SNP
+ Tad/SNP
relative gene expression
125
100
75
50
25
0
*
*
control
control
PKG inh
MEK inh
control
PKG inh
MEK inh
bFGF
TGFβ1
C
w/o Tad/SNP
+ Tad/SNP
SMA
IGFBP3
α-tubulin
control
control
PKG inh
MEK inh
control
PKG inh
MEK inh
bFGF
TGFβ1
D
SMA
IGFBP3
w/o Tad/SNP
+ Tad/SNP
Protein expression
(arbitrary units)
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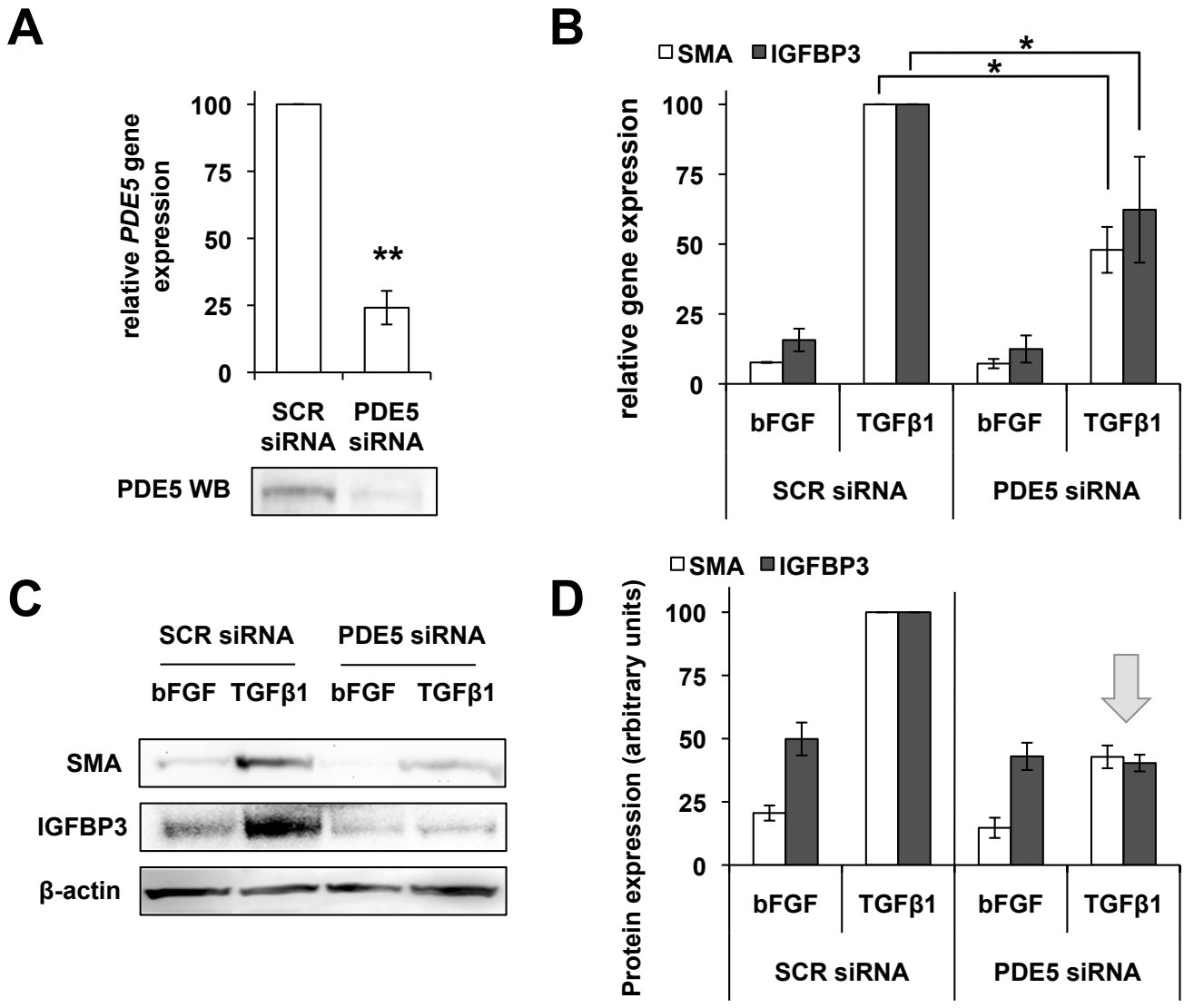
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IGFBP3
