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Regulation of collagen expression using nanoparticle mediated inhibition of TGF-β activation

Binbin Li,[ab] Tristan D Clemons,[c] Vipul Agarwal,[c] Jessica Kretzmann,[c] Michael Bradshaw,[c] Priyanka Toshniwal,[c] Nicole M Smith,[c] Shipu Li,[ab] Mark Fear,[de] Fiona M Wood,[de] K. Swaminathan Iyer[c]*

a. State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Wuhan University of Technology, Wuhan 430070, PR China

b. Biomedical Materials and Engineering Research Center of Hubei Province, Wuhan 430070, PR China.

c. School of Chemistry and Biochemistry, The University of Western Australia, Crawley WA 6009, Australia. E-mail: swaminatha.iyer@uwa.edu.au

d. Fiona Wood Foundation and Burn Injury Research Unit, M318, The University of Western Australia, 35 Stirling Highway, Crawley, WA, 6009 (Australia).

e. Burns Service of Western Australia, Royal Perth and Princess Margaret Hospitals, WA, 6000, Australia.

Supporting information

Materials. All chemicals were purchased from Sigma-Aldrich unless otherwise stated: methyl ethyl ketone (99%, Fisher), chloroform (99%, merck), Pluronic F-108, glycidyl methacrylate, azobisisobutyronitrile, rhodamine B (Fluka), polyethylenimine (50% solution, Mn 1200, Mw 1300). All tissue culture reagents were purchased from Invitrogen unless otherwise stated: Dulbecco's Modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12), PBS, Fetal Bovine Serum, Trypsin/EDTA (Sigma), penicillin/streptomycin (Sigma), human recombinant Transforming growth factor β 1(Sigma), Ascorbic acid (Sigma), MTS.

Preparation of PGMA/PXS64/PEI nanoparticles. Poly-(glycidyl methacrylate) (PGMA) was synthesized by free radical polymerization.[1] In brief, glycidyl methacrylate was polymerized in methyl ethyl ketone (MEK) to give PGMA (Mw= 250,000 g/mol), using azobisisobutyronitrile as initiator. The polymer was purified by multiple precipitations from MEK solution using diethyl ether. Nanoparticle preparation was achieved using an emulsion process described elsewhere.[2] Briefly, PGMA (70 mg) and PXS64 (20 mg) were dissolved in an organic phase of $CHCl_3$ (1.5 mL) and MEK (4.5 mL). The organic phase was then added dropwise slowly into an aqueous solution of Pluronic F-108 (1.25% w/v, 30 mL) under vigorous stirring and then sonicated with a hand held probe on low power for 1 min. After sonication, the organic phase was removed by rotovap at 40°C. The NPs suspension was obtained by centrifugation at 3,000g, 45 min. For PEI surface functionalization, PEI (50% wt) was added in excess to the nanoparticle suspension and left to stir at low speed for 48 h at room temperature. The nanoparticles were then collected by centrifugation (24,000g, 20 min), washed with Milli-Q water and this process repeated for 3x washes, to have a

final stock of PGMA/PXS64?PEI NPs suspended in Milli-Q water.

Footnote 1: For the rhodamine B labeled nanoparticles the PGMA was initially modified with rhodamine B by incubating rhodamine B (20 mg) with PGMA (70 mg) at 70°C in MEK (30 ml) for 5h, before collecting in diethyl ether and following the above protocol for NP synthesis.

Footnote 2: For the PGMA/PEI nanoparticles the PXS64 was left out of the organic phase of the above protocol for NP production, all other steps remained identical.

Dynamic light scattering (DLS) and zeta potential measurements. DLS experiments were performed using a Malvern Zetasizer Nano series. For measuring the size distribution, diluted aliquots of the NP suspensions in milli-Q water were measured over an average of 5 measurements, each of 10 data acquisitions per sample. Zeta potential measurements were recorded in triplicate and 100 data acquisitions were recorded in each measurement. All measurements were recorded at 25^{o}C in Malvern disposable clear Folded Capillary Cells.

Transmission Electron Microscopy (TEM). Synthesized polymer NPs were drop-casted on carbon coated TEM grids and imaged with an accelerating voltage of 120 kV on a JEOL 2100 transmission electron microscope.

High Performance Liquid Chromatography (HPLC). The drug loading rate of PGMA/PXS64/PEI NPs was determined by HPLC. The PXS64 loading rate was calculated based on the standard linear regression equation obtained from the standard curve of the PXS64 concentration and peak area. HPLC analysis was conducted on a Waters 2695 instrument with a Waters 2489 UV/Vis detector. Samples were run on a C18 column with an acetonitrile/water gradient solvent system (both containing 0.1% trifluoroacetic acid) at a flow rate of 1ml/min and absorbance detected at a wavelength of 280nm.

