Mechanisms of Lingual Muscle Regenerative Capacity as a Function of Age and Exercise-Based Therapy

by

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Biomedical Engineering)

At the

UNIVERSITY OF WISCONSIN-MADISON

2019

Date of final oral examination: 6/24/2019

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Abstract

Over the next 30 years, the world population over the age of 60 is projected to be 2.1 billion people. Chronic diseases affect approximately 80% of the aged population globally and many of these illnesses are associated with impairments in swallowing (dysphagia). Of the elderly population, 15-20% will have dysphagia. Because the tongue is a critical participant in the oropharyngeal swallow, age-related degeneration of the tongue muscles may contribute to swallowing deficits in elderly people. However, cellular processes primary to age-related decline in tongue muscle strength and function are relatively unknown. It is also unknown how specific treatments in current clinical use, such as tongue exercise, may affect cellular processes within muscles to reduce age-related decline in function.

The purpose of this research was to investigate cellular mechanisms contributing to age-related tongue muscle degeneration in a rat model, and how tongue exercise may mitigate signs of tongue muscle degeneration. This study had two specific aims: (1) to determine underlying mechanisms of lingual muscle regeneration and to quantify the tongue satellite cell (SC; e.g. adult muscle stem cells) population in aging rats with and without tongue exercise at multiple time points; and (2) to determine the effects of age and tongue exercise on tongue muscle strength.

Our hypotheses were: (1) SC regenerative capacity and maximal voluntary tongue force (MVTF) will be reduced as a function of aging; (2) exercise will enhance the regenerative function of young and aged SCs as manifested by upregulation of SC myogenic transcription factors and increased MVTF at the 2-week and 8-week time points; (3) $p16^{INK4a}$ expression will be up-regulated as a function of aging and down-regulated as a function of exercise in whole muscle and isolated muscle SCs; and (4)

expression of SC myogenic transcription factors will be differentially expressed in the tongue versus limb muscles.

A total of 146 young adult and old rats were randomized into either baseline, 2-week, or 8-week tongue exercise or no exercise control conditions. Tongue strength was determined in the tongue exercise group at the 3 time points. To assess the SC regenerative capacity in tongue muscles and a limb muscle control, western blot was performed to examine protein expression of SC myogenic transcription factors (Pax7, MyoD, myogenin) in tongue and limb whole muscle homogenates at each time point. Pax7 protein expression was also examined in isolated tongue and limb SCs via immunocytochemistry at each time point. At 2-weeks, we examined whole muscle gene expression of SC myogenic transcription factors via RT-qPCR, and location of Pax7-positive SCs according to myofiber type in whole muscle tissue cross sections via immuno-histochemistry. We also examined gene and protein expression of $p16^{INK4a}$, a marker of cellular senescence, in whole muscle and protein expression of $p16^{INK4a}$ in isolated SCs at all 3 time points.

Results of this study indicated that SC regenerative capacity is impaired with age in the tongue and may be related to increased expression of the senescent marker $p16^{INK4a}$. While age-related deficits in tongue strength were not observed, tongue strength increased in young adult and old rats following 2 and 8 weeks of exercise. With tongue exercise, the regenerative potential of SCs increased in both the acute and chronic stages of the tongue exercise program, and was significantly related to the protein expression of SC myogenic transcription factors and $p16^{INK4a}$ in the muscles of the tongue. The location of Pax7-positive SCs according to myofiber type also changed

following 2-weeks of exercise in the tongue. The expression of SC myogenic transcription factors were also different between the tongue and limb muscles (no exercise group), suggesting that the SC regenerative capacity of the tongue muscles is increased in tongue compared to limb.

A clear understanding of underlying skeletal muscle biology is necessary for the development of novel therapeutics for the treatment of age-related musculoskeletal disorders that affect critical life functions, such as swallowing. The results from our study suggest that clinical rehabilitation strategies that strengthen the tongue may increase SC regenerative process within tongue muscles and may be associated with improved tongue muscle function with age.

Acknowledgements

First and foremost, I would like to thank the University of Wisconsin-Madison, as an institution, for providing an incredibly rich and collaborative training environment in which I could learn, grow, and flourish, personally and scientifically.

Secondly, I would like to thank all the wonderful advisors and mentors whom I have had the opportunity to learn from and work with. I will be forever grateful and indebted to my primary PhD advisor, Dr. Nadine Connor, for seeing something in me that I did not see in myself when I first joined her laboratory as a research assistant. Without her mentorship and guidance, I would have never found myself on this journey and in pursuit of a PhD in Biomedical Engineering. Thank you, Nadine, for providing excellent mentorship and unconditional support, being a fantastic role model, and for creating a laboratory environment that I could find my love and passion for biomedical research within.

I would also like to thank Dr. Michelle Ciucci, who has been a phenomenal mentor and incredible friend. Thank you, Michelle, for providing me with never-ending and constant support, both scientifically and personally, and for instilling in me the importance of living a life that has balance.

To Dr. Masatoshi Suzuki, thank you for taking me into your laboratory like I was one of your own students. I will be forever grateful for your mentorship and guidance; you truly enriched my development as a scientist.

I would like to thank the rest of my committee members Drs. Timothy McCulloch, Randolph Ashton, and Nathan Welham, for their guidance and support throughout this process. And a special thank you to Tim, for being a great mentor and for always

stressing the importance of the clinical implications of biomedical research findings. I would also like to thank Dr. Glen Leverson for all the statistical help and advice, and for giving me confidence in the field of biostatistics.

I have had the opportunity to work with a lot of incredible people. I am so thankful for all the colleagues and friends who have supported me scientifically, professionally, and personally throughout graduate school. In particular, I would like to thank my colleagues Dr. John Russell, Dr. Corinne Jones, Dr. Cindi Kelm-Nelson, Dr. Miranda Cullins, Dr. Tiffany Glass, Dr. Allison Schaser, Dr. Matthew Hoffman, Jared Cullen, Brittany Krekeler, and wonderful friends in my PhD program. I have also had the pleasure to mentor and work with four outstanding undergraduates, Sabrina Wang, Patrick Kasl, Allison Hare, and Brittany Ammerman. They provided me an invaluable experience to develop my own mentorship skills, and made coming to lab everyday truly enjoyable.

Lastly, I would like to thank my family and friends. To my mom, Sally, and dad, Bill, thank you for providing me every single opportunity life has had to offer. Thank you for all of your never-ending love and support, and for taking this journey with me. I would also like to thank my older brother, confidante, and best-friend, Billy. I'd also like to thank my grandmother, Barbar, for all of her love and support, and for being the most incredible female role model. I have also been blessed with fantastic step-parents. In particular, I would like to thank John for teaching me the importance of continuing to work on oneself throughout the lifespan and to always chase after your goals. Finally, I would like to thank all of my friends, I would have not been able to make it through this without you!

The work in this dissertation was supported by the National Institutes of Health (grant numbers F31AG054315, T32DC009401, R01DC005935, R01DC008149, R01DC014358, R37CA225608, R21DC016135).

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List of Symbols and Abbreviations

°	Approached Significant, 0.05<p<0.06
*	Statistically Significant, p<0.05
ANOVA	Analysis of Variance
DPBS	Dulbecco's Phosphate-Buffered Saline
EDL	Extensor Digitorum Longus
Ex	Exercise
FACS	Fluorescent Activated Cell Sorting
GG	Genioglossus
HG	Hyoglossus
HSD	Honestly Significant Differences
ICC	Immunocytochemical
IHC	Immunohistochemical
IT	Intrinsic Tongue
MACS	Magnetic Activated Cell Sorting
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MVTF	Maximal Voluntary Tongue Force
MyHC	Myosin Heavy Chain
NA	Not Available
NS.	Not Significant
OCT	Optimal Cutting Compound

PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PSA	Penicillin/Streptomycin/Amphotericin
RT-qPCR	Quantitative Real-Time Polymerase Chain Reaction
SEM	Standard Error of the Mean
SC	Satellite Cell
SG	Styloglossus
Tx	Treatment
Wks	Weeks

Chapter 1. Introduction.

1.1 The Aging Population

By the year 2050, the number of individuals 60 years and older is projected to double in size to more than 2.1 billion people globally, and the number of people age 80 years and over is expected to triple to 425 million.[1] This rapid, worldwide, demographic shift in age has many societal, economic, and public health implications. Burden on health care systems of elderly people has been declared as one of the major public health challenges of the 21st century.[2] Within the United States, 85% of the population over the age of 65 suffers from at least one chronic condition, while 65-75% have multiple chronic illnesses.[3,4] The most common chronic illnesses experienced by the elderly population are cardiovascular disease, diabetes, respiratory disease, cancer, and neurological impairment. Many of these illnesses are associated with impairments in swallowing.[5,6]

1.2 Swallowing Disorders in Elderly People

Of the elderly population, 15-20% will have a swallowing disorder (dysphagia),[7,8] including 50-75% of nursing home residents.[9] In elderly people, dysphagia impedes the ability to eat a meal, a critical function fundamental to quality of life, and has negative effects on health and rehabilitative potential.[10,11] Elderly people with swallowing disorders are 44% more likely to develop aspiration pneumonia due to their inability to swallow safely and 46% more likely to die from that aspiration pneumonia.[12] Despite associated clinical problems, such as malnutrition, depression, impaired airway protection, aspiration pneumonia, and even death,[7,12-16] current knowledge of underlying

mechanisms contributing to the high prevalence of swallowing disorders in older individuals is sparse.

1.3 The Role of the Tongue during the Oropharyngeal Swallow

The degeneration of cranial muscles active during the swallow is one potential pathophysiological mechanism of dysphagia in elderly people. Swallowing is a complex behavior that has been modeled as three interdependent phases that describe the movement or location of the food bolus: the oral, pharyngeal, or esophageal phase.[17,18] The movement of the bolus involves the precision and coordination of more than 30 cranial nerves and muscles.[19] For swallowing actions, the tongue, a muscular organ comprised of both extrinsic and intrinsic muscles, is active during bolus formation in the oral cavity, and in the propulsion and transport of the bolus from the oral cavity to the pharynx.[20-22] The extrinsic tongue muscles (genioglossus [GG], the primary tongue protrusor; styloglossus [SG] and hyoglossus [HG], muscles of tongue retrusion) originate posteriorly on bone and/or cartilage (in rat) and interdigitate with the intrinsic tongue muscles ([IT], the superior and inferior longitudinals, transverse, verticalis), anteriorly (**Fig. 1**; adapted from[23]).[24] Innervated by the hypoglossal nerve (cranial nerve XII), the extrinsic and intrinsic tongue muscles act in concert to shape and position the tongue for bolus formation, propulsion, and transport.[25-28] Understanding the role of the tongue musculature

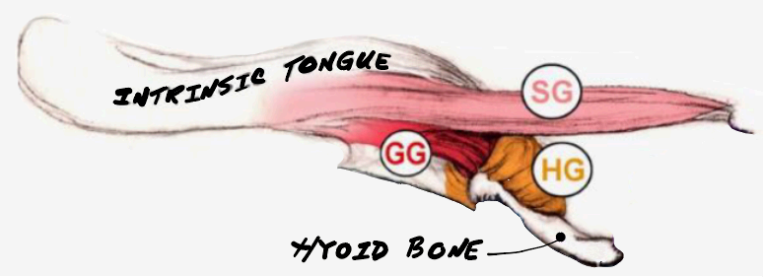

Fig. 1. Tongue Muscles. Illustration, provided by Dr. Tiffany J. Glass, of the extrinsic (genioglossus [GG], styloglossus [SG], hyoglossus [HG]) and intrinsic (IT) tongue muscles involved during swallowing.

during the swallow is a crucial component to the evaluation and diagnosis of dysphagia in elderly individuals.

1.4 Age-Related Changes in Tongue Function During the Oropharyngeal Swallow

Age-related deficits in tongue function may underlie decrements in the aging swallow. Reductions in tongue mass and strength observed in elderly people may contribute to age-related alterations detected in bolus flow in the oral cavity and pharynx.[29-34] Deficits in tongue muscle function may lead to an impaired ability to properly prepare, hold, and propel the food bolus through the oropharynx and is likely related to the increased oral and pharyngeal transit times of old individuals.[35-41] In addition, longer mealtime durations of elderly people are often associated with coughing, choking, and throat clearing, ultimately putting them at an increased risk for the development of aspiration pneumonia.[42,43] Age-related differences observed in elderly individuals as the bolus passes through the pharynx include the accumulation of residue and more variable, and reduced pharyngeal pressures.[44,45] Few studies have examined age-related changes in the human tongue and relevance to swallowing physiology, and have been limited to cadaveric studies of the tongue muscles.[29,46,47] One study reported that the structure of the human tongue musculature changes with age, as evidenced by age-related alterations in myosin heavy chain (MyHC) composition, and another found an increase in the percentage of adipose tissue and amount of connective tissue in the elderly tongue.[29,46,47] Changes observed in the structure and composition of the aged human tongue may impact the oropharyngeal swallow and because of the tongue's role in swallowing, it is an attractive target for rehabilitative therapies.

1.5 Tongue Exercise Can Combat Age-Related Swallowing Changes

Tongue muscle strengthening exercises are in current clinical use for the treatment of dysphagia.[48] Progressive resistance oropharyngeal therapies have been reported to improve tongue strength, increase tongue pressures generated during the swallow, and improve dietary intake and swallowing function in elderly individuals. [48-55] However, the biochemical, molecular, and cellular mechanisms underlying these improvements in tongue strength and swallowing function in elderly people are still relatively unknown. Determination of the biological basis for these changes following tongue muscle strengthening therapies will provide valuable insight into delivery strategies for exercise-based treatments for people with age-related swallowing disorders.

1.6 Age-Related Changes in Skeletal Muscle

Age-related reductions of skeletal muscle mass, strength, and function, termed sarcopenia,[13] in muscles of the tongue may contribute to dysphagia in elderly people. Sarcopenia of the tongue musculature is a likely factor underlying observed functional changes in the aged swallow, manifested as reductions in lingual forces, increased effort and fatigue, increased motor variability in oropharyngeal swallowing pressures, temporal deficits, and alterations in bolus kinematics and biomechanics.[30-32,34,38,45,56-59] However, contributions to sarcopenia are multifactorial and underlying causal mechanisms in the tongue musculature have not been well delineated in the literature. Marked by reductions in muscle strength and mass, the human neuromuscular system begins to steadily decline at the age of 60.[60] This degeneration of the limb neuromuscular system is characterized by muscle atrophy, changes in myosin heavy

chain (MyHC) myofiber type, reductions in motor unit number, alterations in muscle contractility, degeneration of the nervous system, and declines in endocrine function, and is associated with poor nutrition, decreased physical activity, and a rise in chronic illnesses.[61] Another putative mechanism of muscle degeneration suggested by studies performed in the limb is the impaired regenerative capacity of skeletal muscle with age.[61] As muscle cells are lost in the aging process, an inability to regenerate those lost cells could be contributing to reductions in skeletal muscle mass and function.[62,63]

1.7 The Role of Satellite Cells in Muscle Loss and Regeneration

Multiple causal pathways likely exist for the age-related degeneration of skeletal muscle. The reduced ability of aged skeletal muscle to repair and remodel damaged

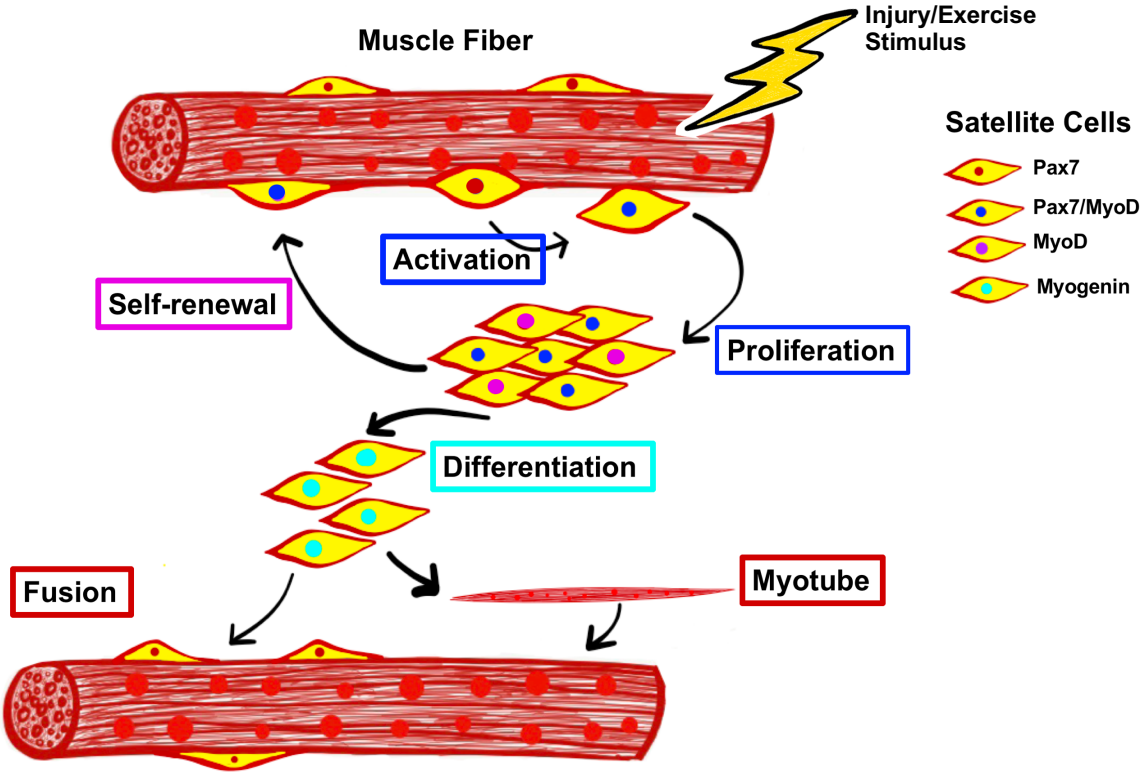

Fig. 2. Satellite Cell (SC) Regenerative Capacity. Schematic illustrating the role of SCs in skeletal muscle regeneration. Specific myogenic transcription factors are expressed during each stage of the SC differentiation cascade. SCs reside in a quiescent state between the sarcolemma and basement membrane of myofibers (Pax7). Following injury to the myofiber, SCs activate (Pax7/MyoD) , proliferate (Pax7/MyoD), and differentiate (myogenin) to repair and remodel damaged myofibers. A subset of SCs self-renew (Pax7) to maintain the quiescent, resident SC pool.

myofibers has been attributed to an age-related functional decline in the regenerative capacity of satellite cells ([SCs], e.g. adult muscle stem cells). [61,63,64] Satellite cell (SC) regenerative capacity refers to the ability or the capacity of SCs to differentiate and self-renew, and to ultimately regenerate damaged skeletal muscle tissue (**Fig. 2)**.[65-68] Post-natal skeletal muscle maintenance, growth, and repair is facilitated by SCs, a population of adult muscle stem cells that reside in the sublaminal niche adjacent to the myofiber.[69-72] Under normal physiological conditions, SCs are mitotically quiescent, arrested in the G_0 phase of the cell cycle, and uniformly express the transcription factor Pax7.[73] In response to injury or disruption of the basal lamina, the membrane surrounding the myofiber, SCs exit the state of quiescence and become activated and proliferate. Activated and proliferating SCs maintain the expression of Pax7 and also upregulate the expression of MyoD. A subset of this population of SCs self-renew to maintain and restore the resident, quiescent SC pool. The other subpopulation of SCs enter a deterministic differentiation pathway through the upregulation of myogenin, and become committed myogenic progenitor cells or adult myoblasts that will facilitate the efficient repair, growth, and regeneration of damaged skeletal muscle.[74] Failure of this regenerative machinery has been linked to the development of sarcopenia in aging limb muscles. However, the SC regenerative capacity in aging tongue muscles has never been examined.[75]

In aging limb muscle, numerous factors have been identified as potential contributors to age-associated dysfunction in homeostatic skeletal muscle tissue maintenance and to the decline in SC regenerative capacity. Age-related reductions in SC number and deficits in SC function have been reported previously in the aging limb

musculature, and have been shown to contribute to the loss in SC regenerative potential following injury- or exercise-induced damage to the myofiber.[76-82] In comparison to the limb, the SC regenerative capacity in aging head and neck muscles has been vastly understudied. The SC pool in the muscles of the intrinsic larynx was shown to decrease with age, and displayed impairments in the their regenerative capacity.[83,84] In studies of aging masseter and extraocular muscles, regenerative capabilities of masseter SCs declined with age, and the SCs from masseter and extraocular muscles displayed altered regenerative responses following myofiber injury when compared to age-matched limb muscles (masseter and extraocular).[85-87] The SC pool in aging tongue muscles has not been quantified. Age-related decline in SC number and function reported in the limb, head, and neck muscles may be related to changes in the extrinsic environment of the SC and to dysregulation of intrinsic mechanisms within the SC.

Changes in the SC extrinsic environmental and SC-intrinsic cues that regulate SC regenerative capacity in response to myofiber injury may also be associated with dysfunction in SC regenerative machinery observed with age.[64,88-95] The extrinsic SC environment includes the basal lamina, the myofiber, local capillaries, endothelial cells, inflammatory cells, and fibroblasts.[70] Age-related modifications in this systemic and local environment may impact the ability of aged SCs to become activated and proliferate. Alterations in systemic circulating factors, disruptions in the composition of the immediate SC microenvironment, alterations in paracrine factors released from the basal lamina and myofiber, and dysregulation in SC-extrinsic signaling pathways have been reported to affect the regenerative capacity of aged SCs in the limb

muscles.[76,82,88,90,96-101] Studies in mice have found improved SC regenerative capacity in aged SCs, when aged SCs are exposed to a young systemic environment.[78,102,103] Even though age-related modifications in the external environment of the SC have been shown to influence the regenerative capacity of aged SCs, SC-intrinsic factors also are reported to change with age and mediate the regenerative response of individual SCs.

At the single cell level, age-related alterations in the intrinsic environment have been shown to impair SC regenerative capacity, and include telomere shortening and epigenetic modifications that alter gene expression.[64,88-95] Dysregulation of the expression of cell cycle regulators in aged SCs are reported to impair SC regenerative capacity in aged skeletal muscle tissue.[63,104-107] The cell cycle and cyclin-dependent kinase inhibitor p16^{INK4a} has been implicated in the age-related loss of SC quiescence and the induction of a state of cellular senescence in aged SCs.[64,106,108-112] Expression of p16^{INK4a}, shown to increase more than 300-fold on a per cell basis with aging, promotes cell cycle arrest and limits the potential for aged SCs to self-renew and contribute to muscle regeneration.[113-116] These studies have shown that the upregulation of p16^{INK4a} contributes to the age-related degeneration in limb skeletal muscle tissue, and in other tissue types. [64,106,108-116] Silencing of p16^{INK4a} expression in a transgenic mouse model of aging rescued satellite cell activation, proliferation, and renewal in limb muscle, making it an attractive therapeutic target in aged skeletal muscle.[106] The age-related effects of p16^{IN4a} gene and protein expression in the tongue musculature and how it may affect the SC regenerative capacity of tongue has never been examined or quantified. Further, the SC regenerative capacity in aging tongue muscles has never been determined, nor have the effects of age-related changes in the

SC external environment or the SC-intrinsic environment on SC function in the aging tongue been examined. A reduction in the SC regenerative capacity likely contributes to the age-related degeneration of the tongue muscles and loss of tongue muscle strength and function, and may contribute to development of swallowing disorders in the elderly.

