

Anti-fibrotic synergy between PDE5 inhibitors and selective oestrogen receptor modulators in Peyronie's disease models

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ABSTRACT:

Background:

Peyronie's disease (PD) is a fibrotic disorder of the penile tunica albuginea (TA), characterized by the formation of a localized fibrous plaque which can lead to deformity and erectile dysfunction. Non-surgical therapeutic options for PD are limited in efficacy and safety. Myofibroblasts are key cells in the pathogenesis of PD and the inhibition of myofibroblast transformation has been suggested as a therapeutic option.

Objective:

To identify potential drugs using a novel phenotypic assay and then to test them in *in vitro* and *in vivo* models of Peyronie's disease.

Design, Setting, and Participants:

We have developed and validated a phenotypic screening assay that measures myofibroblast transformation, by which we tested 21 compounds suggested to be efficacious in treating PD. The successful hits from this assay were further tested *in vitro* and *in vivo* models of PD.

Results and Limitation:

The new assay was able to detect TGF- β 1-induced myofibroblast transformation. Using this assay, phosphodiesterase type 5 inhibitors (PDE5i) and selective oestrogen receptor modulators (SERMs) were identified as significantly inhibiting myofibroblast transformation. A PDE5i (vardenafil) and a SERM (tamoxifen) inhibited myofibroblasts transformation, collagen gel contraction and extracellular matrix production in a synergistic fashion. In a rat model of PD, the anti-fibrotic effect of the combination of vardenafil and tamoxifen was greater than each drug alone. The study is limited by not providing a molecular mechanism for the proposed synergy.

Conclusions:

This is the first demonstration of a synergistic activity between a PDE5i and a SERM discovered through a phenotypic screening approach. Future clinical trials using a combination of these

drugs should be considered during the active phase of PD, given the early evidence of benefit in both *in vitro* and *in vivo* models.

Patient Summary:

This report suggests that the combination of a PDE5i and a SERM may be efficacious in treating PD in its active phase.

Key words:

Peyronie's disease, fibroblast, myofibroblast, fibrosis, fibroproliferative, PDE5 inhibitor, oestrogen receptor modulator, tunica albuginea, phenotypic screening

Take Home Messages:

We have developed a novel phenotypic screening assay measuring myofibroblast transformation and tested 21 compounds. Only PDE5i and SERMs showed significant effect. The drugs exerted a synergistic effect *in vitro* and *in vivo* suggesting that their combination may be clinically efficacious in the active phase of Peyronie's disease.

INTRODUCTION:

Peyronie's disease is a fibrotic disorder characterized by the formation of a fibrous plaque in the connective tissue surrounding the penile erectile tissue, the tunica albuginea (TA). It is a benign condition of unknown aetiology characterized by the formation of localized fibrous plaques, resulting in a penile deformity, manifesting as a curvature, indentation or shortening during erection. The disease has been shown to be prevalent, especially as men get older¹, and affects quality of life, principally through pain during erection, erectile dysfunction, loss of penetrative ability during intercourse and associated psychological stress^{2,3}. Despite the progress in understanding the pathophysiology of PD, there is currently a lack of efficacious medical therapies for PD, with surgery or collagenase injections⁴ being the main treatment options. Although the surgical outcomes following penile straightening are well documented, surgery is invasive, costly and is frequently detrimental to penile size and erectile function⁵.

Myofibroblasts have the features of both fibroblasts and smooth muscle cells and are characterized by the presence of alpha-smooth muscle actin (α -SMA) positive cytoplasmic fibres. These actin fibres contribute to the contractile ability of these cells⁶. Although various progenitor cells for myofibroblasts have been suggested⁷, they most often differentiate from locally residing fibroblasts through normal wound healing signalling, particularly, transforming growth factor- β 1 (TGF- β 1)⁸. Myofibroblasts have been shown to play vital and ubiquitous roles in both normal wound healing and fibrosis. Most important functions include production and remodelling of extracellular matrix (ECM) protein^{6,9}, and the secretion of pro-fibrotic and pro-inflammatory cytokines⁹. These cells have been shown to be present in liver¹⁰, lung¹¹ and kidney¹² fibrosis as well as PD plaques^{13,14}. It is therefore generally agreed that myofibroblasts play a critical role in the pathophysiology of fibrosis. Since the inhibition of myofibroblast transformation has been shown to be effective in preventing fibrosis¹⁵, we aimed to identify if compounds that are suggested for the treatment of PD can inhibit myofibroblast formation using a phenotypic screening assay. Here we report the development of such a phenotypic screening assay through which we identified two classes

of drugs, phosphodiesterase type 5 inhibitors (PDE5i) and selective oestrogen receptor modulators (SERM) that were shown to synergize in both *in vitro* and *in vivo* studies.

We first developed a phenotypic screening assay which is capable of reproducibly measuring transformation of human primary TA-derived fibroblasts to myofibroblasts in high throughput format. We then tested 21 compounds/drugs that have been suggested to efficacious in *in vitro*, *in vivo* animal, and human studies of PD. We identified two classes of drugs, PDE5i and SERMs, from that screen and further tested them alone or in combination in *in vitro* and *in vivo* models of PD.

METHODS:

Acquisition of tunica albuginea samples and isolation of fibroblasts

Tunica albuginea tissue samples were collected from patients undergoing surgery at University College London Hospital (UCLH), London, UK for penile cancer or PD. All patients gave fully informed written consent to the study. The study was approved by independent research ethics committees (NRES Committee East of England 12/EE/0170 and NRES Committee North of Scotland 15/NS/0051). PD plaque tissue was obtained from patients with chronic PD undergoing a Lue procedure (plaque incision and grafting). Plaque tissue would have otherwise been discarded. Non-plaque TA was obtained from PD patients undergoing a Nesbit procedure whereby non-fibrotic TA tissue was excised from the opposite side of the plaque. TA tissue samples from patients with penile cancer were taken from the proximal side away from the tumour, with the tumour showing negative margins on histological examination.

Tissue samples were carefully dissected to ensure that all cavernosal tissue was removed from the tunica albuginea (TA). To establish fibroblast cultures, TA fragments were seeded in 6-well tissue culture plates (Nunc, Fisher Scientific, UK) as described previously¹⁶. The tissue pieces were incubated in DMEM-F12 (GIBCO, Invitrogen, UK) containing 10% FCS (Fisher Scientific, UK) and 1% penicillin-streptomycin (GIBCO, Invitrogen, UK) at 37°C, 5% CO₂ for 5-7 days. Tissue fragments were carefully removed using forceps upon outgrowth of cells. Passages 2 to 4 were used for the rest of the experiments.

In cell ELISA (ICE)

Cells were seeded onto 96 well optical flat bottom black microplates (Nunc, Fisher Scientific, UK) at 5×10^3 cells per well. After overnight attachment they were incubated with or without 10 ng/ml TGF- β 1 for 72 hours. The cells were then fixed using 4% paraformaldehyde, and blocked with 10% donkey serum and 0.1% Triton X-100 in PBS. The cells were then incubated with anti- α -SMA antibody (1:3,000; Sigma Aldrich, UK) for 2h. Afterwards cells were incubated with donkey anti-mouse secondary antibody conjugated to an infrared dye which emits at 800 nm (1:500; IRdye 800CW; Li-Cor, UK) and a nuclear counterstain at that emits at 700 nm (1:1,000; DRAQ5, Biostatus, UK) for 1h. The plate was scanned using an infrared imaging system (Odyssey CLx imager, LI-COR, UK) at both the 700 nm and 800 nm wavelengths.

Collagen gel contraction assay

Cell Contraction Assay (Cell Biolabs Inc, CBA-201) was used according to the manufacturer's instructions. Briefly, 10,000 cells/well were mixed with collagen solution and DMEM with or without 10 ng/ml TGF- β 1 and then plated into 96 well plates. Cultures were incubated for 3 days at 37 °C, 5% CO₂ and lattices were released from the walls of the wells using a sterile spatula or needle. Contraction of the collagen lattices was observed for 8h and documented using a digital camera (Canon Digital IXUS 55, 5.0 mega pixels). Images were analysed using ImageJ software by measuring the surface area of the contracting lattice. Contraction was calculated as percentage of the surface of the unreleased lattice. Data is shown as percentage of maximum contraction of vehicle control.

ECM production assay

Cells were seeded onto 96 well optical flat bottom black microplates (Nunc, Fisher Scientific, UK) at 5×10^3 cells per well. After overnight attachment, they were stimulated with or without 10 ng/ml TGF- β 1 and/or compounds for 7 days. DRAQ5 in PBS (1:1,000) was added and cells were incubated for 5 min at 37 °C, 5% CO₂ before scanning the plate to obtain nuclear staining. Cells were then lysed using ammonium hydroxide as described previously¹⁷ and ECM was fixed using a solution containing 50% methanol and 7.5% acetic acid for 1h at -20°C. Afterwards ECM was stained either with Coomassie Blue (total ECM) overnight at 4°C or with primary antibodies (collagen I, abcam; collagen III, Millipore; collagen V, abcam; fibronectin, Millipore) at 1:1000 for 1h 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.

Animal treatment

The animal model for PD was first described by El-Sakka and Lue¹⁸ and modified by Bivalacqua and Hellstrom¹⁹. Male Sprague-Dawley rats (10-12 weeks old) were housed in a regulated environment with a 12-hour light/dark cycle in a standard experimental laboratory. The animals had free access to food and water *ad libitum*. Fifty male Sprague-Dawley rats were divided into 5 groups: A: sham (injection of vehicle citrate buffer), B: TGF- β 1 injection (1 μ g in 100 μ l citrate buffer (10mM, Sigma-Aldrich, UK)), C: TGF- β 1 injection + tamoxifen (5 mg/kg/day; i.p.), D: TGF- β 1 injection + vardenafil (1.5 mg/kg/day; drinking water), E: TGF- β 1 injection + tamoxifen + vardenafil. Treatment was initiated at the day after injury and continued for 5 weeks followed by a 48 hours wash-out period.

Assessment of erectile function

At the end of wash off period, under ketamine (100 mg/kg) and xylazine (10 mg/kg) anaesthesia, the major pelvic ganglion (MPG) and cavernous nerve (CN) were exposed bilaterally via midline laparotomy. A 25 G butterfly needle, filled with 250 U/ml heparin solution, was inserted into the proximal left corpus cavernosum and connected to a pressure transducer for ICP measurement. The ICP was recorded at a rate of 25 samples per second. A bipolar stainless-steel hook electrode was used to stimulate the CN directly via a signal generator and custom-built constant-current amplifier generating monophasic rectangular pulses with stimuli of 5, 7.5, 10, and 15V. Depending on the anatomical positioning and accessibility of the nerve, the stimulations were performed on either the left or right MPG/CN. The maximal amplitude of ICP during nerve electrostimulation was calculated from baseline value and included for statistical analysis in each animal.

Systemic blood pressure was recorded by inserting a PE-50 polyethylene tubing into the right common carotid artery. After functional testing, animals were euthanized by cervical dislocation. Following these measurements, the penis was harvested for histological, molecular and transcriptional analysis.

qPCR

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used according to the manufacturer's instructions to transcribe RNA to cDNA. One µg in 10 µl were added to 10 µl of the master mix for reverse transcription for a total reaction volume of 20 µl. After reverse transcription the cDNA was diluted 1:10 for qPCR. The Applied Biosystems™ TaqMan™ Fast Advanced Master Mix was used for qPCR. Gene specific primer pairs for GAPDH, beta-actin, alpha smooth muscle actin, elastin, and collagens I, III, and V were purchased from Applied Biosystems. StepOnePlus™ Real-Time PCR System and software were used for the experiments and data analysis. Data was analysed using the $2^{-\Delta\Delta C_t}$ method for relative quantifications.

