

Phosphodiesterase Type 5 Inhibitors and Selective Estrogen Receptor Modulators Can Prevent But Not Reverse Myofibroblast Transformation in Peyronie's Disease

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ABSTRACT

Background: Myofibroblast transformation is a key step in the pathogenesis of Peyronie's disease (PD). Phosphodiesterase type 5 inhibitors (PDE5is) and selective estrogen receptor modulators (SERMs) can prevent the formation of fibrosis in in vitro and in vivo models of PD. However, it is unknown whether these drugs can also reverse established fibrosis.

Aim: To investigate whether PDE5is and SERMs can reverse transforming growth factor beta 1 (TGF- β 1)—induced myofibroblast transformation and determine the point of no return.

Methods: In-Cell enzyme-linked immunosorbent assay was used to quantify TGF- β 1—induced myofibroblast transformation of human primary fibroblasts isolated from tunica albuginea (TA) of patients undergoing surgery for treatment of PD. Extracellular matrix production and collagen contraction assays were used as secondary assays. Reverse transcription—quantitative polymerase chain reaction and In-Cell enzyme-linked immunosorbent assay were used to measure drug target expression. PDE5i (vardenafil) and SERM (tamoxifen) were applied at various time points after TGF- β 1.

Outcomes: Reversibility of myofibroblast transformation and drug target expression were investigated in a time-dependent manner in TA-derived fibroblasts.

Results: Vardenafil or tamoxifen could not reverse the myofibroblast traits of alpha-smooth muscle actin expression and extracellular matrix production, whereas only tamoxifen affected collagen contraction after 72 hours of TGF- β 1 treatment. Phosphodiesterase 5A and estrogen receptor (ER)- β were downregulated after 72 hours, and estrogen receptor - α protein could not be quantified. Tamoxifen could prevent myofibroblast transformation until 36 hours after TGF- β 1 treatment, whereas vardenafil could prevent only 24 hours after TGF- β 1 treatment. This was mirrored by downregulation of drug targets on mRNA and protein level. Furthermore, antifibrotic signaling pathways, peroxisome proliferator-activated receptor gamma and betaglycan (TGFB receptor III), were significantly downregulated after 36 hours of TGF- β 1 exposure, as opposed to upregulation of profibrotic thrombospondin-1 at the same time point.

Clinical Translation: This study suggests that using PDE5is and SERMs might only help for early-phase PD and further highlights the need to test drugs at the appropriate stage of the disease based on their mechanism of action.

Strengths & Limitations: The study uses primary human TA-derived fibroblasts that enhances translatability of the results. Limitations include that only 1 example of PDE5i- and SERM-type drug was tested. Time course experiments were only performed for marker expression experiments and not for functional assays.

Conclusion: This is the first study to demonstrate that timing for administration of drugs affecting myofibroblast transformation appears to be vital in in vitro models of PD, where 36 hours of TGF- β 1 treatment can be suggested as a “point of no return” for myofibroblast transformation. **Ilg MM, Stafford SJ, Mateus M, et al. Phosphodiesterase Type 5 Inhibitors and Selective Estrogen Receptor Modulators Can Prevent But Not Reverse Myofibroblast Transformation in Peyronie's Disease. J Sex Med 2020;XX:XXX–XXX.**

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INTRODUCTION

Fibrosis is defined as the excess accumulation of extracellular matrix (ECM) proteins in response to chronic injury or inflammation.¹ Fibrotic disorders encompass a wide range of clinically relevant diseases, affecting any organ or system in the body, such as the skin, liver, kidney, or lung.^{2–5} Peyronie's disease (PD) is characterized by the formation of a fibrotic plaque in the penile tunica albuginea (TA) leading to pain, curvature, and erectile dysfunction.⁶ Despite affecting 0.3–9% of men worldwide,^{7–9} current medical treatment is very limited. Specifically, there are no approved drugs for the early, unstable phase of PD. To aid drug development endeavors and preclinical research, *in vitro* modeling of PD has often been attempted by using TA-derived fibroblasts from patients suffering from PD.

Myofibroblasts are highly contractile and proliferative, α -smooth muscle actin (ASMA)—positive, ECM-producing cells. They are derived from quiescent tissue resident fibroblasts¹⁰ and play a key role in the formation and pathophysiology of PD and fibrosis in general.¹¹ While being vital in the physiological wound healing response, they are responsible for the plaque formation and subsequent contraction in PD.^{12,13} Furthermore, owing to their role as main modulators of tissue remodeling and matrix homeostasis, they have been described as critical effectors in fibrosis where their persistence leads to resistance to apoptosis.^{14,15} While multiple origins of the myofibroblast have been proposed, the most common source remain locally resident fibroblasts undergoing transformation to myofibroblasts.¹⁶

Transforming growth factor beta 1 (TGF- β 1) is a crucial regulator of fibroblast phenotype and function and is the main effector cytokine in myofibroblast transformation.¹⁷ The canonical signaling pathway for TGF- β 1 activates Smad (mothers against decapentaplegic homolog) transcriptional activator-dependent that regulates myofibroblast transformation by directly influencing the expression of ACTA2, the gene for the myofibroblast marker ASMA.¹⁸ In addition, TGF- β 1 can act via non-canonical ways, independent of Smad signaling and through crosstalk with other signaling pathways.^{19,20}

Along with directly targeting TGF- β 1-signaling, the inhibition of myofibroblasts has been suggested as a therapeutic approach, and recent research has focused on preventing their formation.^{21–23} Previously, the antifibrotic effect of phosphodiesterase type 5 inhibitors (PDE5is) and selective estrogen receptor modulators (SERMs) has been demonstrated in models of PD.²³ This study showed that PDE5is and SERMs were able to prevent TGF- β 1-induced myofibroblast transformation on a

phenotypic (reduced ASMA expression) and functional level (reduced ECM formation and contractile ability) in TA-derived cells. However, this study did not address whether the drugs had the ability to reverse already formed fibrosis, for example, by inducing dedifferentiation, which has been proposed as a promising alternative in facing the challenges of drug development to tackle fibrotic diseases.²⁴ Several agents, such as capsaicin,²⁵ CU/Zn SOD,²⁶ or S-nitrosothiols,²⁷ have been reported to achieve a return to non-myofibroblast state, but no study has looked at the direct influence of TGF- β 1 exposure time on preventing myofibroblast transformation when testing drugs. It is unclear whether drug treatment targeting myofibroblasts might even be more successful during the process of myofibroblast differentiation, as opposed to before or after. Consequently, the primary aim of this study was to investigate whether the previously discovered antimyofibroblast effect of PDE5is and SERMs is limited to preventing myofibroblast transformation or whether they can also reverse already formed myofibroblasts. A second aim was to investigate the efficacy of PDE5is and SERMs after various times of TGF- β 1 exposure and determine a point of no return after TGF- β 1 treatment, where it has become impossible for the drugs to reverse or prevent the myofibroblast state.

METHODS

Sample Acquisition

TA tissue samples that would have otherwise been surgically discarded were acquired from patients undergoing corrective surgery for PD at University College London Hospital (UCLH), United Kingdom. Non-plaque TA was obtained from patients with PD undergoing a Nesbit procedure whereby nonfibrotic TA tissue was excised from the opposite side of the plaque. The patients enlisted for this study were aged between 18 and 75 years, able to understand the patient information sheet, and able to give written consent. Ethical approval was obtained by independent research ethics committees (NRES committee East of England [12-EE-0170] and NRES committee North of Scotland [15-NS-0051]).

Isolation of Fibroblasts From TA Tissue

Isolation of fibroblasts was performed as previously described.^{22,23} Tissue samples were dissected into small pieces to ensure that the corpus cavernosum was removed, submerged in culture media (DMEM [GIBCO, Invitrogen, Waltham, Massachusetts], 10% fetal calf serum [Fisher Scientific, Loughborough, UK], and 1% Pen/Strep [GIBCO, Invitrogen]) in 6-well plates, and incubated at 37°C, 5% CO₂ in a humidified atmosphere for 5–7 days until cellular outgrowth was observed.

Tissue was removed, cells were washed with phosphate-buffered saline (PBS), and fresh, warm medium was added. Cells were incubated at 37°C until they reached 50–70% confluence, after which cultures were expanded. Cells were characterized as previously described.^{22,23} Briefly, cells were subjected to reverse transcription–quantitative polymerase chain reaction, immunocytochemistry, In-Cell enzyme-linked immunosorbent assay (ELISA) (ICE), and Western blot to measure expression of mesenchymal marker vimentin, in absence of smooth muscle marker desmin with expression of myofibroblast marker ASMA in TGF- β 1 concentration–dependent manner. Passages 2–6 were used throughout these experiments.