The development of therapies that target the underlying age-related failure of SC regenerative processes in the tongue muscles may ameliorate swallowing disorders in elderly people. In limb muscle, rehabilitative exercise has been shown to improve the regenerative capacity of SCs and to enhance muscle function.[117,118] Resistance exercise programs are capable of generating significant muscle strain and subsequent myofiber injury in target muscles and may enhance the regenerative potential of skeletal muscle through the stimulation and activation of the resident SC pool.[119-121] Studies to assess SC regenerative capacity following exercise of the limb musculature, in both human and mouse models, have observed increases in SC activation and SC content.[119-122] Exercise has also been suggested to downregulate the expression of $p16^{IN4a}$ by preventing the accumulation of senescent cells.[123,124] Although tongue strengthening therapies for the treatment of dysphagia are in current clinical use, the link between these therapies and the promotion or rescue of the SC regenerative capacity in lingual muscles has not been studied.

1.8 Differences Exist Between Cranial and Limb Muscles

Although possible causal pathways of age-related muscle decline and reduced capacity for regeneration are suggested by recent findings in the limb musculature, direct study of the lingual muscles is warranted and necessary due to a number of differences between cranial and limb muscle anatomy and physiology. Specifically,

distinctions between limb and cranial muscles are found in development,[125-127] morphology,[29,128-130] myosin heavy chain (MyHC) composition,[131-133] innervation patterns,[134,135] vascularization,[136] SC regenerative capacity,[75] and contractile properties.[137] Because swallowing disorders are so prevalent in elderly people, investigation of mechanisms contributing to age-related lingual muscle degeneration and how this may be treated with an exercise therapy is highly relevant to human health, aging research, and to the development of future targeted treatments.

1.9 The Rodent as a Model to Study Mechanisms of Age-Related Tongue Degeneration

Because the use of human subjects is often precluded in the examination of age-related degeneration of the cranial muscles, aging rodent models have been used to study mechanisms of tongue and swallowing dysfunction and to investigate the potential of exercise therapy to reverse/restore tongue muscle function. The rat and human tongue, pharynx, and larynx share many structural, anatomical, and functional similarities despite differences in size and posture.[24,138] Degeneration of muscles involved in swallowing have been reported in aged rodents, and include alterations in the myosin heavy chain (MyHC) isoform and myofiber type composition, atrophy and death of myofibers and myo-nuclei, increased regions of muscle fibrosis, fragmentation of the neuromuscular junction, and impaired regenerative potential.[83,131-133,139-155] This degradation of the cranial musculoskeletal system may be associated with age-related alterations in muscle contractile properties and contribute to weakness and fatigue in the aged tongue, masseter, and pharynx.[75,83,85,125,131,133,145,147,149,153-158] These biological and physiological changes that occur within head and neck muscles are likely

contributors to alterations in swallowing kinematics and biomechanics of aged rodents,[159-162] and probable mechanisms underlying functional swallowing deficits observed in the elderly.

1.10 Tongue Exercise in a Rodent Model

Our laboratory has developed an aging rat model of progressive resistance tongue exercise analogous to clinical tongue strengthening therapies implemented by speech-language pathologists for the treatment of dysphagia in elderly patients.[48,54] Similar to tongue strengthening therapies where humans are trained to progressively increase lingual forces over the course of many weeks or months by pressing the tongue against an oral device (Iowa Oral Performance Instrument, SwallowSTRONG),[54,163] our rats are trained to press the tongue against a force incremented disk for a water reward, and forces to attain that reward are progressively increased over a period of 8 weeks.[132] Comparable gains in tongue strength following progressive resistance tongue exercise therapies have been observed in both humans and rats.[54,131,132,148,151,164] Previous research from our laboratory has shown that 8 weeks of progressive resistance tongue exercise induces considerable musculoplastic changes in the tongues of old rats. Alterations in protrusive tongue muscle contractile properties (increased maximal twitch tension; reduction in fatigue) were detected following the 8-week exercise program.[131,132] We also observed exercise-induced biochemical and morphological changes in protrusive muscles of the extrinsic (genioglossus) and intrinsic (superior and inferior longitudinal, transverse, verticalis) tongue muscles, such as alterations in the myosin heavy chain (MyHC) isoform composition and myofiber type, and a trend towards increased myofiber size.[131,132,164]

Additionally, our exercise paradigm induced neuroplastic effects at the level of the primary motor cortex (increased motor map size for tongue) and the hypoglossal nucleus (changes in neurotrophin concentration), the location of tongue muscle motor nuclei.[151,165] Clearly, tongue strengthening therapies are capable of inducing neural, physiological, biochemical, and morphological changes in associated regions of the motor cortex, brain nuclei, and tongue musculature. However underlying cellular mechanisms of tongue muscle degeneration and regeneration, specifically the contribution of lingual SCs, with age and exercise that accompany MyHC isoform and myofiber type transition, alterations in tongue muscle contractile properties, and changes in tongue muscle function, have not been elucidated.

1.11 Purpose, Aims, and Hypotheses

The purpose of this research was to investigate cellular mechanisms contributing to age-related tongue muscle degeneration in a rat model, and how tongue exercise may mitigate signs of tongue muscle degeneration.

This study had two specific aims:

Aim 1: To determine underlying mechanisms of lingual muscle regeneration and to quantify the tongue SC population in aging rats with and without tongue exercise at multiple time points. SC transcription factors (Pax7, MyoD, Myogenin) and p16^{INK4a} expression were quantified in whole muscle and isolated SCs from the genioglossus (GG), styloglossus and hyoglossus (SG and HG), intrinsic tongue (IT), and extensor digitorum longus (EDL; limb muscle control) of young adult and old rats randomized into either a no exercise or tongue exercise condition at baseline, 2-weeks,

or 8-weeks. We also examined the SC content and location of SCs in relation to myofibers (Type IIb, IIx, IIa, I) and non-myofibers (vessels and nerves) in the GG and SG muscle mid-belly at the 2-week time point.

Aim 2: To determine the effects of age and exercise on tongue muscle strength. Assays of tongue strength (maximal voluntary tongue force [MVTF]) were performed at baseline, 2-week, and 8-week time points in all rats randomized into the tongue exercise condition. Rationale for selection of these time point is found in Methods.

Our hypotheses were: (1) SC regenerative capacity and maximal voluntary tongue force (MVTF) will be reduced as a function of aging; **(2)** exercise will enhance the regenerative function of young and aged SCs as manifested by upregulation of SC myogenic transcription factors and increased MVTF at the 2-week and 8-week time points; **(3)** $p16^{INK4a}$ expression will be up-regulated as a function of aging and down-regulated as a function of exercise in whole muscle and isolated muscle SCs; and **(4)** expression of SC myogenic transcription factors will be differentially expressed in the tongue versus limb muscles.

Chapter 2: Methods.

2.1 Animals

This research was performed in compliance with the *NIH Guide for the Care and Use of Laboratory Animals, 8th Edition* (2011) and the Public Health Service policy on care and use of laboratory animals. The animal care and use protocol was approved by the Animal Care and Use Committee at the University of Wisconsin School of Medicine and Public Health.

A total of 146 male Fisher 344/Brown Norway rats were obtained from the National Institute on Aging Aged Rodent Colony (Charles River Laboratory, Raleigh, NC). The Fisher 344/Brown Norway rat is the most studied and well characterized aging rat strain, with a median life expectancy of 36 months.[166] Young adult (7 or 9 months old; n=73) and old (30 or 32 months old; n=73) rats were randomized into either baseline, 2-week, or 8-week tongue exercise or no exercise, control conditions (10 - 14 young adult; 10 - 14 old rats per each time point by exercise condition).

2.1.1 Time Points

As shown **Figure 3**, three time points were chosen to assess: (1) the acute phases of SC regenerative capacity at baseline and after 2-weeks of exercise; and (2) the chronic phase of SC regenerative capacity following 8-weeks of tongue exercise.[120,121,131,132,164,167-172] The three tongue exercise conditions according to time point were defined as: (1) baseline exercise group (Acute = 2 weeks of acclimation, including 3 days of maximal voluntary tongue force [MVTF] testing); (2) 2-week exercise group (Acute = [a] 2 weeks of acclimation, including 3 days of MVTF testing, [b] 2 weeks of exercise, including 3 days of MVTF testing); and (3) 8-week exercise group (Chronic

= [a] 2 weeks of acclimation, including 3 days of MVTF testing, [b] 2 weeks of exercise, including 3 days of MVTF testing, [c] 6 weeks of exercise, including 3 days of MVTF testing**)**.

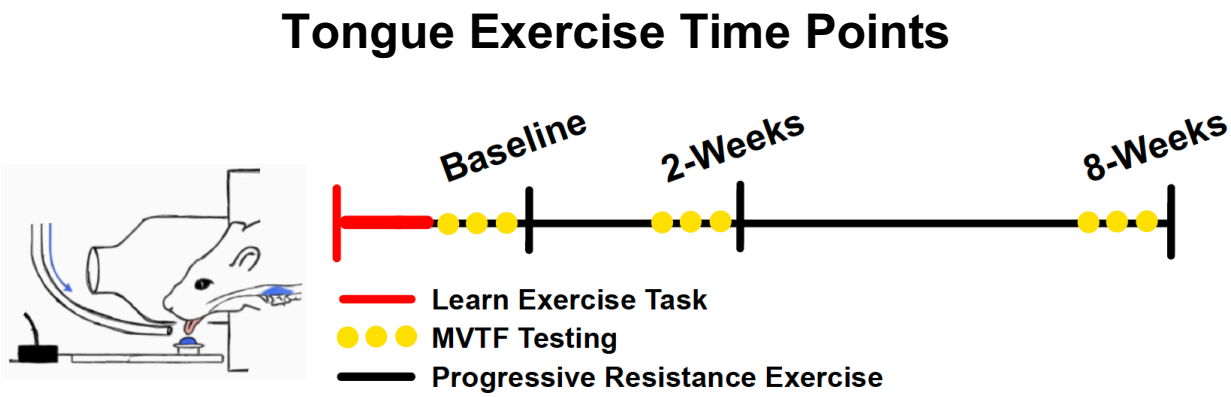

Fig. 3. Tongue Exercise Time Points. Schematic of tongue exercise timeline for baseline, 2-week, and 8-week time points. MVTF = maximal voluntary tongue force

2.1.2 Tongue Exercise and No Exercise Conditions

To address the Institute of Medicine's recommendation to improve translation of research from animals and humans,[173] our laboratory has developed and validated a rodent model of tongue exercise analogous to that used in human clinical care of dysphagia.[48,50,51,54,55,132,174]

Immediately upon arrival at the vivarium, rats were randomized into age by time point by exercise conditions (**Fig. 4)**, and acclimated to the vivarium and the 12:12 hour light-dark reverse light cycle to ensure that exercise is provided at the time of most activity. Rats were housed in pairs in standard polycarbonate cages.

Rats in the tongue exercise condition were limited to only 3 hours of water a day during the acclimation period to establish a water reward system for achievement of tongue force goals during the exercise treatment period. Over the course of two weeks, for 10 minutes each day, rats were trained to press the tongue against a 18 mm

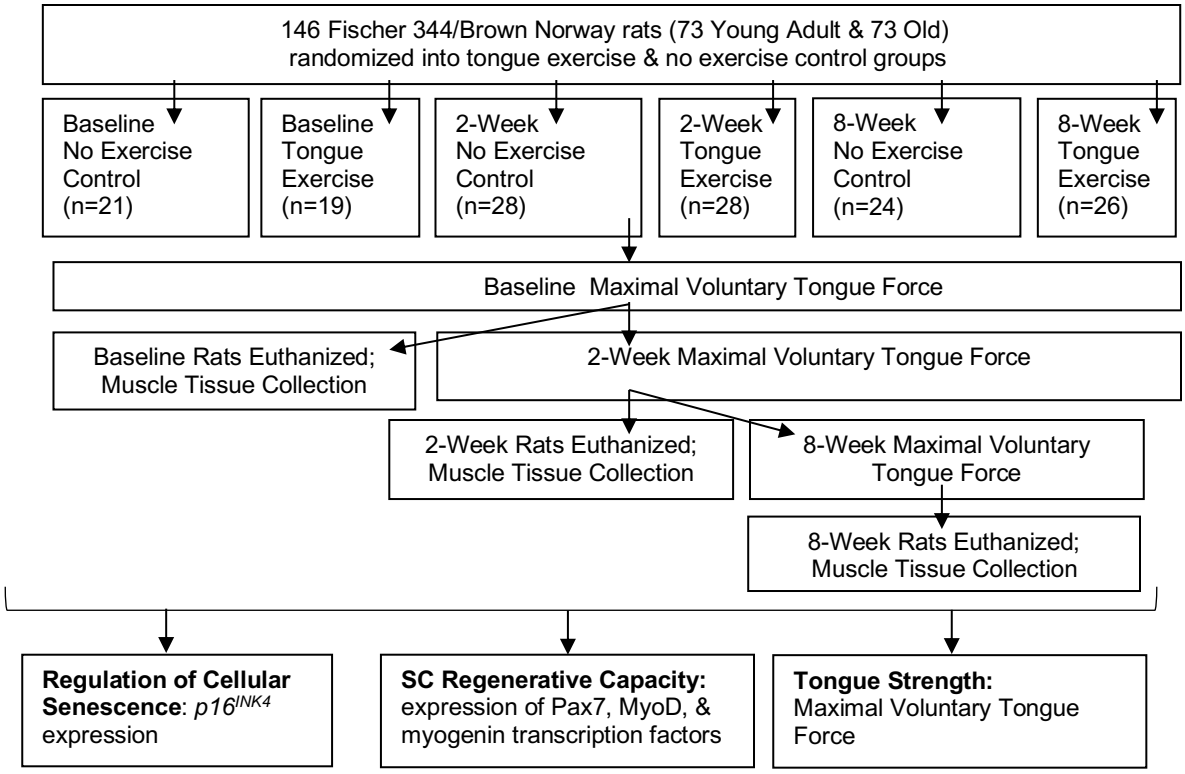

Fig. 4. Data Collection Flow Chart. Rats randomized into groups (20-28 per condition; 10-14 young adult, 10-14 old). Baseline Maximum Voluntary Tongue Force (MVTF; mN) was determined in all rats in the exercise condition, followed by 2-weeks or 8-weeks of tongue exercise and a 2-week or 8-week MVTF. Age-matched, no exercise control rats were also included for each of the 3 time points. All rats were euthanized within 72 hours of the last baseline, 2-week, or 8-week exercise time point. Assays were then performed to determine and quantify mechanisms regulating cellular senescence and SC regenerative capacity in the genioglossus (GG), styloglossus (SG), hyoglossus (HG), intrinsic tongue (IT), &extensor digitorum longus (EDL) muscles, and tongue strength analyzed (MVTF).

aluminum disk fitted with a force transducer (Sensotec load cell, 0 - 245.17 mN) at a force of ~2 mN to receive a water reward of ~0.10 mL, using a variable ratio 5 (VR5) reward schedule (**Fig. 5**). Upon removal from the tongue exercise operandum, rats drank water *ad libitum* from a water dish for 3 hours. Following the two-week acclimation period, a baseline maximal voluntary tongue force (MVTF; mN) was determined by averaging the 10 maximum tongue force values obtained by each rat over the course of 3 days. Baseline MVTF was determined for all rats randomized into either the baseline, 2-week, or 8-week tongue exercise conditions, and was used to set individual training goals across the 2- or 8-week exercise treatment period. Tongue

exercise was performed 5 days/week for 10 minutes a day. Rats in the 2-week exercise group were trained at 50% MVTF (weeks 1 and 2), and a 2-week MVTF was determined. For those rats in the 8-week exercise group, force increments to obtain a water reward were increased every two weeks to mimic a progressive resistance exercise training program: 50% MVTF (weeks 1 and 2), 60% MVTF (weeks 3 and 4), 70% MVTF (weeks 5 and 6), and 80% MVTF (weeks 7 and 8), and 2-week and 8-week MVTF were determined.

No exercise control rats were handled in an identical manner to the tongue exercise group, were regulated to 3 hours of water access each day, and did not participate in any tongue exercise training for the duration of the experimental period (baseline, 2-weeks, or 8-weeks).

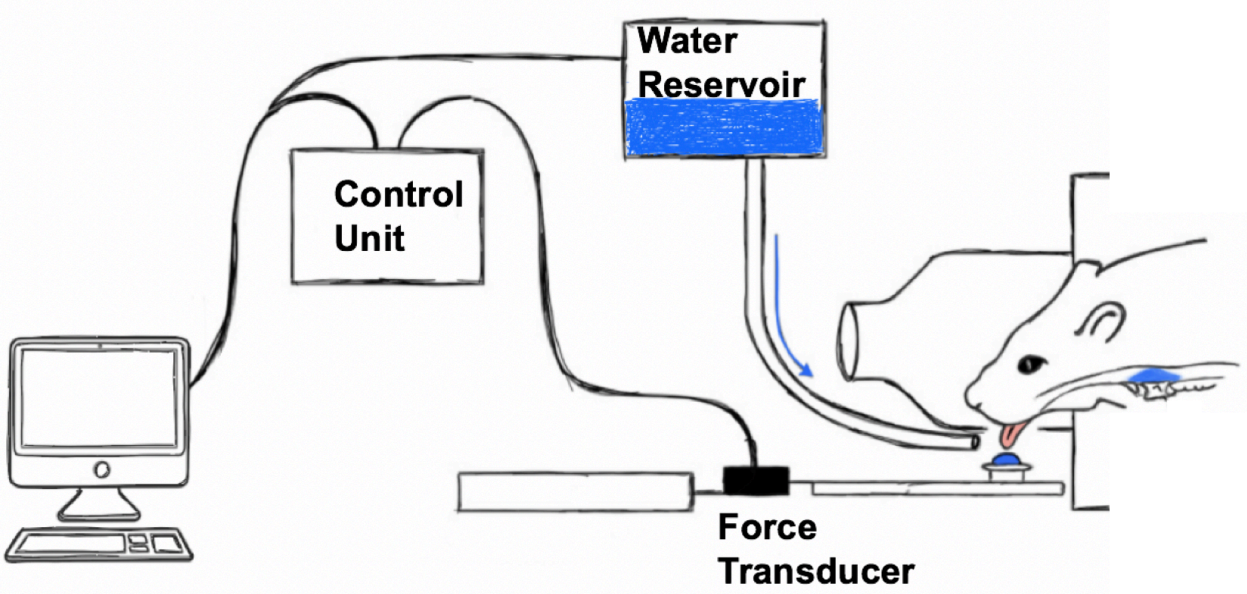

Fig. 5. Schematic of Tongue Exercise Operandum.

2.2 Muscle Tissue Harvest

Following the baseline, 2-week, or 8-week experimental duration, rats were deeply anaesthetized by isoflurane and euthanized by an overdose of Euthasol (0.3 mL,

ic injection). The exercise group was euthanized 72 hours following the last day of MVTF testing. [169]The extrinsic tongue muscles (genioglossus [GG], styloglossus [SG], hyoglossus [HG]), the whole intrinsic tongue (IT), and the extensor digitorum longus (EDL) muscles were extracted and: 1) enzymatically and mechanically dissociated for satellite cell (SC) isolation and immunocytochemical (ICC) analysis, or 2) either snap frozen by liquid nitrogen in a microcentrifuge tube, or embedded in optimal cutting temperature compound (OCT, Tissue-Tek) and frozen in 2-methylbutane cooled by liquid nitrogen, and stored in a -80° freezer for quantitative real-time polymerase chain reaction (RT-qPCR), western blot, or immunohistochemical (IHC) analysis.

2.3 Satellite Cell Analyses

2.3.1 Satellite Cell Isolation

Rats were soaked in 70% ethanol following euthanasia, and tongue (GG, SG, HG, IT) and limb (EDL) muscles were harvested using surgical loupes in a laminar flow hood under sterile conditions. All connective and non-muscle tissue was then removed from the skeletal muscle, and muscle was washed 6x in sterile DPBS (Dulbecco's Phosphate-Buffered Saline, Gibco). Tissue was then weighed and finely minced with sterile surgical scissors in a biosafety cabinet, and digested with a warm protease solution (1.25 mg/mL, Protease from *Streptomyces griseus*, Sigma-Aldrich, St. Louis, MO) in sterile 15 mL conical tubes. Tissue was incubated in the protease solution for 1.5 hours in a 37°C water bath, and agitated (shaken and vortexed) every 15 minutes. Following the 1.5 hour incubation, conical tubes were centrifuged for 4 minutes at 4,000 rpm. Supernatant was then poured off into a new conical tube and reserved (GG,

combined SG and HG, EDL) or discarded (IT). Warm DPBS (5mL) was added to conical tubes containing each tongue or limb muscle, and vortexed for 20 seconds. Tubes were then centrifuged for 10 minutes at 1,000 rpm. Supernatant from all skeletal muscle was poured off and reserved. Warm DPBS (5mL) was again added to conical tubes containing tissue, vortexed for 20 seconds, and centrifuged for 8 minutes at 1,000 rpm. Supernatant was then reserved, warm DPBs added to the tissue, vortexed for 20 seconds, and centrifuged for 5 minutes at 1,000 rpm. Supernatant was reserved, and the tissue pellet placed aside. All tubes containing reserved supernatant were centrifuged for 5 minutes at 3,000 rpm to pellet cells. Previous studies indicate that mechanical and enzymatic dissociation of skeletal muscle tissue yields a cell preparation that is highly enriched for SCs.[175-181]

The supernatant was then carefully suctioned off, and 5 mL of culture media (DMEM + Glutamax, 10% FBS, 1% penicillin/streptomycin/amphotericin B [PSA]) added. The cell suspension was then incubated for a minimum of 25 minutes in a 37°C water bath, and passed through a 40-μm, single cell filter (Falcon-Corning) to generate a single-cell suspension. The filter was then rinsed with 5 mL of culture media. The single-cell suspension was then centrifuged at 3,000 rpm for 5 minutes, and the supernatant aspirated off. Culture media (~125 μL) was then added to the cell pellet, and the single cell suspension plated down on glass coverslips coated with PLL (poly-l-lysine) and fibronectin (40% in DMEM + Glutamax). Four hours after cells were plated down, 300 μL of culture media was added to each well. Cells were cultured for a total of 6 hours in a humidified incubator at 37°C in 5% CO_2 to maintain a quiescent state.[182-186]

2.3.2 Immunocytochemical Staining

Following the incubation period (6 hours [quiescent]), cells were washed in warm DPBS, fixed in 4% PFA (paraformaldehyde in phosphate buffered saline [PBS]) at room temperature for 10 minutes, washed 3x in PBS, and stored in PBS at 4°C for immunocytochemical detection of $p16^{INK4a}$ and Pax7 or desmin. Fixed cells were blocked in 5% NGS (normal goat serum) and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS for 1 hour, and then rinsed in PBS. Cells were incubated with primary antibodies CDKN2A/$p16^{INK4a}$ (ab211542, 1:100, rabbit monoclonal IgG; Abcam, Cambridge, MA) and Pax7 (6 hours incubation; Pax7, 1:40, mouse monoclonal IgG1; Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA) overnight at 4°C. Following incubation with primary antibodies, cell cultures were rinsed 3x for 5 minutes in PBS at room temperature, then incubated with secondary antibodies (A11034 goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary alexa fluor 488, 1:250; A21124, goat anti-mouse IgG1 cross-adsorbed secondary antibody, alexa fluor 568, 1:500 (Pax7); Invitrogen, Waltham, MA). After completion of secondary antibody incubations, cells were rinsed 3x in PBS for 5 minutes, and cover-slipped with ProLong Gold Antifade Mountant with DAPI (Invitrogen). Negative control coverslips were stained following the identical procedure with omission of the primary antibody.