Western Blot

20-30 µg of protein was mixed 1:1 with 2x laemmli buffer (Bio-Rad) under reducing or non-reducing conditions and heat denatured at 95°C for 4 minutes. Samples were loaded onto an Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad) along with 5 µl of a protein ladder (Bio-Rad). After the gel electrophoresis the transfer onto a methanol activated PVDF membrane (Bio-Rad) was achieved by wet blotting for 1 hr at 350 mA. Membranes were washed before blocking the unspecific binding with 10% (w/v) non-fat dried milk (NFD, Marvel) in 0.1% TBS-T for 1h. Primary antibodies were diluted in 5% NFD in 0.1% TBS-T and incubated O/N at 4°C on a shaker. Subsequently membranes were washed 4x with 0.1% TBS-T and blocked again. After this, the secondary antibodies were added in a dilution of 1:3,000 in 5% NFD with 0.1% TBS-T and incubated for 1h on a shaker in the dark. Four minutes washes with 0.1% TBS-T for 5 minutes were followed with a 5 minutes incubation of an enhancer solution (Supersignal west dura, Thermo Fisher). Blots were visualized using a Syngene documentation system and Genesys Software.

Statistical Analysis

Data analysis was performed using Microsoft Excel 2013 or GraphPad Prism 7 software. The differences in between multiple groups in *in vivo* RT-qPCR and Western blot quantification experiments were compared using analysis of variance, one way ANOVA. The differences in between multiple groups in ICP/MAP and the *in vitro* ICE synergy experiments were compared using analysis of variance, two way ANOVA. Student's t-test for unpaired means (two-sided) was used to compare the difference between two groups in *in vitro* ICC and RT-qPCR experiments. Prior to performing this calculation, F-test of equality of variances was

performed, to ensure equal variance could be assumed when performing Student's t-test. A P value less than 0.05 was considered statistically significant. All *in vitro* experiments were performed in at least triplicate of three experiments (n=9) using samples from 3 patients (N=3). Results from the nine experiments were pooled and the mean values and standard errors of mean were used for statistical analysis. 8 rats were used in each group in *in vivo* experiments. Results from the rats were pooled in each group and the mean values and standard errors of mean were used for statistical analysis.

Z-factor (Z') was used to measure the statistical effect size of TGF- β 1-induced myofibroblast transformation, to evaluate its reproducibility, variability and potential use as a high throughput screening (HTS) assay. The formula for Z' factor is given in Supplementary methods.

RESULTS:

Development and validation of the phenotypic assay:

We have isolated primary fibroblasts from the plaque and non-plaque TA of patients with PD. We also isolated primary fibroblasts from TA of patients with penile cancer as non-fibrotic controls (Suppl Figure 1). The primary fibroblasts were similar in morphology and function (as shown by their response to TGF- β 1) in all three groups: fibroblasts derived from plaque of PD patients, fibroblasts derived from non-plaque TA of PD patients and fibroblasts derived from TA of patients with penile cancer. Based on this similarity, we have utilised fibroblasts derived from non-plaque TA of PD patients throughout this manuscript since they would be more representative of fibroblasts that have not been exposed to pro-fibrotic environment. The fibroblast identity of the TA-derived cells was validated (Suppl Figure 2).

Upon exposure to TGF- β 1 (10 ng/ml for 72 hrs), we observed a significant 8-fold increase in α -SMA expression in both mRNA and protein levels in TA-derived fibroblasts (Figure 1). We then developed a phenotypic screening assay in a 96-well plate format using in-cell ELISA (ICE) where the cell viability and α -SMA protein expression can be simultaneously measured in a reproducible manner (Z'=0.89; Figure 1). The assay was further validated using vehicle control and a TGF- β 1 receptor antagonist (SB505124) where the cells remained viable in up to 1% DMSO and SB505124 inhibited TGF- β 1-induced myofibroblasts transformation in a concentration-dependent manner (IC50=0.6 μ M; Suppl Figure 3).

Hit identification:

We then tested twenty-one compounds/drugs (Suppl. Table 1) which have been suggested to be efficacious in PD based on *in vitro* and *in vivo* studies and/or early-phase clinical studies. Out of these 21 drugs only 2 classes, selective oestrogen receptor modulators (SERMs) and PDE5 inhibitors (PDE5i), showed significant inhibition on myofibroblast transformation. Full concentration response curves were constructed for these hits, which yielded inverse sigmoid curves with an upper and lower plateau without affecting the cell viability (Figure 2). The following molecules were used to investigate the two classes of drugs: vardenafil, sildenafil and tadalafil as PDE5i (IC_{50} = 30 μ M, 15 μ M and 3.5 μ M respectively) and tamoxifen and raloxifene as SERMs (IC_{50} = 11.9 μ M and 7 μ M respectively). When a PDE5i, vardenafil and a SERM, tamoxifen were tested in combination, a synergy between the two drugs became apparent (the observed inhibition was greater than the arithmetic sum of each) (Figure 2, Suppl. table 2).

Functional assays:

The two classes of drugs were then tested in functional assays. Firstly, we tested their efficacy in inhibiting collagen gel contraction as a measure of myofibroblast contractility; a characteristic function of myofibroblasts, which separates them from fibroblasts. Collagen gels were loaded with fibroblasts and contraction was measured after stimulation with TGF- β 1 as described before^{20,21}. Both vardenafil and tamoxifen inhibited TGF- β 1-induced contraction of collagen at concentrations of 10 μ M and 1 μ M respectively (Figure 3). Secondly, we tested the drugs' efficacy in inhibiting ECM protein production, which is again one of the critical characteristic functions of myofibroblasts. Again, both vardenafil and tamoxifen inhibited TGF- β 1-induced ECM protein production (collagens I, III, V and fibronectin) (IC_{50} =23 μ M, 17 μ M, 23 μ M, and 44 μ M for vardenafil and 7 μ M, 5.3 μ M, 6.7 μ M and 1.6 μ M for tamoxifen, respectively; Figure 4).

***In vivo* testing of the two hits:**

To elucidate whether the drugs could also prevent fibrosis *in vivo*, they were taken further to be tested in an animal model for PD. Five weeks after TGF- β 1 injection into the rat TA, with or without treatment with vardenafil, tamoxifen or their combination, the rats were subjected to erectile function measurement (intracavernous pressure measurement; ICP) before harvesting the penis for molecular analysis. The ICP measurement revealed that TGF- β 1 injection led to a decrease in erectile function by 55% which was prevented in all treatment

groups. Treatment groups showed no significant differences in ICP compared to the vehicle-injected group (Figure 5). Subsequent mRNA expression analysis of the penile tissue harvested from the rats showed that expression of collagens I, III, and V were significantly upregulated in the TGF- β 1 injection group, but not in the vehicle or treated groups. Treatment with vardenafil or tamoxifen therefore prevented upregulation of TGF- β 1-induced increase in collagen expression. Interestingly, the combination of vardenafil and tamoxifen acted synergistically on the down-regulation of elastin (Figure 5). The formation of fibrosis in response to TGF- β 1 injection into the penis was further confirmed by measuring α -SMA in the corpus cavernosum (as a measure of loss of smooth muscle mass due to fibrotic tissue) using Western blot and immunohistochemistry. The results showed a significant loss of smooth muscle which was prevented in the treatment groups (Figure 5). Furthermore, immunohistochemistry using H&E and Masson's Trichrome staining showed increased infiltration of inflammatory cells, formation of fibrosis and loss of smooth muscle in the TGF- β 1 injected group; effects which were prevented in the treatment groups (Figure 6).

DISCUSSION:

In contrast to the single-target approach, the phenotypic screening seeks to find compounds that target a phenotype rather than a single molecular target. In this case we have chosen transformation of fibroblasts to myofibroblasts as the target phenotype and developed an assay which can quantify inhibition of myofibroblast transformation in a reproducible manner. Using this assay, we then tested 21 compounds/drugs that have been suggested as potentially anti-fibrotic agents. Among this cohort, only two groups were able to inhibit myofibroblast transformation: PDE5i and SERMs. When applied together, the two classes showed synergistic activity both *in vitro* and *in vivo*.

To our knowledge this is the first study to show a synergy between PDE5i and SERMs. PDE5i have previously been suggested to be effective anti-fibrotic agents *in vitro* using TA-derived fibroblasts²². This was further confirmed in an animal model for PD with long term vardenafil treatment^{23,24}. Furthermore, *in vivo* studies led to PDE5i being proposed as a potential treatment for other fibrotic disorders such as muscle fibrosis in a Duchenne muscular dystrophy mouse model²⁵ and for prevention of cardiac fibrosis and its underlying cardiac

fibroblast activation²⁶. Tamoxifen has been shown to be effective in animal models for renal tubulo-interstitial fibrosis and peri-portal hepatic fibrosis^{27,28}. The anti-myofibroblast effect has also been reported in models utilizing TGF- β 1-mediated activation of primary human dermal and breast fibroblasts²⁹. Previous research suggested that the effect of tamoxifen on fibroblast-mediated collagen contraction is either due to downregulation of TGF- β 2³⁰ or a change in morphology of fibroblasts³¹. Oestradiol has been shown to inhibit transformation of TA-derived fibroblast to myofibroblasts³². However, there is no previous study that has investigated the effect of tamoxifen in an animal model of PD.

Previous clinical studies have shown mixed results using PDE5i and SERMs^{33–37}. An open label single-arm study with tamoxifen showed a positive effect³³ while a later placebo-controlled study showed no effect with tamoxifen³⁴. However, the open label study noted that tamoxifen showed some improvement in patients with early PD³³; while all of the patients in the latter study were in the late phase of the disease³⁴. Similarly, in studies with PDE5i, the results have been mixed^{35–37}. This is not surprising since the drugs were tested on patients with established plaque-fibrotic tissue. Our results suggest that PDE5i or SERMs inhibit myofibroblast transformation and ECM production; they would not be able to reverse the established / preformed fibrosis. We are therefore proposing that the combination of PDE5i and SERMs will inhibit myofibroblast transformation, hence new fibrosis formation and prevent new plaque formation.

We believe that more and more men are presenting at early stages of the disease as there are now better information resources and access to healthcare to have symptoms investigated. Indeed, others have reported that 30-40% of patients present with progressing deformity^{38–40}. This drug combination may be more effective in patients in the acute phase where penile pain or the onset of a nodule would be an indication for referral. This will be an area for patient and primary care education.

The doses of vardenafil and tamoxifen used in our animal model are representative of their clinical doses; tamoxifen 20 mg twice daily and vardenafil 20 mg daily (please see Supplementary methods that explains the rationale behind the dose selection).

This study is limited by not providing a mechanism of action for the proposed synergistic effect of the drugs, something that is currently being investigated. The number of compounds/drugs that have been screened also limits the study; other SERMs that are used in treatment of male fertility would be very interesting to test. A larger phenotypic screening campaign is planned, hopefully providing us with more hits that might reveal new treatment strategies not only for PD but for fibrosis in general.

Another limitation to the study is that the drugs were tested in only one of the several animal PD models⁴¹. Although TGF- β 1 injection model is the most widely used model, it may not necessarily be the most clinically relevant animal model for example it does not develop curvature of the penis. Further studies are required to test this combination in other animal models of PD.

Conclusions

In summary, this is the first study to demonstrate a synergistic anti-fibrotic effect of a combination of PDE5i and SERMs in *in vitro* and *in vivo* disease models. Future prospective clinical trials using a combination of these drugs should be considered during the active phase of PD, given the early evidence of benefit in both *in vitro* and *in vivo* models. We also envisage that the combination will be more efficacious than using either of the drugs as a monotherapy. We will be investigating such a combination in men with early PD in the near future. These results are likely to lead to further research into the interaction between the two pathways and development of novel therapeutic approaches for the prevention and/or treatment of other fibrotic diseases.