In-Cell ELISA

The expression of ASMA and other protein markers was quantified in 96-well plates using the ICE technique, as previously described.^{22,23} Fibroblasts were either untreated or pretreated with 10 ng/mL of TGF- β 1 for 72 hours to generate myofibroblast cultures. Cells were seeded onto 96-well optical flat-bottom black microplates (Nunc, Rochester, New York) at 5.0×10^3 cells/well and incubated overnight at 37°C, 5% CO₂. Media were replaced with fresh media with and without TGF- β 1 (10 ng/ml) for the indicated time points hours. In addition, a SERM (tamoxifen; Sigma-Aldrich, Gillingham, UK) or PDE5i (vardenafil; Sigma-Aldrich, UK) was added at different concentrations (0.1 μ M–1,000 μ M for PDE5i and 0.018 μ M–54 μ M for SERM). SB-505124, a transforming growth factor beta receptor II (TGFBR2) inhibitor, was used as control. The compounds were dissolved in 100% dimethyl sulfoxide to stock concentration. After incubation, cells were fixed using 4% paraformaldehyde for 20 minutes at room temperature, washed with 0.1% Triton X-100 in PBS, and blocked for 90 minutes using 10% donkey serum plus 0.1% Triton X-100 in PBS. Primary antibody solution diluted in PBS (1:3,000 dilution of mouse monoclonal anti-ASMA antibody, 1:100 dilution of rabbit monoclonal anti-phosphodiesterase 5A (PDE5A), 1:500 dilution of rabbit polyclonal estrogen receptor [ER] alpha, or 1:500 dilution of rabbit polyclonal ER beta) was added and incubated for 2 hours at room temperature. In addition, blocking peptides specific to antibodies were used to ensure specificity of antibodies. Blocking peptides were obtained by Abcam (Cambridge, UK). Cells were subjected to 3 washing steps using 0.1% Tween 20 in PBS after which the secondary antibody and nuclear stain were added (donkey anti-mouse or donkey anti-rabbit at 1:500 that emits at 800 nm; IRdye 800CW [LI-COR, Cambridge, UK]; nuclear counterstain at 1:1,000 that emits at 700 nm [DRAQ5, Biostatus, Loughborough, UK]) After 1 hour incubation, cells were washed thrice with 0.1% Tween 20 in PBS, and the plate was scanned using an infrared imaging system (Odyssey CLx imager; LI-COR, UK) at both 700 nm and 800 nm wavelengths.

Immunocytochemistry

Cells were seeded at 5.0×10^4 cells/well into wells of a 6-well plate containing sterile glass coverslips. After overnight incubation, media

were replaced with either fresh media or media containing TGF- β 1 (10 ng/mL; Sigma-Aldrich, UK) for 72 hours. Cells were fixed using ice-cold methanol at –25°C. Coverslips were incubated with 10% donkey serum (Millipore, Burlington, Massachusetts) in PBS and then with a mouse monoclonal anti- α -SMA antibody (1:1,000; Sigma-Aldrich, UK), a monoclonal anti-vimentin antibody (1:1,000; Abcam, UK) or a monoclonal anti-desmin antibody (1:100; Abcam, UK). The secondary antibodies used were a donkey anti-mouse secondary antibody (1:250; Millipore, UK) and donkey anti-rabbit secondary antibody (1:250; Millipore, UK). Images were captured using a Zeiss LSM 510 confocal microscope. Images were quantified where applicable by using ImageJ software to assess the corrected total cell fluorescence (CTCF). The formula for CTCF is as follows:

$$CTCF = \text{integrated density} - (\text{area of cell} * \text{mean fluorescence background reading})$$

CTCF results were normalized as percentage of untreated control cells.

Collagen Gel Contraction Assay

Cell contraction assay (Cell Biolabs Inc, San Diego, California) was used as per the manufacturer's instructions as previously described.²³ Cells were treated with 10 ng/mL of TGF- β 1 before the experiments. Contraction experiments lasted over a period of 8 hours, and images were taken every hour using a digital camera (Canon Digital IXUS 55, 5.0 megapixels). Image analysis was performed using ImageJ software, measuring the surface area of the contracting collagen lattice. Contraction was calculated as percentage of the surface of the unreleased lattice. Data are shown as percentage of maximum contraction of vehicle control.

ECM Production Assay

ECM production assays were performed as previously described.²³ Cells were treated with 10 ng/mL of TGF- β 1 before the experiments. Cells were seeded at 5×10^3 cells/well onto 96-well optical flat-bottom black microplates (Nunc, Fisher Scientific, UK). After overnight attachment, they were stimulated with compounds for 7 days. DRAQ5 in PBS (1:1,000) was added, and cells were incubated for 5 minutes at 37°C, 5% CO₂, before scanning the plate to obtain nuclear staining. Cells were then lysed using ammonium hydroxide, and ECM was fixed using a solution containing 50% methanol and 7.5% acetic acid for 1 hour at –20°C. Afterward, ECM was stained with primary antibodies (collagen I, Abcam; collagen III, Millipore; collagen V, Abcam; fibronectin, Millipore) at 1:1,000 for 1 hour on a shaker, followed by incubation with secondary antibody and scanning the plate using an infrared imaging system (Odyssey CLx imager, LI-COR, UK) at both the 700 nm and 800 nm wavelengths. Results were normalized to the cell number before lysis.

RNA Isolation and Quality Assessment

Cells were seeded at 5.0×10^4 cells/well into 6-well plates (Nunc, Fisher Scientific, UK) and incubated with or without 10 ng/ml

TGF- β 1 for the indicated time points. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, UK), as per the manufacturer's instructions. RNA was resuspended in 40 μ L of water and stored at -80°C . RNA was quantified using a Nanodrop (ThermoFisher Scientific, UK), and its integrity was assessed using an Agilent Bioanalyzer (Agilent Scientific Instruments, Didcot, UK). All RNA samples had RNA integrity number values of 9 and more.

RT Reactions

RNA extracted from 2 patients was used, with RNA from 1 patient subjected to replicate RT reactions. RNA aliquots (100 ng) were reverse transcribed using 2 reverse transcriptases (SuperScript IV [Thermo Fisher Scientific, UK] and UltraScript 2.0 [qPCRBIOS, London, UK]) in 20- μ L reactions using random priming and conditions specified by the manufacturers' RT protocols on a thermocycler (G-Storm, Pickmere, Knutsford, Cheshire, UK) with the heated lid set to 112°C . For subsequent qPCR reactions, the cDNA preparations were diluted 5 times with RNase-free water.

qPCR

qPCR assays were carried out in 5- μ L reactions containing 1x SensiFast SYBR (Bioline, London, UK) master mixes, with primers at 300 nmol final concentration and 1 μ L of diluted cDNA. Thermal cyclers used were either a CFX Connect (Bio-rad, Watford, UK) or an Eco (PCRMax, Stone, Staffordshire, UK) programmed as follows: enzyme activation at 95°C for 30 seconds, followed by 40 cycles of 95°C for 2 seconds, 60°C for 2 seconds, and 72°C for 5 seconds, with fluorescence collection at 72°C . Absence of PCR inhibition was checked by using the SPUD assay to detect changes in C_q with the SPUD artificial template in the reactions containing sample DNA compared with water controls.²⁸

Analysis of qPCR Data

Standard curves were prepared for each assay using 10-fold serial dilutions of PCR amplicons, with amplification efficiencies calculated from the slopes of the dilution curves. Fold changes were calculated using the $\Delta\Delta C_q$ method, modified to include actual amplification efficiencies as recommended by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.²⁹ The statistical analyses for data sets were analyzed and graphed using Prism for Mac OS X, version 9.0 (Graphpad Software, San Diego).

Statistical Analysis

Data analysis was performed using Microsoft Excel 2013 or GraphPad Prism 7 software. Statistical significance, unless otherwise stated, was calculated using one-way analysis of variance and Student's *t*-test for unpaired means (2 sided). A *P* value less than .05 was considered statistically significant.

The differences between multiple groups in collagen contraction and time course In-Cell ELISA quantification experiments were compared using one-way analysis of variance. Student's *t*-test for unpaired means (2 sided) was used to compare the difference between 2 groups in immunocytochemistry and ICE. Before performing this calculation, F test of equality of variances was performed, to ensure that equal variance could be assumed when performing Student's *t*-test. A *P* value of $<.05$ was considered statistically significant. Experiments were performed in at least 3 independent times using samples from at least 3 patients in triplicate wells ($N = 3$). Cells derived from the same 3 patients were used in all experiments. Results from all experiments were pooled, and the mean values and standard errors of mean were used for statistical analysis.

RESULTS

PDE5i and SERM Cannot Reverse TGF- β 1-Induced Myofibroblast Marker Expression

We have previously shown that PDE5is and SERMs can prevent transformation of TA-derived fibroblasts to myofibroblasts in vitro.²³ The effect of a PDE5i (vardenafil) and SERM (tamoxifen) on myofibroblast transformation in cells that have been pretreated with TGF- β 1 for 72 hours was investigated by measuring ASMA protein expression using the ICE method. After 72 hours of TGF- β 1 treatment, the cells were treated with varying concentrations of vardenafil or tamoxifen for 72 hours to assess whether the drugs could reverse the expression of the myofibroblast marker ASMA. Figure 1A shows that an exemplar vardenafil only had a minor effect on the expression of ASMA and only at the highest concentration (1,000 μM) where a decrease in cell viability was evident. The effect of tamoxifen on ASMA expression in TGF- β 1 pretreated cells is illustrated in Figure 1B. A decrease in ASMA expression was only observed at the highest concentration of tamoxifen (54 μM), which also showed reduced cell viability. These results suggest that vardenafil or tamoxifen are not able to reverse myofibroblast transformation.