Immunocytochemical images were acquired using an Olympus BX53 Upright Epifluorescence Microscope with fully automated xyz stage control, and a DP80 Digital Camera (Olympus, Tokyo, Japan). Photographs of 4-8 random nonoverlapping images at an objective magnification of 20x were acquired. Counts of isolated SCs from the GG, SG and HG, IT, and EDL were performed using cellSens (Olympus, Tokyo, Japan) or

Fiji (LOCI, University of Wisconsin-Madison) software. In each field of view the percentage of DAPI, $p16^{INK4a}$, and Pax7 stained nuclei were determined and averaged from. The intensity of $p16^{INK4a}$+ cells that were also Pax7+ SC was also determined. All cell count data are reported as mean ± SEM.

2.4 Whole Muscle Analyses

2.4.1 RT-qPCR

Quantitative real-time polymerase chain reaction (RT-qPCR) was performed to evaluate the gene expression of Pax7, MyoD, Myogenin, and $p16^{INK4a}$ in the GG, combined SG and HG, IT and EDL muscles at the 2-week study time point. Tissue snap-frozen in liquid nitrogen was weighed, finely minced with surgical scissors, and sonicated on ice in PureZOL RNA reagent (Bio-Rad, Hercules, CA). Total RNA was then extracted with the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad) per the manufacturer's instructions. Total RNA was measured (ng/µL; 260/280, 260/230) using a Nanodrop system (Thermo Fisher Scientific) and the DNase-treated RNA (100 ng/µL per cDNA reaction per cDNA reaction) was converted into single-stranded cDNA using the SuperSricpt III First-Strand Synthesis System (Invitrogen, Carlsband, CA).

NCBI Primer Blast was used to design and confirm primers for control reference genes (*Ywaz* and *GAPDH*) and genes of interest (**Table 1**[187-189]; *Pax7*, *MyoD*, *Myogenin*, *p16^{INK4a}*) using the rat (*Rattus norvegicus*) genome. Additionally, Netprimer (PREMIER Biosoft, Palo Alto, CA) was used to examine the secondary structure of all primers, to avoid primer products. Non-template controls were run with each primer pair to check for formation of primer-dimers, as well as nonspecific products. Specificity for

each primer pair was confirmed using melt curve analysis; all primer runs yielded single-peak melt curves indicating amplification of single gene products. Furthermore, to confirm sequence identity, the qPCR reaction product for each gene was sequenced using Sanger sequencing with both forward and reverse primers at the University of Wisconsin Biotechnology Center. Using NCBI BLAST all sequences were confirmed to match the intended targets.

Muscle PCR Data

Gene Name	Gene Abbreviation	Gene Link	Accession Number	NetPrimer Score	Forward Sequence	Reverse Sequence	Melt Temperature (C)	CT Threshold	Produce Size (bp)	Primer Sequencing Reference
Gapdh glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	http://www.ncbi.nlm.nih.gov/gene/24383	NM_017008.4	100	GGATACTGAGAGCAAGAGAGA	TTATGGGGTCTGGGATGGAA	60	14	106	(Kelm-Nelson, Stevenson et al. 2016)
Ywhaz tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	*Ywaz*	http://www.ncbi.nlm.nih.gov/gene/25578	NM_013011.3	100	GGCAGAGCGATACGATGAC	AGACGACCCTCCAAGATGAC	60	19	143	n/a
Myod1 myogenic differentiation 1	*Myod*	https://www.ncbi.nlm.nih.gov/gene/337868	NM_176079.1	100	GCTCTGATGGCATGATGGATGTGGAGATGCGCTCCACTAT	GCTCTGATGGCATGATGGATGTGGAGATGCGCTCCACTAT	60	24	174	(Rossi, Pozzobon et al. 2010)
Myog myogenin	*Myog*	https://www.ncbi.nlm.nih.gov/gene/29148	NM_017115.2	100	ACTACCCACCGTCCATTCACTCGGGGCACTCACTGTCTCT	ACTACCCACCGTCCATTCACTCGGGGCACTCACTGTCTCT	60	21	159	(Rossi, Pozzobon et al. 2010)
Cdkn2a cyclin-dependent kinase inhibitor 2A	*P16 Ink4a*	https://www.ncbi.nlm.nih.gov/gene/25163	NM_031550.1	100	GTGCGGTATTTGCGGTATCT	AGGAGAAAAAGGAGGGCTGA	55.5	25	154	(Zhang, Xu et al. 2018)
Pax7 paired box 7	*Pax*	https://www.ncbi.nlm.nih.gov/gene/500574	NM_001191984.1	100	CACGGTGCCCTCAGTGAGTTTCTCGCCATCTTCTTCCTTTTT	CACGGTGCCCTCAGTGAGTTTCTCGCCATCTTCTTCCTTTTT	54.5	29	190	(Rossi, Pozzobon et al. 2010)

Gene	Temp	Elongation	Melt Curve	Runs @CT	Stds to Use
Gapdh	59/60°C	72°C/30sec	88°C	14	HG std-super undil
Ywaz	60°C	72°C/30sec	88°C	19	HG std-super undil
Myod	60°C	72°C/45sec	95°C	24	HG std-super undil
Myog	60°C	72°C/45sec	95°C	21	HG std-super undil
P16 Ink4a	55.5°C	72°C/45sec	95°C	25	Brain std-super (1:5)
Pax	54.5°C	72°C/45sec	95°C	29-30	Brain std-super (1:5)

Table 1. RT-qPCR Information.

Relative gene expression was normalized to *GAPDH* and *Ywaz* genes, and determined using RT-qPCT following the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines for quantitative real-time PCR. Samples were prepared in reaction tubes containing the respective sample cDNA, nuclease-free water, characterized forward and reverse primers (5 µM concentration; Integrative DNA Technologies, Coralville, IA) and SsoFast EvaGreen Supermix, Bio-Rad, Hercules, CA).[190] On each qPCR plate, five standards were run (1:10 serial dilutions, starting at 500 ng/µL) with a non-template negative control. Samples and

standards were run in triplicate. Plates were read with the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each qPCR run entailed of an initiation step at 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, a 30 second annealing phase, a 45 second elongation phase at 72°C, and a melt curve from 60 to 95°C, 0.5 degrees for each 5 second step. All plates were read following each elongation and melt curve stages.

Data inclusion criteria consisted of run efficiencies between 90% and 110% as well as an R^2 of at least 0.99. The mean Ct value for each cycle was defined as the average cycle number at which each sample triplicate crossed the amplification threshold (set *a priori* to 200 RFU). Th mean Ct values for each sample were transformed via the Pfaffl Method.[191]

2.4.2 Western Blot

Western Blot analyses were performed to determine the protein expression of Pax7, MyoD, myogenin, and p16^{INK4a} in the GG, combined SG and HG, IT, and EDL muscles. Snap-frozen tissue was weighed, finely minced with surgical scissors and homogenized via sonication on ice in RIPA buffer (diluted 1x in milli-q H_2O; Cell Signaling Technology, Danvers, MA) supplemented with Halt phosphatase and protease inhibitor cocktails, and EDTA (Thermo Fisher Scientific; Waltham, MA). Following sonication, tissue was placed on ice on an orbital shaker at 4°C for 1 hour. The tissue suspension was then centrifuged at 13,000 rpm for 15 minutes, the supernatant collected and reserved for quantification of total protein concentration (DC Protein Assay; Bio-Rad, Hercules, CA). Stock was then diluted with the homogenization

buffer to a working concentration (8 µg/µL), aliquoted, and stored at -80°C until use for western blot.

Protein (70 µg) from each tissue sample was loaded into a precast 4-12% Criterion XT Bis-Tris Protein Gel, and gel electrophoresis was performed. A positive internal control skeletal muscle tissue lysate (70 µg; ab29711, Mouse Skeletal muscle tissue lysate-total protein; Abcam, Cambridge, MA) and prestained protein standard (Precision Plus Protein Kaleidoscope Prestained Protein Standard; Bio-Rad) were also loaded into each gel. Proteins were then transferred to a nitrocellulose membrane. Following the transfer, total protein on the membrane was detected (Pierce Reversible Protein Stain Kit for Nitrocellulose Membranes; Thermo Fisher Scientific) per manufacturer's instructions. The membrane was then blocked for 1 hour at room temperature in filtered 5% nonfat dry milk in TBST (Tris buffered saline, 0.15% Tween 20), and incubated with primary antibodies overnight at 4°C on a revolving tube rotator (Pax7, 1:250, mouse monoclonal IgG1, DSHB; MyoD, MA1-41017, 1:250, mouse monoclonal IgG1, Invitrogen; Myogenin, F5D, 1:250, mouse monoclonal IgG1, DSHB; $p16^{INK4a}$, PA130670, 1:250, rabbit polyclonal IgG, Invitrogen).

Following primary antibody incubation, membranes were washed 5x for 5 minutes in TBST, and then incubated for 1 hour at room temperature in appropriate horseradish peroxidase-conjugated secondary antibodies (hrp linked anti-mouse IgG1 cross-adsorbed, hrp, Invitrogen; hrp linked anti-rabbit IgG, Cell Signaling Technology). Membranes were then washed in TBST, and developed (SuperSignal West Pico Plus Chemiluminescent Substrate, Thermo Fisher Scientific) using a ChemiDoc-It2 Imaging System (UVP) and imaged, and analyzed using VisionWorks Software (Analytik Jena).

2.4.3 Immunohistochemistry

Tissue (GG, SG, EDL) embedded in optimal cutting temperature compound (OCT, Tissue-Tek) was cut from the mid-belly of the muscle into serial 10μm cross-sections using a -16°C cryostat (Leica CM 1850, Leica Biosystems) and mounted on slides. Tissue cross-sections were then fixed in cold acetone for 10 minutes at 4°C, and sections washed in PBS 3x for 2 minutes. Slides were blocked for 1 hour on an orbital platform shaker at room temperature in 10% NGS, 0.1% Tritron X-100, and PBS. Tissue cross-sections were then incubated overnight at 4°C on an orbital platform shaker in primary antibodies Pax7 (Pax7, 1:10, mouse monoclonal IgG1; Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA), myosin heavy chain (MyHC) Type I (BA-F8, 1:50, mouse monoclonal IgG2b; DSHB), MyHC Type IIx (6h1, 1:90, mouse monoclonal IgM; DSHB), MyHC IIb (BF-F3, 1:200, mouse monoclonal IgM; DSHB, and Laminin (L9393, 1:1000, rabbit polyclonal; Sigma-Aldrich, St. Louis, MO). Myosin heavy chain type IIa fibers were identified by an absence of staining. After primary antibody incubation, tissue cross-sections were washed in PBS 3x for 5 minutes at room temperature. Sections were then incubated in secondary antibodies for 1 hour at room temperature on an orbital shaker (A21140, 1:200, goat anti-mouse IgG2b cross-adsorbed alexa fluor 350; A21121, 1:500, goat anti-mouse IgG1 cross-adsorbed alexa fluor 488; A21426 goat anti-mouse IgM cross-adsorbed alexa fluor 555; A21071, 1:500 goat anti-rabbit IgG (H+L) cross-adsorbed alexa fluor 633; Invitrogen, Waltham, MA). Following 3, 5 minute PBS washes, slides were mounted ProLong Gold Antifade with DAPI to visualize nuclei.

Tissue cross-sections were imaged using a 20x objective (Olympus BX53 Upright Epifluorescence Microscope with fully automated xyz stage control; DP80 Digital Camera; Olympus, Tokyo, Japan), and 4 to 6 random nonoverlapping images were taken. Fiji (LOCI, University of Wisconsin-Madison) was used to analyze images for quantification of Pax7-positive staining nuclei (nuclei located in the basal lamina staining positively for both DAPI and Pax7) by MyHC fiber type. In each field of view the percentage of Pax7 positive nuclei by fiber type was determined and averaged from the GG, SG, and EDL. The percentage of centralized nuclei by MyHC fiber type was also determined from each image. In addition, the co-localization of Pax7+ nuclei with non-muscle tissue structures (vasculature, nerves, non-muscle connective tissue bodies) was also determined. All cell count data are reported as mean ± SEM. The HG and IT were not immunostained due to the complexity of the muscle fiber orientation and the inability to obtain true cross-sections and identify the localization of Pax7+ nuclei by fiber type.

2.5 Statistical Analysis

All statistical analyses were conducted with GraphPad Prism version 8.1.2 for Mac and Windows (GraphPad Software, La Jolla, CA). The critical value for obtaining statistical significance was set at $\alpha=0.05$. Dr. Glen Leverson, lead biostatistician in the Department of Surgery at the University of Wisconsin School of Medicine and Public Health, served as a consultant for all statistical analyses.

2.5.1 Tongue Strength

Mixed-model repeated measures 2-way analysis of variance (ANOVA) was used to examine main effects for age (Young Adult, Old), time point (baseline, 2-weeks, and 8-weeks), and their interaction on Maximal Voluntary Tongue Force (MVTF; mN), percent change in MVTF, and variability in MVTF. The Geisser-Greenhouse correction was used to account for the value of epsilon. Post-hoc testing was completed using Tukey's honestly significant differences (HSD) to correct for multiple comparisons.

2.5.2 Satellite Cell Regenerative Capacity and Cellular Senescence

Two-way ANOVA was used to examine main effects for age (Young Adult, Old), treatment (Exercise, No Exercise), and interaction effects on Pax7, MyoD, myogenin, and $p16^{INK4a}$ gene expression in the GG, combined SG and HG, IT, and EDL and on the colocalization of Pax7 by myofiber type in the GG and SG at the 2-week experimental time point. The total percentage of Pax7-positive (Pax7+) nuclei and centralized nuclei at 2-weeks in the GG and SG was also examined by 2-way ANOVA. To correct for multiple comparisons, post-hoc testing was completed using Tukey's HSD. T-tests were used to compare colocalization of Pax7 by myofiber type, and the total percentage of Pax7+ nuclei and centralized nuclei between the young adult and old age group in the EDL muscle at the 2-week time point.

To examine differences in protein expression of Pax7, MyoD, myogenin, and $p16^{INK4a}$ among the muscles of the tongue and limb in the no exercise group at the 2-week time point, a 2-way ANOVA was performed to examine main effects for age (Young Adult, Old) and muscle (GG, combined SG and HG, IT, EDL), and interaction

effects. Post-hoc testing was completed using Tukey's HSD to correct for multiple comparisons.

Three-way ANOVA was used to examine main effects for age (Young Adult, Old), treatment (Exercise, No Exercise), time point (baseline, 2-weeks, and 8-weeks), and their interactions on Pax7, MyoD, myogenin, and $p16^{INK4a}$ protein expression in the GG, combined SG and HG, IT, and EDL. To correct of multiple comparison, post-hoc testing was completed using Tukey's HSD.

In isolated SCs from the GG, combined SG and HG, IT, and EDL, three-way ANOVA was used to examine main effects for age (Young Adult, Old), treatment (Exercise, No Exercise), time point (baseline, 2-weeks, and 8-weeks), and their interactions on the percentage of Pax7 positive SCs (Pax7+ SCs), the percentage of $p16^{INK4a}$ positive/Pax7 positive SCs ($p16^{INK4a}$+/Pax7+ SCs) and the intensity of $p16^{INK4a}$ expression in Pax7+ SCs. Post-hoc testing was completed using Tukey's HSD to correct for multiple comparisons.

2.5.3 Relationship Between Muscle Regenerative Capacity and Tongue Strength

Multiple linear regressions were performed to examine the degree to which protein expression of Pax7, MyoD, myogenin, or $p16^{INK4a}$ in the GG, SG and HG, or IT could predict Maximal Voluntary Tongue Forces at the baseline, 2-week, and 8-week time points.

2.5.4 Missing Data

For some statistical comparisons, missing data resulted in a smaller sample size, reflected in smaller degrees of freedom. Unexpected animal expiration (n=2; 1 old rat at

baseline, 1 old rat at 2-weeks) accounted for missing MVTF data and gene expression data. All other available data were used in all statistical analyses.

Chapter 3. Results.

3.1 Tongue Strength

3.1.1 Maximal Voluntary Tongue Force (MVTF)

Rats in the tongue exercise group completed either the baseline, 2-week, or 8-week progressive resistance tongue exercise treatment (**Fig. 6A)**. That is, separate groups of young adult and old rats were used for each of the three time points. On the mixed-model repeated measures 2-way ANOVA, no significant interaction effects were noted between age group and time point ($F_{2,75}$=0.871, p=4.23; **Fig. 6B**). Within the tongue exercise groups, MVTF (mN) was significantly increased after the 2-week and 8-week tongue exercise treatment periods ($F_{1.462,54.81}$=131.9, p<0.001; **Fig. 6B**). Percent change in MVTF between age groups was not significantly different ($F_{1,51}$=0.004, p=0.950). After the 8-week exercise period, MVTF increased an average of 118.5% in the young adult group and an average of 141.7% in the old group. The greatest gains in MVTF occurred in the young adult group at both the 2-week (mean = 70.44% [young adult] vs mean = 68.76% [old]) and 8-week time points (mean = 51.81% [young adult] vs mean = 45.63% [old]). There were not significant differences in variability in MVTF between the young adult and old groups at any time point (Age, $F_{1,2}$=16.80, p=0.055; Time, $F2_{,2}$=16.05, p=0.059; **Fig. 6C**).

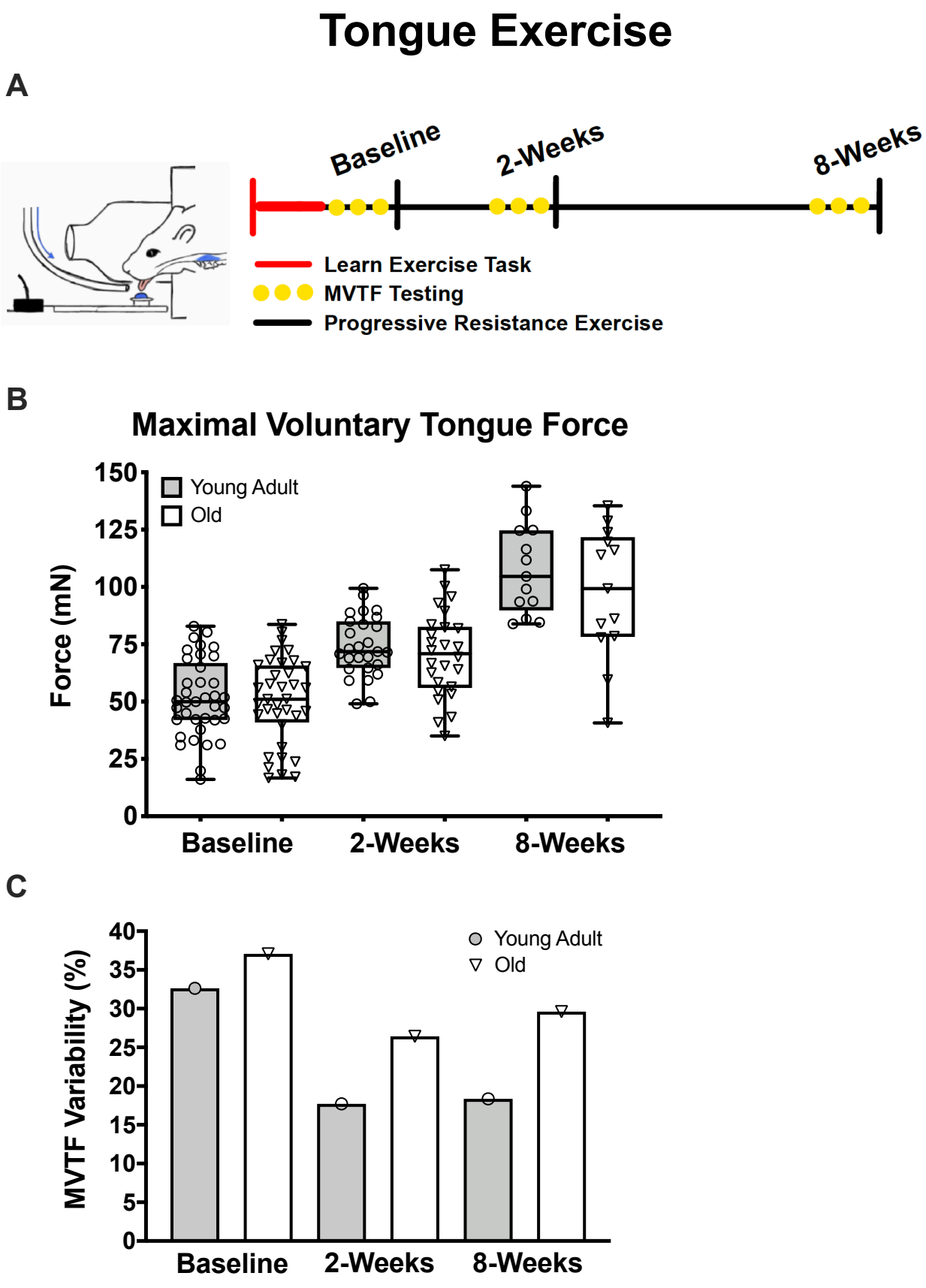

Fig. 6. Tongue Exercise. (A) Schematic of tongue exercise timeline. (B) Maximal voluntary tongue force significantly improved following 2-weeks and 8-weeks of tongue exercise. (C) The old rat group had greater variability in MVTF at each time point [approached significance]. MVTF = maximal voluntary tongue force

3.2 Satellite Cell Regenerative Capacity

3.2.1 Pax7

3.2.1.1 Whole Muscle Pax7 Gene Expression at 2 Weeks

As shown in Figure 6, significant age-related alterations in Pax7 gene expression were found in tongue and EDL muscles in the young adult and old groups in the absence of a significant interaction with treatment (tongue exercise, no exercise; GG, $F_{1,11}$=0.006, p=0.942; EDL, $F_{1,11}$=0.715, p=0.416). Expression of the Pax7 gene significantly decreased with age in the GG ($F_{1,11}$=5.633, p=0.037; **Fig. 7A**) and EDL ($F_{1,11}$=10.92, p=0.007; **Fig. 7D**) muscles of the old group versus the young adult group. No significant age-related differences in Pax7 gene expression were observed in the SG and HG ($F_{1,10}$=0.402, p=0.540; **Fig. 7B**) or IT ($F_{1,11}$=0.174, p=0.685; **Fig. 7C**) muscles. No significant exercise effects were observed in the GG ($F_{1,11}$=0.053,p=0.821), combined SG and HG ($F_{1,10}$=0.375, p=0.554), IT ($F_{1,11}$=1.492, p=0.247), or EDL ($F_{1,11}$=0.079, p=0.784) muscles.