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FIGURE LEGENDS

Figure 1: TGF- β 1 induces myofibroblast transformation which can be measured by high-throughput screening assay. Fibroblasts were exposed to TGF- β 1 (10 ng/ml) for 72 hours. Representative images of α -SMA staining in (A) untreated TA-derived cells and (B) TA-derived cells exposed to TGF- β 1. Images were captured at 200x magnification. Scale bars 50 μ m. C: Quantification of α -SMA positive cells. D: The mRNA levels of α -SMA were determined using the $2^{-\Delta\Delta C_t}$ method. Data points plotted as mean \pm SEM, N=3 patients for each group; n=9. *P<0.05 vs negative control. (E) Representative Western blot for α -SMA content in protein lysates from untreated cells and cells exposed to 10 ng/ml TGF- β 1 for 72h: 20 μ g of protein was loaded under reducing conditions. Lower bands (35kD) represent GAPDH loading control, higher bands (42kD) represent α -SMA. Lane 1: protein ladder, Lanes 2, 4, 6, 8: untreated TA-derived cells. Lanes 3, 5, 7, 9: cells exposed to 10 ng/ml TGF- β 1. (F) Statistical validation of the ICE method. Positive controls correspond to wells exposed to TGF- β 1, negative controls correspond to wells exposed to media only. Data normalized to nuclear dye intensity. Validation for high throughput screening by calculation of Z' comparing negative control wells to positive control wells, yielding a Z' value of 0.89.

Figure 2: Concentration response curves for hits acquired from screening campaign. Effect of PDE5is vardenafil, sildenafil, and tadalafil (A) and SERMs tamoxifen and raloxifen (B) on TGF- β 1-induced myofibroblasts transformation. Cells derived from TA tissue were exposed to a range of concentrations of PDE5is between 0.03 and 100 μ M in co-incubation with 10 ng/ml TGF- β 1 for 72 hours. Data points were plotted as average \pm SEM of the percentage of maximum response of the α -SMA/DNA staining ratio, N=3; n=9.

Figure 3: Effect of compounds on TGF- β 1-induced myofibroblast collagen contraction in fibroblast populated collagen lattices (FPCL). (A) Representation of the contraction of the fibroblast populated collagen lattices. Top row: example for no contraction after release. Bottom row: example for uniform contraction after release from wall of the well. FPCLs were exposed to 10 ng/ml TGF- β 1 and various concentrations of tamoxifen (B) or vardenafil (C). FPCLs were released after 72h and contraction was observed for 8h. Data presented as percentage of maximum collagen contraction compared to vehicle control (DMSO) in cells exposed to tamoxifen/vardenafil. Data points were plotted as mean \pm SEM, N=3; n=9. *P<0.05 vs vehicle control at the same time point.

Figure 4: Effect of compounds on TGF- β 1-induced myofibroblast ECM production. (A) Cells derived from TA tissue were exposed to a range of concentrations of vardenafil, tamoxifen or SB-505124 in co-incubation with 10 ng/ml TGF- β 1 for 7 days. ECM was stained for collagen I (Col I; A); collagen III (Col III; B); collagen V (Col V; C) and fibronectin (D) after cell lysis. Data

points were plotted as average \pm SEM of the percentage of maximum response of protein/pre-lysis DNA staining ratio, N=3; n=9.

Figure 5: Vardenafil, tamoxifen and their combination ameliorate penile fibrosis in an animal model for PD. (A) Δ ICP measurement for different stimulation voltages in all the treatment groups. Intracavernous pressure (ICP) change from baseline to peak ICP (Δ ICP). Data plotted as mean \pm SEM, N=7. (B) mRNA levels of α -SMA (ACTA2) were determined using the $2^{-\Delta\Delta C_t}$ method. Data points plotted as mean \pm SEM, N=4. *P<0.05 vs TGF- β 1 injected group. (B) mRNA levels of Col I were determined using the $2^{-\Delta\Delta C_t}$ method. Data points plotted as mean \pm SEM, N=4. *P<0.05 vs TGF- β 1 injected group. (C) mRNA levels of Col III were determined using the $2^{-\Delta\Delta C_t}$ method. Data points plotted as mean \pm SEM, N=4. *P<0.05 vs TGF- β 1 injected group. (D) mRNA levels of Col V were determined using the $2^{-\Delta\Delta C_t}$ method. Data points plotted as mean \pm SEM, N=4. *P<0.05 vs TGF- β 1 injected group. (E) mRNA levels of elastin were determined using the $2^{-\Delta\Delta C_t}$ method. Data points plotted as mean \pm SEM, N=4. *P<0.05 vs TGF- β 1 injected group or in between groups. (F) Western blot quantification of α -SMA. Data shown as fold change between injected and uninjected side of the penis. Data points plotted as mean \pm SEM, N=3. *P<0.05 vs TGF- β 1 injected group or in between groups. (G) Western blot quantification of α -SMA/Col I ratio. Data shown as ratio of fold change between injected and uninjected side of the penis for α -SMA and Col I. Data points plotted as mean \pm SEM, N=3. *P<0.05 vs TGF- β 1 injected group or in between groups. (H) Western blot quantification of α -SMA/Col III ratio. Data shown as ratio of fold change between injected and uninjected side of the penis for α -SMA and Col III. Data points plotted as mean \pm SEM, N=3. *P<0.05 vs TGF- β 1 injected group or in between groups. (I) Western blot quantification of Col I/Col III ratio. Data shown as ratio of fold change between injected and uninjected side of the penis for Col I and Col III. Data points plotted as mean \pm SEM, N=3. *P<0.05 vs TGF- β 1 injected group or in between groups. TGF- β 1=TGF- β 1 injected group, vehicle=vehicle injected group, tamoxifen=TGF- β 1 injected + tamoxifen treatment group, vardenafil= TGF- β 1 injected + vardenafil treatment group, combined=TGF- β 1 injected plus combined treatment group.

Figure 6: Immunohistochemical staining for different treatment groups. Representative images of Masson's Trichrome staining and H&E staining in whole sections of rat penis for: (A) TGF- β 1 injected group, left: Masson's Trichrome staining for injected side (4x magnification), right: H&E staining for injected side (4x magnification). (B) TGF- β 1 injected group treated with vardenafil, left: Masson's Trichrome staining for injected side (4x magnification), right: H&E staining for injected side (4x magnification). (C) TGF- β 1 injected group treated with tamoxifen, left: Masson's Trichrome staining for injected side (4x magnification), right: H&E staining for injected side (4x magnification). (D) TGF- β 1 injected group treated with combination of vardenafil and tamoxifen, left: Masson's Trichrome staining for injected side (4x magnification), right: H&E staining for injected side (4x magnification). Black arrow indicates smooth muscle. Orange box indicates collagenous fibrotic plaque. Blue arrows indicate nuclei (cellular infiltration due to inflammation).

Supplementary Figure 1: TGF- β 1 induces myofibroblast transformation in TA-derived cells from all three tissue sources. No significant difference was observed between the three cell

groups in morphology or in their response to TGF- β 1. Fibroblasts were exposed to TGF- β 1 (10 ng/ml) for 72 hours. A, C, E: Representative images of α -SMA staining in untreated cells derived from PD plaques (A), penile cancer TA (C) and TA opposite the PD plaque (E). B, D, F: Representative images of α -SMA staining in TGF- β 1-treated cells derived from PD plaques (B), penile cancer TA (D) and TA opposite the PD plaque (F). Images were captured at 200x magnification. Scale bars 50 μ m. G: Effect of TGF- β 1 on TA-derived cells. Cells were exposed to a range of concentrations of TGF- β 1 between 0.001 and 30 ng/ml for 72 hours. Data points were plotted as mean \pm SEM of the percentage of activity achieved, N=3. Light blue=TA from non-plaque PD, purple=TA from penile cancer, red=PD plaque

Supplementary Figure 2: Vimentin and desmin quantification in cells derived from non-plaque TA tissue from patients with Peyronie's disease. Quantification of vimentin (A) and desmin (B) mRNA expression of TA-derived cells using qPCR. Fibroblasts were exposed to TGF- β 1 (10 ng/ml) for 72 hours before RNA isolation. Data points were plotted as mean \pm SEM, N=3 patients for each group. n.d.=not detectable C,D,E,F: Representative images of (C) vimentin stained TA-derived cells exposed to control conditions. (D) Vimentin stained TA-derived cells exposed to TGF- β 1. (E) Desmin stained TA-derived cells exposed to control conditions. (F) Desmin stained TA-derived cells exposed to TGF- β 1. Images were captured at 200x magnification. Quantification of vimentin (G) and desmin (H) protein expression in TA-derived cells using ICE. Data points were plotted as mean \pm SEM, N=3 patients for each group.

Supplementary Figure 3: Further validation of In Cell ELISA assay. (A) Effect of TGF- β 1 on TA-derived cells. Cells were exposed to a range of concentrations of TGF- β 1 between 0.001 and 30 ng/ml for 72 hours. Data points were plotted as mean \pm SEM of the percentage of activity achieved, N=3. (B) Effect of DMSO on TA-derived cells. Cells were exposed to a range of concentrations of DMSO between 0.0003 and 9% in co-incubation with 10 ng/ml TGF- β 1 for 72 hours. Data points were plotted as mean \pm SEM, N=3. (C) Effect of TGF- β 1 receptor inhibitor SB-505124 on TA-derived cells. Cells were exposed to a range of concentrations between 0.003 and 100 μ M in co-incubation with 10 ng/ml TGF- β 1 for 72 hours. Data points were plotted as mean \pm SEM, N=3.

Supplementary Figure 4: Effect of SB505124 on TGF- β 1-induced myofibroblast collagen contraction in fibroblast populated collagen lattices (FPCL). (A) FPCLs were exposed to 10 ng/ml TGF- β 1 and various concentrations of SB-505124. FPCLs were released after 72h and contraction was observed for 8h. Data presented as percentage of maximum collagen contraction compared to vehicle control (DMSO) in cells exposed to SB-505124. Data points were plotted as mean \pm SEM, N=3. *P<0.05 vs vehicle control at the same time point.

Supplementary Figure 5: Vardenafil, tamoxifen and their combination ameliorate penile fibrosis in an animal model for PD. Representative examples of MAP (blue) and ICP (red) during electrostimulation at different voltages (15, 10, 7.5, 5V) for TGF- β 1 injected rats (A), vehicle injected rats (B), TGF- β 1 injected rats treated with tamoxifen (C), TGF- β 1 injected rats treated with vardenafil (D), for TGF- β 1 injected rats treated with tamoxifen and vardenafil (E). Note the MAP dropping during ICP peaks, indicating an erection. (F) Representative western

blot for Col I content in protein lysates from injected and uninjected corpora of rats from different treatment groups: 20 µg of protein was loaded under non-reducing conditions. Lower bands (35kD) represent GAPDH loading control, higher bands (130kD) represent Col I. (G) Representative western blot for Col III content in protein lysates from injected and uninjected corpora of rats from different treatment groups: 20 µg of protein was loaded under non-reducing conditions. Lower bands (35kD) represent GAPDH loading control, higher bands (130kD) represent Col III. (H) Representative western blot for α-SMA content in protein lysates from injected and uninjected corpora of rats from different treatment groups: 20 µg of protein was loaded under reducing conditions. Lower bands (35kD) represent GAPDH loading control, higher bands (42kD) represent α-SMA. Lane 1: MW protein ladder, lane 2: vehicle injected group, lane 3&4: TGF-β1 injected group, lanes 5&6: TGF-β1 injected group with vardenafil treatment, lanes 7&8: TGF-β1 injected group with tamoxifen treatment, lanes 9&10: TGF-β1 injected group with combined treatment.

Supplementary methods:

Immunocytochemistry

Cells were seeded into wells of a 6-well plate containing sterile glass coverslips at 5.0×10^4 cells/well. After overnight incubation, media was replaced with either fresh media or media containing TGF-β1 (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 (EMD-Millipore, UK) 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.

Real-time RT-PCR (RT-qPCR)

Cells were seeded into 6-well plates (Nunc, Fisher Scientific, UK) at 1.0×10^5 cells/well and incubated with or without 10 ng/ml TGF-β1 for 72 hours. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, UK), according to the manufacturer's instructions. First strand cDNA was synthesized from 500 ng of RNA by reverse transcription (RT) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, UK), following the

manufacturers protocol. Real-time PCR was performed utilizing the QuantiTect® SYBR® Green PCR kit (Qiagen, UK). PCR reactions were carried out on the Rotor-Gene Q (Qiagen, UK). Relative mRNA levels were determined using the $2^{-\Delta\Delta C_t}$ method and presented as fold change of target gene in the test sample relative to the calibrator sample and normalized to the expression of the reference genes (EIF4A2 and TOP1).