Not All Myofibroblast Functions Can Be Reversed by PDE5is and SERMs

To further investigate whether the PDE5i and SERM influence the myofibroblast functions, the effect of the drugs on the myofibroblast function was assessed. Typical myofibroblast functions include contraction and ECM formation,¹⁰ and functional assays to measure these have been described previously.²³ Cells were pretreated with TGF- β 1 for 72 hours in the experiments as shown in Figures 2 and 3. The effect of vardenafil, tamoxifen, and a TGFBR2 inhibitor (SB-505124) on production of ECM components collagen I, III, and V and fibronectin is shown in Figure 2. Neither the PDE5i nor the SB-505124 compound had any effect on ECM formation at any concentration (Figure 2A–D), whereas the tamoxifen only had an effect at the highest concentration, which has previously been shown to affect cell viability. Collagen contraction assays were

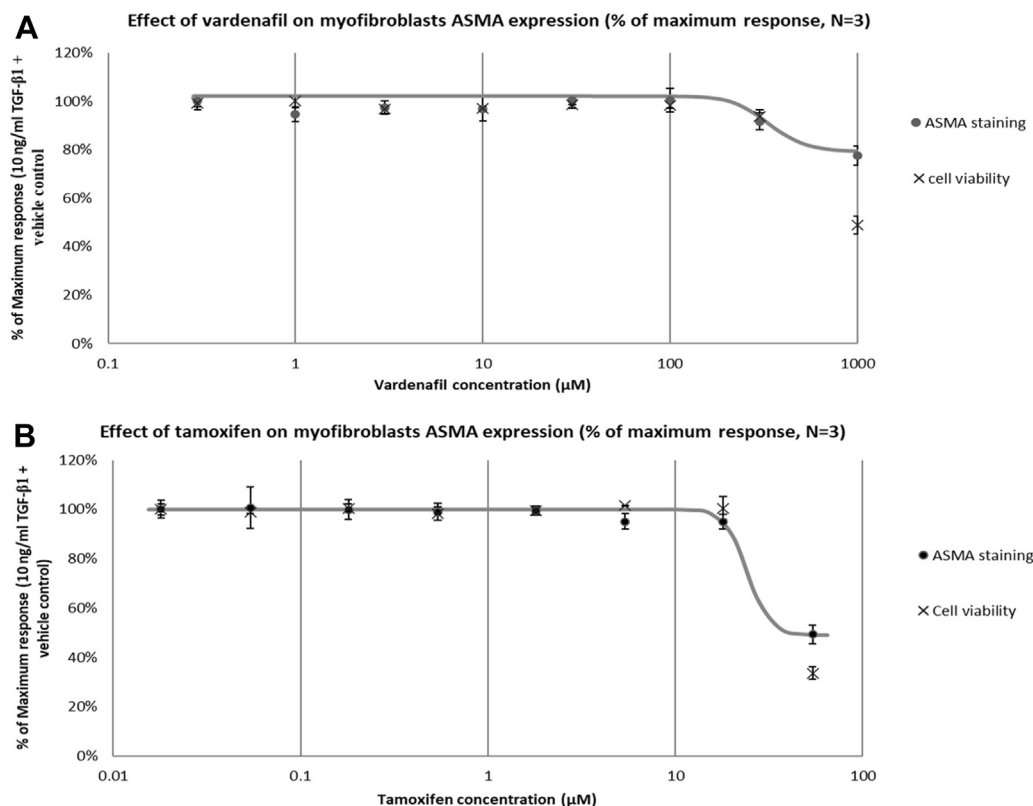


Figure 1. PDE5i and SERM cannot reverse ASMA expression after 72 hours of TGF- β 1 pretreatment of TA-derived cells. (A) Effect of vardenafil on ASMA expression. TA-derived cells were pretreated with 10 ng/mL of TGF- β 1 for 72 hours and then exposed to various concentrations (0.1–1,000 μ M) of vardenafil. (B) Effect of tamoxifen on ASMA expression. TA-derived cells were pretreated with TGF- β 1 for 72 hours and then exposed to various concentrations (0.018–54 μ M) of tamoxifen. ASMA staining was normalized to DNA staining (cell viability). Data points were plotted as average \pm SEM of ASMA/DNA staining ratio obtained from a Odyssey infra-red imager. N = 3. ASMA = alpha-smooth muscle actin; PDE5i = phosphodiesterase type 5 inhibitor; SEM = standard error of mean; SERM = selective estrogen receptor modulator; TA = tunica albuginea; TGF = transforming growth factor.

used to further assess the effect of vardenafil and tamoxifen on myofibroblast function in cells pretreated with TGF- β 1. The TGFBR2 inhibitor SB-505124 did not inhibit contraction of myofibroblast-populated collagen lattices at any concentration over the course of the experiment (Figure 3A). The same observation could be made for the vardenafil that did not decrease the contraction at any concentration over 8 hours (Figure 3B). Surprisingly, any of the tested tamoxifen concentrations (1, 3, and 10 μ M) caused a significant ($P < .05$) decrease in collagen lattice contraction over the course of 8 hours (Figure 3C).

Completed Myofibroblast Transformation Leads to Downregulation of Drug Targets

To elucidate whether this lack of antimyofibroblast activity is related to the expression of the drug targets, the expression of PDE5A and ER α and β was compared between untreated TA-derived fibroblasts and cells exposed to TGF- β 1 for 72 hours. The ICE method was used in conjunction with antibody-specific blocking peptides to quantify protein expression reliably (Figure 4), whereas ICC was used to confirm the findings

(Supplementary Figure 1). TGF- β 1 exposure caused a significant ($P < .05$) downregulation of PDE5A in TA-derived fibroblasts compared with untreated cells (Figure 4A, B; Supplementary Figure 1A–C). MCF7 cells were used as a positive control and showed expression of PDE5A, whereas the blocking peptide confirmed specificity of the antibody against PDE5A (Figure 4A, B). Expression of ER- α could not be detected on protein level in TA-derived fibroblasts that were untreated or treated with TGF- β 1 for 72 hours and could only be shown in the positive control MCF7 (Figure 4C; Supplementary Figure 1, D–F). Protein expression of ER- β was significantly downregulated ($P < .05$) in TA-derived fibroblasts after 72 hours of TGF- β 1 exposure (Figure 4C, F; Supplementary Figure 1G–I). Specificity of the antibody was ensured via the antibody-specific blocking peptide (Figure 4C). MCF7 cells were used for ICC as positive control for PDE5A and ER- β (Supplementary Figure 1J, K).

Effect of PDE5is and SERMs on Myofibroblast Transformation After Limited TGF- β 1 Exposure

To assess whether there was a specific time point between initial TGF- β 1 exposure and fully completed myofibroblast

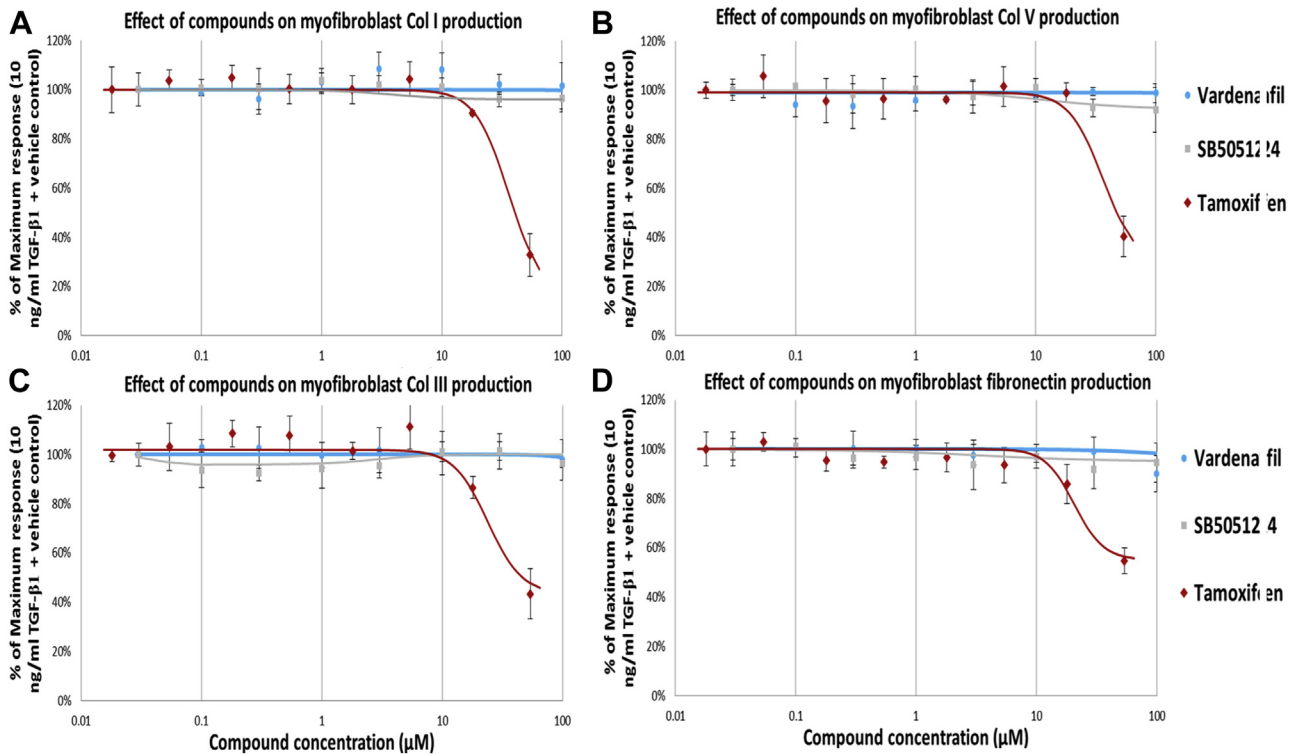


Figure 2. PDE5i and SERMs do not reduce ECM production after 72 hours of TGF- β 1 pre-treatment of TA-derived cells. TA-derived cells were pretreated with 10 ng/mL of TGF- β 1 for 72 hours and then exposed to various concentrations of vardenafil (0.03–100 μ M; blue), tamoxifen (0.018–54 μ M; red), or SB-505124 (0.03–100 μ M; gray). 8 days after treatment, cells were lysed, and ECM content was quantified. ECM proteins included Col I (A), Col V (B), Col III (C), and fibronectin (D). Protein was normalized to DNA staining before lysis. Data points were plotted as average \pm SEM of protein/DNA staining ratio obtained from a Odyssey infra-red imager. N = 3. ECM = extracellular matrix; PDE5i = phosphodiesterase type 5 inhibitor; SEM = standard error of mean; SERM = selective estrogen receptor modulator; TA = tunica albuginea; TGF = transforming growth factor. Figure 2 is available in color online at www.jsm.jsexmed.org.