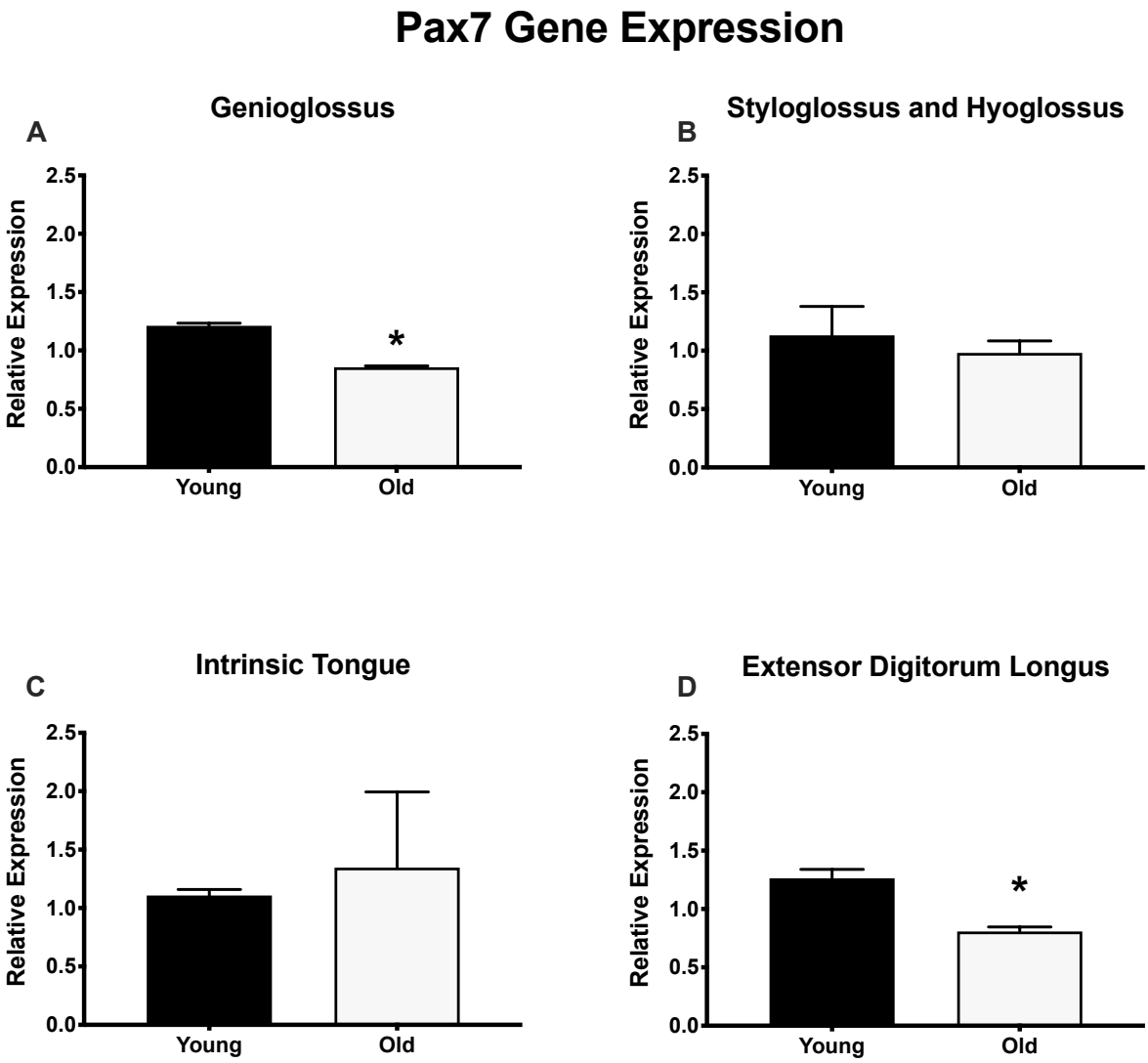

Fig. 7. The Effect of Age on Pax7 Gene Expression. With age, Pax7 gene expression was significantly reduced in the genioglossus (A) and in the extensor digitorum longus (D) muscles of the old group in comparison to the young adult group. No significant changes in Pax7 gene expression were observed in the combined styloglossus and hyoglossus (B), or intrinsic tongue (C) muscles.
* = $p<0.05$

3.2.1.2 Whole Muscle Pax7 Protein Expression

Significant alterations in Pax7 protein expression in the GG, EDL, and IT muscles were observed with age and time point, in the absence of significant interaction effects (tongue exercise, no exercise; GG, $F_{2,36}$=0.391, p=0.679; EDL, $F_{1,24}$=0.015, p=0.905; IT, $F_{2,36}$=0.390, p=0.680; **Fig. 8**). Pax7 protein expression in the GG was significantly reduced in the old compared to young adult group ($F_{1,36}$=5.154, p=0.029; **Fig. 9A**), and was significantly elevated at the 2-week time point in comparison to baseline and 8-week time points ($F_{2,36}$=39.13, p<0.001**; Fig. 10A**). In the EDL muscle, Pax7 protein expression significantly increased with age ($F_{1,24}$=21.56, p<0.001; **Fig. 9D**), and was greatest at the 8-weeks ($F_{1,24}$=6.452, p=0.018; **Fig. 10D**). Expression of the Pax7 protein in the IT was significantly higher at the 8-week timepoint ($F_{2,36}$=9.105, p<0.001; **Fig. 10C**). No exercise effects were observed in GG ($F_{1,36}$=0.874, p=0.356), combined SG and HG ($F_{1,36}$=0.899, p=0.349), IT ($F_{1,36}$=0.651, p=0.425), or EDL ($F_{1,24}$=0.499, p=0.487).

Significant differences in Pax7 protein expression were observed among the GG, combined SG and HG, IT, and EDL muscles in the no exercise group at the 2-week time point (Muscle x Age; $F_{3,24}$=7.888, p<0.001; **Fig. 8**). Pax7 protein expression was significantly greater in the GG of the young and old no exercise group in comparison to the combined SG and HG, IT, and EDL muscles.

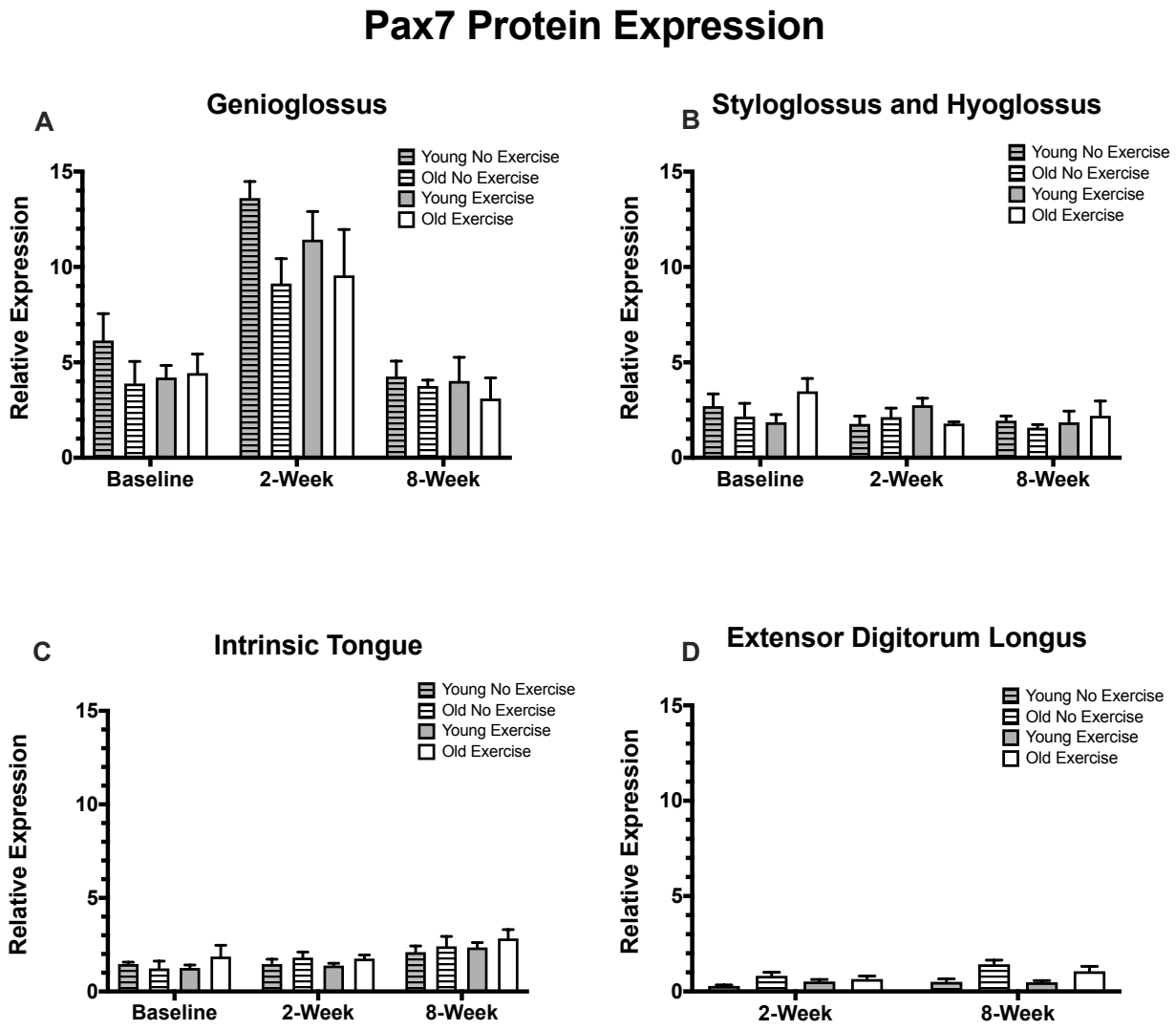

Fig. 8. Pax7 Protein Expression. Pax7 protein expression was elevated in the tongue muscles (A-C) compared to limb (D). No significant interaction effects were observed in Pax7 protein expression in the genioglossus (A), combined styloglossus and hyoglossus (B), intrinsic tongue (C), or extensor digitorum longus (D) muscles.

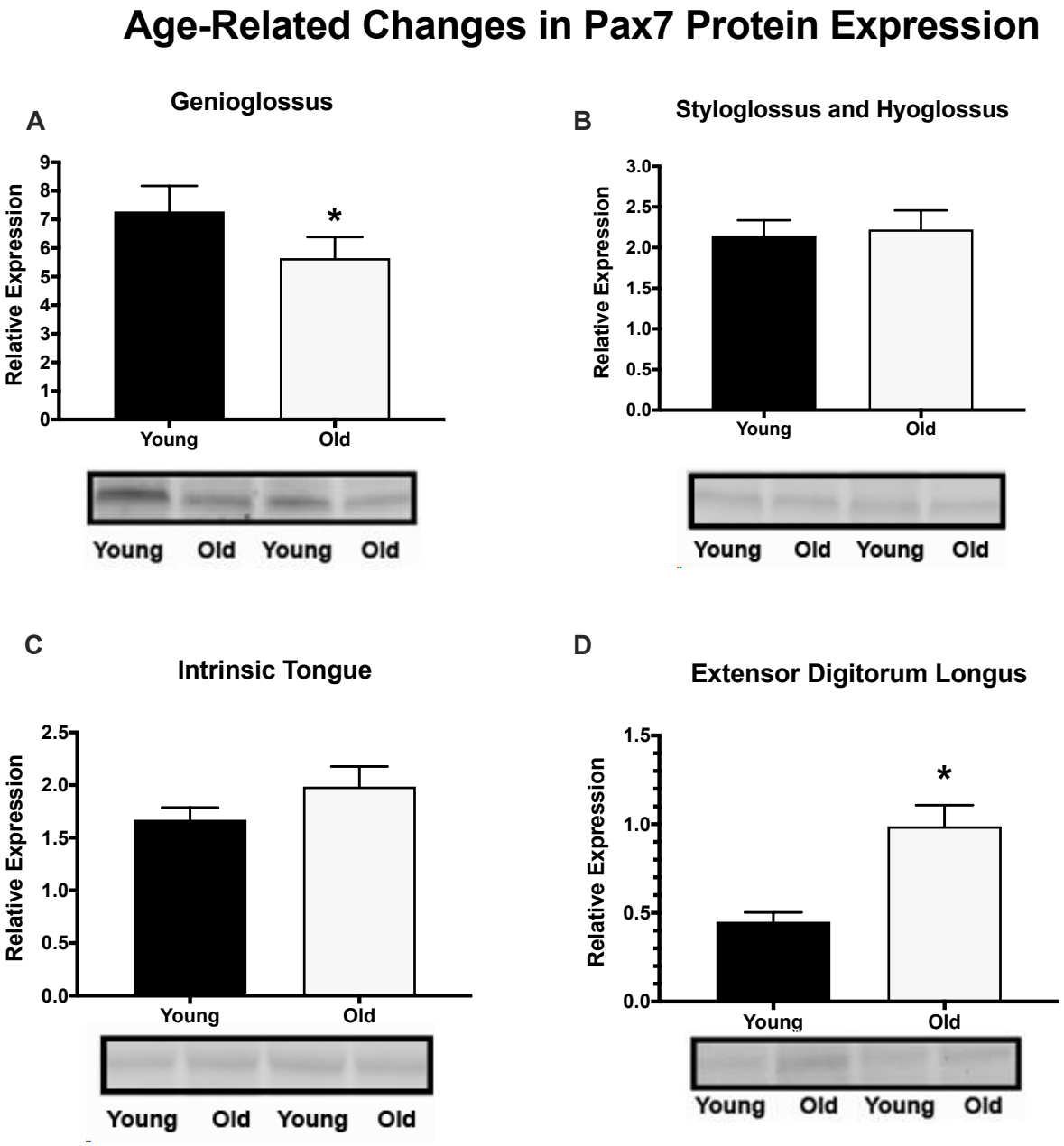

Fig. 9. The Effect of Age on Pax7 Protein Expression. With age, Pax7 protein expression was significantly reduced in the genioglossus (A) and was significantly elevated in the extensor digitorum longus (D) muscles of the old group in comparison to the young adult group. No significant changes in Pax7 protein expression were observed in the combined styloglossus and hyoglossus (B), or intrinsic tongue (C) muscles. * = $p<0.05$

Time-Related Changes in Pax7 Protein Expression

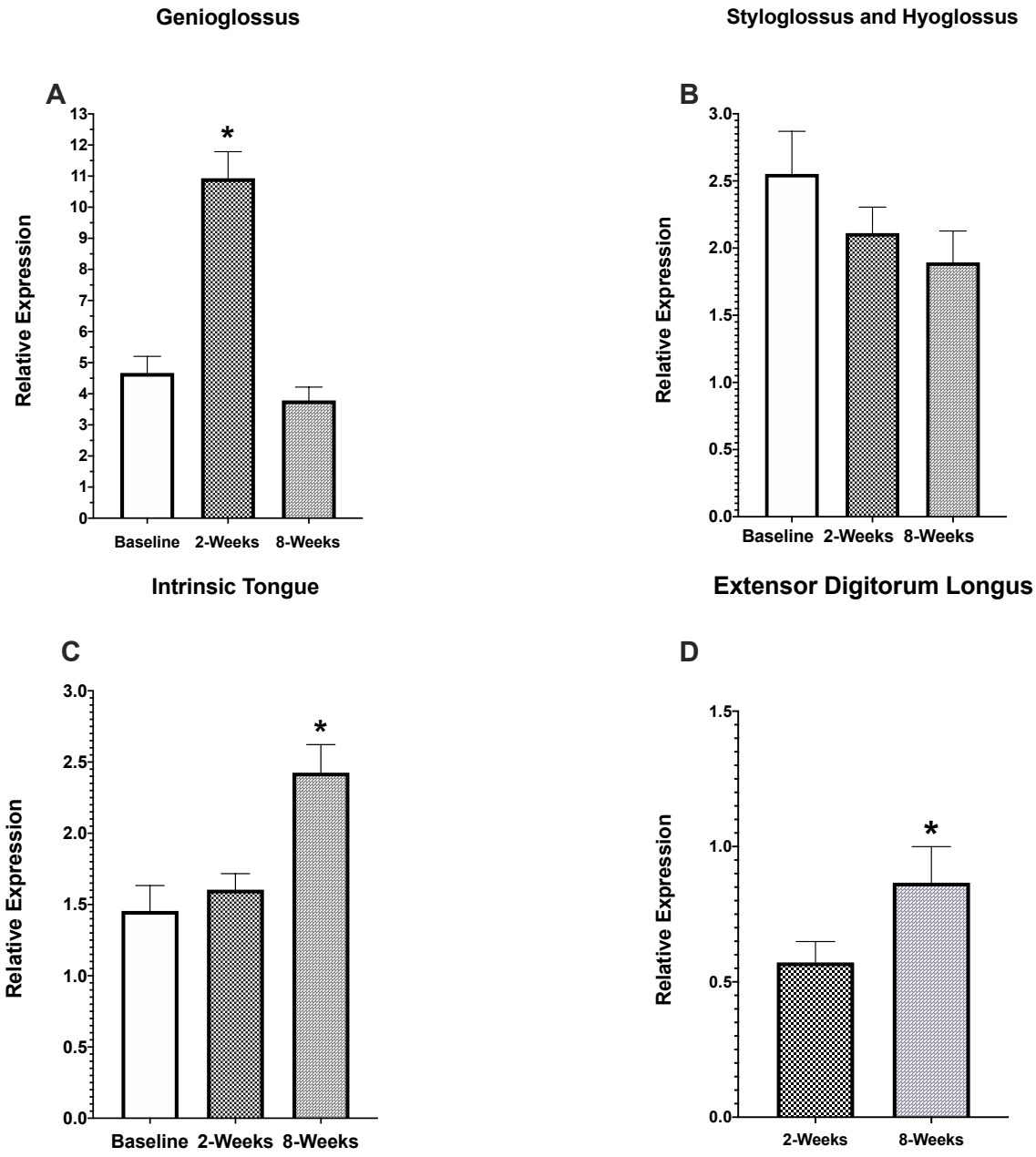

Fig. 10. The Effect of Time on Pax7 Protein Expression. Pax7 protein expression was significantly elevated in the genioglossus at the 2-week time point (A), and at the 8-week time point in the intrinsic tongue (C) and the extensor digitorum longus (D) muscles. * = $p<0.05$

3.2.1.3 Pax7 Colocalization by Myofiber Type at 2 Weeks

A significant interaction effect was found for the percentage of Pax7+ SCs in the GG muscle (Age x Treatment; $F_{1,20}$=12.47, p=0.002; **Fig. 11B**). SC content significantly increased following 2 weeks of tongue exercise in the young adult group (p=0.005; **Fig. 11B**) compared to the no exercise young adult group. The old no exercise group had a significantly greater percentage of Pax7+ SCs compared to the young adult, no exercise group (p=0.021; **Fig. 11B**). The percentage of Pax7 SCs colocalizing near vessels and/or nerves in the GG muscle significantly increased following 2 weeks of tongue exercise ($F_{1,20}$=7.631, p=0.012; **Fig. 14F**) in the absence of an interaction effect ($F_{1,20}$=1.565, p=0.225; **Fig. 13F**). A significant interaction effect was observed for the percentage of Pax7 SCs colocalizing around myosin heavy chain (MyHC) type IIa myofibers ($F_{1,20}$=6.092, p=0.0227; **Fig. 13D**) in the GG muscle. The no exercise, young adult and old groups had a significantly greater percentage Pax7 SCs near MyHC IIa myofibers in comparison to the young adult exercise (p=0.018 [young, no exercise]; p<0.001 [old, no exercise]) and the old exercise (p<0.001 [young, no exercise]; p<0.001 [old, no exercise]) groups. For the colocalization of Pax7 SCs near MyHC IIb and IIx myofibers, near MyHC IIb, IIx, or IIa myofibers, or around MyHC type I myofibers in the GG muscle there were no significant age (MyHC IIB and IIx, $F_{1,20}$=1.039, p=0.320; MyHC IIB, IIx, and IIa, $F_{1,20}$=0.514, p=0.482; MyHC I, $F_{1,20}$=1.912, p=0.182), treatment (MyHC IIB and IIx, $F_{1,20}$=0.434, p=0.518, **Fig. 14B**; MyHC IIB, IIx, and IIa, $F_{1,20}$=2.139, p=0.159, **Fig. 14C**; MyHC I, $F_{1,20}$=0.937, p=0.345, **Fig. 14D**), or interaction effects (MyHC IIB and IIx, $F_{1,20}$=1.721, p=0.205, **Fig. 13B**; MyHC IIB, IIx, and IIa, $F_{1,20}$=0.691, p=0.416, **Fig. 13C**; MyHC I, $F_{1,20}$=0.113, p=0.741; **Fig. 13D**).