Calculation of Z-factor

Z-factor (Z') was used to measure the statistical effect size of TGF-β1-induced myofibroblast transformation, to evaluate its reproducibility, variability and potential use as a high throughput screening (HTS) assay. The following formula was used to calculate the Z':

$$1 - \frac{3 - (\sigma_p - \sigma_n)}{|\mu_p - \mu_n|}$$

Whereby σ_p and σ_n refer to the standard deviation of the positive and negative controls, respectively and μ_p and μ_n stand for the means of positive and negative controls respectively.

Tamoxifen and vardenafil administration

Although tadalafil was the most potent PDE5i in our *in vitro* experiments, the water solubility of tadalafil is poor. Therefore, we chose vardenafil for the animal model because vardenafil can be administered in the drinking water. Tamoxifen was administered once daily via intraperitoneal injection (i.p.) at a dose of 5 mg/kg/day. Vardenafil (1.5 mg/kg/day) was administered orally daily by adding the drug to the drinking water (containing 1% citric acid). Administration of the two drugs was initiated at the day of TGF-β1 injection and continued for five weeks at the end of which the animals could have 48 hours wash-out period.

Dose selection:

In this study, the dose selection for tamoxifen and vardenafil was based on the body surface area normalization method⁴², yielding an animal dose equivalent to the doses used in the clinics. Using this method, the animal equivalent dose for tamoxifen was calculated from the dose used in the clinical study that indicated an effect for tamoxifen in the early stage of PD³³,

in which the patients were given 20 mg of tamoxifen twice a day. The calculated animal equivalent dosage for rats would be 4.2 mg/kg/day of tamoxifen which is also within the ethically acceptable limits for tamoxifen treatment in this species. For vardenafil the highest dose used in humans was selected as a starting point. 20 mg a day in humans correspond to a dose of 2 mg/kg/day in rats which is higher than the dose of 1.5 mg/kg/day that was utilized in this study, limited by the water solubility of the drug. Increasing the concentration of vardenafil would have meant further acidifying the drinking water, which would have had a negative effect on drinking behaviour and consequently animal welfare. No effect on drinking behaviour could be observed with the protocol chosen for this study (data not shown). As a result of these calculations and observations, doses to be used in the animal model were finalised as 5 mg/kg/day and 1.5 mg/kg/day for tamoxifen and vardenafil respectively.

RNA isolation

RNA isolation was performed using a phenol/chloroform-based protocol using the TriPure reagent. Briefly, tissue samples were transferred to tubes containing ceramic beads (Precellys) and 500 µl of TriPure (Sigma) was added and the tissue was minced twice using a Precellys homogenizer (2 x 30s, 6500 rpm). 0.1 mL of chloroform was added and mixed vigorously. After 10 min of incubation the mixture was centrifuged for 15 min at 12,000 g at 4°C. The aqueous phase was transferred to a new tube and RNA was precipitated using 0.5 mL isopropanol. After inversion and incubation for 15 min the tubes were centrifuged for 10 min at 12,000 g at 4°C and the supernatant was discarded. The RNA pellet was washed in 75% ethanol and after another centrifugation step the RNA was resuspended in 40 µl of RNase-free water. RNA quality was checked using the Experion RNA StdSens Analysis Kit (Bio-Rad) and only RNA of a RNA integrity number (RIN) of 8 or higher was used.

Protein extraction

Tissue samples were transferred to tubes containing ceramic beads (Precellys) and 200 µl of lysis buffer was added. After 1 minute of incubation the tissue was minced twice using a Precellys homogenizer (2 x 30s at 6,500 rpm) and 200 µl of sucrose buffer was added. This was followed by 15 min of centrifugation at 3,000 g at 4°C. The supernatant was transferred

to a new reaction tube and the concentration was measured using a BCA assay according to the manufacturer's instructions.

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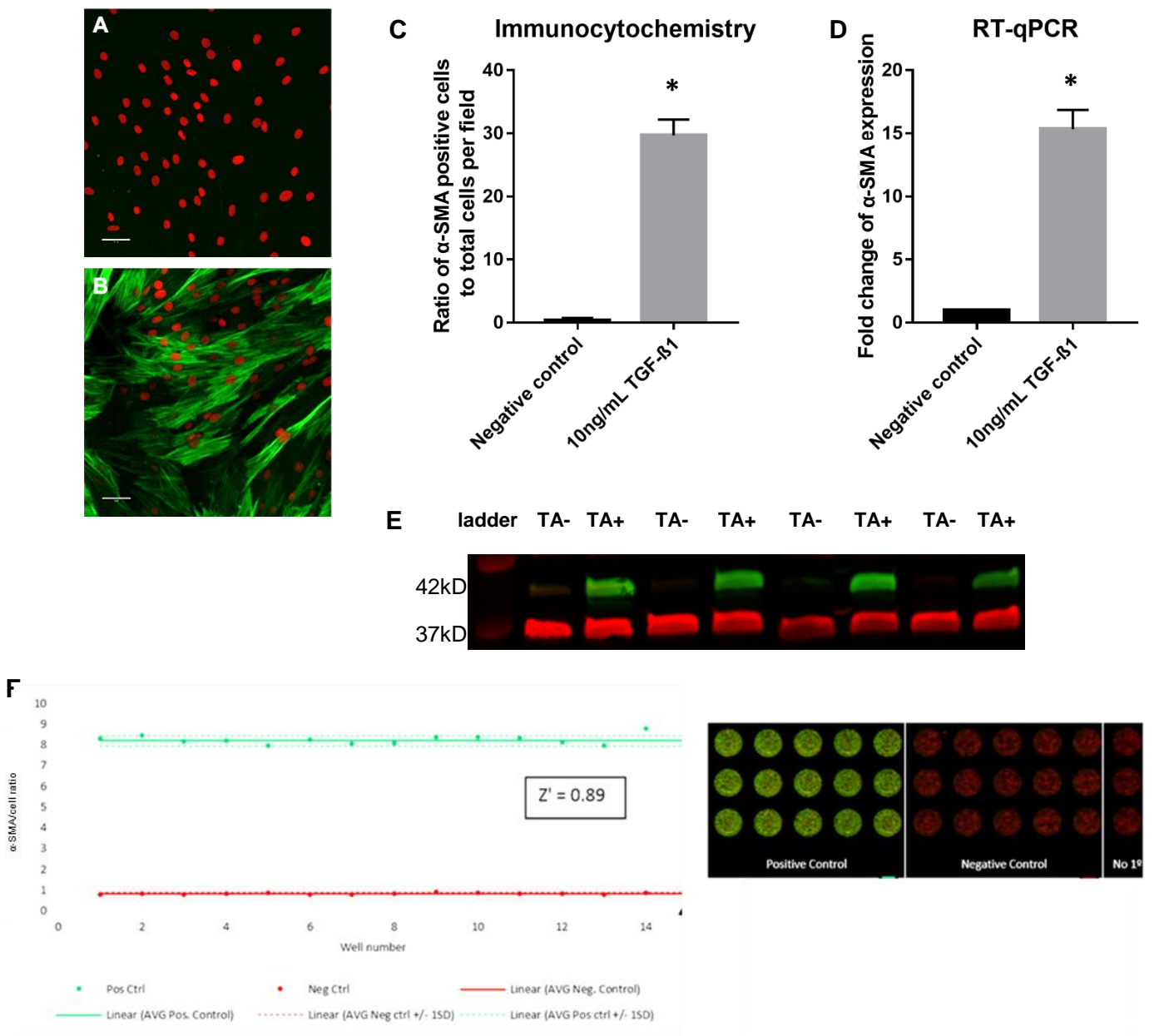


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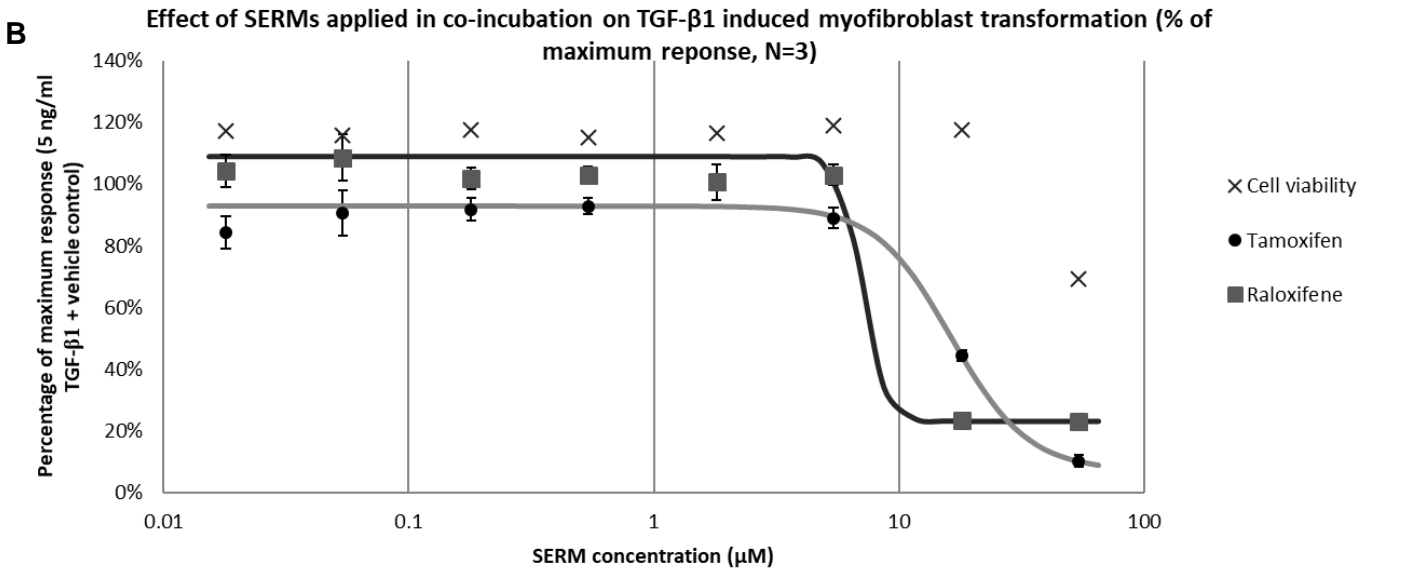
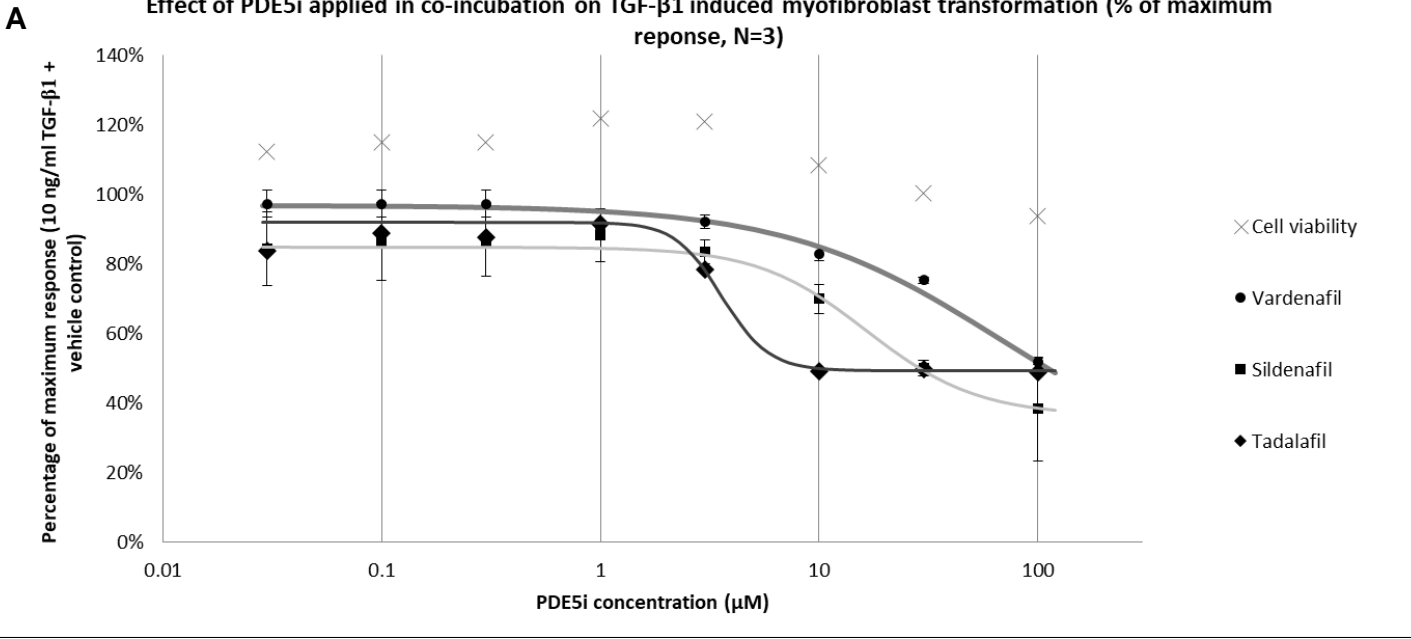
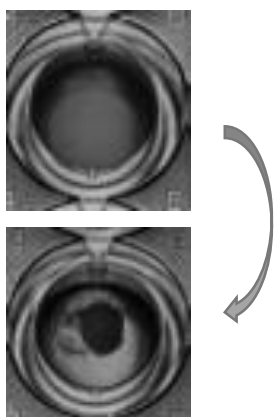
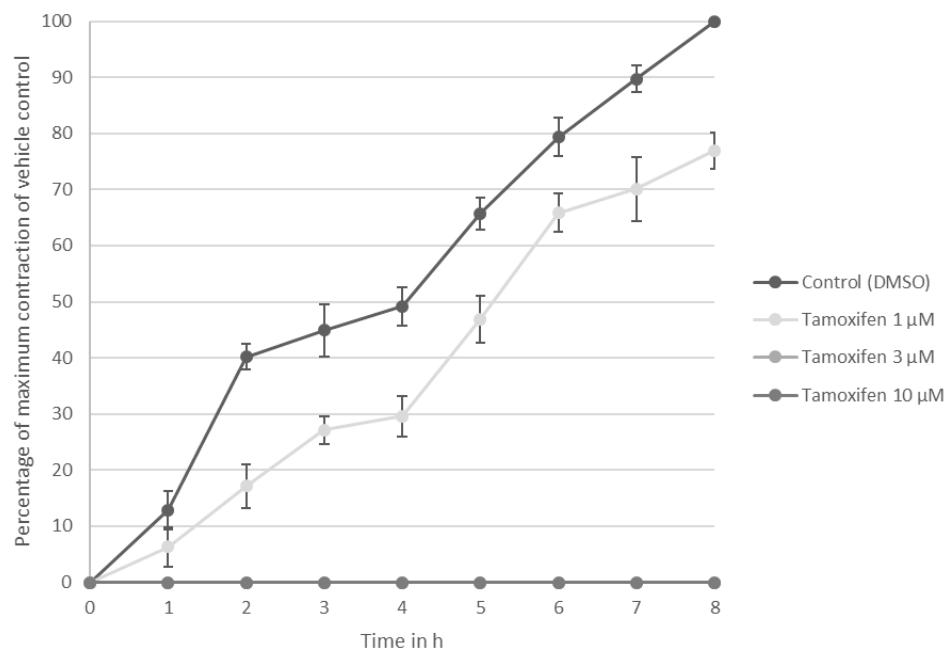