transformation at which PDE5is or SERMs could affect or reverse ASMA expression, time course experiments were performed. It is known that myofibroblast transformation requires a total of 72 hours for completion.³⁰ To this end, cells were treated with TGF- β 1 for 24, 36, or 48 hours after which it was removed and replaced with one of the drugs for the rest of the 72-hour incubation. To ensure that limited exposure to TGF- β 1 would still lead to complete myofibroblast transformation, TA-derived fibroblasts were used, and TGF- β 1 was removed after 24, 36, 48, and 72 hours after which ASMA expression was quantified at the end of the 72-hour period (Supplementary Figure 2). As can be seen, there was no difference in ASMA expression between the different time points, meaning only the initial 24-hour exposure to TGF- β 1 is needed to complete full myofibroblast transformation within a 72-hour period, so cells do not need to be exposed to TGF- β 1 for the entire 72 hours.

Figure 5 depicts the effect of PDE5is on myofibroblast transformation after different TGF- β 1 exposure times. When only treated with TGF- β 1 for 24 hours, a subsequent 48-hour incubation with vardenafil caused a decrease of ASMA expression, as is evidenced by the inverse sigmoid curve with upper and lower plateau in Figure 5A. PDE5i treatment could not prevent ASMA expression after 36 or 48 hours of TGF- β 1 exposure (Figure 5B, C).

Tamoxifen could be shown to affect myofibroblast transformation after 24 and 36 hours of TGF- β 1 exposure (Figure 6A, B) but not after 48 hours (Figure 6C). The anti-myofibroblast effect seen when using 5.4, 18, and 54 μ M of tamoxifen gradually gets weaker with longer TGF- β 1 pretreatment. Although the highest concentration (54 μ M) still had some effect (9% inhibition) after 48 hours of TGF- β 1 treatment, lower concentrations did not inhibit ASMA expression anymore.

To confirm the findings, a complete time course of TGF- β 1 with SB-505124 was performed (Supplementary Figure 3). SB-505124 has previously been shown to prevent myofibroblast transformation in vitro.³¹ In our setting, SB-505124 was incapable of affecting ASMA expression in cells that were pretreated with TGF- β 1, regardless of dose or treatment time point, confirming that main response to TGF- β 1 signaling takes place within the first 24 hours.

Time Course Experiments Suggest Point of Return for Myofibroblast Transformation Is After 36 Hours

To further investigate the effect of TGF- β 1 on the expression of drug targets for PDE5is and SERMs, cells were exposed to TGF- β 1 for varying time points, and mRNA levels of specific

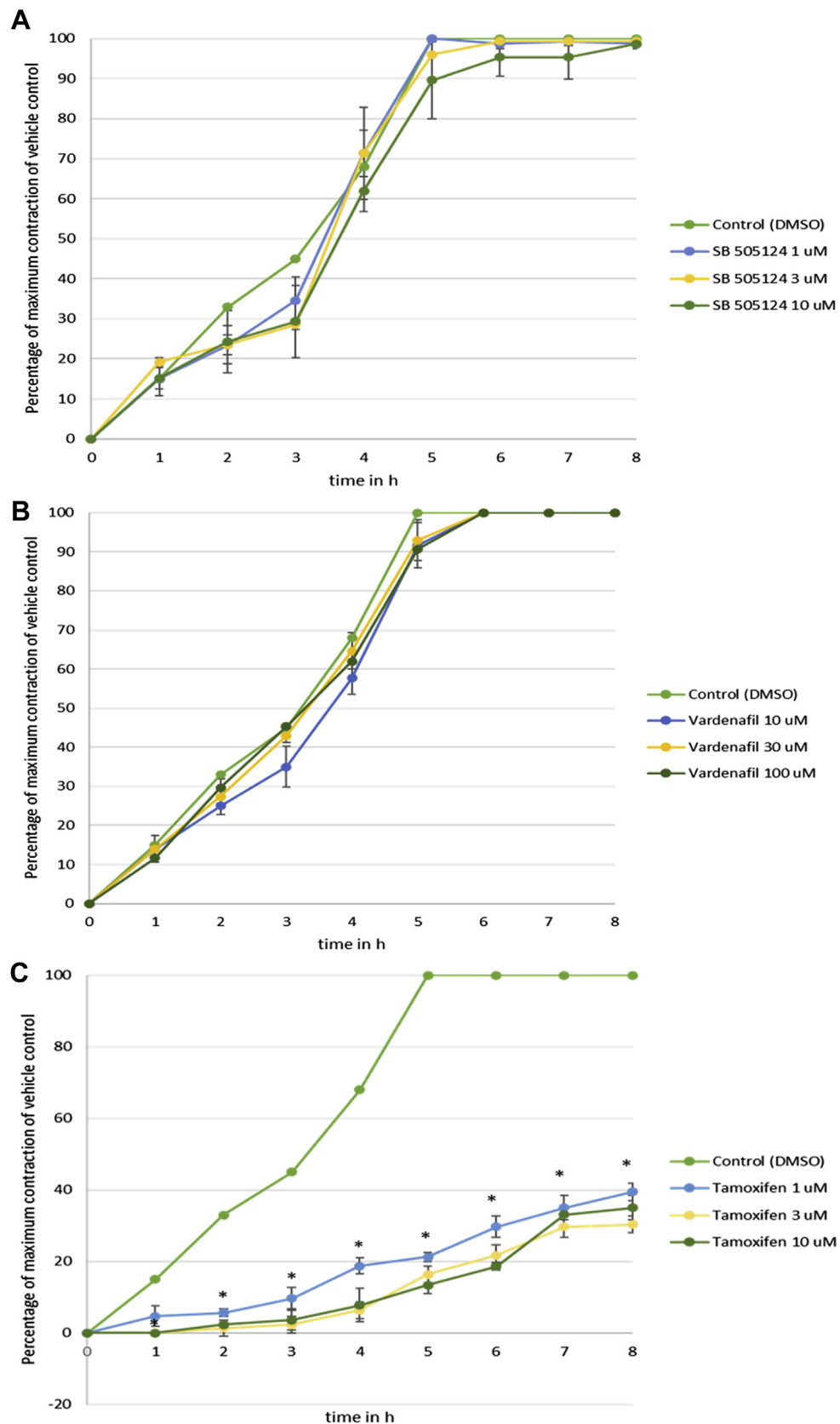


Figure 3. SERM but not PDE5i can reduce collagen contraction after 72 hours of TGF- β 1 pretreatment of TA-derived cells. TA-derived cells were pretreated with 10 ng/mL TGF- β 1 for 72 hours and then exposed to various concentrations of (A) SB-505124 (1, 3, and 10 μ M), (B) vardenafil (10, 30, and 100 μ M), (C) tamoxifen (1, 3, and 10 μ M). 3 days after treatment, collagen lattices were released from the wall of the well, and contraction was observed over an 8-hour period. Data presented as percentage of maximum collagen contraction compared with

genes were quantified and compared with those of untreated cells. GAPDH was validated as a suitable reference gene, as its expression varied little between control samples and those subjected to TGF- β 1 treatment RNA samples, as shown in [Supplementary Figure 4](#). *ACTA*, the gene for ASMA, was significantly upregulated at 24, 36, 48, and 72 hours of TGF- β 1 treatment ([Figure 7A](#)). Significant downregulation of mRNA upon TGF- β 1 treatment could be observed for *PDE5A*. The mRNA levels were significantly lower than the level of technical noise at any point of TGF- β 1 treatment ([Figure 7B](#)). Despite not being able to measure protein levels of ER- α , the mRNA levels of *ER1* were quantified. As can be seen in [Figure 7C](#), the fold change for expression ranged within the levels of technical noise for the 24- and 36-hour treatment groups, whereas the 48-hour treatment group showed significant downregulation, which returned to levels of technical noise after 72 hours ([Figure 7C](#)). *ER2*, the gene for ER- β , showed significant upregulation of mRNA 24 hours after TGF- β 1 treatment. After 36 hours of TGF- β 1 treatment, there was no significant differences compared with untreated cells (within the 3-fold level for technical noise), whereas *ER2* mRNA was significantly downregulated after 48 and 72 hours of TGF- β 1 treatment ([Figure 7D](#)).

These findings were complemented with protein quantification using ICE. ASMA, PDE5A, and ER- β were quantified after exposing cells to TGF- β 1 for 24, 36, 48, and 72 hours. While mRNA was upregulated after 24 hours, protein levels of ASMA were only significantly upregulated ($P < .05$) after 36 hours and reached their maximum after 72 hours ([Figure 7E](#)), with no significant differences between 48 and 72 hours. This suggests that its transcriptional and translational regulation is decoupled. In contrast, the protein levels of PDE5A were immediately significantly reduced ($P < .05$) after 24 hours ([Figure 7F](#)), in line with the significantly lower levels of mRNA. Longer TGF- β 1 treatment lead to further reduction, as levels after 36 hours were significantly lower than those after 24 hours, and levels after 72 hours were significantly lower than those after 36 and 48 hours. As the cells used in these experiments do not seem to express ER- α ([Figure 4](#), [Supplementary Figure 1](#)), only levels of ER- β were quantified. Protein levels of ER- β were significantly reduced ($P < .05$) after 48 and 72 hours compared with those of untreated cells, while there appeared to be a trend for reduced expression after 36 hours that did not reach significance ([Figure 7G](#)).