A significant treatment effect was found for the percentage of Pax7 SCs in the SG muscle ($F_{1,20}$=6.889, p=0.016; **Fig. 12C**) in the absence of an interaction effect (Age x Treatment; $F_{1,20}$=0.331, p=0.571; **Fig. 11C**). Pax7 SC content significantly increased following 2 weeks of tongue exercise in comparison to the no exercise group in the SG muscle. A significant interaction effect was observed for the percentage of Pax7 SCs colocalizing near vessels and/or nerves in the SG muscle ($F_{1,20}$=4.971, p=0.037; **Fig. 15F**). Pax7 SC content was increased in the old no exercise group and the young adult and old tongue exercise groups compared to the young adult no exercise group. A significant increase in the percentage of Pax7 SCs cells colocalizing near MyHC type IIb and IIx myofibers was observed following 2 weeks of tongue exercise compared to the no exercise group ($F_{1,20}$=8.692, p=0.008; **Fig. 16B**), in the absence of an interaction effect ($F_{1,20}$=0.586, p=0.453; **Fig. 15B**). While a significant reduction in the percentage of Pax7 SCs colocalizing around MyHC type IIb, IIx, and IIa myofibers was observed following 2-weeks of tongue exercise in comparison to the no exercise group ($F_{1,20}$=6.901, p=0.016; **Fig. 16C**), in the absence of an interaction effect ($F_{1,20}$=2.238, p=0.150; **Fig. 15C**). There were no significant age ($F_{1,20}$=0.024, p=0.879), treatment ($F_{1,20}$=1.185, p=0.289**; Fig. 16D**), or interaction effects ($F_{1,20}$=0.185, p=0.671; **Fig. 15D**) for the colocalization of Pax7 SCs around MyHC IIa myofibers. No MyHC Type I myofibers were observed in the SG of the young adult or old groups in either condition (Exercise, No Exercise**; Fig. 15D, Fig. 16E**).

In the EDL, no significant age effects were observed in Pax7 SC content (t_{10}=0.727, p=0.484; **Fig. 11D**). An increase in the percentage of Pax7 SCs colocalizing near MyHC type I myofibers (t_{10}=2.970, p=0.014; **Fig. 17E**) was observed in the old

group compared to the young adult group in the EDL (t_{10}=2.970, p=0.014). There were no significant age effects in the colocalization of Pax7 SCs near MyHC IIb and IIx myofibers (t_{10}=0.692, p=0.505; **Fig. 17B**), MyHC type IIb, IIx, and IIa myofibers (t_{10}=0.513, p=0.619; **Fig. 17C**), MyHC type IIa myofibers (t_{10}=1.592, p=0.142; **Fig. 17D**), or near non-myofibers (t_{10}=1.157, p=0.274; **Fig. 17F**) in the EDL muscle.

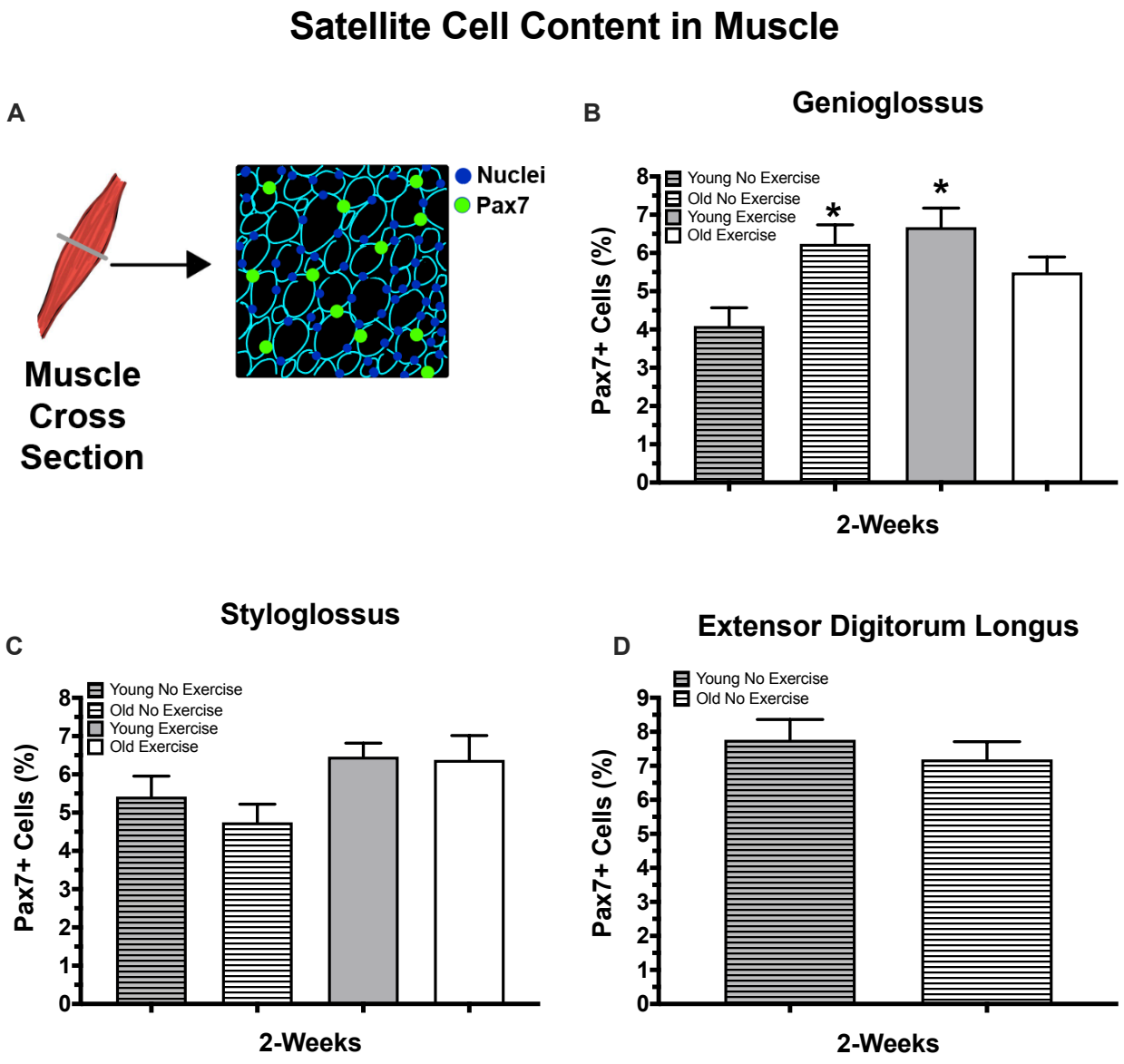

Fig. 11. Satellite Cell (SC) Content in Tongue and Limb. (A) Schematic of skeletal muscle tissue cross section location (gray line) to identify colocalization of Pax7+ SCs and myonuclei. In the genioglossus muscle, the percentage of Pax7+ SCs was significantly increased in the old no exercise group. Following 2 weeks of tongue exercise the percentage of Pax7+ SCs significantly increased in the young adult group (B). No other significant interaction effects were observed. * = $p<0.05$

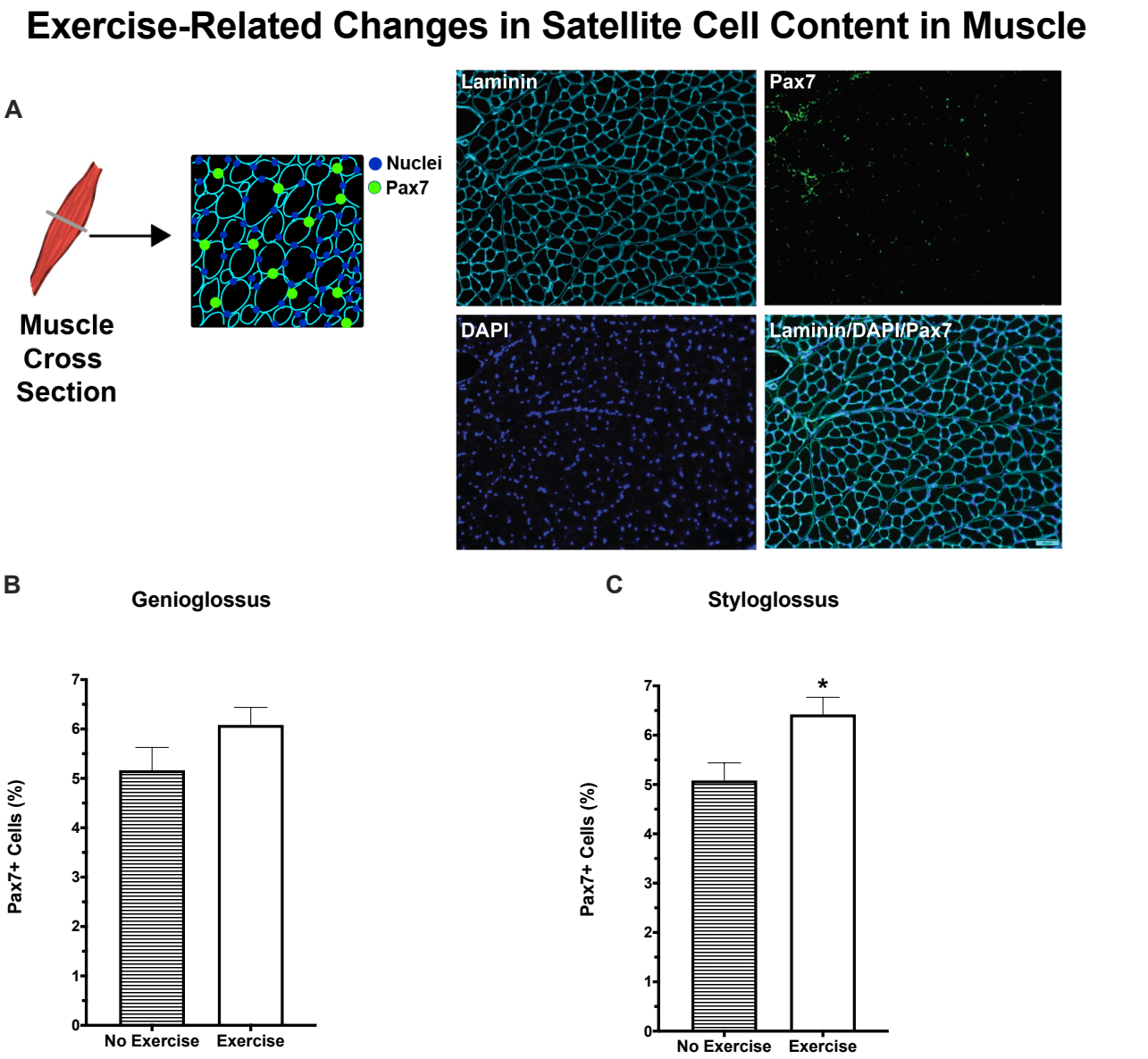

Fig. 12. The Effect of Exercise on Satellite Cell (SC) Content. (A) Schematic of skeletal muscle tissue cross section location (gray line) to identify colocalization of Pax7 MuSCs and myonuclei. Representative image from exercise rat depicting Laminin, DAPI, and Pax7 immunostaining. Following 2 weeks of exercise, the percentage of Pax7+ SCs significantly increased in the styloglossus muscles (C). * = $p<0.05$

Location of Pax7 Satellite Cells in the Genioglossus

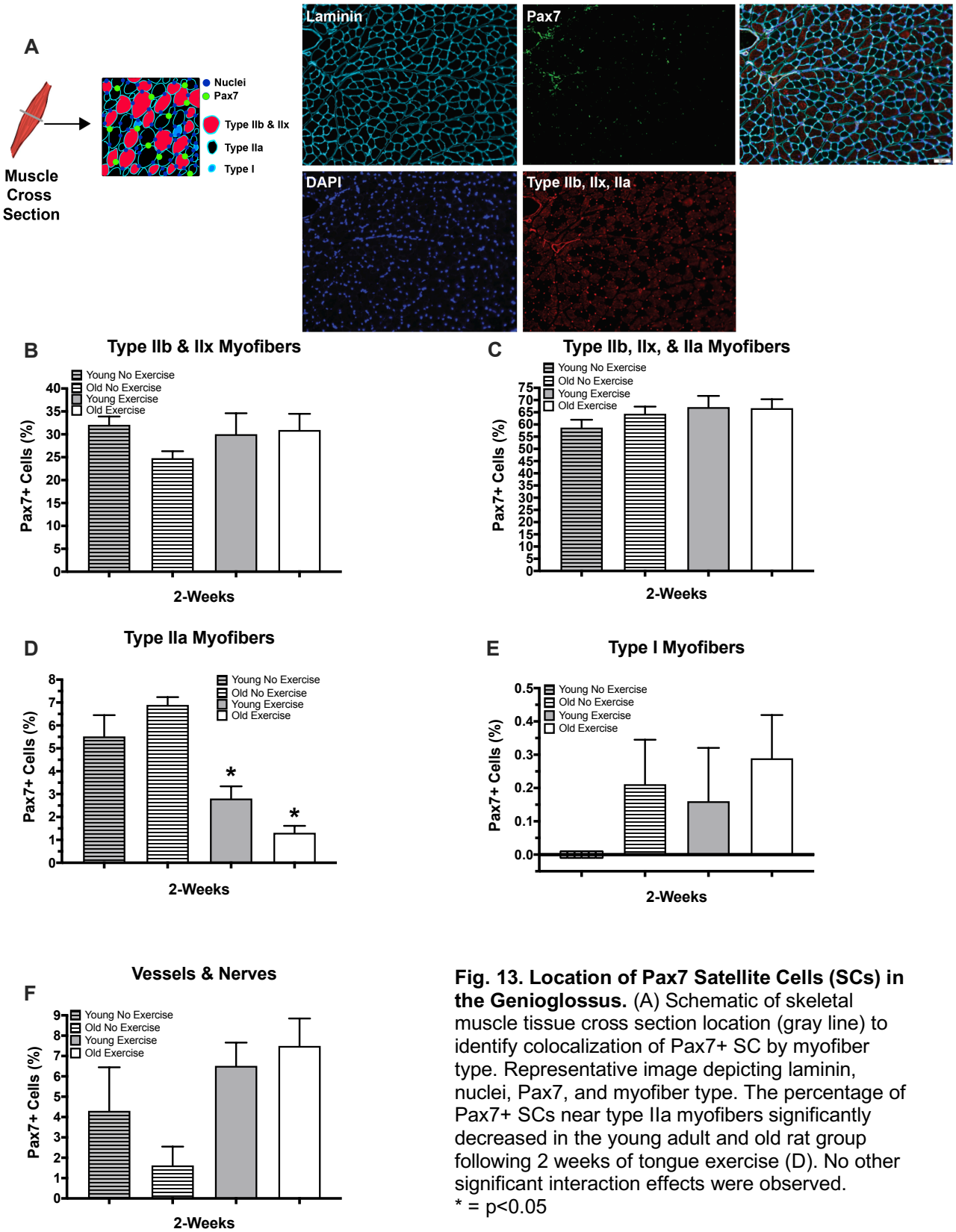

Fig. 13. Location of Pax7 Satellite Cells (SCs) in the Genioglossus. (A) Schematic of skeletal muscle tissue cross section location (gray line) to identify colocalization of Pax7+ SC by myofiber type. Representative image depicting laminin, nuclei, Pax7, and myofiber type. The percentage of Pax7+ SCs near type IIa myofibers significantly decreased in the young adult and old rat group following 2 weeks of tongue exercise (D). No other significant interaction effects were observed.
* = $p<0.05$

Exercise-Related Changes in Pax7 Location in the Genioglossus

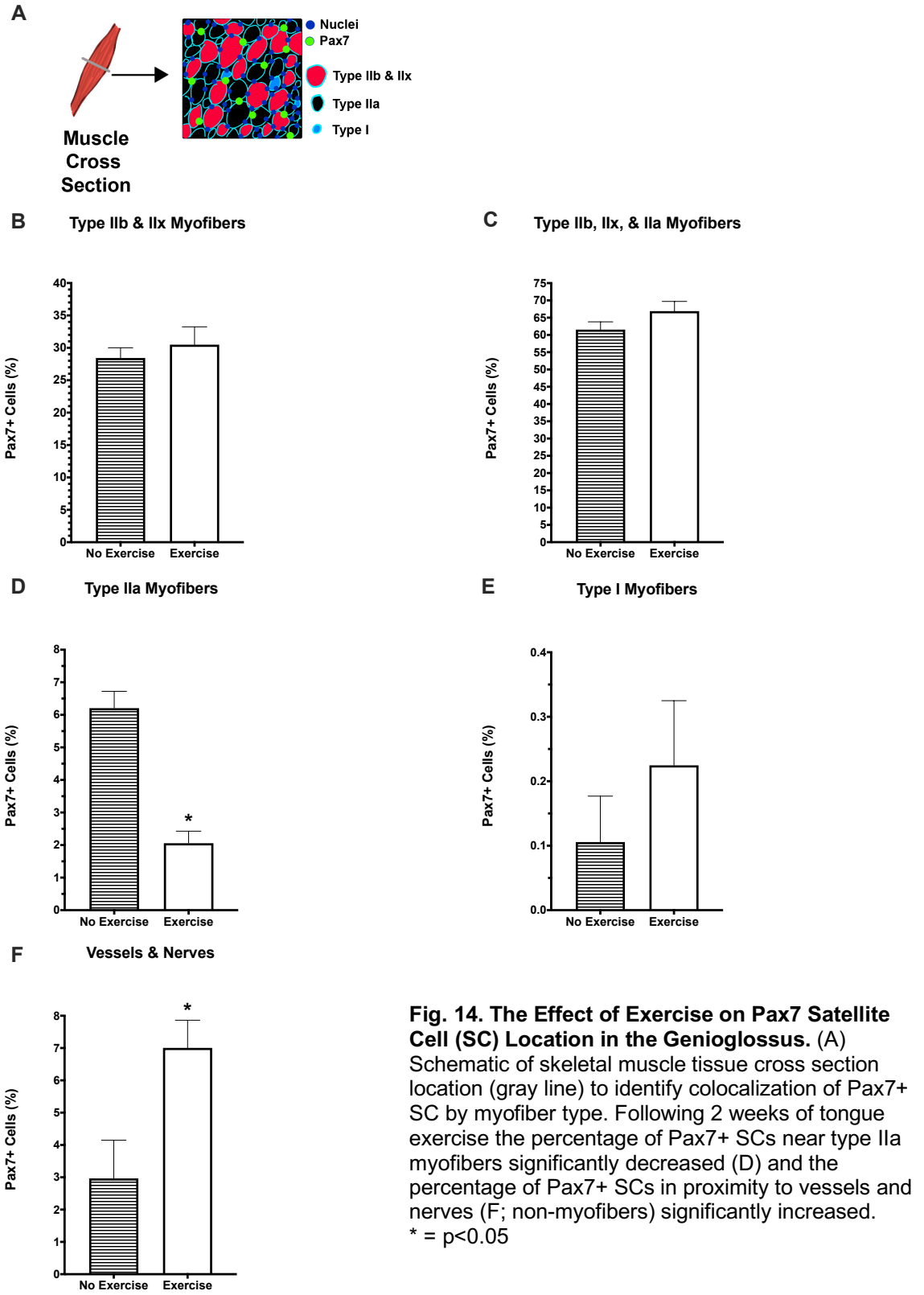

Fig. 14. The Effect of Exercise on Pax7 Satellite Cell (SC) Location in the Genioglossus. (A) Schematic of skeletal muscle tissue cross section location (gray line) to identify colocalization of Pax7+ SC by myofiber type. Following 2 weeks of tongue exercise the percentage of Pax7+ SCs near type IIa myofibers significantly decreased (D) and the percentage of Pax7+ SCs in proximity to vessels and nerves (F; non-myofibers) significantly increased. * = $p<0.05$

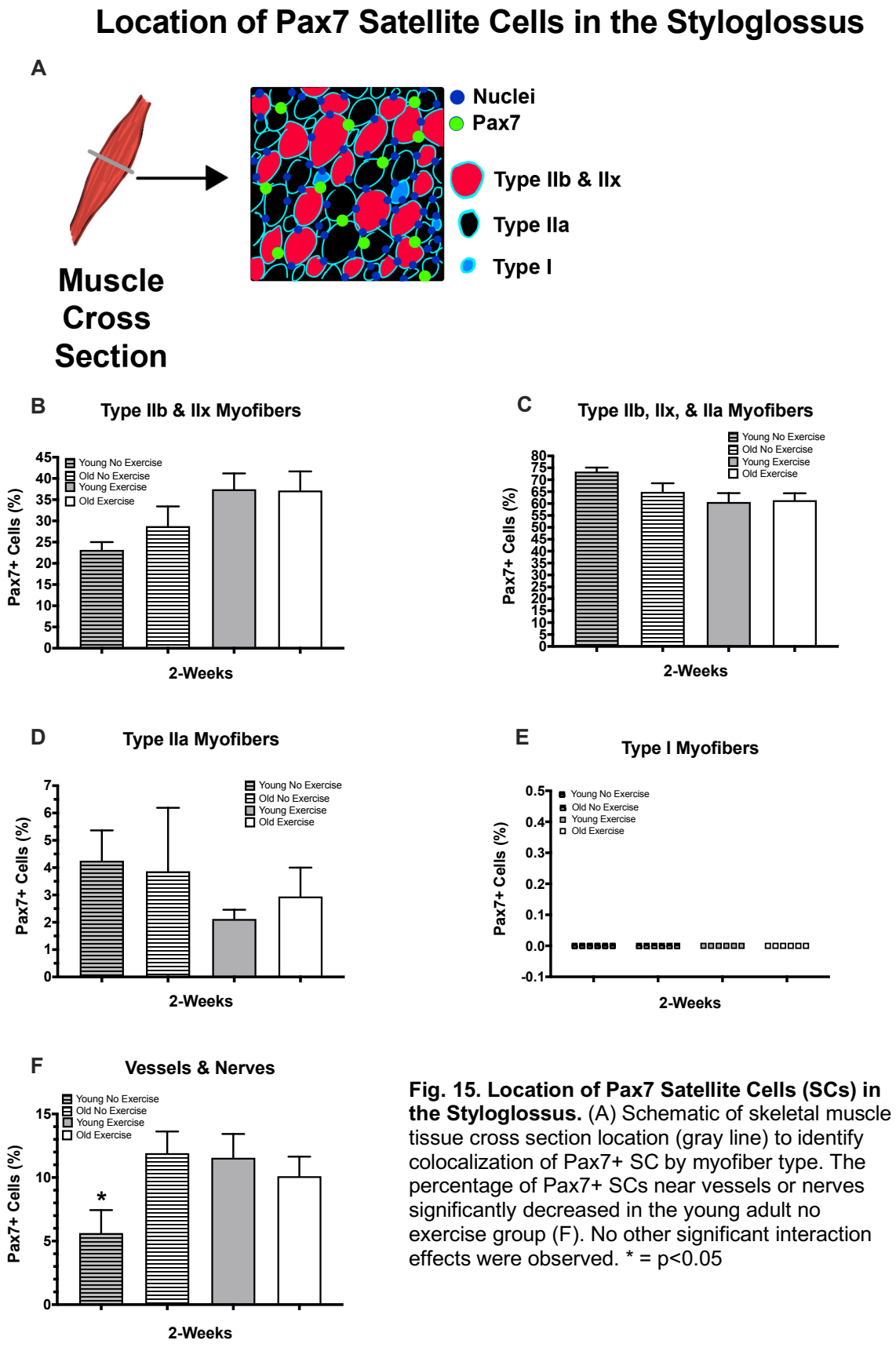

Fig. 15. Location of Pax7 Satellite Cells (SCs) in the Styloglossus. (A) Schematic of skeletal muscle tissue cross section location (gray line) to identify colocalization of Pax7+ SC by myofiber type. The percentage of Pax7+ SCs near vessels or nerves significantly decreased in the young adult no exercise group (F). No other significant interaction effects were observed. * = $p<0.05$