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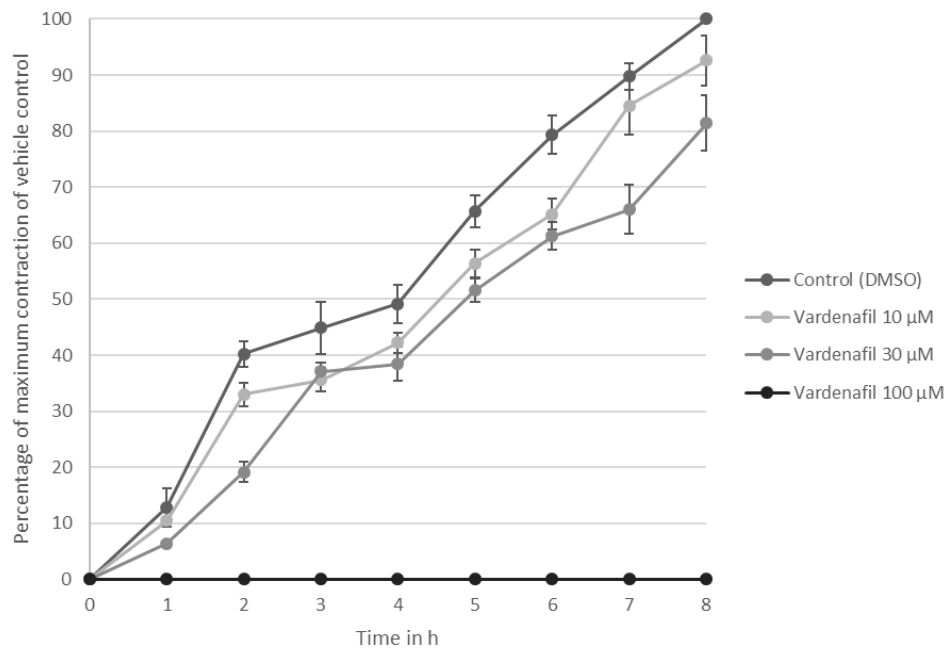
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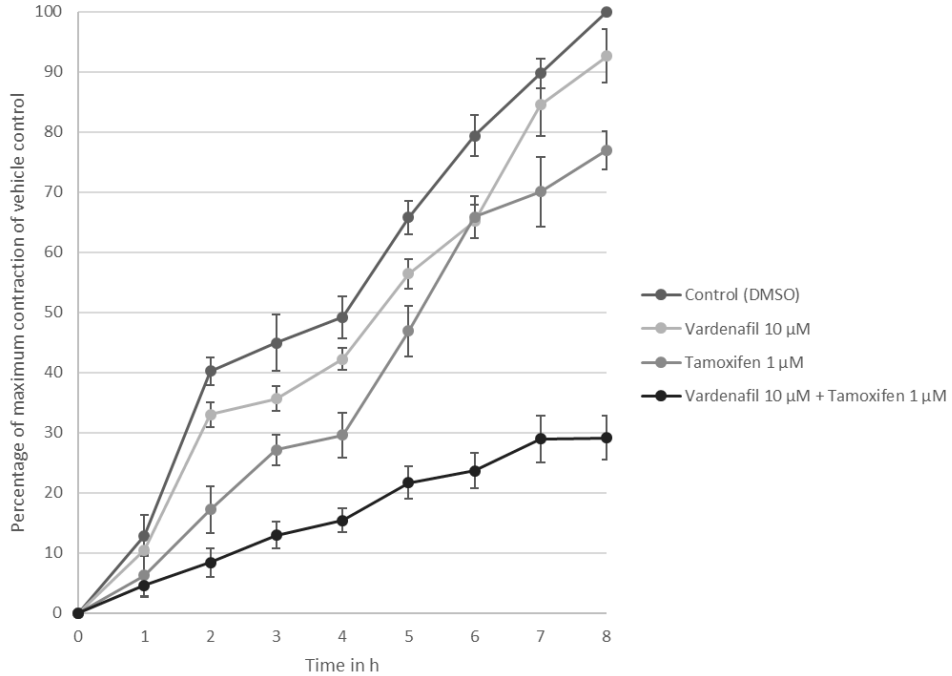


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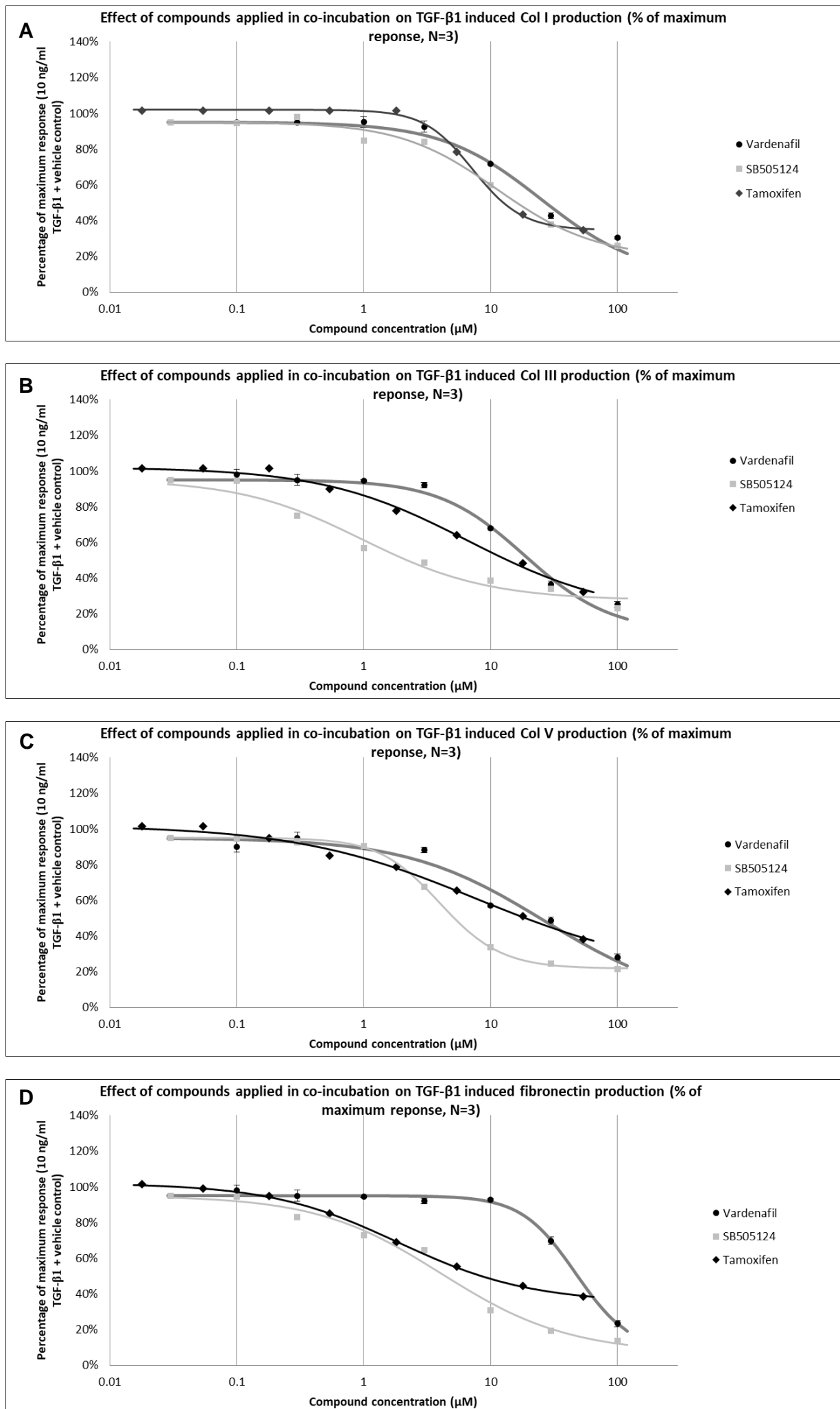