Confocal and Fluorescence Microscopy Analysis. Immunocytochemistry samples were analyzed using confocal and fluorescence microscopy. Confocal microscopy was carried out using a Leica TCS SP2 AOBS Multiphoton Confocal microscope and fluorescence microscopy with a Diaplan fluorescence microscope.

Cell Culture. Human primary fibroblast scar cells used herein were cultured in T75 flasks in a humidified atmosphere containing 5% CO_2 at 37 ^{o}C, and maintained in DMEM/F12 medium containing fetal bovine serum (10% v/v) and penicillin/streptomycin (1% v/v). The cell culture medium was changed every 3 days and all experiments were conducted on cells between passages P3-P6. Cells were collected, with UWA ethics approval and informed consent from all patients in accordance with the NHMRC national statement on ethical conduct in human research.

Cell viability measurement. Primary dermal scar fibroblasts of passage 6 were cultured and seeded for 24 hours in a 96-well plate at 37°C, 5% CO_2. The cell seeding density used was 8×10^4 cells/mL. Separate 96-well plates (Greiner Bio-One) were prepared for each sampling time point (24 and 48 hours). 100µL of cells (8×10^4 cells/mL) in DMEM media were seeded in each well. The plates were left to incubate overnight (37°C, 5% CO_2). Three hours before each sampling, 60µL of CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS; Promega) solution was added to every well of the plate for the particular time point. The plate was further incubated (37°C / 5% CO_2) for three hours. At each sampling time point, the absorbance for the plate was read at 490 nm (BMG Labtech, FLUOstar OPTIMA). Viability was determined at 2 time points (24, 48 hours) for a range of concentrations of PXS64 free drug (0, 1, 5, 10, 20 µM), PGMA/PEI (0, 20, 100, 200, 400 ug/mL) and PGMA/PXS64/PEI (0, 20, 100, 200, 400 ug/mL). Each measurement was carried out in triplicate.

Scar-in-a-Jar. Cells were seeded at a density of $5x10^4$ cells per well in a 4-well chamber slide and incubated for 6 days in 'crowded medium' (mixed with Ficoll 400 (25 mg/ml) and 70 (37.5 mg/ml), TGFβ1 (5 ng/ml), FBS (0.5%) and L-Ascorbic Acid (100 µM)) and with different concentrations of treatments (either PXS64 or NP formulations) in a humidified atmosphere containing 5% CO_2 at 37 ^oC. After incubation, the cells were fixed with 4% paraformaldehyde, and immunohistochemistry for collagen-1 and nuclei performed. 6-10 regions of interest were randomly selected from each well and the area of collagen I and nuclei number in that region of interest was analysed. This analysis was then converted into area of collagen per cell and averaged across the regions for each treatment. All immunocytoochemical analyses were conducted by a single investigator, ensuring constant selection criteria for each region of interest were maintained across all samples.

Immunocytochemistry. The scar cells were treated for collagen 1 visualization following standard immunocytochemistry procedures. The cells were first fixed in 4 % paraformaldehyde (30 min), then blocked in 5% normal goat serum (30 min) at room temperature. Primary incubation with the primary collagen 1 antibody (in 3 % BSA, 1:1000, mouse anti-human, Santa Cruz) at 37 ^oC for 90 min, followed by secondary antibody incubation (Alexa Fluor 488 in 3% BSA, goat anti-mouse, 1:500, 30 min, 37 ^oC, Life Technologies). Cell nuclei were stained with Hoechst 33258 (1:1000) for 10 min at room temperature. Cells were washed 3 times with PBS after every step. Slides were mounted with Dako Fluorescent Mounting Medium (Dako, USA) and covered standard glass coverslips. All experiments were performed in triplicate. Images were acquired using the Nikon TE300 microscope with a 4X (Plan Apo, NA 1.3) phase-contrast objective lens, CoolSnap-FX CCD camera, and NIS-Elements software.

Image and Statistical Analysis. All results are displayed as means ± SD. Data was analysed using the GraphPad Prism version 6.0 data management software to conduct ANOVA on groups of data. Statistically significant differences between each treatment

were determined using Bonferroni post hoc tests ($p \leq 0.05$).

