Mild cold exposure improves fibrosis in aged mouse regeneration and human fibrogenic differentiation

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INTRODUCTION: In response to muscle injury, bidirectional interactions between FAPs and other muscle-resident cells coordinate regeneration while regulating ECM production to preserve tissue mechanics and function. With aging, this regulatory cycle becomes disrupted and an increased number of FAPs differentiate into myofibroblasts creating a fibrotic environment which blunts efficient regeneration. Therapeutic cooling has been studied for its effect of tissue physiology (White et. al, 2013). Sustained exposure to mild cold *in vitro* promoted myogenic differentiation in C_2C_{12} myoblasts and in primary muscle cells from aged mice and partially restored regenerative capacity (Dey et. al, 2024). We hypothesize that mild cold exposure will reduce fibrosis in aged mouse muscle regeneration and reduced temperature *in vitro* will suppress fibrosis markers in human myofibroblasts.
METHODS: All mice and human samples were males, to limit the systemic differences between sex and sample availability. Aged 22-month-old mice were incubated at either thermoneutral (30°C) or mild cold (17°C) for 7 days. The tibialis anterior was injured via barium chloride ($BaCl_2$) and after 8 days, the muscles were extracted and stained with collagen VI (marker of fibrosis). Human muscle samples were obtained from the Orthopaedic Institute at Mission Bay, UCSF as medical waste (IRB approval, UCSF). Human muscle tissue was mechanically digested in 1mg/mL collagenase XI, washed with PBS and 0.25% trypsin. Digested tissue was filtered (40µm), and erythrocytes were lysed in ACK lysing buffer. Viable hFAPs ($CD31^-$/$CD45^-$/$CD45^-$/$CD29^-$/$CD56^-$/$CD34^+$/$PGFR^+$) were isolated via fluorescent activated cell sorting and cryopreserved. FAPs were thawed, seeded onto Matrigel coated T-25 flasks, and expanded in F10 media (10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin (Penstrep), and 5ng/mL human basic fibroblast growth factor (bFGF)) at 37°C. Once cultures reached 80% confluence, cells were reseeded into 48 well plates at 30,000 cells/well and grown until 80% confluent. The experiment consisted of two media conditions (F10 and fibrogenic media (F10, 10ng/µL TGF-β, 10% fetal bovine serum (FBS), 1% Penstrep, and 5ng/mL bFGF) each at two temperatures (32°C and 37°C). Cultures were differentiated for 6 days and Collagen 1A1 gene expression was quantified using qPCR and cells were fixed in 4% paraformaldehyde and stained for α-smooth muscle actin (α-SMA).
RESULTS: Seven days of mild cold exposure in aged mice significantly reduced muscle fibrosis by 10% (Fig 1A, B). In human FAPs after 6 days of fibrogenic differentiation, Collagen 1A1 expression was significantly reduced at 32°C vs 37°C temperature (Fig 2). In addition, there was a significant reduction in α-SMA in both F10 media and Fibrogenic media after 6 days of differentiation in 32°C vs 37°C degree temperature (Fig 3A-C).
DISCUSSION: Our data demonstrate that mild cold exposure in aged mice can reduce excess fibrosis, which is known to blunt muscle regeneration. However, understanding the potential mechanisms and translating this approach to humans is challenging. Therefore, we developed a proof-of-concept *in vitro* regime on human cells where we reduced the cell culture temperature by 5°C, to mimic the *in vivo* mouse experiment. FAPs are known to differentiate into myofibroblasts which create a severe fibrotic environment. Using this mild cold *in vitro* approach, after 6 days of differentiation in high serum (F10 media) and fibrogenic media we demonstrated that markers of fibrosis were blunted at the cooler temperature. Future experiments will explore the further molecular mechanisms to provide therapeutic targets and investigate the effect on aged human cells where fibrotic conditions are elevated.
SIGNIFICANCE: Excessive fibrosis in aged individuals is related to many pathophysiological conditions and specifically impairs musculoskeletal recovery after injury. Mild cold exposure has the potential to reduce this fibrotic environment and thus improve recovery following injury.
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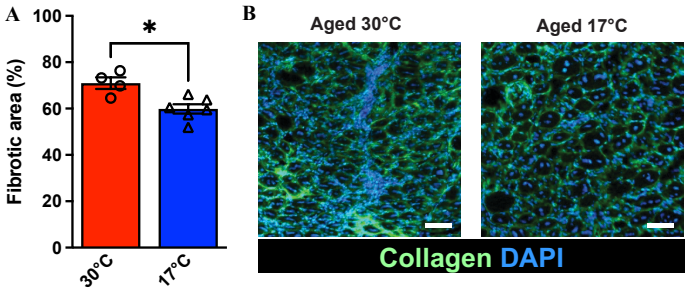

Fig 1. A-B: Fibrotic area and representative collagen VI staining of TA from aged mice after TN and MC exposure and BaCl injury. Mean±SEM, N=4-6. *P<0.05

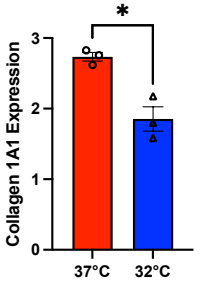

Fig 2. Collagen 1A1 gene expression in human FAPs after 6d of fibrogenic differentiation. Mean±SEM, N=2-3, *P<0.05

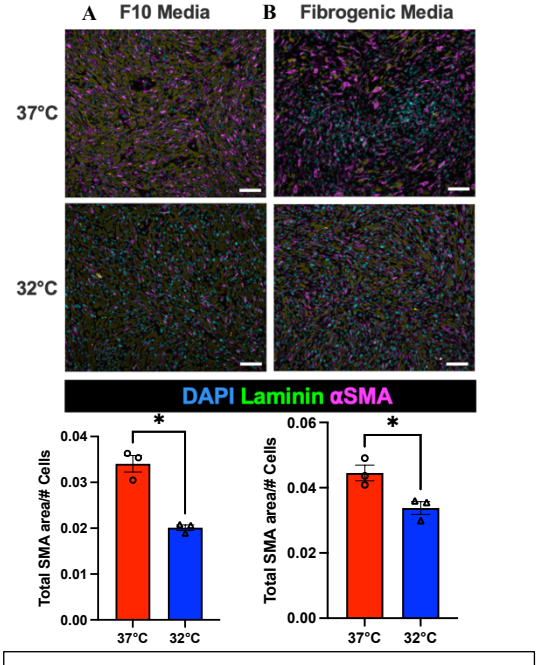

Fig 3. Representative images of total α-SMA area per cell in human FAPs after 6d in F10 media (**A**) fibrogenic (**B**). Mean±SEM, N=2-3, *p<0.05