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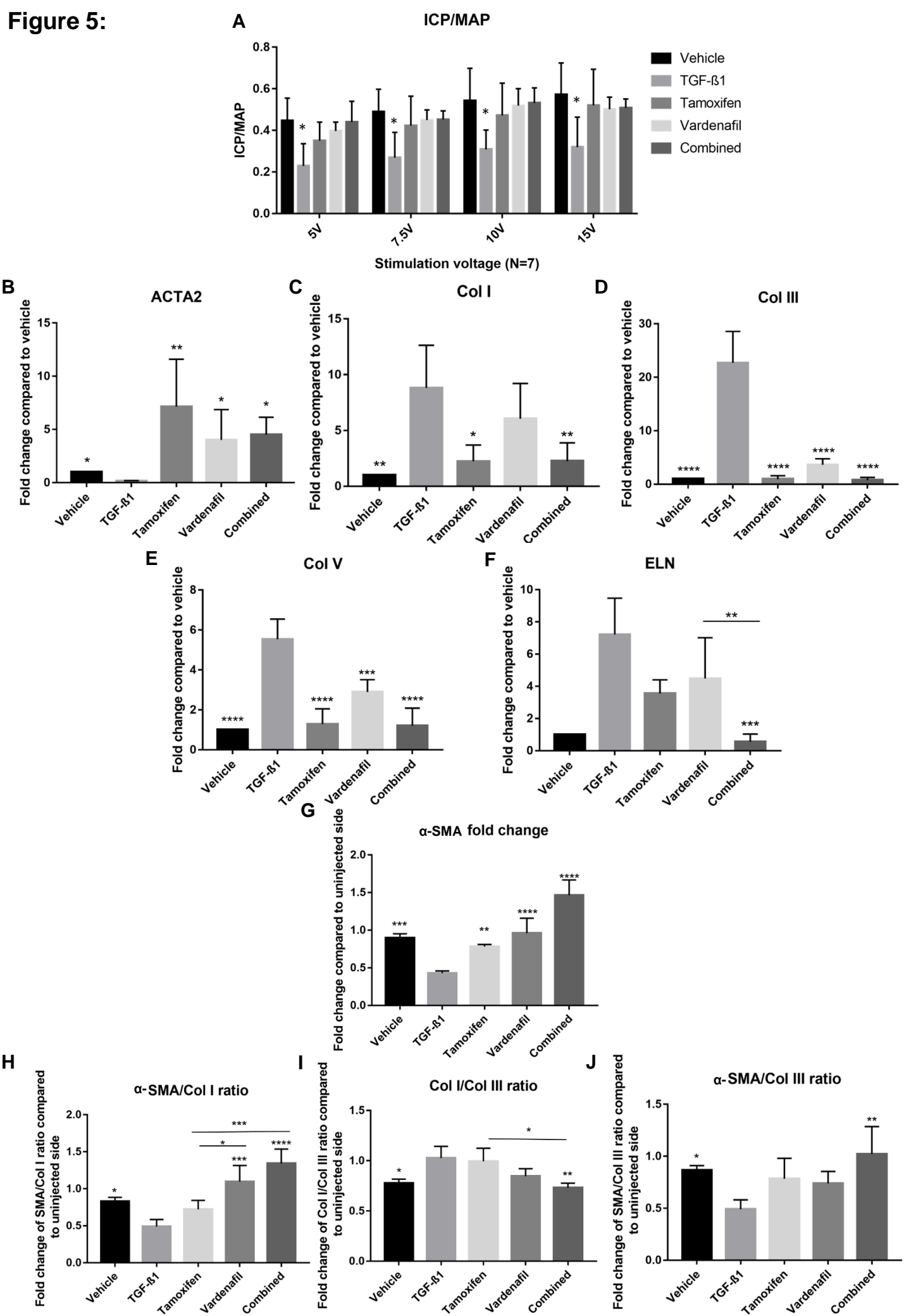
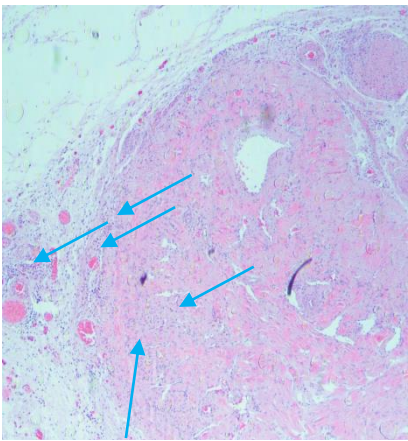
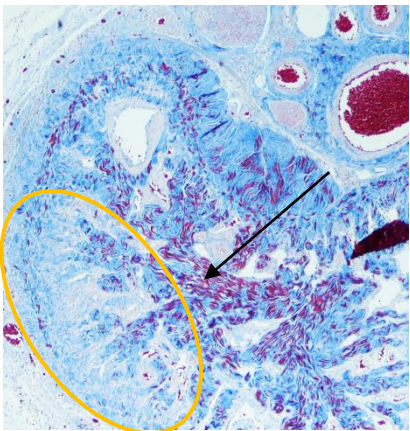
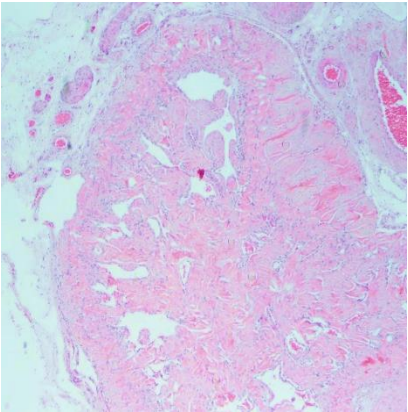
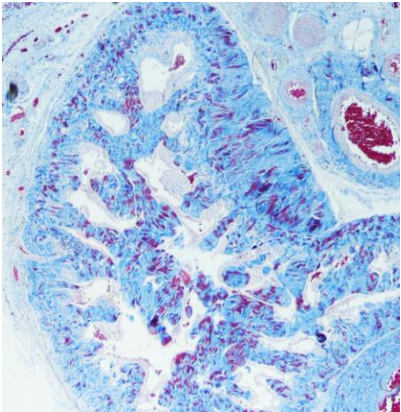


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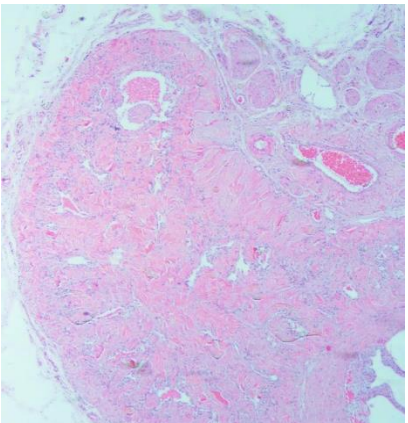
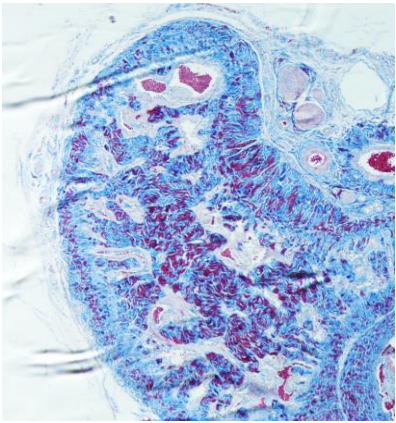
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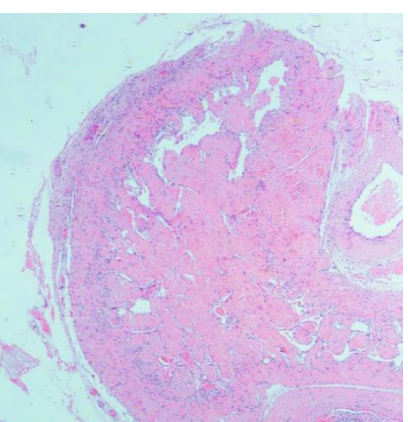
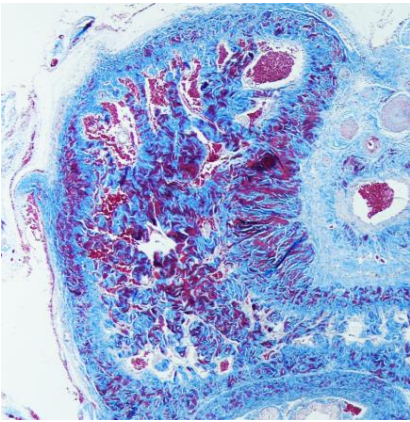
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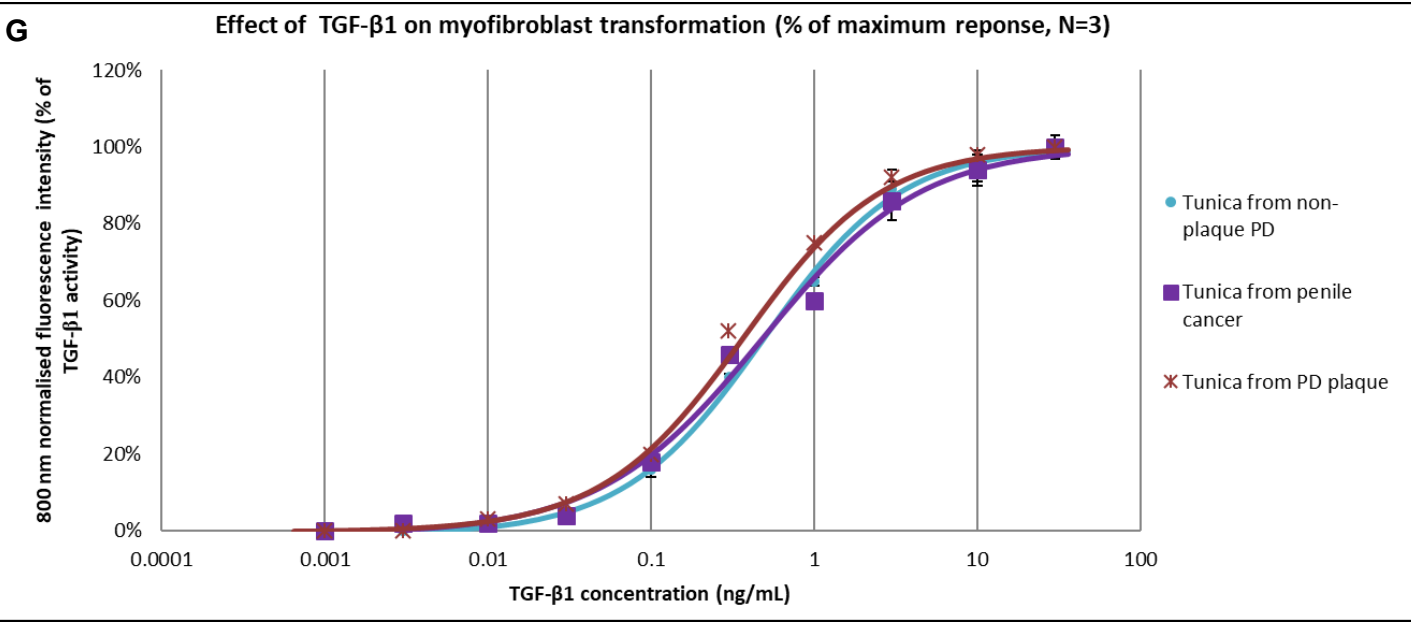
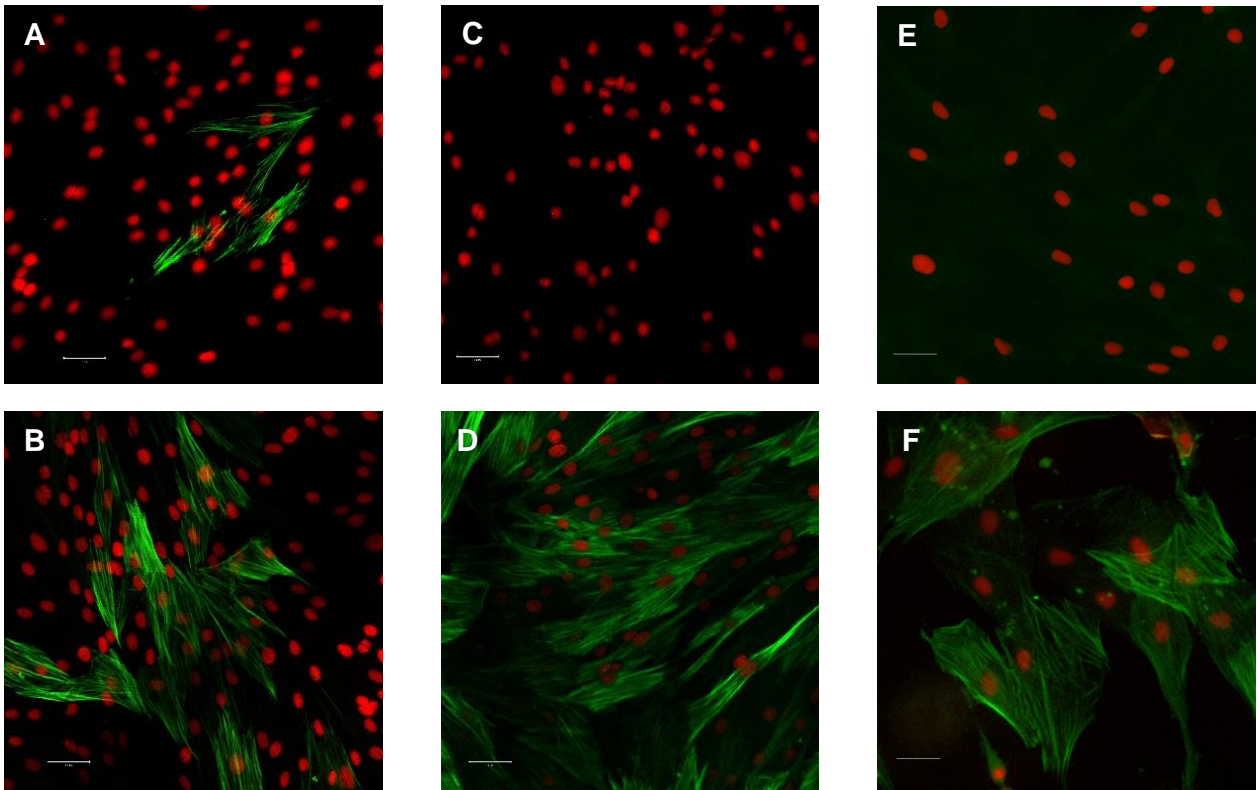
Supplementary table 1: Compounds and drugs tested with the phenotypic screening assay. Full concentration response curve with IC50 values were only constructed for preliminary hits in the screening campaign. Hits highlighted in red