Supporting Figures:

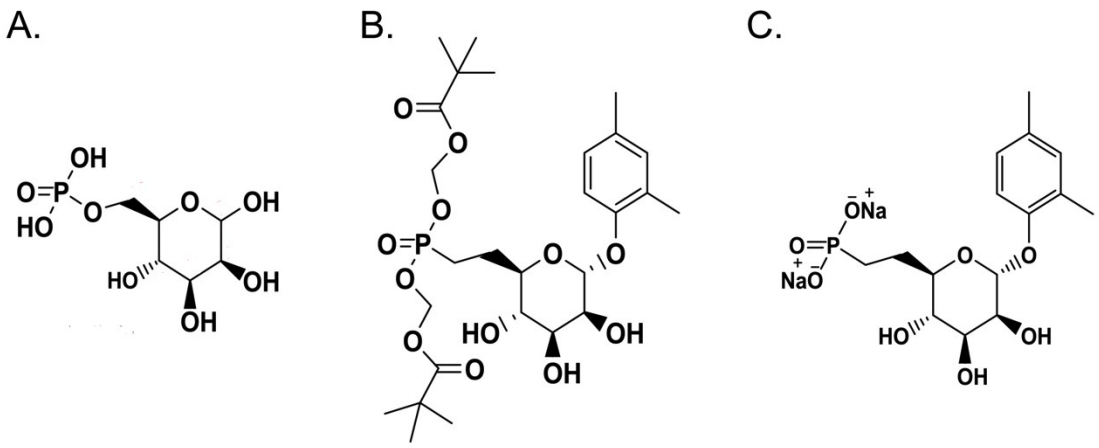

Figure S1. A. Mannose-6-phosphate, B. PXS64 chemical structure, and C. the active degradation product of PXS64 following esterase cleavage.

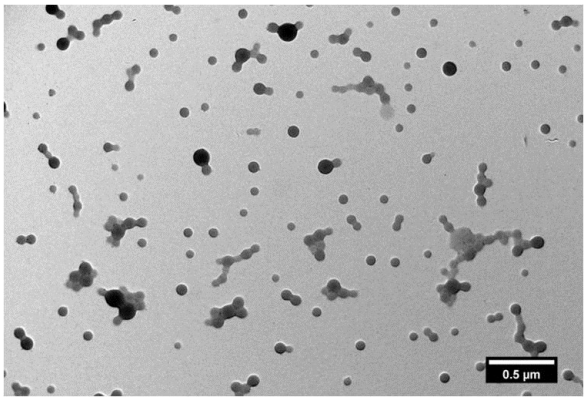

Figure S2. TEM image of PGMA/PXS64/PEI NPs in low magnification.

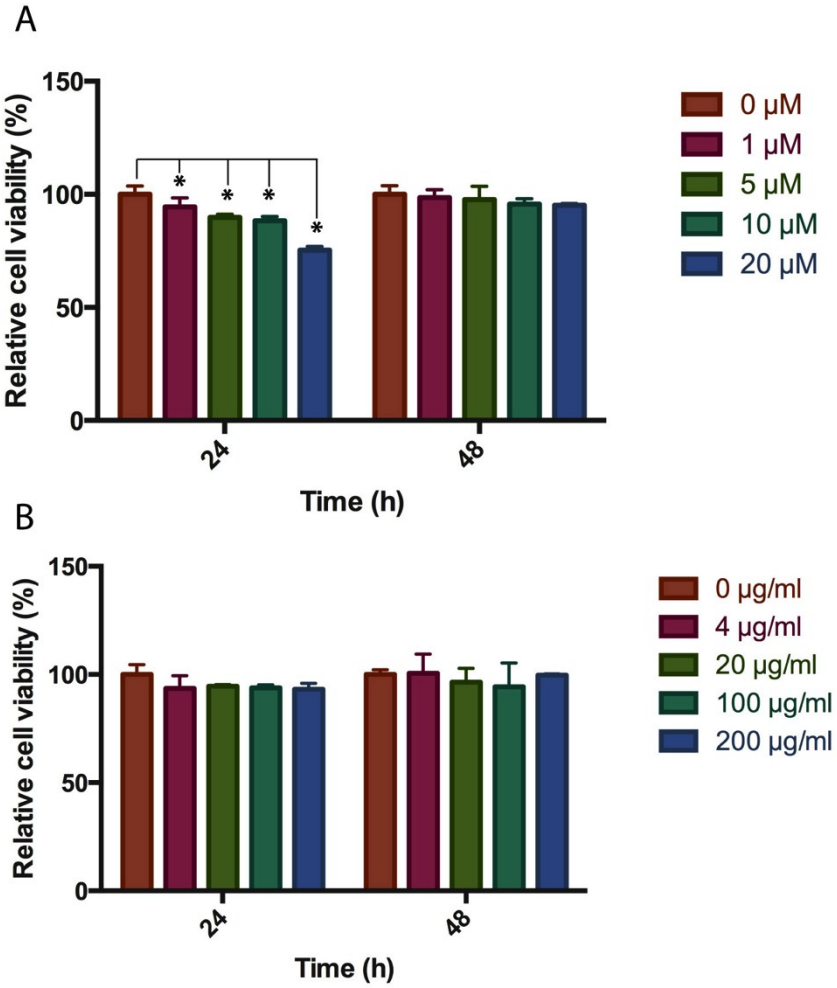

Figure S3. MTS cell viability assays. A. Cell viability after treatment with varying concentrations of PXS64 at 24 and 48h. B. Cell viability after treatment with different concentrations of PGMA/PEI NPs (no PXS64) at 24 and 48h. Data displayed as relative cell viability compared to control for each time point ± standard deviation, significance assessed after an ANOVA followed by a Bonferroni post-hoc test with $p<0.05$.