Gene Expression Analysis Reveals Three Additional Genes With Differential Regulation After 36 Hours

Because the protein data for ASMA, PDE5A, and ER- β suggested a significant event between 36 and 48 hours, further

profibrotic and antifibrotic signaling pathways were investigated via reverse transcription—quantitative polymerase chain reaction. GAPDH was validated as a reference gene ([Supplementary Figure 4](#)). A panel of various marker genes was interrogated ([Figure 8](#), [Supplementary Figure 5](#)). 3 genes were found to follow the pattern of 36-hour point of no return: peroxisome proliferator-activated receptor- γ (PPAR γ), transforming growth factor beta receptor III (TGFBRIII), and thrombospondin-1 (THSB-1). [Figure 8A, B](#) depict the expression of PPAR γ and TGFBRIII at various time points after TGF- β 1 exposure. Both genes were significantly downregulated after 36 hours, strengthening the case for a point of no return after 36 hours of TGF- β 1 treatment. The gene for TSP-1 (*THSB-1*) was significantly upregulated after 36 hours of TGF- β 1 treatment ([Figure 8C](#)). The other markers tested in this study were either unchanged (*BIRC5*, *GPER2*) over the entire time course or significantly upregulated (*IGF1*, *IGFBP3*) or downregulated (*GPER4*) 48 hours after TGF- β 1 insult ([Supplementary Figure 5](#)).

DISCUSSION

This study reports 2 major findings: (1) the anti-fibrotic effects of the PDE5i and SERM are dependent on the time of TGF- β 1-exposure, as longer TGF- β 1-exposure leads to a decrease in drug efficacy and (2) there is a point of no return, which occurs around 36 hours after TGF- β 1 treatment that can be delineated by upregulation and downregulation of key marker proteins and genes.

Previously, both PDE5is and SERMs have been suggested as a treatment for PD.^{32–37} We have shown that the combination of both drug classes exerted a synergistic effect in preventing myofibroblast transformation.²³ Interestingly, in our experiments, PDE5i treatment did not reduce ASMA expression in cells pretreated with TGF- β 1 for longer than 24 hours. This indicates that the drugs can inhibit myofibroblast transformation only at the start of the process and cannot reverse it. It also contradicts a previously published result, where treatment using various concentrations of vardenafil could reverse TGF- β 1-induced prostate-derived myofibroblast state.³⁸ However, that study did not quantify protein levels, and cells were treated in the same culture dish for a total of 144 hours in presence of 1 ng/mL of TGF- β 1. In contrast, our protocol included a 72-hour pretreatment period with 10 ng/ml TGF- β 1 after which cells were seeded onto appropriate plates for treatment with the PDE5i or SERM. This difference makes any comparison difficult and could explain differences in outcomes between the studies. Myofibroblast function such as contraction and ECM formation were also unaffected by PDE5i treatment, which is conclusive in

vehicle control (DMSO), data points plotted as mean \pm SEM. Statistical analysis using Student's *t*-test with $*P < .05$ vs vehicle control. N = 3. DMSO = dimethyl sulfoxide; PDE5i = phosphodiesterase type 5 inhibitor; SEM = standard error of mean; SERM = selective estrogen receptor modulator; TA = tunica albuginea; TGF = transforming growth factor. [Figure 3](#) is available in color online at www.jsm.

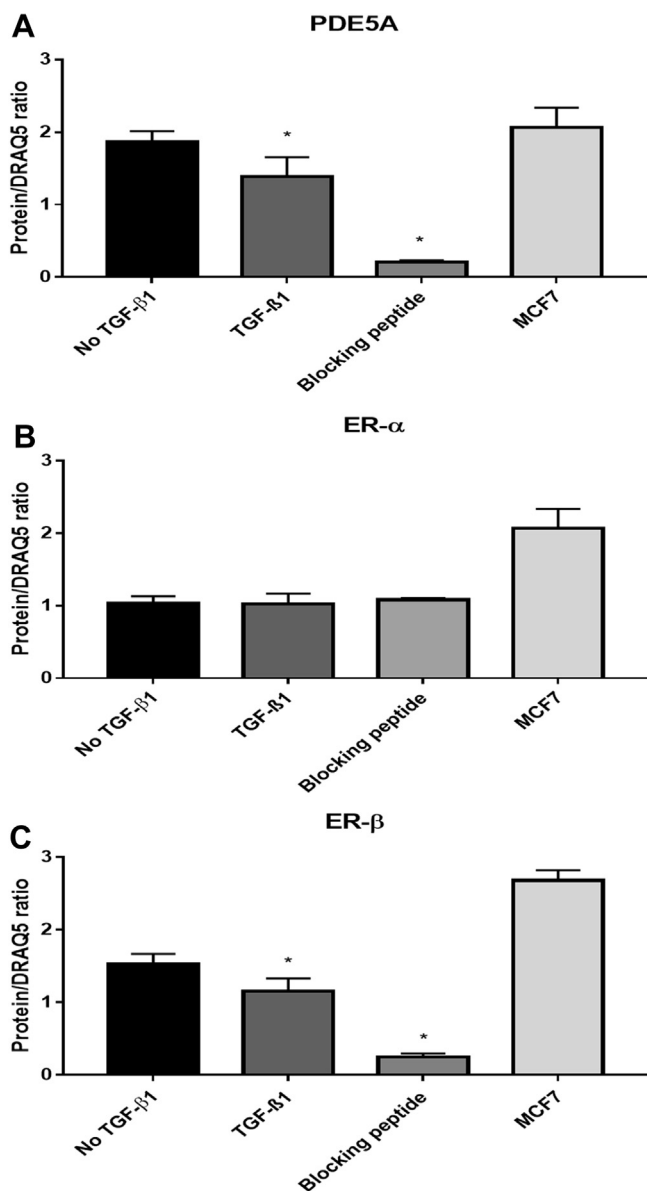


Figure 4. Quantification of PDE5A, ER- α and ER- β after 72 hours of TGF- β 1 treatment of TA-derived cells. TA-derived cells were left untreated or treated with 10 ng/mL of TGF- β 1 for 72 hours. (A) Quantification of PDE5A using ICE. Data points plotted as mean \pm SEM. Blocking peptide and positive control cell line MCF7 used for accuracy. Statistical analysis using Student's *t*-test with $*P < .05$ vs untreated. $N = 3$. (B) Quantification of ER- α using ICE. Data points plotted as mean \pm SEM. Blocking peptide and positive control cell line MCF7 used for accuracy. Statistical analysis using Student's *t*-test with $*P < .05$ vs untreated. $N = 3$. (C) Quantification of ER- β using ICE. Data points plotted as mean \pm SEM. Blocking peptide and positive control cell line MCF7 used for accuracy. Statistical analysis using student's *t*-test with $*P < .05$ vs untreated. $N = 3$. ER = estrogen receptor; ICE = In-Cell enzyme-linked immunosorbent assay; PDE5A = phosphodiesterase 5A; PDE5i = phosphodiesterase type 5 inhibitor; SEM = standard error of mean; SERM = selective estrogen receptor modulator; TA = tunica albuginea; TGF = transforming growth factor.

the context of PDE5i preventing rather than reversing myofibroblast transformation.

Previously, it has been suggested that TA-derived myofibroblast transformation can be prevented by estrogen signaling through inhibition of Smad and Rho.³⁹ Previous studies have reported SERMs as a potential antifibrotic treatment strategy with evidence gathered from in vitro and in vivo models suggesting reduction of hepatic⁴⁰ and renal⁴¹ fibrosis, while improved wound healing could be observed in skin fibrosis.⁴² Mechanistically, it has been proposed that in rats, SERMs decrease the production of TGF- β 1⁴³ and inhibit the canonical Smad signaling in mice, thereby suppressing myofibroblast transformation.⁴⁴ Furthermore, a non-Smad mechanism of action has been proposed via targeting of ERK1/2 and AP-1 transcription factor signaling in primary human skin fibroblasts.⁴⁵ Given that these suggested mechanisms all involve pathways that are critical for the induction of myofibroblast transformation but are not indispensable for the maintenance of the myofibroblast state, our observations in the time course experiments (prevention until the 36-hour mark) can be partially explained.