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*
relative gene expression
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50
25
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bFGF
TGFβ1
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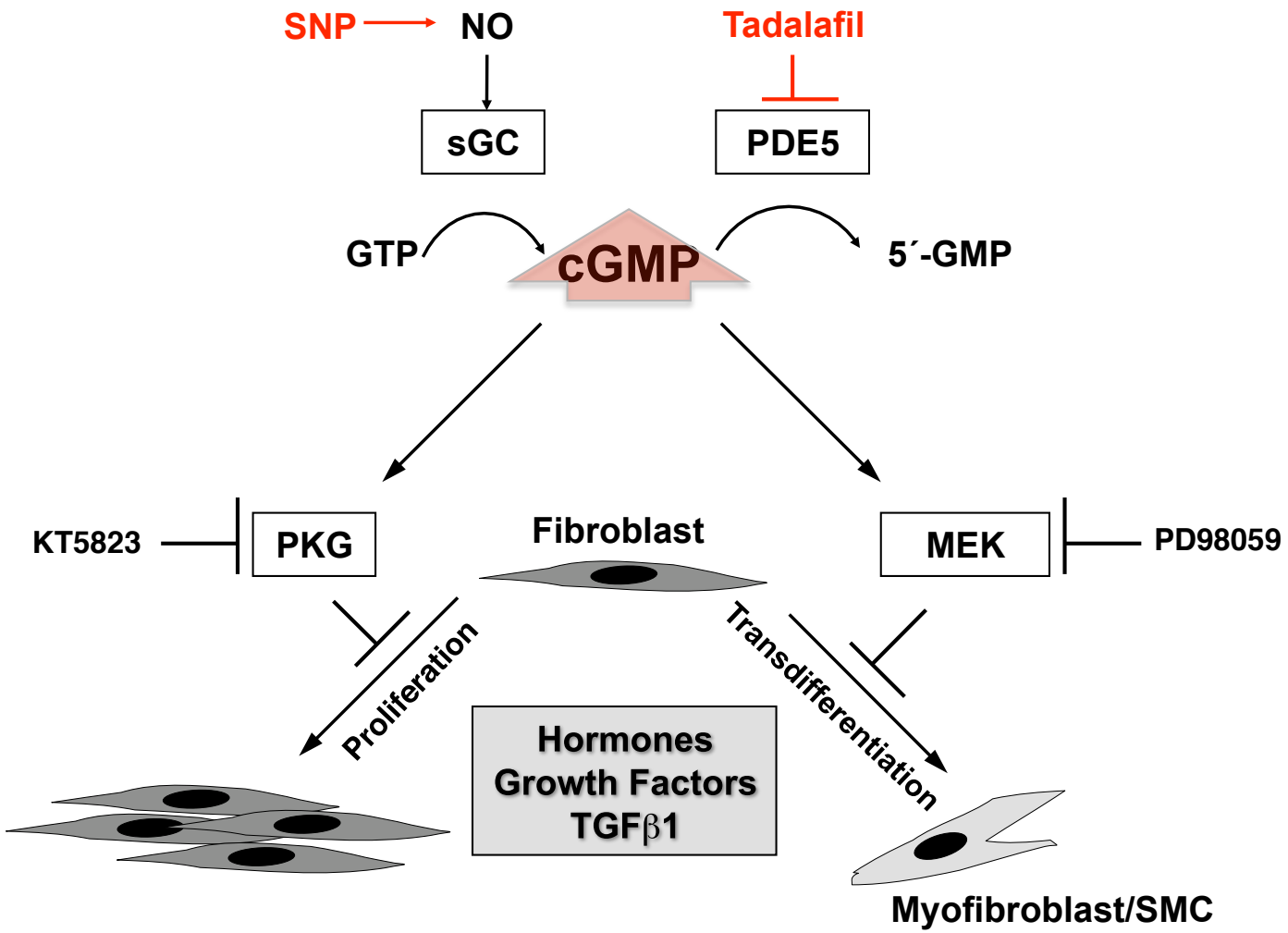
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Proliferation
Transdifferentiation
Hormones
Growth Factors
TGFβ1
Myofibroblast/SMC