ERRγ Overexpression in Skeletal Muscle Reduces Cellular Senescence in Aged Mice

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INTRODUCTION

Estrogen related receptor gamma is specific to slow-twitch muscle fibers also known as oxidative fibers (1). Oxidative fibers require a well vascularized environment due to their higher demand for oxygen and have been found to upregulate vascular endothelial growth factor (VEGF) for enhanced skeletal muscle vascularization (1,2). Generating higher VEGF levels by selectively overexpressing ERR-γ in the skeletal muscle is believed to improve overall vascularization which offers imperishable benefits to age-related health concerns. Our preliminary data has already suggested that the overexpression of ERR-γ in skeletal muscle (TG mice) maintains musculoskeletal health throughout the aging process. Cellular senescent levels, which increase with age, are detrimental to a healthy skeletal muscle niche (3). Considering the correlation between aging and senescent-like-cells, the selective estrogen related receptor gamma overexpression in skeletal muscle may reduce senescent levels by improving vascularization. In this study we measured baseline and after-injury cellular senescent levels in both WT and TG mice at 22 months of age to determine if the positive impact ERR-γ overexpression has on aging is related to reduced senescence. We posit that the TG mouse model has enhanced vascularization capable of mitigating cellular senescent levels in both the skeletal muscle and systemic circulation. The significance of these findings is potentially linked to developing a therapeutic approach for preventing age-associated musculoskeletal decline.

METHODS

Animals: The TG colony was obtained from Dr. Narkar's laboratory, and the animal protocol used within was approved by Colorado State University's Animal Care and Use Committee. **Muscle injury:** Both wild-type mice (WT) and TG mice were aged up to 22-months-old and subjected to cardiotoxin (CTX) injuries in the right gastrocnemius muscle (GM). The mice were euthanized 5 days after CTX injury and both the left (control) and right (injured) gastrocnemius muscles were harvested for cellular senescence analysis. After flash-freezing the GMs in liquid nitrogen-cooled 2-methylbutane they were cryo-sectioned (10μM) for immunohistochemistry. **C_{12}FDG Staining and Flowcytometry:** The peripheral blood from 22-month-old WT and TG mice was collected for analysis of cellular senescence in peripheral blood mononuclear cells (PBMCs). The PBMCs were processed according to a recently optimized detection strategy, and then stained for senescent cell marker C_{12}FDG, and finally measured with flowcytometry by adhering to the protocol as previously described (4). **Immunohistochemistry:** The GM cryo-sections were fixed with 4% PFA, blocked with 10% donkey serum and then incubated with primary antibodies for CD31 (endothelial cell marker) and dystrophin (to show the muscle fibers) to determine levels of vascularization. An antibody against Beta-galactosidase (β-gal, senescent marker) was used to evaluate cellular senescence. Alexa fluor 594 conjugated donkey anti-rabbit IgG and Alexa fluor 488 conjugated donkey anti-rat IgG were used as secondary antibodies. The number of CD31+ cells and β-gal+ cells were analyzed using ImageJ software. Analysis of C_{12}FDG flowcytometry was performed using GuavaSoft software. **Statistical analysis:** All results are presented as mean ± standard deviation (SD). Means from the non-injured and CTX injured gastrocnemius of WT and TG mice were compared using Student's t-test with pairwise comparisons and Two-way ANOVA with Tukey's multiple comparisons test; significance is indicated by a p value < 0.05.