However, the data of the functional collagen contraction assay need a more critical review. It was unexpected to observe that tamoxifen was able to influence collagen contraction after TGF- β 1 pretreatment, in context of the potential mechanism of action (preventing rather than reversing myofibroblast transformation as outlined previously). As the other myofibroblast hallmarks of ASMA expression (an important contractile feature) and ECM production have not been reversed by SERM treatment, it is reasonable to conclude that inhibition of contraction alone is not sufficient evidence to suggest that myofibroblast transformation was reversed. We thereby hypothesize that tamoxifen affected only the contractile mechanism of the cells, without actually reversing their myofibroblast state. The literature provides examples for SERM influencing collagen contraction.^{46,47} Furthermore, using cells derived from the palmar fascia of patients with Dupuytren's disease and control patients, it could be shown that tamoxifen was able to inhibit the contraction of the cells in both, fibrotic and non-fibrotic settings.⁴⁸ Others used fibroblasts from Dupuytren's disease and carpal tunnel-affected fascia and observed a higher basal level of contraction in the diseased cells indicating higher myofibroblast baseline count.⁴⁹ This study demonstrated an inhibition of contraction after SERM treatment, which was ascribed to a decrease in TGF- β 2 expression. Mechanistically, it has been proposed that treatment with SERM leads to a change of morphology that results in a lack of contraction in cells such as fibroblasts⁴⁶ but also cancer cells.^{50,51} Mechanical inactivation of fibroblast-like cells by tamoxifen has been described to be mediated via GPER/RhoA with subsequent inactivation of YAP.⁵² Given the down-regulation of ERs after 72 hours of TGF- β 1 treatment, the

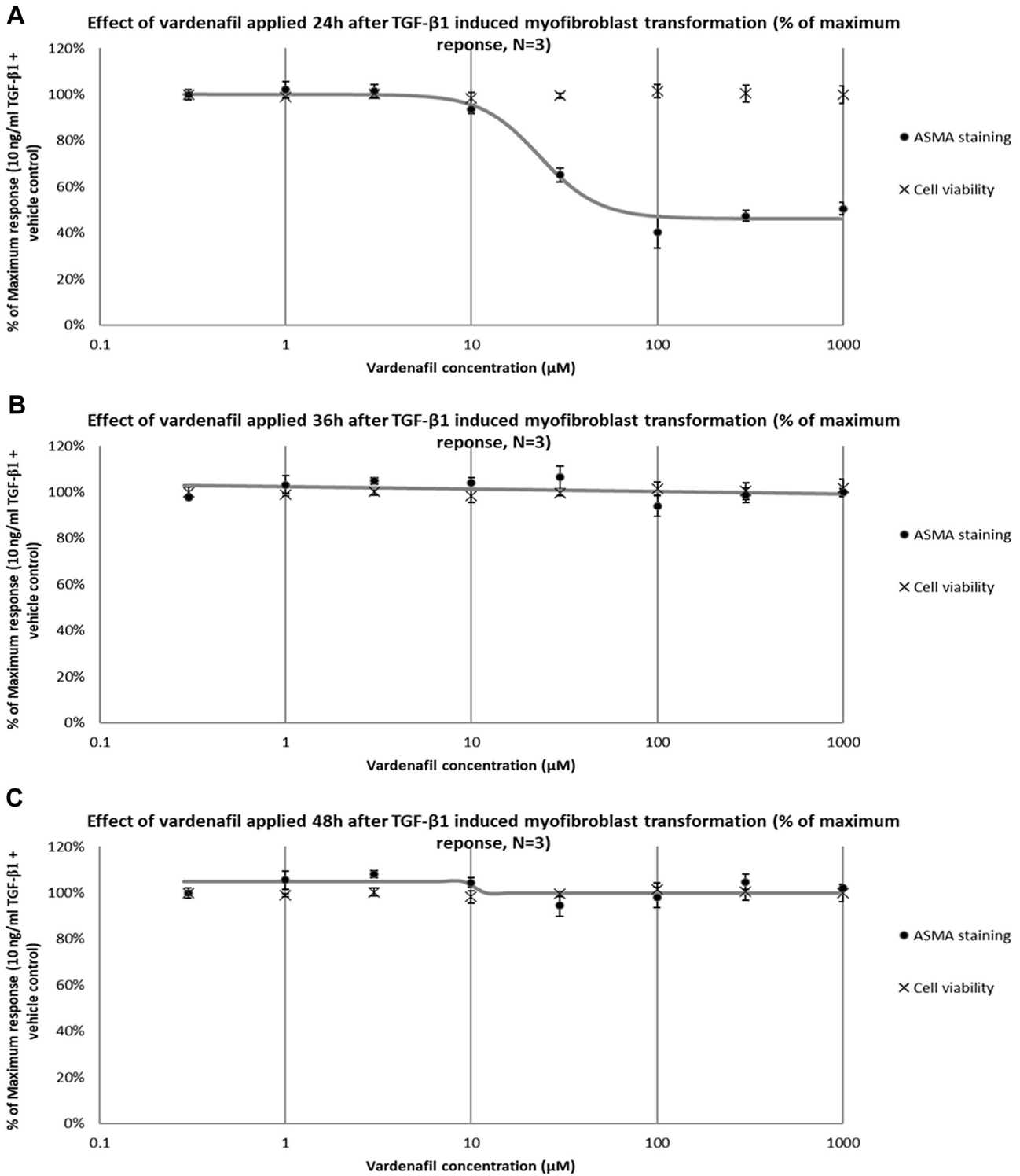


Figure 5. Effect of PDE5i at various time points after TGF- β 1 treatment. TA-derived cells were treated with TGF- β 1 for 24 hours (A), 36 hours (B), or 48 hours (C). After indicated incubation period, TGF- β 1 was removed, and cells were treated with various concentrations (0.1–1,000 μ M) of vardenafil. ASMA staining was assessed 72 hours after initial TGF- β 1 treatment. ASMA staining was normalized to DNA staining (cell viability). Data points were plotted as average \pm SEM of ASMA/DNA staining ratio obtained from a Odyssey infra-red imager. N = 3. ASMA = alpha-smooth muscle actin; PDE5i = phosphodiesterase type 5 inhibitor; SEM = standard error of mean; TA = tunica albuginea; TGF = transforming growth factor

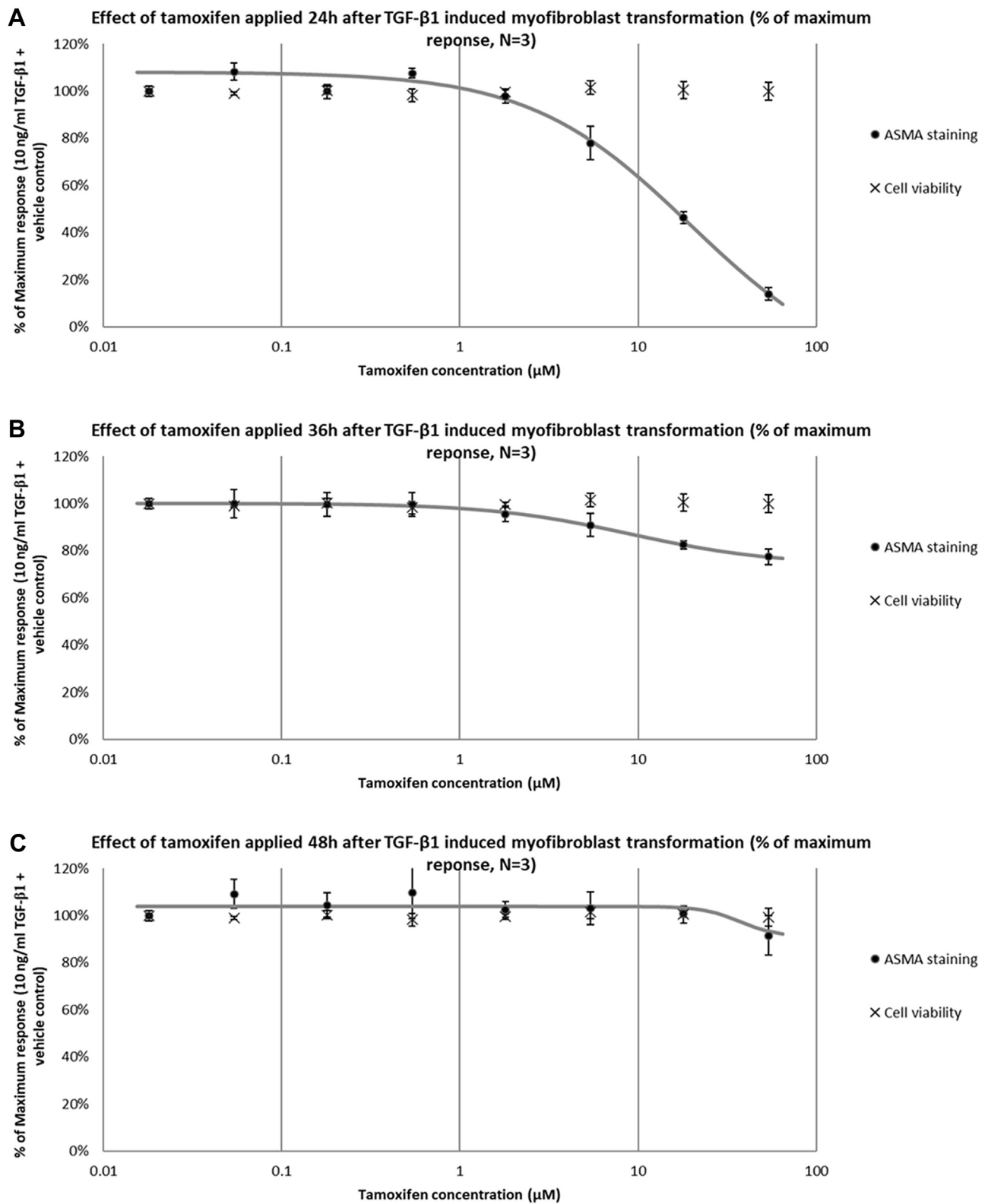


Figure 6. Effect of SERM at various time points after TGF- β 1 treatment. TA-derived cells were treated with TGF- β 1 for 24 hours (A), 36 hours (B), or 48 hours (C). After indicated incubation period, TGF- β 1 was removed, and cells were treated with various concentrations (0.018–54 μM) of tamoxifen. ASMA staining was assessed 72 hours after initial TGF- β 1 treatment. ASMA staining was normalized to DNA staining (cell viability). Data points were plotted as average \pm SEM of ASMA/DNA staining ratio obtained from Odyssey infra-red imager. N = 3. ASMA = alpha-smooth muscle actin; SEM = standard error of mean; SERM = selective estrogen receptor modulator; TA = tunica albuginea; TGF = transforming growth factor.