Exercise-Related Changes in Pax7 Location in the Styloglossus

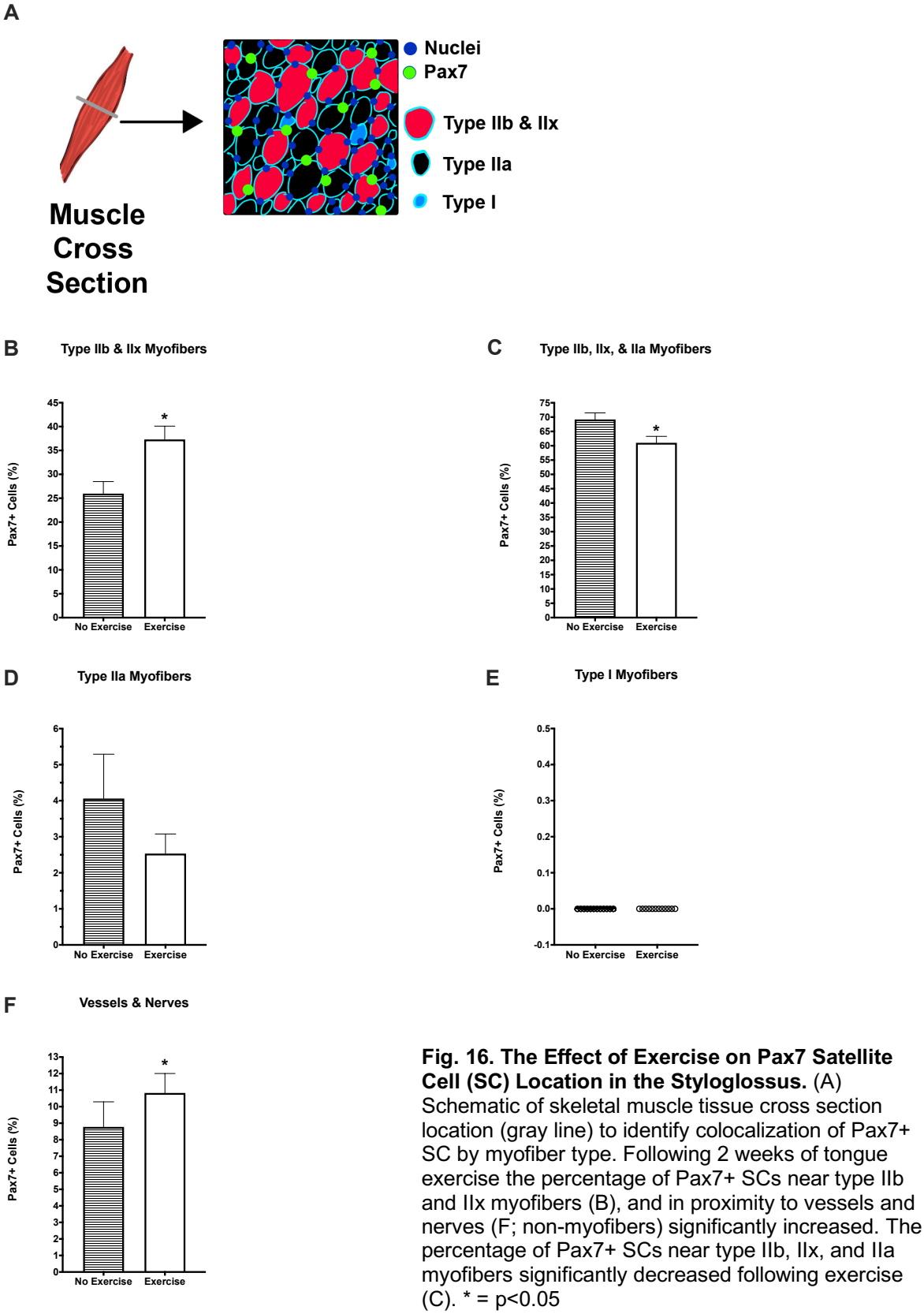

Fig. 16. The Effect of Exercise on Pax7 Satellite Cell (SC) Location in the Styloglossus. (A) Schematic of skeletal muscle tissue cross section location (gray line) to identify colocalization of Pax7+ SC by myofiber type. Following 2 weeks of tongue exercise the percentage of Pax7+ SCs near type IIb and IIx myofibers (B), and in proximity to vessels and nerves (F; non-myofibers) significantly increased. The percentage of Pax7+ SCs near type IIb, IIx, and IIa myofibers significantly decreased following exercise (C). * = $p<0.05$

Localization of Pax7 Satellite Cells in the Extensor Digitorum Longus

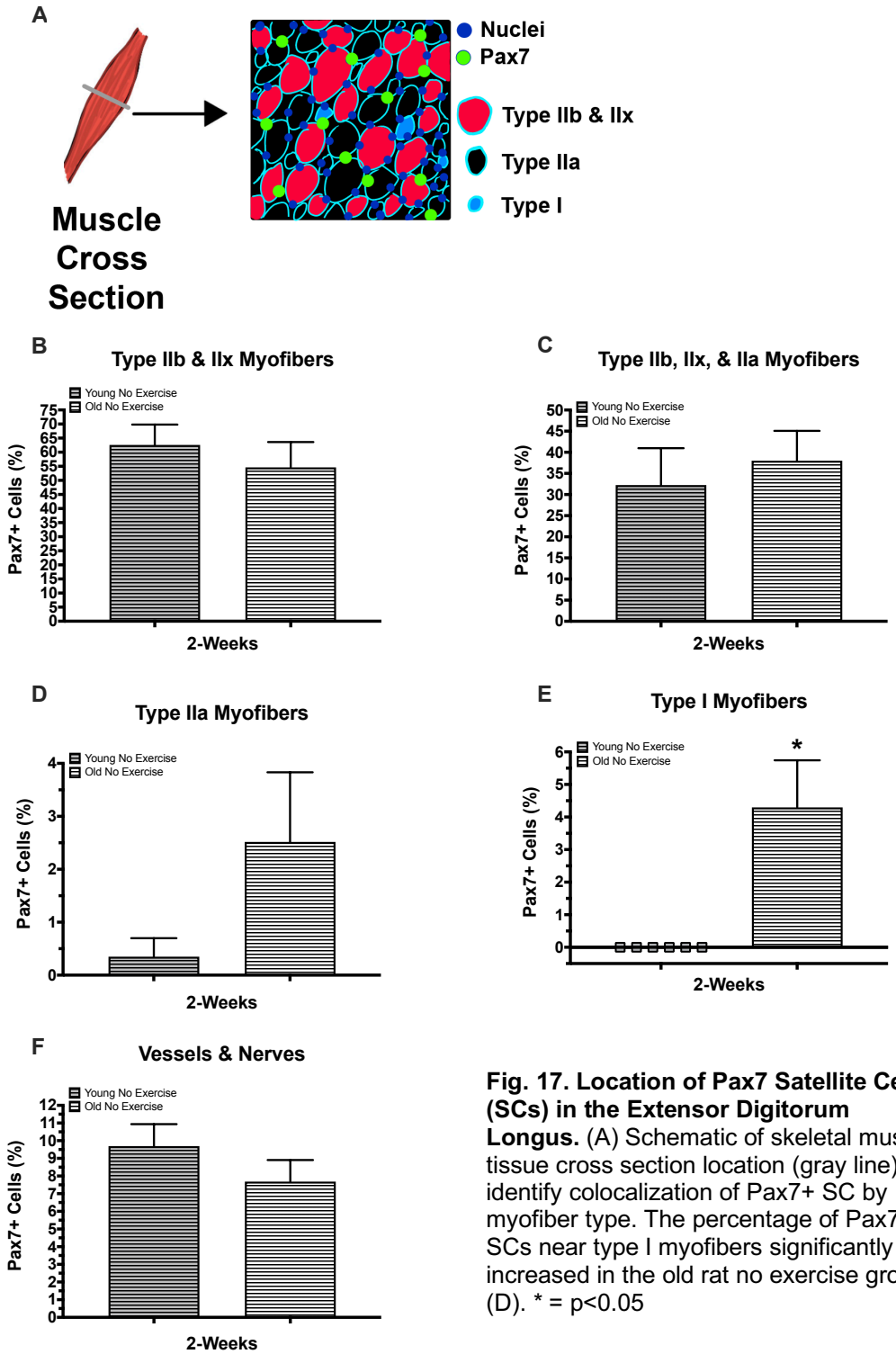

Fig. 17. Location of Pax7 Satellite Cells (SCs) in the Extensor Digitorum Longus. (A) Schematic of skeletal muscle tissue cross section location (gray line) to identify colocalization of Pax7+ SC by myofiber type. The percentage of Pax7+ SCs near type I myofibers significantly increased in the old rat no exercise group (D). * = $p<0.05$

3.2.1.4 Myofiber Repair and Remodeling at 2 Weeks

In the GG muscle, the percentage of centralized nuclei within a myofiber significantly increased with age ($F_{1,20}$=7.204, p=0.014; **Fig. 19B**) and following 2 weeks of tongue exercise ($F_{1,20}$=5.244, p=0.033; **Fig. 20B**), in the absence of an interaction effect ($F_{1,20}$=3.036, p=0.097; **Fig. 18B**). The percentage of centralized nuclei within a myofiber in the SG significantly increased with age ($F_{1,20}$=8.178, p=0.001; **Fig. 19C**), in the absence of treatment ($F_{1,20}$=4.040, p=0.058; **Fig. 20C**) or interaction effects ($F_{1,20}$=2.005, p=0.172; **Fig. 18C**). In the EDL muscle, the percentage of centralized nuclei within a myofiber also significantly increased with age (t_{10}=2.290, p=0.045; **Fig. 18D, Fig. 19D**).

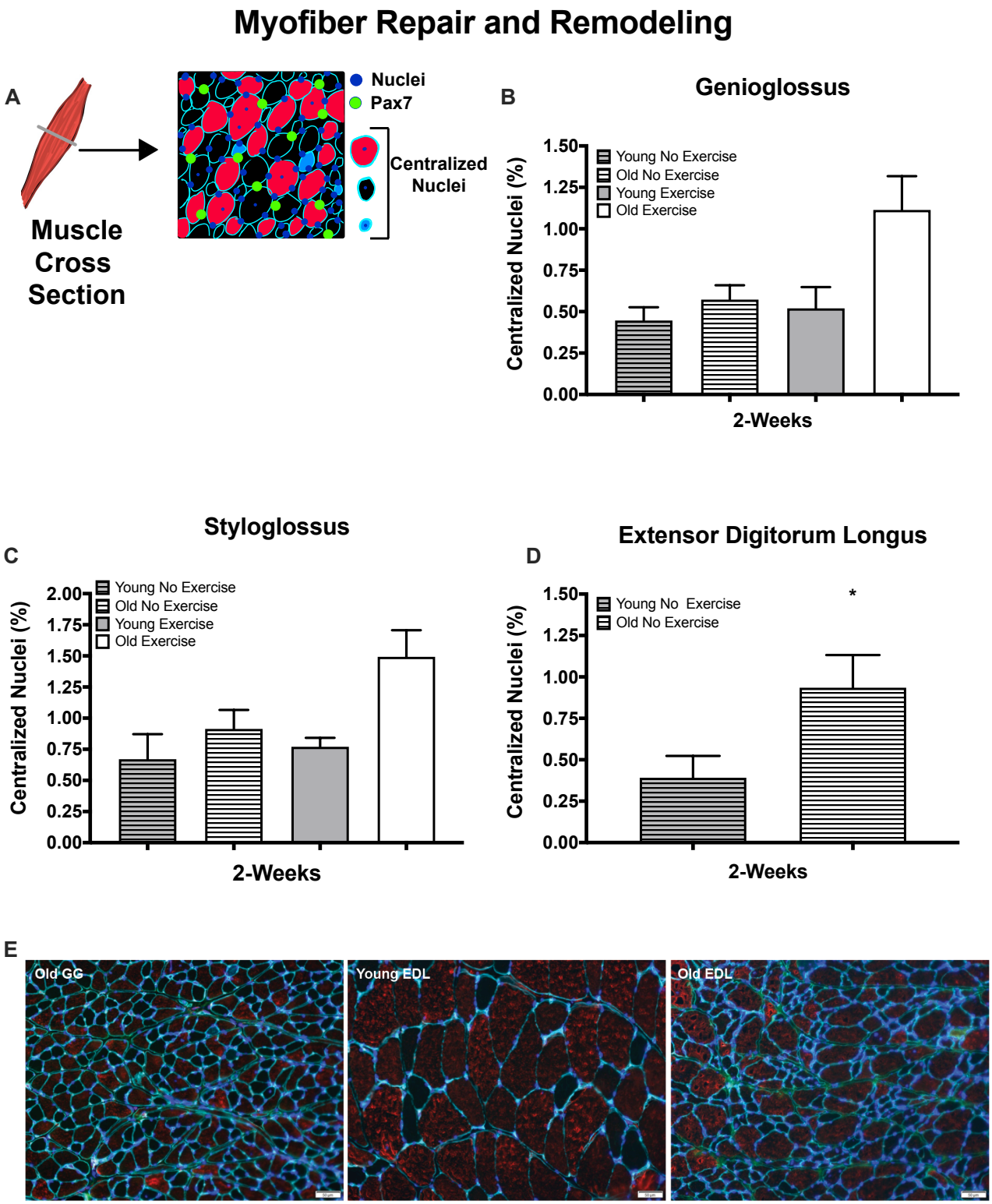

Fig. 18. Myofiber Repair and Remodeling. (A) Schematic of skeletal muscle tissue cross section location (gray line) to identify centralized nuclei in genioglossus, styloglossus, and extensor digitorum longus myofibers. The percentage of centralized nuclei significantly increased in the old group and following 2 weeks of tongue exercise. No significant interaction effects were observed. (E) Representative images of centralized nuclei from an Old GG muscle, Young EDL muscle, and Old EDL muscles.

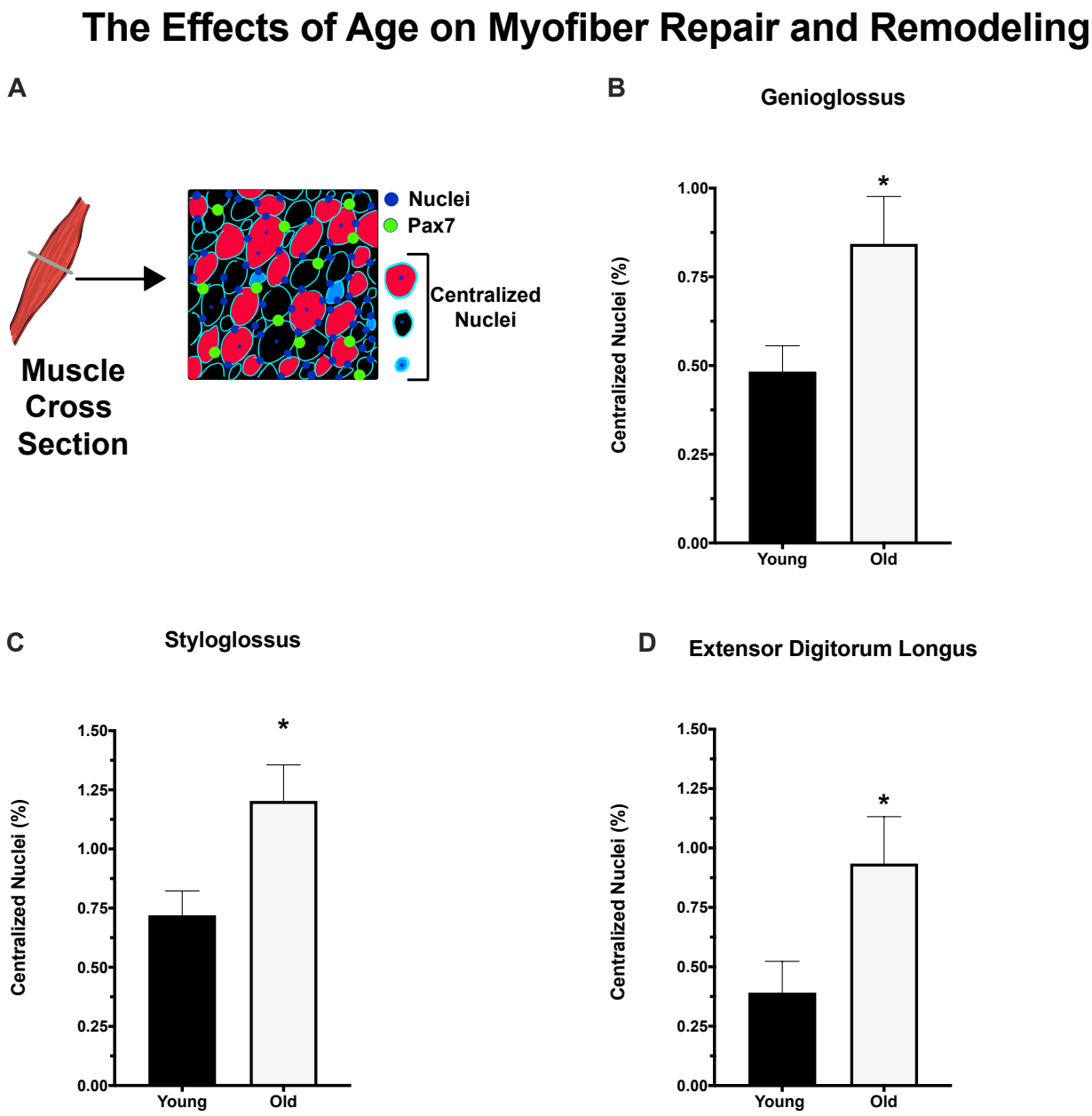

Fig. 19. The Effect of Age on Myofiber Repair and Remodeling. (A) Schematic of skeletal muscle tissue cross section location (gray line) to identify centralized nuclei in genioglossus, styloglossus, and extensor digitorum longus myofibers. With age, the percentage of centralized nuclei significantly increased in the genioglossus (B), styloglossus (C), and extensor digitorum longus (D) muscles. * = $p<0.05$

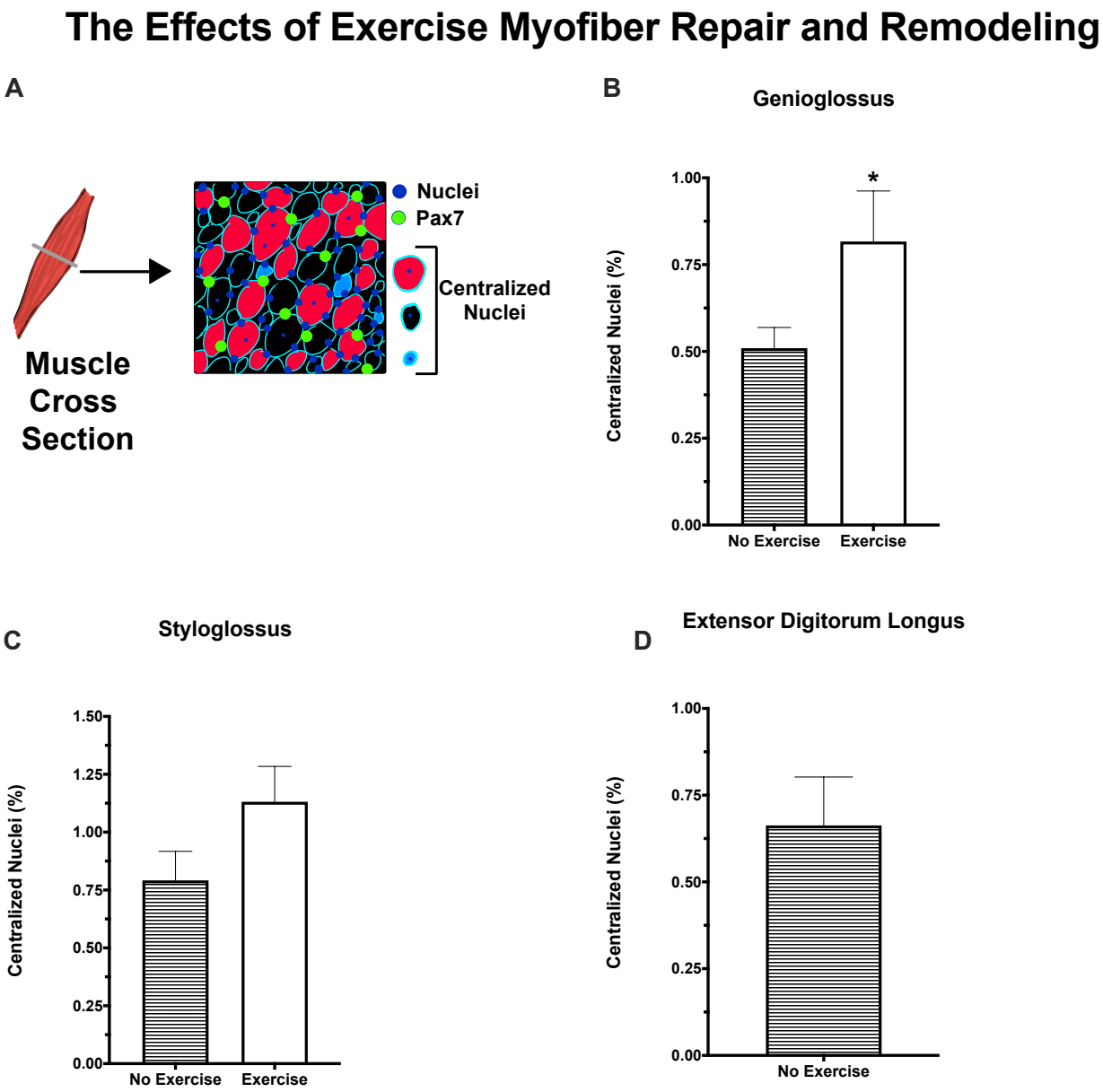

Fig. 20. The Effect of Exercise on Myofiber Repair and Remodeling. (A) Schematic of skeletal muscle tissue cross section location (gray line) to identify centralized nuclei in genioglossus, styloglossus, and extensor digitorum longus myofibers. Following 2 weeks of tongue exercise, the percentage of centralized nuclei significantly increased in the genioglossus (B) and styloglossus (C) muscles. * = $p<0.05$

3.2.1.5 Pax7-Positive (Pax7+) Satellite Cells

A significant reduction in the percentage of Pax7+ SCs isolated from the SG and HG muscles was observed at the 2-week time point (Time, $F_{2,36}$=37.52, p<0.001, **Fig. 22B**; Age, $F_{1,36}$=0.326, p=0.572; Treatment, $F_{1,36}$=0.040, p=0.843). No significant interactions among age, treatment, or time were observed ($F_{2,36}$=1.803, p=0.179; **Fig. 21D**). No significant main effects for age ($F_{1,36}$=0.107, p=0.746), treatment ($F_{1,36}$=0.464, p=0.500), or time point ($F_{2,36}$=0.869, p=0.428; **Fig. 22A**) were observed in the percentage of Pax7+ SCs isolated from the GG muscle.