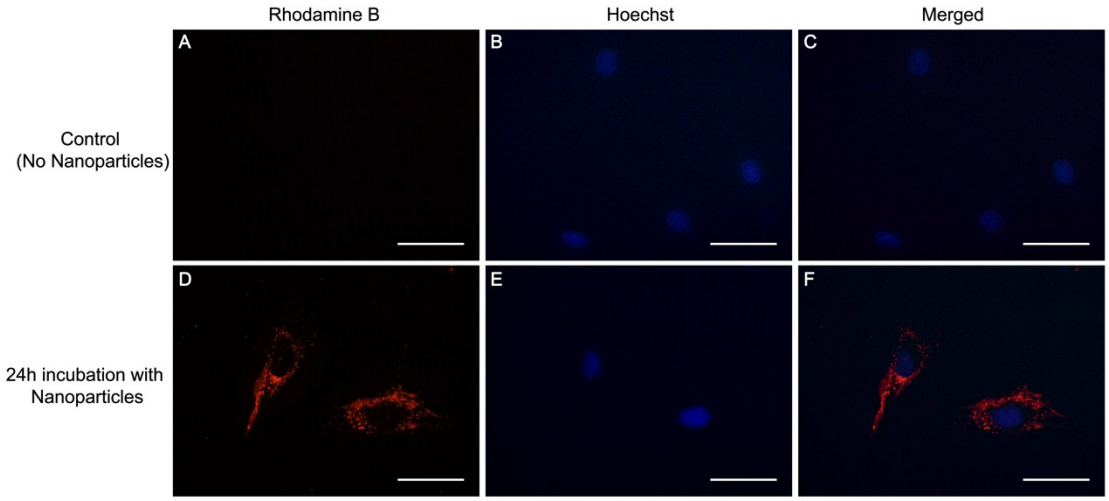

Figure S4. Fluorescent microscopy analysis of primary fibroblasts incubated with or without nanoparticles for 48h. A – C, control (no nanoparticles), A. Rhodamine B, B. Hoechst and C, merged. D – F, cells incubated for 48h with nanoparticles, D. Rhodamine B, E. Hoechst and F. merged. All scale bars are 50 μm.

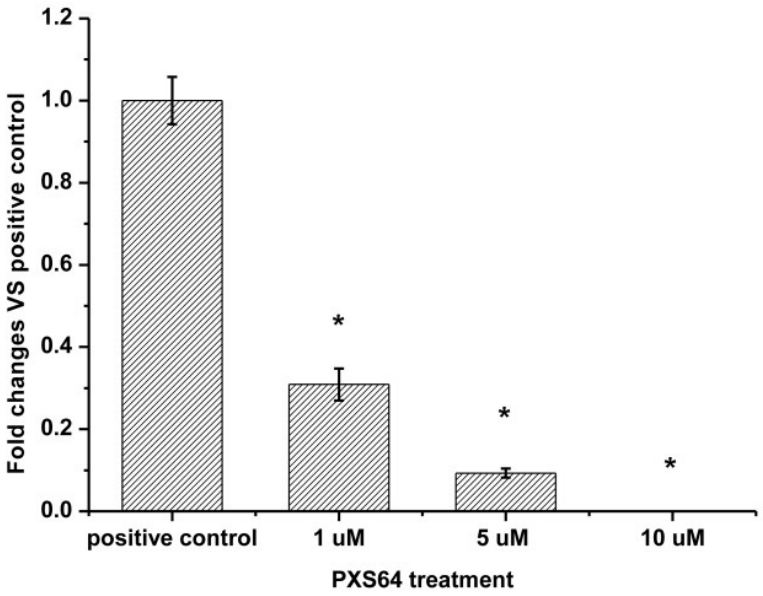

Figure S5. Scar in a jar *in vitro* analysis of collagen deposition per cell (area deposited) treated with different PXS64 concentrations.

References

1. V. Tsyalkovsky, V. Klep, K. Ramaratnam, R. Lupitskyy, S. Minko and I. Luzinov, *Chem. Mater.*, 2008, **20**, 317-325.
2. C. W. Evans, M. Fitzgerald, T. D. Clemons, M. J. House, B. S. Padman, J. A. Shaw, M. Saunders, A. R. Harvey, B. Zdyrko, I. Luzinov, G. A. Silva, S. A. Dunlop and K. S. Iyer, *ACS Nano*, 2011, **5**, 8640-8648.