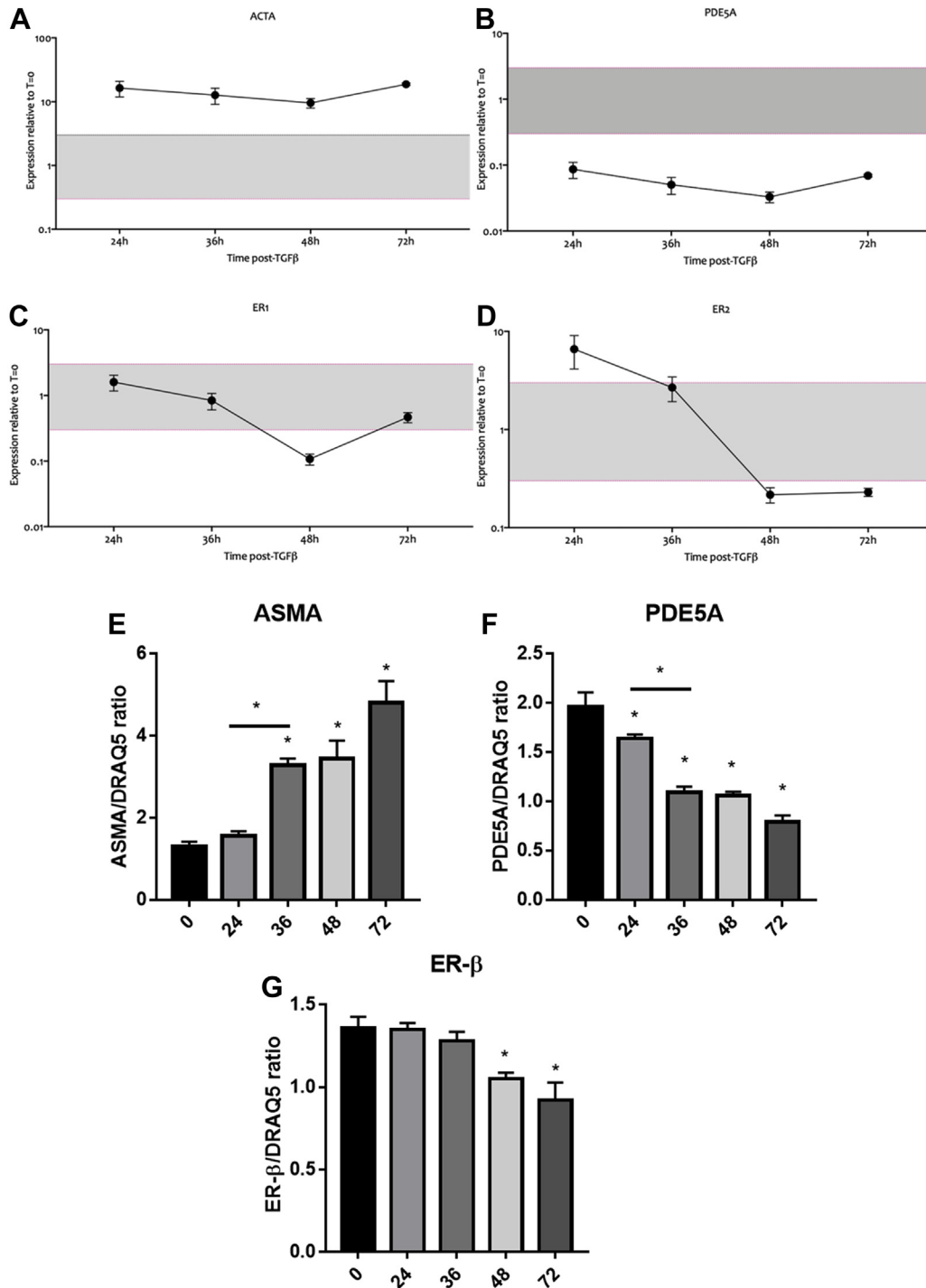


Figure 7. Effect of TGF- β 1 exposure time on mRNA and protein expression of ASMA, PDE5A, and ERs. TA-derived cells were treated with TGF- β 1 for 24, 36, 48, or 72 hours. Expression of mRNA was assessed for ACTA (A), PDE5A (B), ER1 (C), and ER2 (D). Expression patterns of genes at 24, 36, 48, and 72 hours. Results are plotted as expression fold difference compared with $t = 0$ in response to TGF- β relative to the expression of the reference gene GAPDH. Pink horizontal lines mark the 3-fold upregulation or downregulation levels, as previously suggested as limits of significance. Each time point represents the mean fold difference \pm range. Protein expression of ASMA (E), PDE5A (F), and ER- β (G) was quantified. ICE was used to determine Protein/DNA staining ratio. Data points were plotted as average \pm SEM of ASMA/DNA staining ratio obtained from Odyssey infra-red imager. Statistical analysis performed using one-way ANOVA with multiple comparisons. * $P < .05$ vs control column. # $P < .05$ vs 24 h column. $N = 3$. ANOVA = analysis of variance; ASMA = alpha-smooth muscle actin; ER = estrogen receptor; ICE = In-Cell enzyme-linked immunosorbent assay; PDE5 = phosphodiesterase type 5; PDE5A = phosphodiesterase 5A; SEM = standard error of mean; SERM = selective estrogen receptor modulator; TA = tunica albuginea; TGF = transforming growth factor. Figure 7 is available in color online at www.jsm.jsexmed.org.

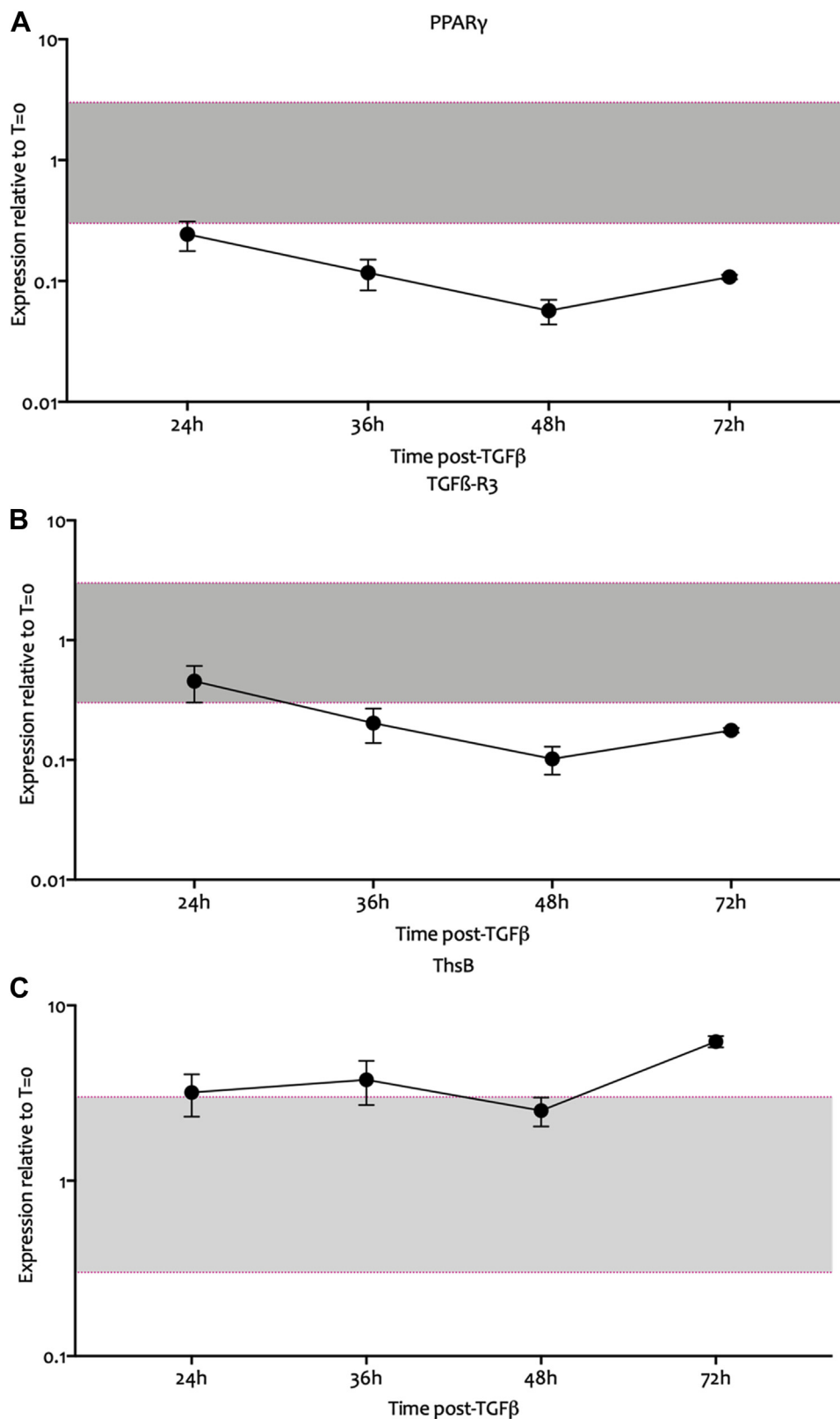


Figure 8. Effect of TGF- β 1 exposure on various target genes. Expression patterns of PPAR γ (A), TGF β -R3 (B), and THSB (C) at 24, 36, 48, and 72 hours. Results are plotted as expression fold difference compared with t = 0 in response to TGF- β relative to the expression of the reference gene GAPDH. Pink horizontal lines mark the 3-fold upregulation or downregulation levels, as previously suggested as limits of significance. Each time point represents the mean fold difference \pm range. PPAR γ = peroxisome proliferator-activated receptor- γ ; TGF = transforming growth factor; THSB = thrombospondin. Figure 8 is available in color online at www.jsm.jsexmed.org.

possibility of an additional, ER-independent or GPER-driven mechanism of SERM that mainly affects cell morphology, cytoskeletal makeup (rearrangement of intermediate and actin filaments/stress fibers⁵⁰), or mechanosensing of the cells to prevent contraction cannot be excluded.