A significant interaction effect for the percentage of Pax7+ SCs from the IT was found (Time x Treatment x Age; $F_{2,36}$=4.441, p=0.019; **Fig. 21E**). Post-hoc testing revealed that at the 2-week time point the percentage of Pax7+ SCs from the old exercise group was significantly decreased in comparison to baseline (p=0.001) and 8-week (p=0.008) old exercise groups. In addition, the percentage of Pax7+ SCs isolated cells from the IT of young adult exercise group was significantly increased compared to old exercise group at 2-weeks (p=0.003; **Fig. 22C**).

A significant interaction effect (Time x Age; $F_{2,36}$=5.464, p=0.008; **Fig. 21F**) for the percentage of Pax7+ SCs from the EDL was observed in the old group at the two-week time point. Post-hoc testing revealed that the percentage of isolated SCs expressing Pax7 was reduced with age in the EDL at the 2-week time point in comparison to the baseline and 8-week time points.

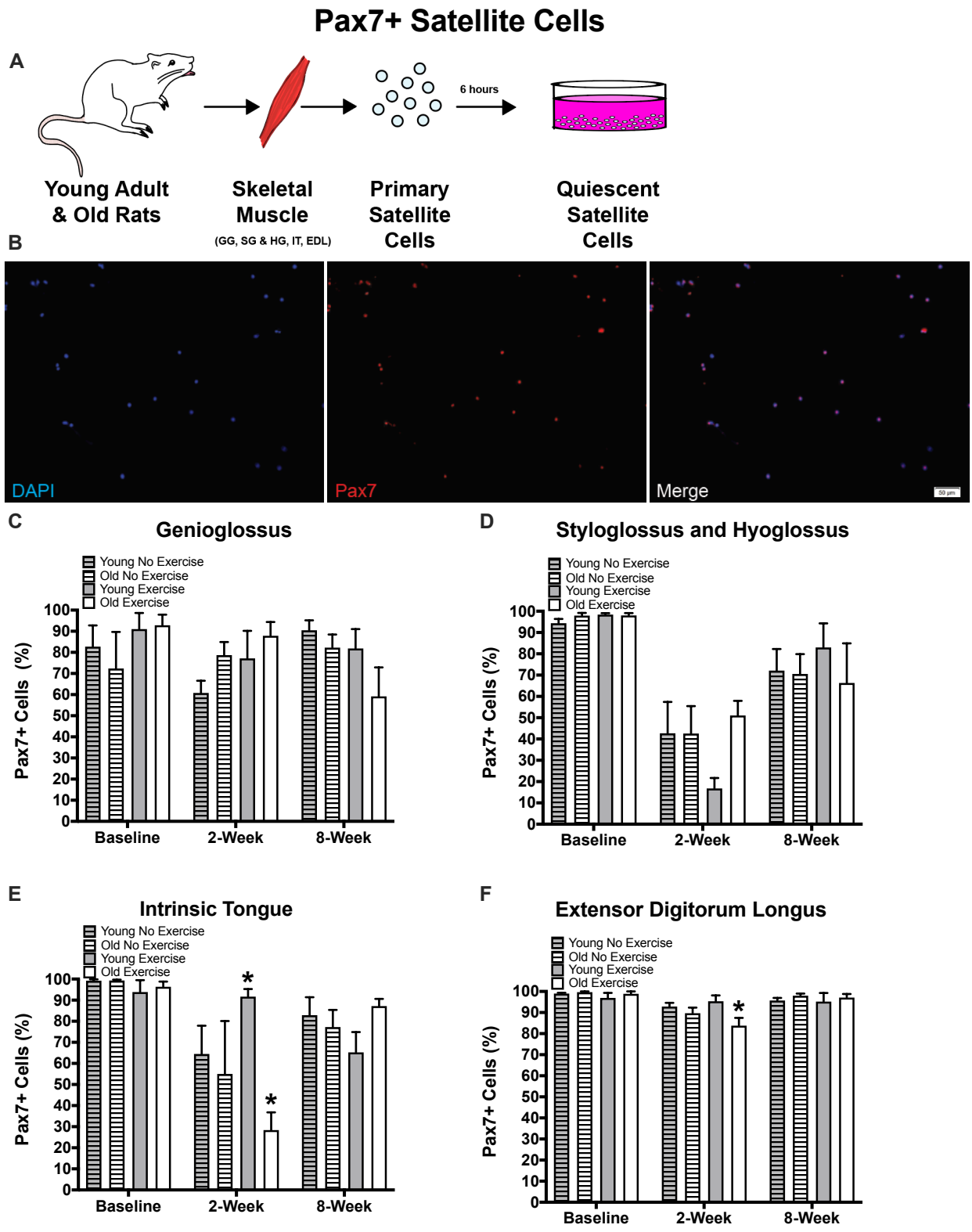

Fig. 21. Pax7-positive (Pax7+) Satellite Cells (SCs). (A) Schematic diagram of *ex*-vivo primary SC culture from young adult and old F344/BN rats. (B) Representative images of Pax7+ SCs isolated from the combine SG and HG muscles. In the intrinsic tongue at the 2-week time point, the percentage of Pax7+ SCs from the young adult exercise group significantly increased, and significantly decreased in the old exercise group (D). The percentage of Pax7+ SCs from the extensor digitorum longus of the old exercise group significantly decreased (F). * = $p<0.05$

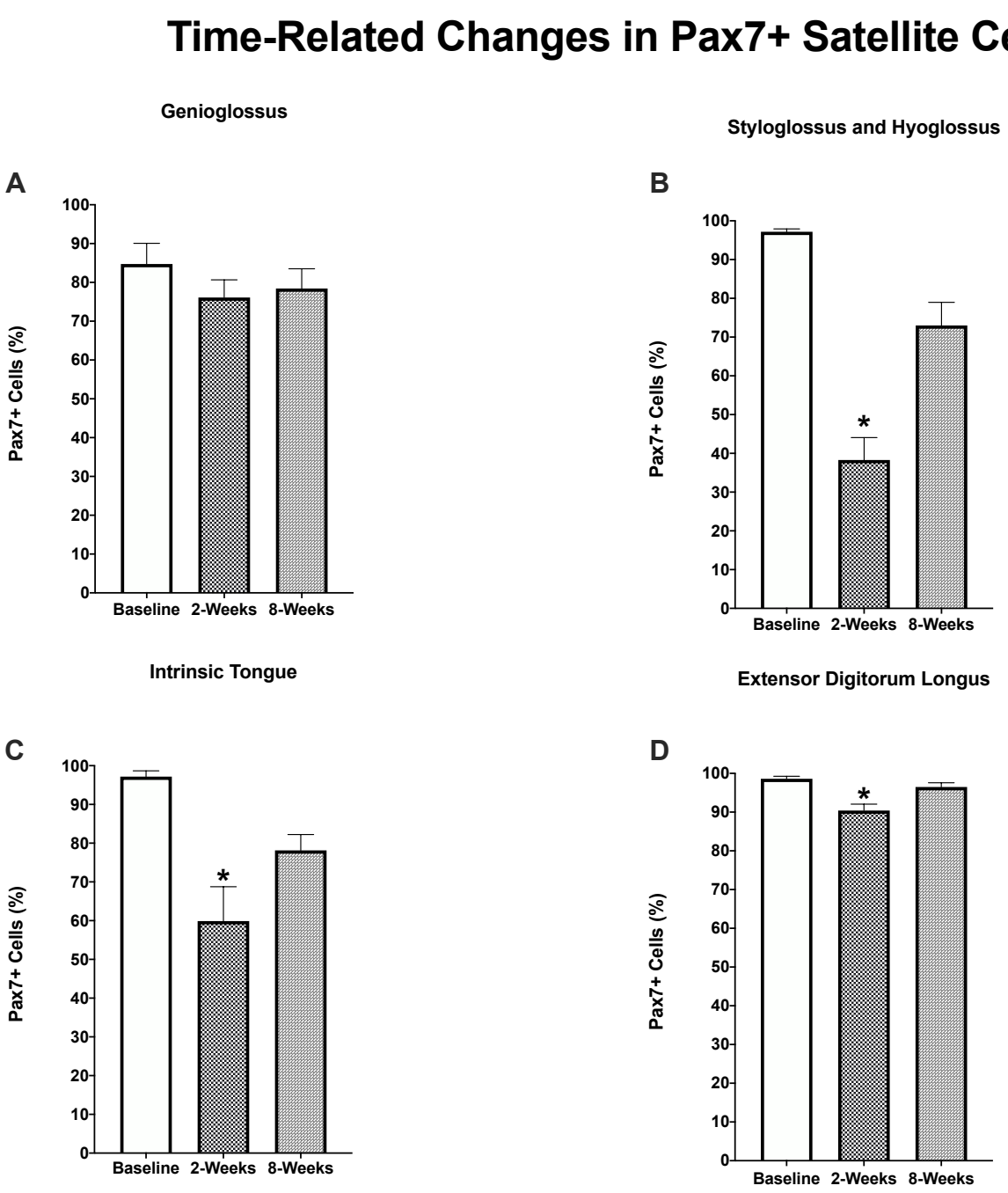

Fig. 22. The Effect of Time on Isolated Pax7-Positive (Pax7+) Satellite Cells (SCs). The percentage of Pax7+ SCs isolated from the combined styloglossus and hyoglossus (B), intrinsic tongue (C), and extensor digitorum longus (D) muscles significantly decreased at the 2-week time point. * = $p<0.05$

3.2.2 MyoD

3.2.2.1 Whole Muscle MyoD Gene Expression at 2 Weeks

Significant age-related alterations in MyoD gene expression were observed in the EDL muscle ($F_{1,11}$=5.990, p=0.032; **Fig 23D**). No significant interaction effects between treatment and age were noted ($F_{1,11}$=0.936, p=0.354). Specifically, MyoD was significantly increased in the EDL of the old rat group. No significant age-related differences in MyoD gene expression were observed in the tongue musculature (GG, $F_{1,11}$=2.671, p=0.131, **Fig 23A**; combined SG and HG, $F_{1,10}$=2.905, p=0.119, **Fig 23B**; IT, $F_{1,10}$=0.970, p=0.348; **Fig 23C**). No significant exercise effects were observed in the GG ($F_{1,11}$=0.137, p=0.718), combined SG and HG ($F_{1,10}$=1.496, p=0.249), IT ($F_{1,10}$=1.401, p=0.264), or EDL ($F_{1,11}$=0.079, p=0.784) muscles.

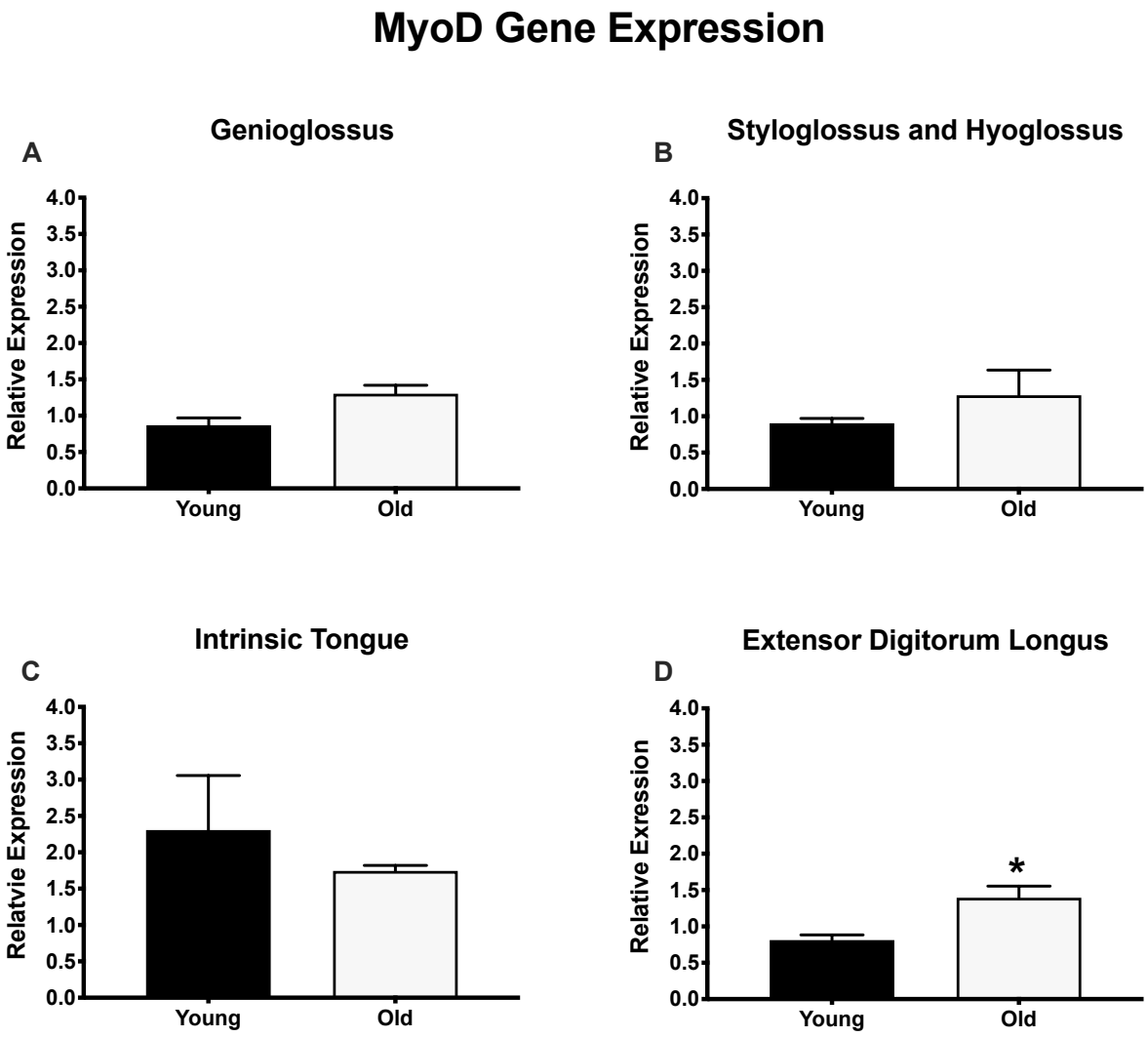

Fig. 23. The Effect of Age on MyoD Gene Expression. With age, MyoD gene expression was significantly elevated in the extensor digitorum longus muscles of the old group in comparison to the young adult group (D). No significant changes in MyoD gene expression were observed in the genioglossus (A), combined styloglossus and hyoglossus (B), or intrinsic tongue (C) muscles. * = $p<0.05$

3.2.2.2 Whole Muscle MyoD Protein Expression

Alterations in MyoD protein expression were observed in the combined SG and HG, IT, and EDL muscles (**Fig. 24**). A significant interaction effect (Time x Age x Treatment; $F_{2,36}$=3.900, p=0.029; **Fig. 24B**) in MyoD protein expression of the combined SG and HG muscle was observed. MyoD protein expression at baseline in the young adult no exercise combined SG and HG muscles was significantly greater compared to old no exercise group at the 2-week time point (HSD p=0.022) and to all conditions at the 8-week time point (young adult no exercise, HSD p=0.006; young adult exercise, HSD p=0.016; old no exercise, HSD p=0.019; old exercise, HSD p=0.004).

Significant main effects for time were observed for MyoD expression in the IT and EDL muscles (**Fig. 25)**. MyoD expression was significantly reduced in the IT at 2-week and 8-week time points compared to baseline ($F_{2,36}$=18.72, p<0.001; **Fig. 25C**). At 8 weeks, MyoD expression in the EDL was significantly elevated ($F_{1,24}$=10.94, p=0.003; **Fig. 25D**). No effects of age ($F_{1,36}$=0.575, p=0.453), exercise ($F_{1,36}$=0.089, p=0.768), or time point ($F_{2,36}$=0.887, p=0.421; **Fig. 25A**) were observed in MyoD protein expression in the GG muscle.

Significant differences in MyoD protein expression were observed among the GG, combined SG and HG, IT, and EDL muscles in the no exercise group at the 2-week time point (Muscle x Age; $F_{3,24}$=4.718, p=0.010; **Fig. 24**). MyoD protein expression was significantly greater in the combined SG and HG of the young no exercise group in comparison to the GG, IT, and EDL muscles.

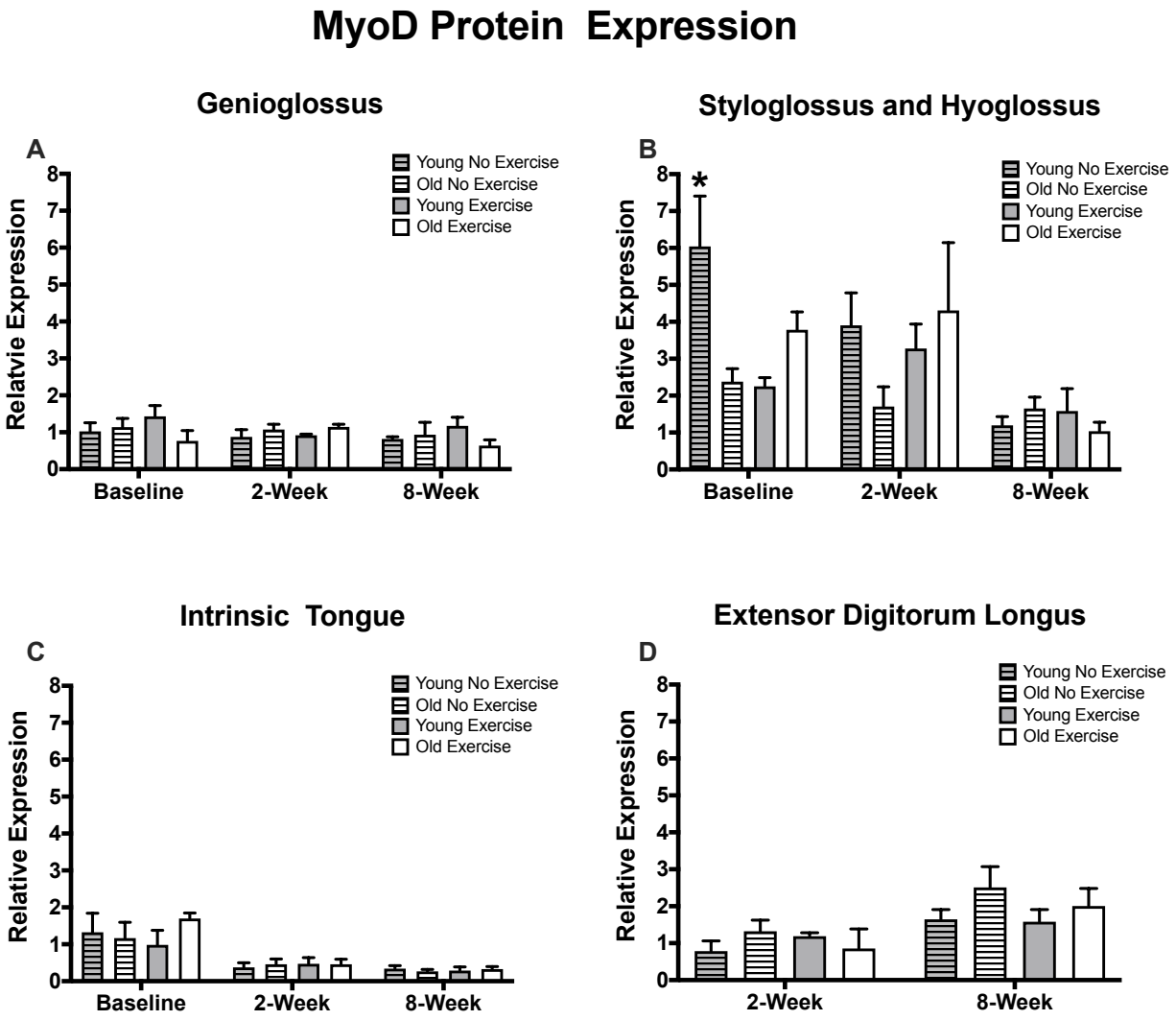

Fig. 24. MyoD Protein Expression. MyoD protein expression was elevated in the combined styloglossus and hyoglossus (B) in comparison to the genioglossus (A), intrinsic tongue (C), and extensor digitorum longus (D) muscles. MyoD expression was highest in the styloglossus and hyoglossus muscles of the young adult, no exercise group at the baseline time point. * = $p<0.05$