RESULTS

TG mice have increased vascularity in their skeletal muscle compared to WT mice. We first compared the vascularity in skeletal muscle between WT and TG mice. As expected, the results showed that there are significantly higher amounts of CD31+ (endothelial marker) cells per myofiber in aged TG mice compared to age-related WT mice **(Fig. 1, p<0.0178)**. ***Aged TG mice have less cellular senescence in their skeletal muscle than age-matched WT mice.*** To determine the cellular senescence in the muscle of aged mice without injury β-gal staining was performed. By counting the number of β-gal+ cells per image, we observed that although senescent cells were low in aged muscle without injury, the aged TG mice have a significantly lower number of senescent cells compared to age-matched WT mice **(Fig. 2A and C, p< 0.0086)**. ***Cellular senescent levels remain significantly reduced after acute CTX injury in aged TG mice.*** Increased cellular senescence after acute muscle injury has been reported (3). To conclude if the aged TG mice can resist the influx of cellular senescent levels after injury, we performed a CTX injury on both the aged TG and WT mice, and assessed the senescence levels again with β-gal staining. Although cellular senescent levels demonstrated a 50%-fold increase in both the WT and TG mice, the senescent levels after-injury in the TG mice remained significantly lower than WT after injury and did not surpass the baseline senescent levels of WT mice **(Fig. 2B-C)**. ***Aged TG mice also demonstrated lower levels of senescent-like-PBMCs***. To further investigate if overexpression ERR-γ in skeletal muscle would affect the number of senescence cells in peripheral blood, we collected and isolated PBMCs from aged TG and age-matched WT mice after injury and performed flowcytometry analysis for C_{12}FDG detection. According to flowcytometry data, gating places the majority of brightly fluoresced C_{12}FDG+ PBMCs in the relative range of mature granulocytes for both models **(Fig. 3A-B)**. When analyzing the percentage of C_{12}FDG+ PBMCs against the total number of their similar cell population, a significant decrease in cellular senescent levels is again evident in the TG mice compared to the WT mice after injury **(Fig. 3C, p<0.0028)**. These significant findings indicate that the benefits of ERR-γ overexpression are not restricted to skeletal muscle and may systemically reduce cellular senescence by enhancing vascular function.

DISCUSSION

Skeletal muscle vascularity is typically jeopardized during the aging process (1,2,3). However, the TG model mechanistically resists age-related declines in musculoskeletal-vascular health most likely by the upregulation of VEGF. Skeletal muscle composes approximately 40% of the human body (5). Reasonably, maintaining skeletal muscle vascularity throughout the aging process may be an underlying biological factor for reducing age-related concerns that extend beyond the musculoskeletal system. A systemic concern related to aging is the accumulation of senescent cells that secrete cell-arresting inflammatory cytokines capable of interfering with cellular communication required for tissue maintenance and repair (3,4). Our results indicate ERR-γ overexpression can mitigate cellular senescent levels not only within the skeletal muscle niche but also offer an overall systemic reduction in senescence. Systemically preventing the age-related accumulation of senescent cells may preserve tissue regeneration potential throughout the aging process.

SIGNIFICANCE. Drug induced ERR-γ activation may target senescent cells offering a multi-mechanistic modality capable of addressing the wide berth of age-related decline.

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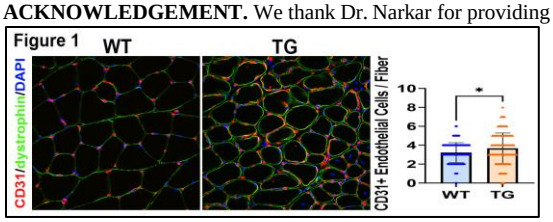

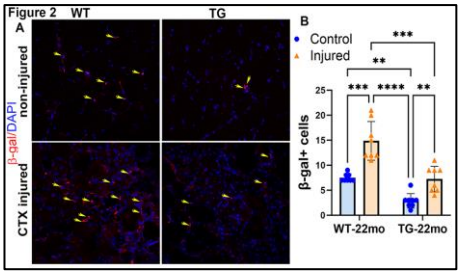

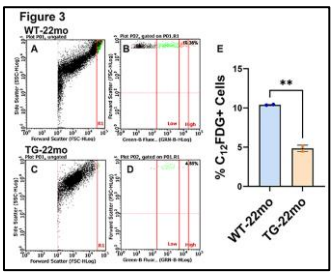

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