Collectively, the data point toward PDE5is and SERMs only preventing but not reversing TGF- β 1-induced myofibroblast transformation of TA-derived cells in vitro. To investigate potential underlying reasons for this effect, the expression of drug targets was quantified in a TGF- β 1-dependent and time-dependent manner. We hypothesized that exposure to TGF- β 1 would lead to a downregulation of PDE5A and ER α and β , which would prevent the drugs from exerting their antifibrotic action owing to a lack of drug target availability.

Our results demonstrate that TGF- β 1 induces a significant downregulation of PDE5A on mRNA and protein level after 72 hours of TGF- β 1 treatment, which may explain the loss of PDE5i efficacy caused by lack of target expression after TGF- β 1 exposure. Interestingly, while the mRNA downregulation is already apparent after 24 hours, the most significant downregulation of PDE5A protein appears after 36 hours, which coincides with a more significant upregulation of ASMA protein levels. Expression of PDE isoforms PDE4 and PDE5 could previously be determined in fibroblast cultures established from human PD plaques, normal TA, and rat TA.⁵³ Our data are in conflict with those of a previous study conducted by Zenzmaier et al³⁸ that examined the consequences of lentivirus-mediated shRNA knockdown of PDE5 in prostate-derived fibroblasts. That study reported that loss of PDE5 lead to a reversal of the myofibroblast state, whereas we consider the loss of PDE5 as a marker for myofibroblast transformation. Expression of PDE5 has been reported in both rat and human fibroblasts where it was shown that cells at higher passage, which are more susceptible to spontaneous myofibroblast transformation, show a decreased PDE5 expression.⁵⁴ Furthermore, one study showed that TGF- β 1 treatment lead to a significant downregulation of PDE5A protein expression in HFL-1 cells (roughly 50% reduction),⁵⁵ which corroborates our expression results and thereby implies the loss of PDE5i efficacy after TGF- β 1 pretreatment is caused by lack of target expression.

While we can detect expression of ER- α and ER- β mRNA in our primary fibroblasts, only ER- β is expressed at the protein level. This narrows down the potential drug target of SERMs in TA-derived fibroblasts. Protein expression of ER- α could only be demonstrated in MCF7 (a breast cancer cell line isolated from a Caucasian woman) cells that have been reported to express ER- α mRNA and protein.⁵⁶ This is in line with previous studies that showed expression of ER- β only in adult human mammary fibroblasts in absence of ER- α .⁵⁷ Exposure to TGF- β 1 for 72 hours leads to a significant downregulation of ER- β at the protein level. 24 hours of TGF- β 1 lead to a significant upregulation of ER- β mRNA that did not translate to protein level but could be an attempt of the cell to compensate for or counteract the

TGF- β 1 signaling. During the time course of TGF- β 1 treatment, it was indicated that expression of the receptor was significantly downregulated after 48 hours both on mRNA and protein level, which explains why tamoxifen still had a minor effect on myofibroblast transformation after 36 hours of TGF- β 1 exposure.

Collectively, this supports the hypothesis of TGF- β 1-mediated downregulation of the drug targets as the main reason for PDE5is and SERMs being unable to reverse full myofibroblast transformation that is reinforced by the observations of the drugs being able to prevent myofibroblast transformation until the expression of the drug targets is too low (after 36 hours for PDE5is and 48 hours for SERMs). Furthermore, the lack of ER- α expression suggests that the antifibrotic effect of SERMs may be mediated via ER- β -related signaling, a notion supported by evidence in breast cancer in general⁵⁸ and reports the tamoxifen response in ER- α -negative cancer being ER- β dependent.⁵⁹ In turn, other reports suggest that tamoxifen acts through other intracellular signaling cascades, independent of ER-mediated signaling, such as Smad, ERK,^{44,45} or the suppression of TGF- β 1 transcription.⁴³ Studies into the precise mechanism of action are needed to resolve which signaling cascade is responsible for the antifibrotic effect of SERM in TA-derived cells.

The results of our study may have clinical implications, as they suggest that treatment with PDE5is or SERMs may be useful in patients with early-stage PD and that fully formed plaques are unlikely to be affected by either treatment, as myofibroblast elimination or reversal cannot be achieved. Fibrotic conditions such as PD are characterized by their relatively slow progression, which poses an inherent issue for early treatment. However, reports suggest that patients do present at earlier stages (within the first 3 months) and not just with fully formed plaques.^{60–62}

The significant downregulation of key antifibrotic proteins and upregulation of myofibroblast markers at 36 hours lead us to use reverse transcription-quantitative polymerase chain reaction to investigate the expression of additional genes involved in the fibrotic process. In doing so, our study is the first one to try and establish a point of no return in TGF- β 1-induced myofibroblast transformation.

We analyzed a panel of genes that are involved in the fibrotic process and that were differentially regulated after TGF- β 1 treatment. Within this panel of profibrotic and antifibrotic genes, we observed genes that were unchanged (BIRC5, GPER2), immediately strongly upregulated (IGF1, IGFBP3), and downregulated after 48 hours. In addition, we observed that 3 genes had a differential expression pattern after 36 hours: PPAR γ , TGFBR1, and THSB-1 that followed the pattern of point of no return we have described previously. These 3 genes have strong evidence of influence in the fibrotic process. We have shown that PPAR γ and TGFBR1 mRNA levels were significantly decreased 36 hours after TGF- β 1 exposure. Decreased PPAR γ levels have been suggested as a potential biomarker in skin

fibrosis,⁶³ whereas the antifibrotic effects of L-carnitine, a drug suggested as treatment for PD,⁶⁴ are derived from its ability to upregulate PPAR γ .⁶⁵ Furthermore, PPAR γ agonists have been demonstrated to decrease fibrotic responses by opposing TGF- β 1 profibrotic signaling (including myofibroblast differentiation)⁶⁶ suggesting a vital role of PPAR γ in the fibrotic process. TGFBR3 has been shown to be downregulated in fibrotic tissue, whereas its upregulation has been proposed as an antifibrotic strategy.⁶⁷ This underlines the significance of the downregulation of the receptor after 36 hours of TGF- β 1 exposure, as TGFBR3 can act as an accessory coreceptor to modulate TGF- β 1 signaling.⁶⁸ Our results showed a significant upregulation of THSB-1 mRNA 36 hours after TGF- β 1 exposure. THSB-1 perpetuates fibrotic signaling by activating latent TGF- β 1 and has therefore been proposed as a target for antifibrotic therapy,^{69,70} and its upregulation has been reported to predict hypoxia as well as fibrosis.^{71,72} In penile tissues, THSB-1 upregulation was attributed to fibrotic changes observed in patients with erectile dysfunction after undergoing radical prostatectomy.⁷³ The upregulation of this gene is therefore an important event in the development of fibrosis, and alongside, the downregulation of antifibrotic genes at the same time point further underlines the importance of the 36-hour mark outlined in this manuscript. Defining what leads to this key event that promotes profibrotic genes and at the same inhibits antifibrotic genes after 36 hours might be a novel target to tackle not only PD but fibrosis in general.

Given that fibrosis has a complex pathology with multiple genes and pathways involved, it will be necessary to confirm this proposed point of no return by obtaining more complete transcriptomic and proteomic data. This should result in the establishment of gene signatures that will help reveal the full mechanism underlying myofibroblast transformation and lead to the discovery of new druggable targets. We acknowledge this lack of high-volume experimentation as one of the limitations of this study, along with the lack of confirmation on protein level. Furthermore, this study is limited by only testing 1 exemplar PDE5i and SERM and not investigating the effect the drugs might have on the expression of their respective targets. In addition, it is evident that all in vitro models are inherently limited in fully representing the complexity and physiology of fibrosis. Ideally, the data would be supported by both, experimentation in 3-dimensional multicellular models using multi-cytokine insults and in vivo experiments.

CONCLUSIONS

This study suggests that PDE5is and SERMs cannot reverse already formed fibrosis and should therefore only be used in early-stage patients with PD to obtain an antifibrotic effect. We also demonstrate a point of no return for myofibroblast transformation after 36 hours of TGF- β 1 treatment. It is worth investigating whether this point of no return can be a viable novel target for further drug development endeavors, as opposed

to only preventing formation of or reversing fully formed fibrosis. Our data further demonstrate the need to design clinical trials that take the mechanism of action of the drug into account, as PDE5is and tamoxifen have only been shown to be successful in the trials that recruited patients with early-phase PD, which as these data demonstrate will respond better to the drugs.

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STATEMENT OF AUTHORSHIP

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jsxm.2020.06.022>.