Compound	Supplier	IC50
Sildenafil	Sigma-Aldrich, UK	15 μM
Quercetin	Sigma-Aldrich, UK	No effect at 30 μ M
Apigenin	Sigma-Aldrich, UK	33 μ M
Suramin sodium salt	Sigma-Aldrich, UK	No effect at 50 μ M
Tranilast	Sigma-Aldrich, UK	502 μ M
Pirfenidone	Sigma-Aldrich, UK	No effect at 500 μ M
Raloxifene	Sigma-Aldrich, UK	12 μM
P144	GenScript	No effect at 50 μ g/mL
TRAIL	Peptotech, USA	No effect at 100 ng/mL
SLV 320	TOCRIS, USA	362 μ M
Coenzyme Q10	Sigma-Aldrich, UK	No effect at 10 μ M
Tadalafil	Sigma-Aldrich, UK	3.5 μM
Verapamil hydrochloride	Sigma-Aldrich, UK	118 μ M
Interferon α 2B human	Sigma-Aldrich, UK	999 ng/mL
4-Aminobenzoic acid potassium salt (PABA)	Sigma-Aldrich, UK	No effect at 100 μ M
L-Carnitine hydrochloride	Sigma-Aldrich, UK	No effect at 1 mM
Pentoxifylline	Sigma-Aldrich, UK	905 μ M
Tamoxifen citrate salt	Sigma-Aldrich, UK	12 μM
α -Tocopherol	Sigma-Aldrich, UK	No effect at 50 μ M
Linoleic acid	Sigma-Aldrich, UK	No effect at 20 μ M
Vardenafil hydrochloride	Sigma-Aldrich, UK	30 μM

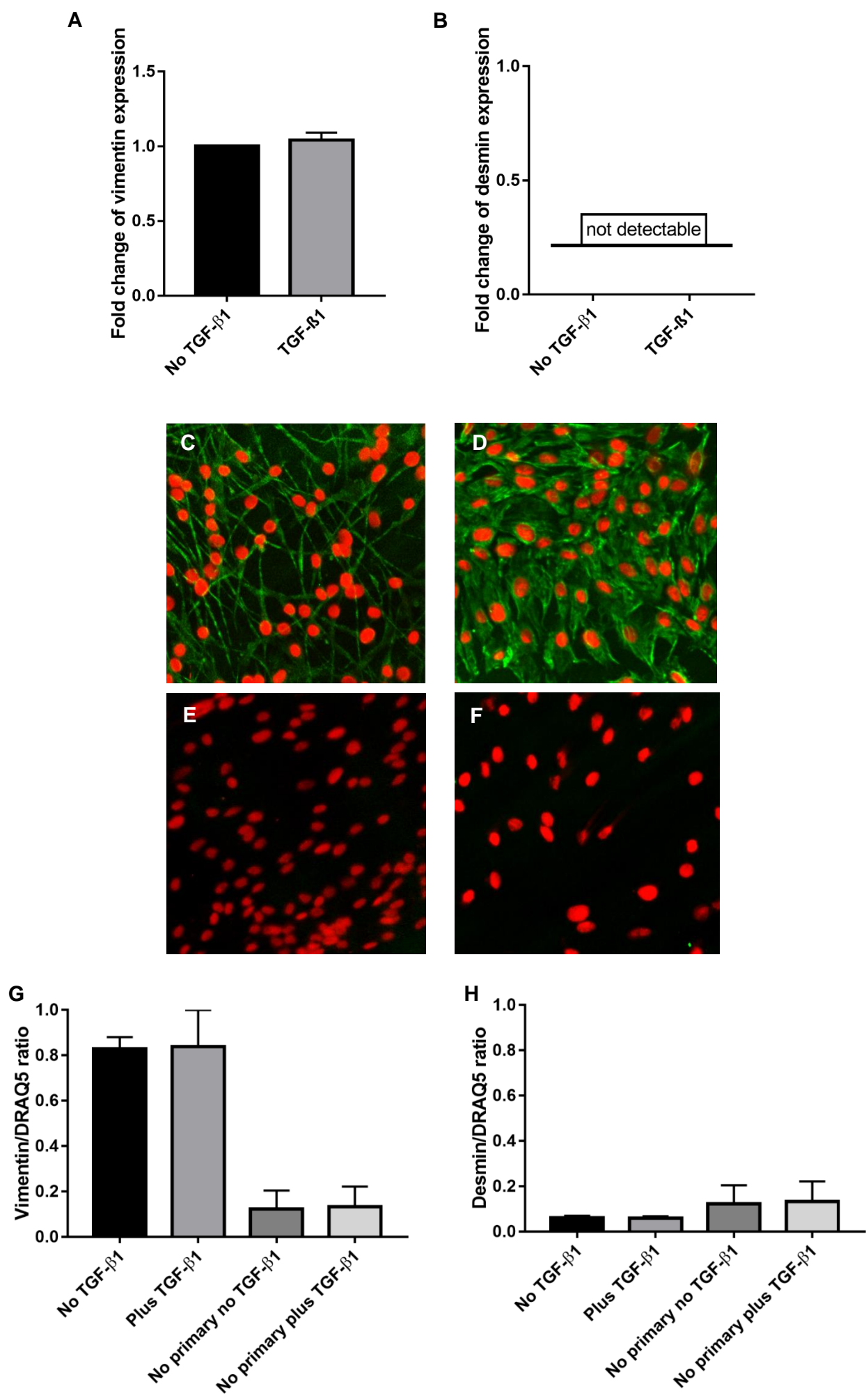
Supplementary table 2: Synergistic effect of vardenafil in co-incubation with tamoxifen on TGF- β 1-induced myofibroblasts transformation. Cells derived from non-PD TA tissue were exposed to a range of concentrations of vardenafil between 0.03 and 100 μ M in co-incubation with 10 ng/ml TGF- β 1 for 72 hours. Additionally, 1 μ M of tamoxifen was added, N=9. Statistical significance determined by using Sidak's multiple comparison test, *P<0.05 vs predicted additive effect.

Percentage of inhibition of TGF- β 1 induced myofibroblast transformation					
	Tamoxifen only	Vardenafil only	Vardenafil plus 1 μ M tamoxifen: Predicted additive effect	Vardenafil plus 1 μ M tamoxifen: Observed synergistic effect	P value
0.3μM	10 \pm 2.6%	2.6 \pm 3.0%	13 \pm 5.0%	22 \pm 1.5% *	<0.0001
1μM	10 \pm 2.0%	10 \pm 1.2%	20 \pm 3.2%	23 \pm 1.6% *	0.001
3μM	10 \pm 3.3%	8 \pm 1.6%	18 \pm 3.6%	24 \pm 2.3% *	<0.0001
10μM	25 \pm 2.9%	17 \pm 1.9%	27 \pm 3.9%	29 \pm 4.0%	0.47
30μM	80 \pm 2.1	25 \pm 0.72%	35 \pm 2.7%	38 \pm 0.62% *	0.004
100μM	cell death	48 \pm 0.84%	58 \pm 2.6%	55 \pm 1.0% *	0.001

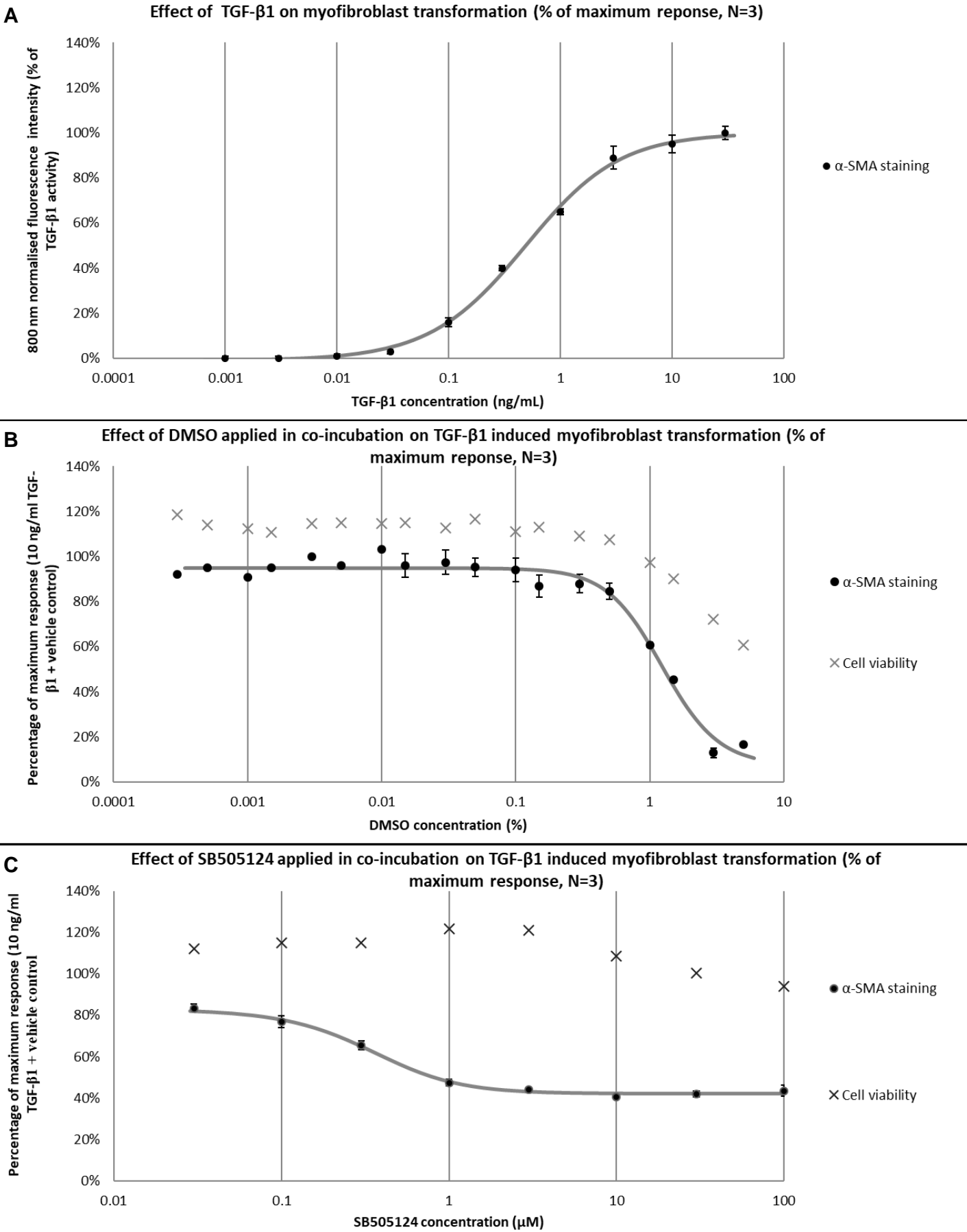
Supplementary Figure 1:



