# Pulmonary Arterial Fibroblast Activation in Response to Dynamic Microenvironmental Stiffness is Sex-Specific

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**Statement of Purpose**: Pulmonary arterial hypertension (PAH) is the stiffening and narrowing of the arteries in the lung. PAH is a non-reversable condition that most commonly affects females between the ages of 30 – 60 years old.[1] Many factors, including genetics and endogenous sex hormones may contribute to the initiation of pulmonary vascular remodeling, although, it is not well understood why PAH is four times more likely to impact females versus males. There are three cell types that are responsible for vascular remodeling: smooth muscle cells, endothelial cells, and fibroblasts. Our studies focus on fibroblast activation in the pulmonary arterial walls, which causes increased production of extracellular matrix (ECM) proteins.[1,2] These aberrant increase in ECM deposition results in vessel stiffening. By looking at sex-specific fibroblast activation in dynamically stiffening microenvironments, we can further our understanding of sex differences in PAH. We used phototunable dynamic hydrogels to observe the effects of microenvironment stiffness on HPAAFs (human pulmonary arterial adventitial fibroblasts). This investigation of the differences in sex-specific fibroblast activation demonstrated that male and female cells responded differently to changes in microenvironmental stiffness.

**Methods**: *PEGαMA Hydrogel:* Eight-arm 10kg $mol^{-1}$ polyethylene glycol-α methacrylate (PEGαMA) macromers were synthesized as previously described.[3] DTT and KCGPQGIWGQCK (MMP-2 degradable peptide) were used as crosslinkers and a fibronectin mimetic peptide (CGRGDS) was used to enhance cell adhesion to the hydrogel. For rheology gels, the precursor solution was placed in 40 µL drops between two glass slides covered in parafilm, and allowed to polymerize for 30 min. The hydrogels were swelled PBS with LAP overnight. The hydrogels were stiffened under UV light (365 nm) for 5 min. For cell culture gels, the precursor solution was placed in 90 µL drops between a silanated 18-mm glass coverslip and a glass slide treated with SigmaCote and allowed to polymerize for 30 min. The hydrogels were soaked in HPAAF medium overnight at 37ºC to swell. *Cellular Activation:* Male and female HPAAFs were seeded at 20,000 cells/$cm^2$ onto the hydrogels. HPAAF medium with fibroblast growth supplement, 1% FBS, and penicillin/streptomycin was used to supplement the cells. HPAAFs were grown out on the soft platform for 7 days. For stiffened conditions, LAP was added to the solution on day 6 and UV light was applied on day 7 to stiffen hydrogels. HPAAFs were grown on stiffened platform for 2 more days. Samples were immunostained for α-smooth muscle actin (αSMA; mouse anti-human αSMA monoclonal antibody, goat anti-mouse Alexa Fluor-555), f-actin (ActinGreen 488 Ready Probes), and nuclei (DAPI), imaged using fluorescent microscopy, and analyzed using ImageJ.

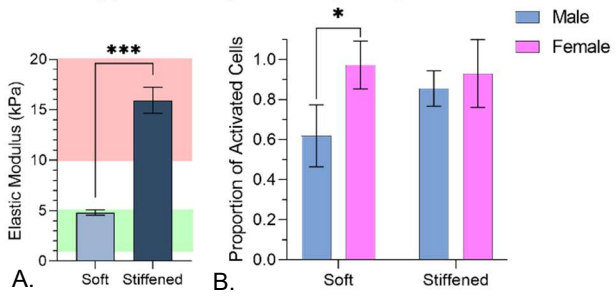


**Figure 1.** Sex-specific fibroblast activation on soft and stiffened hydrogels. A) Rheology data of elastic modulus of soft hydrogels and stiffened hydrogels; $p < 0.0002$. B) Proportion of cells that tested positive for the presence of αSMA on soft or stiffened hydrogels; $p < 0.05$.

**Results**: Soft hydrogels were fabricated with an elastic modulus of 4.82 ± 0.28 kPa which accurately replicated the modulus of healthy lung tissue (1-5 kPa).[4] (Fig. 1A) The hydrogel then underwent a secondary polymerization in the presence of a photoinitiator and UV light, increasing the crosslinking density therefore increasing the stiffness of the hydrogel to 15.94 ± 1.3 kPa to replicate the stiffness of hypertensive lung tissue (> 10 kPa).[4] (Fig. 1A) The HPAAFs were grown on soft hydrogels for 7 days as a healthy model, and for a PAH model the HPAAFs were grown on the soft hydrogel for 7 days and stiffened on day 7 and grown for 2 more days on the stiff hydrogel. Fibroblast activation was quantified by counting the proportion of αSMA-positive cells.[2] (Fig.1B) There was no difference in female HPAAF activation between the healthy or PAH model, but the male HPAAFs were less activated on the soft hydrogels than on stiffened hydrogels. The male HPAAFs were also significantly less activated on the soft hydrogel than the female HPAAFs (Fig.1B).

**Conclusions**: These phototunable PEGαMA hydrogels mimicked the microenvironment stiffnesses of healthy or hypertensive lung tissue. When male and female HPAAFs were grown on the soft and stiffened hydrogels, the female HPAAF activation was not different on soft versus stiffened substrates, but male HPAAFs seemed to be more sensitive to the changes in microenvironmental stiffness than the female cells. Ongoing experiments will evaluate how these cells respond when grown in sex-specific serums as well.

**References**: [1] Prins KW. Cardiol Clin. 2016 Aug;34(3):363-74 [2] Baum J. J Cardiovasc Pharmacol. 2011 Apr;57(4):376–379 [3] Petrou CL. J. Mater. Chem. B,2020,8,6814-6826 [4] Liu F. JCI Insight. 2016;1(8):e86987