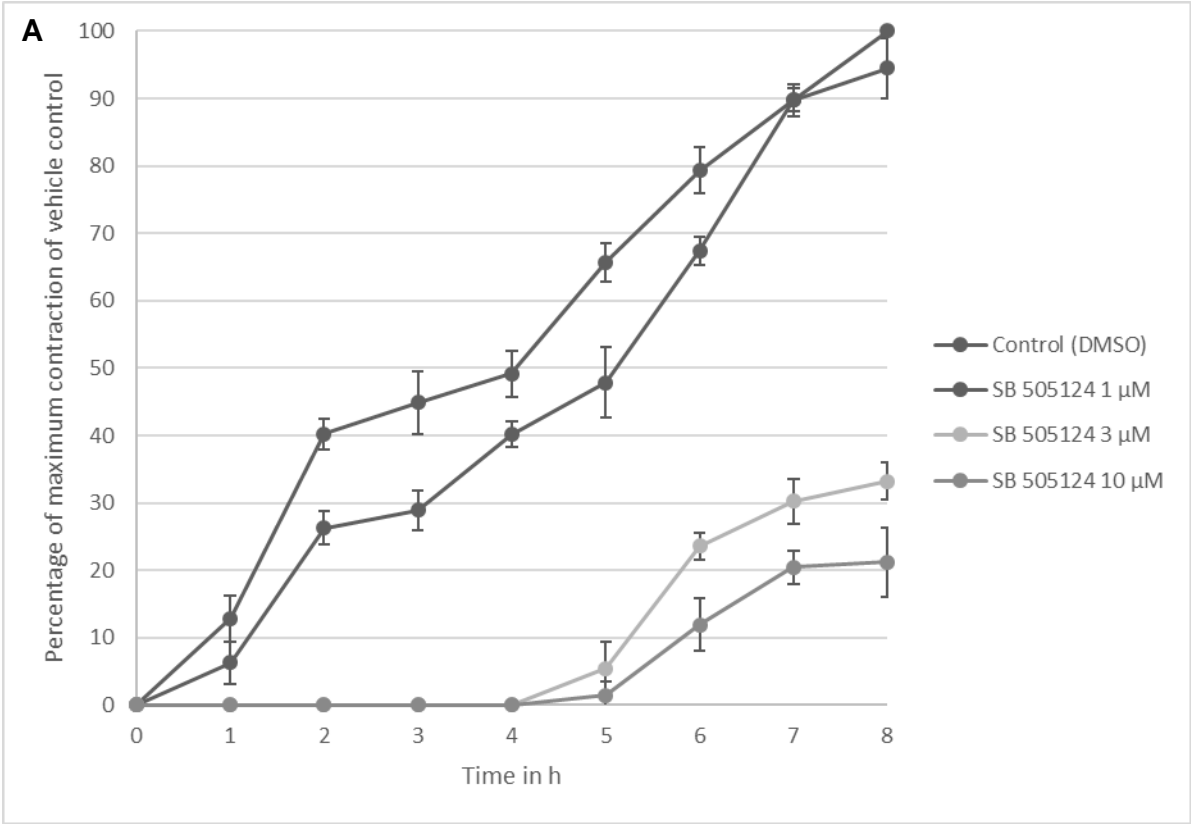
Supplementary Figure 2:



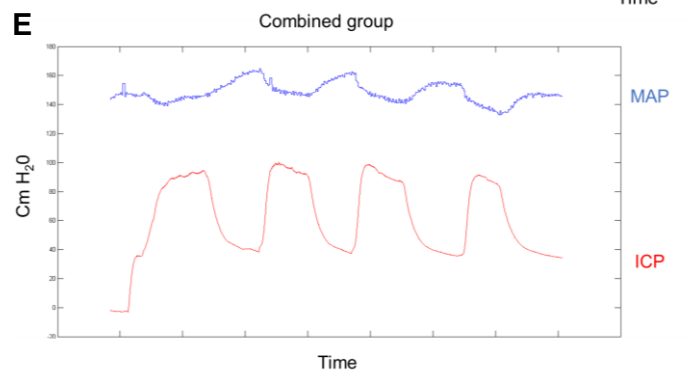
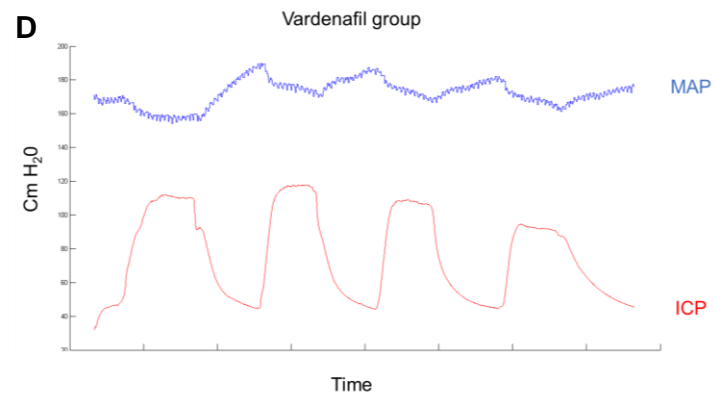
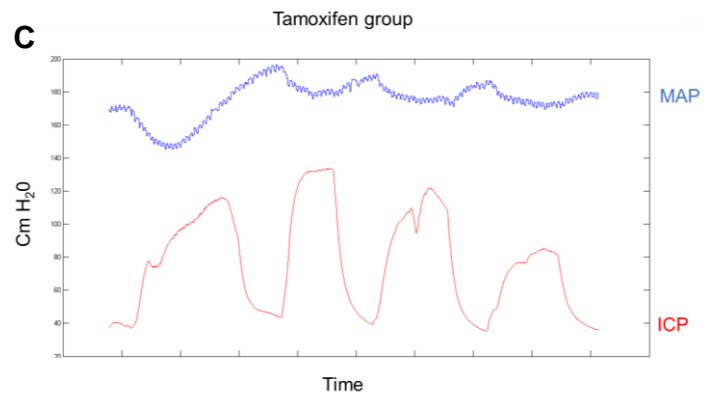
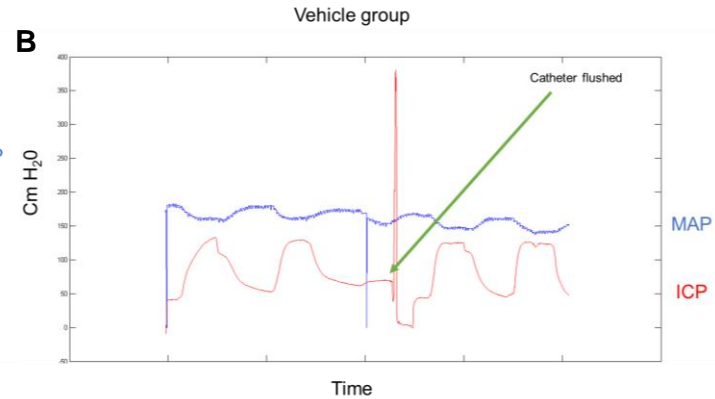
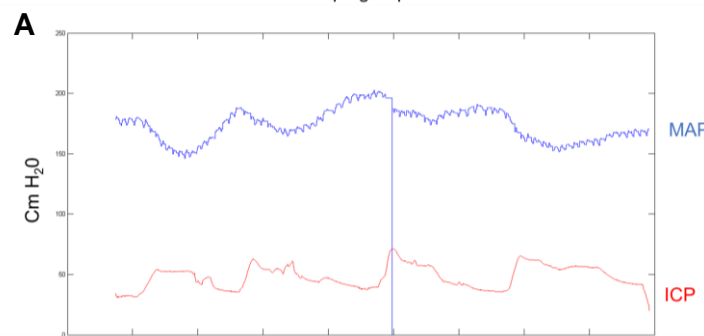
Supplementary Figure 3:



Supplementary Figure 4:

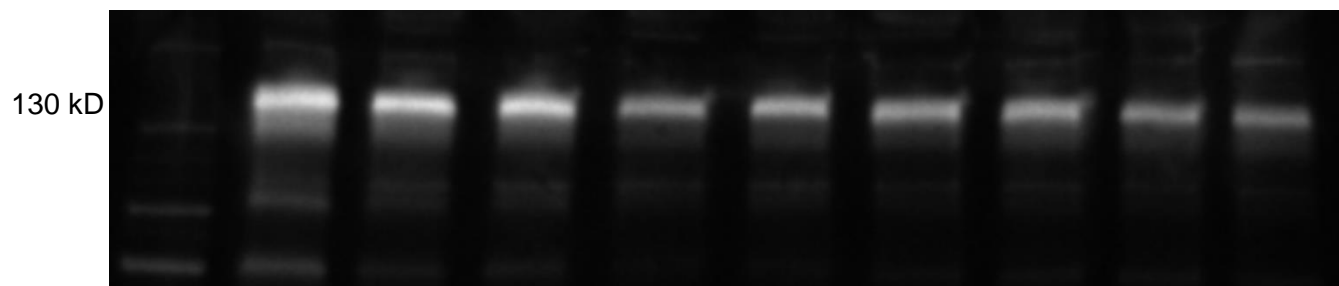


Supplementary Figure 5: TGF- β 1 group

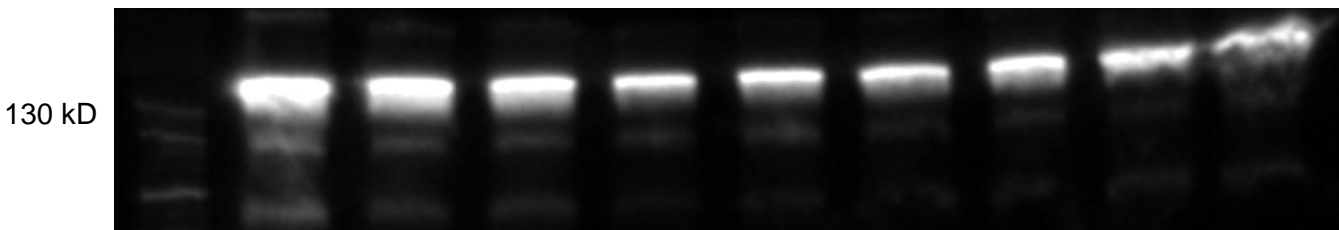


Veh inj TGF- β 1 uninj TGF- β 1 inj Var uninj Var inj Tam uninj Tam inj Com uninj Com inj

F



G



H