Time-Related Changes in MyoD Protein Expression

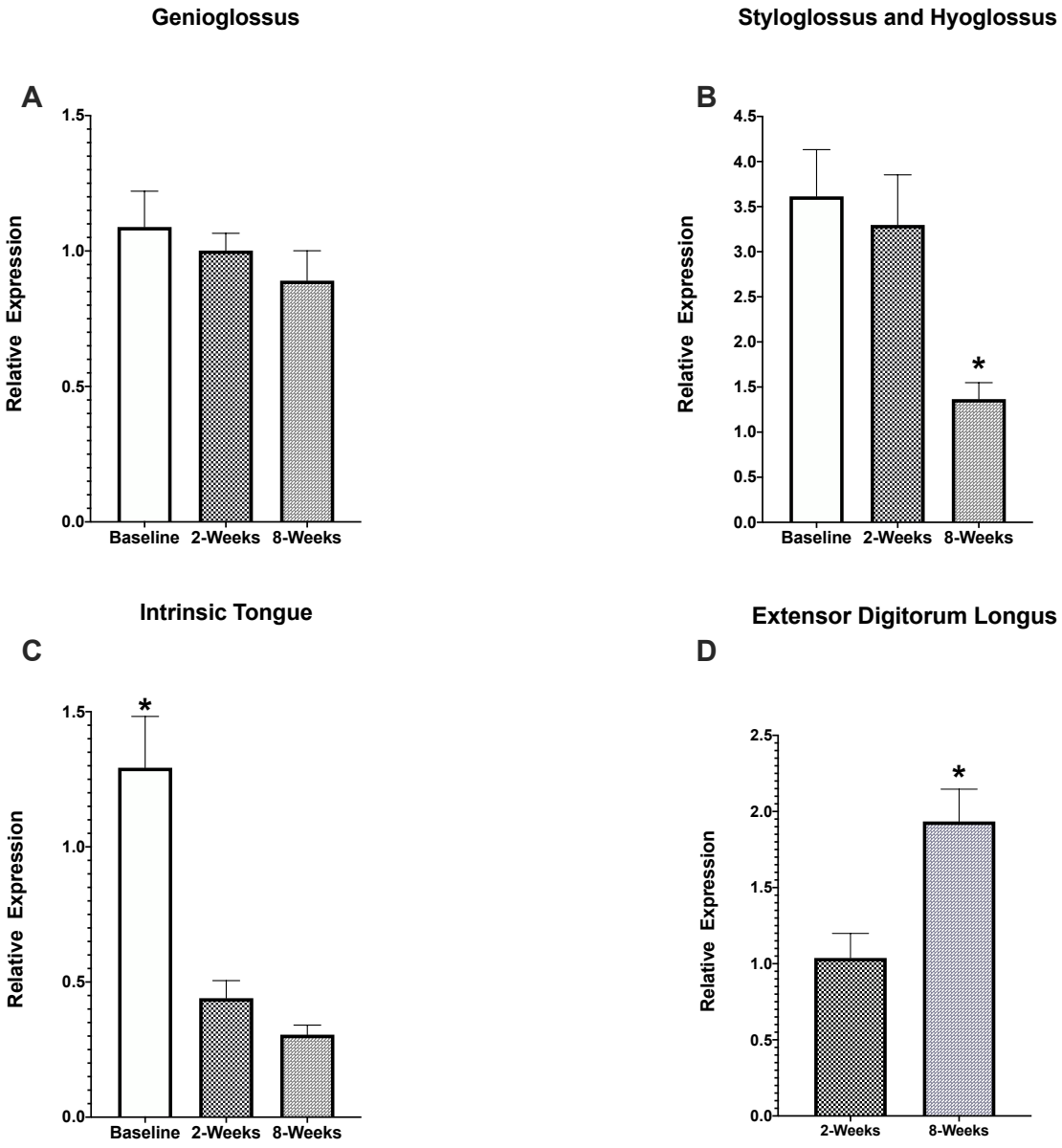

Fig. 25. The Effect of Time on MyoD Protein Expression. MyoD protein expression was significantly reduced in the styloglossus and hyoglossus at the 8-week time point (B). MyoD protein expression was significantly elevated in the intrinsic tongue at the Baseline time point (A) and at the 8-week time point in the extensor digitorum longus (D). * = $p<0.05$

3.2.3 Myogenin

3.2.3.1 Whole Muscle Myogenin Gene Expression at 2 Weeks

A significant age-related increase in myogenin gene expression was observed in the EDL muscle of old versus young adult groups ($F_{1,11}$=9.088, p=0.012; **Fig. 26D**). No interaction effects between treatment and age were observed ($F_{1,11}$=1.057, p=0.326). No age-related differences in myogenin gene expression were observed in the tongue musculature (GG, $F_{1,11}$=1.696, p=0.220, **Fig. 26A**; combined SG and HG, $F_{1,11}$=0.488, p=0.499, **Fig. 26B**; IT, $F_{1,10}$=1.429, p=0.260; **Fig. 26C**). No significant exercise effects were observed in myogenin gene expression in the GG ($F_{1,11}$=0.338, p=0.573), combined SG and HG ($F_{1,11}$=0.347, p=0.568), IT ($F_{1,10}$=1.429, p=0.260), or EDL ($F_{1,11}$=1.062, p=0.325) muscles.

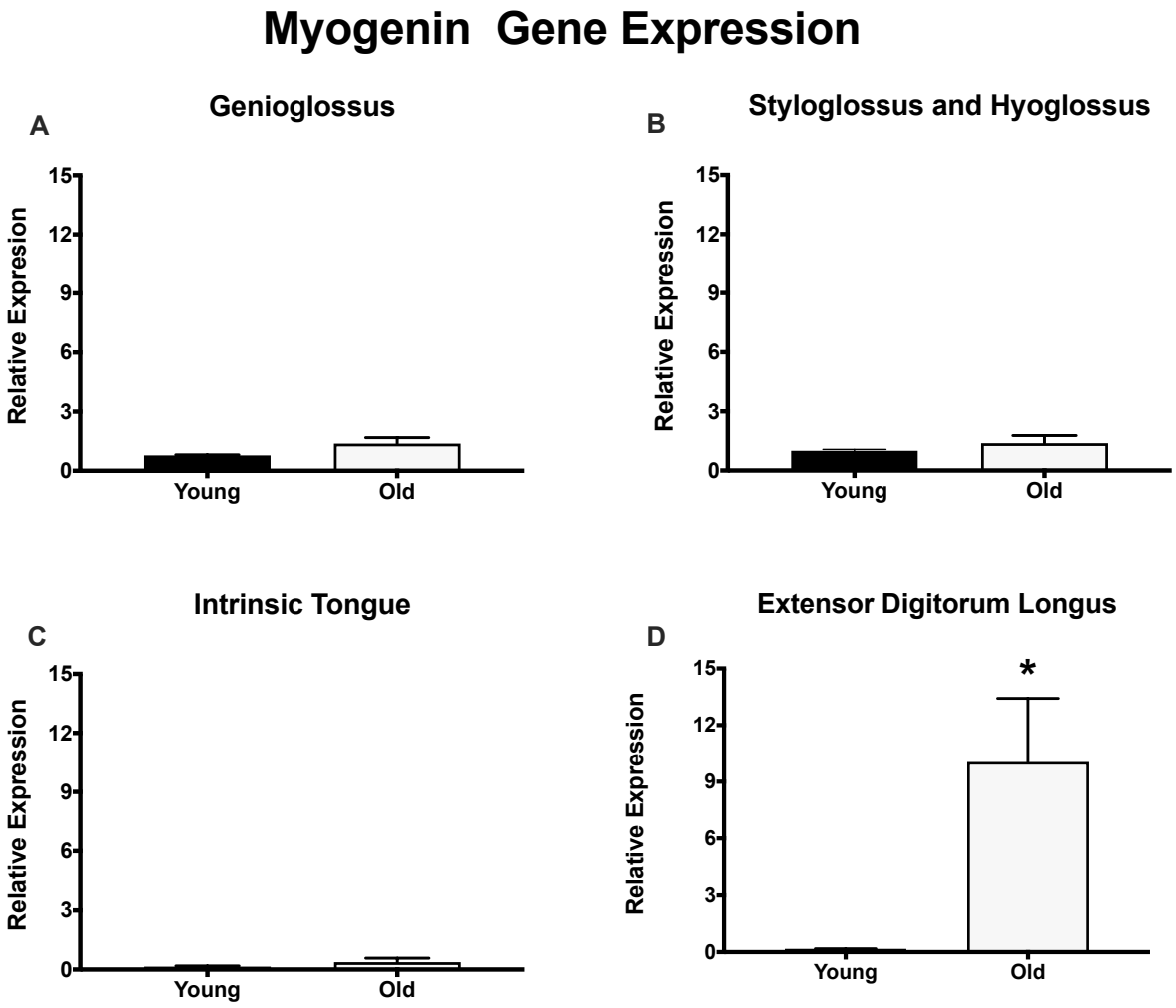

Fig. 26. The Effect of Age on Myogenin Gene Expression. With age, myogenin gene expression was significantly elevated in the extensor digitorum muscles of the old group in comparison to the young adult group (D). No significant changes in myogenin gene expression were observed in the genioglossus (A), combined styloglossus and hyoglossus (B), or intrinsic tongue (C) muscles.
* = $p<0.05$

3.2.3.2 Whole Muscle Myogenin Protein Expression

Changes in myogenin protein expression were observed in the combined SG and HG, and the IT muscles. No significant interaction effects were observed (combined SG and HG, $F_{2,36}$=0.942, p=0.399, **Fig. 27B**; IT, $F_{2,36}$=1.317, p=0.280, **Fig. 27C**). A significant treatment effect was found in the combined SG and HG muscles ($F_{1,36}$=8.798, p=0.005; **Fig. 28B**). Myogenin protein expression significantly increased following progressive resistance tongue exercise compared to the no exercise control condition in the combined SG and HG muscles. At the 2-week and 8-week time points, myogenin protein expression was significantly greater in the IT compared to baseline ($F_{2,36}$=12.66, p<0.001; **Fig. 29C**). No alterations in myogenin protein expression were observed with age (GG, $F_{1,36}$=0.017, p=0.896; EDL, $F_{1,24}$=1.848, p=0.187), exercise (GG, $F_{1,36}$=0.543, p=0.7467, **Fig. 28A**; EDL, $F_{1,24}$=0.120, p=0.659, **Fig. 28D**), or time point (GG, $F_{2,36}$=0.241, p=0.787, **Fig. 29A**; EDL, $F_{1,24}$=2.576, p=0.122, **Fig. 29D**) in the GG and EDL muscles.

Significant differences in myogenin protein expression were observed among the GG, combined SG and HG, IT, and EDL muscles in the no exercise group at the 2-week time point (Muscle; $F_{3,24}$=31.18, p<0.001; **Fig. 27**) in the absence of an interaction effect (Muscle x Age; $F_{3,24}$=2.756, p=0.064). Myogenin protein expression was significantly greater in the GG of the young and old no exercise group in comparison to the combined SG and HG, IT, and EDL muscles.

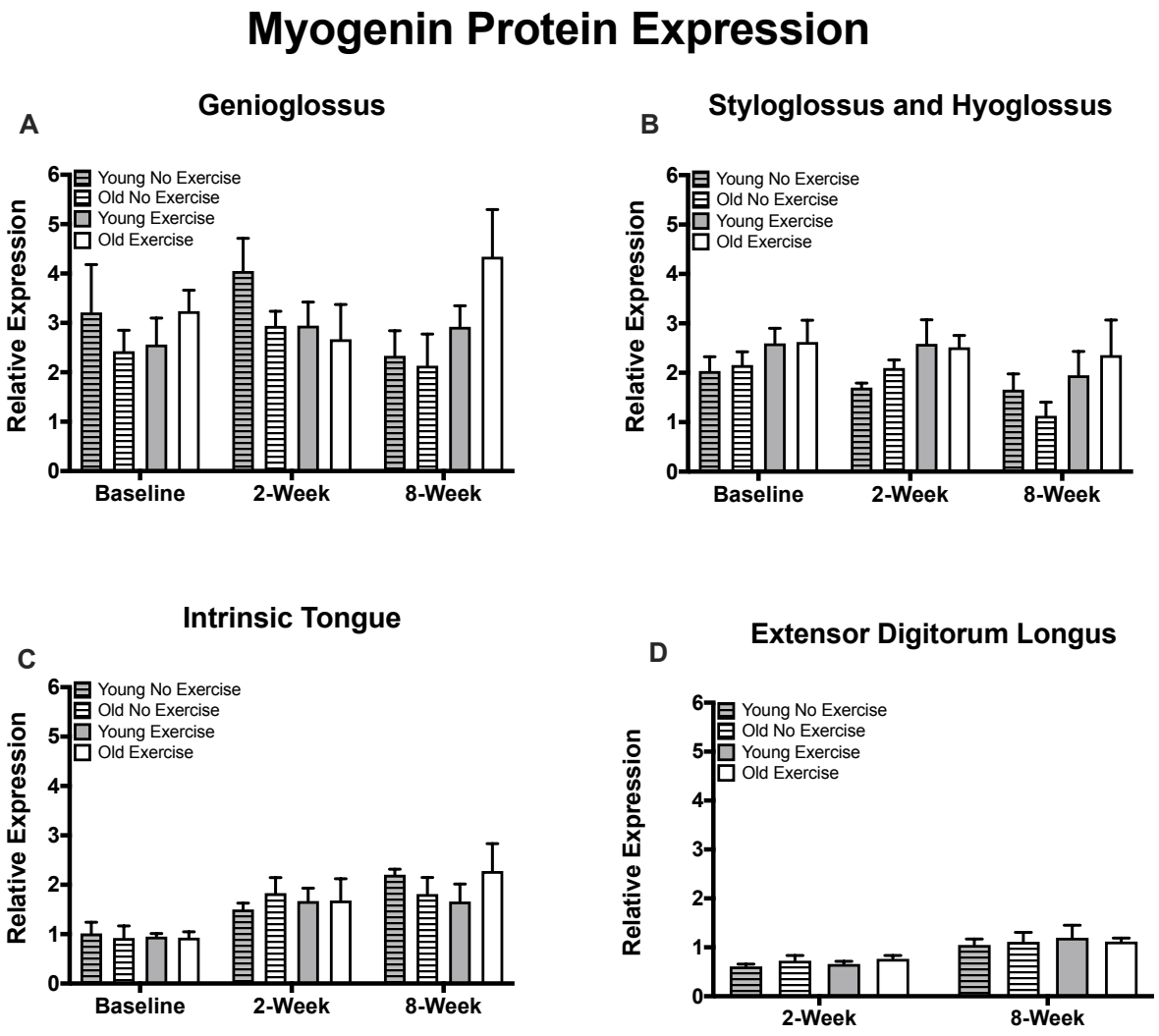

Fig. 27. Myogenin Protein Expression. Myogenin protein expression was elevated in the tongue muscles (A-C) compared to limb (D). No significant interaction effects were observed in myogenin protein expression in the genioglossus (A), combined styloglossus and hyoglossus (B), intrinsic tongue (C), or extensor digitorum longus (D) muscles.

Exercise-Related Changes in Myogenin Protein Expression

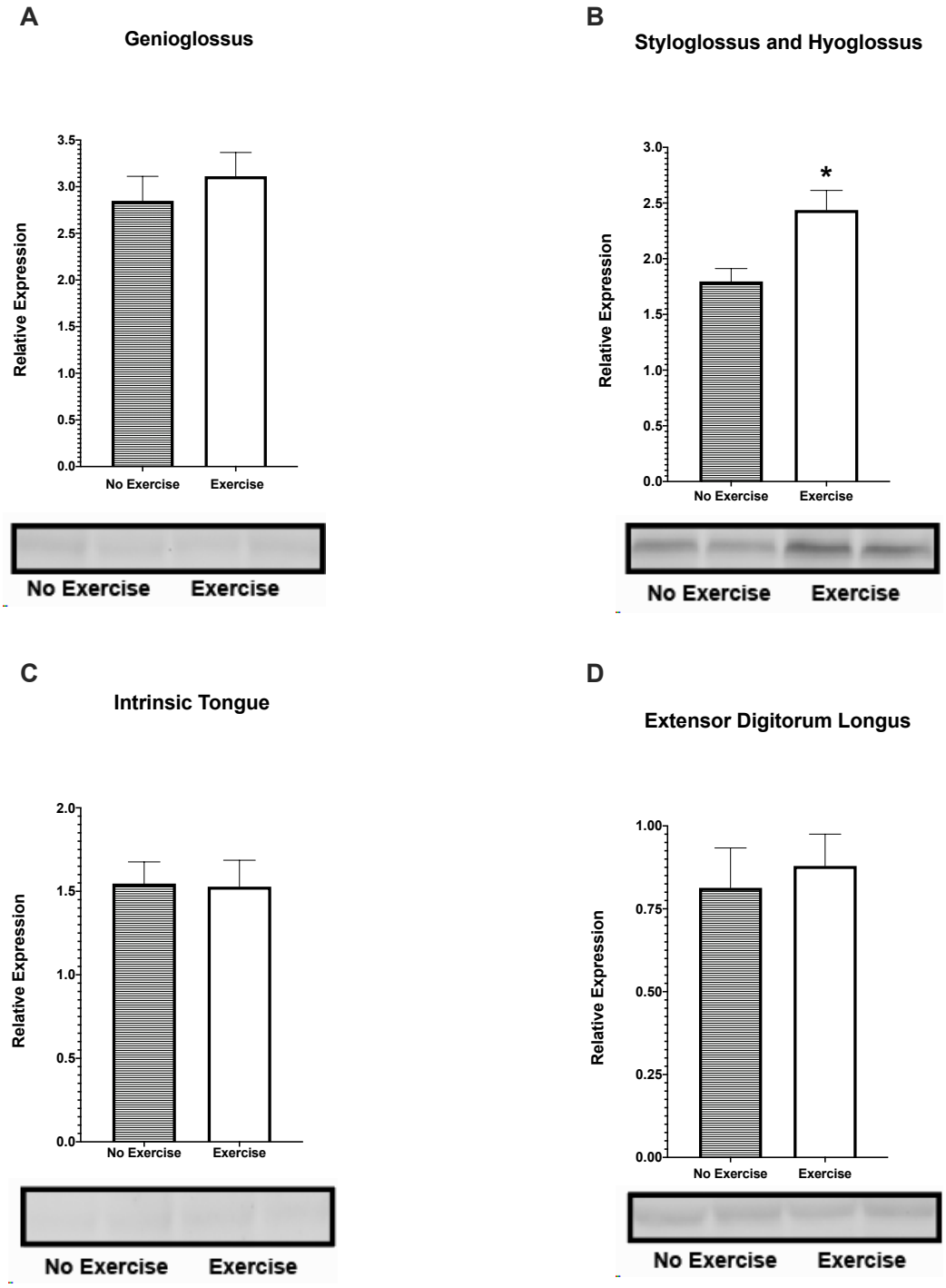

Fig. 28. The Effect of Exercise on Myogenin Protein Expression. With exercise, myogenin protein expression was significantly elevated in the combined styloglossus and hyoglossus muscles (B). No exercise-induced changes in myogenin protein expression were observed in the genioglossus (A), intrinsic tongue (C), or extensor digitorum longus (D) muscles. * = $p<0.05$

Time-Related Changes in Myogenin Protein Expression

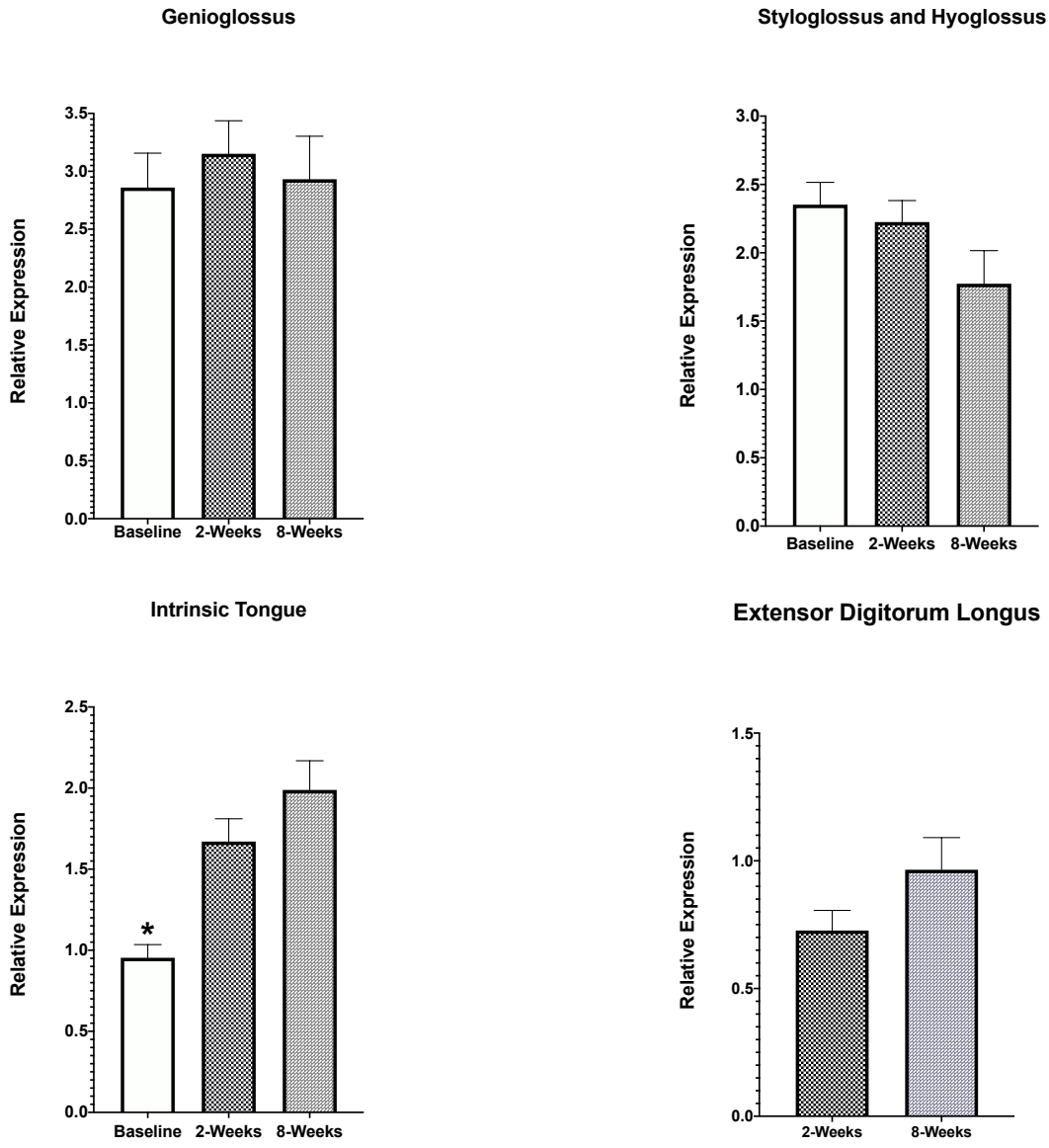

Fig. 29. The Effect of Time on Myogenin Protein Expression. Myogenin protein expression was significantly reduced in the intrinsic tongue at the Baseline time point (C). * = $p<0.05$

3.3 Mechanisms of Cellular Senescence

3.3.1 p16^{INK4a}

3.3.1.1 Whole Muscle p16^{INK4a} Gene Expression at 2 Weeks

A significant age-related increase in p16^{INK4a} gene expression was observed in the GG of the old compared to the young adult groups ($F_{1,11}$=8.704, p=0.013; **Fig. 30A**). No significant interaction effects were observed ($F_{1,11}$=0.007, p=0.933). No significant age-related differences in p16^{INK4a} gene expression were observed in the combined SG and HG ($F_{1,10}$=0.227, p=0.644; **Fig. 30B**), intrinsic tongue ($F_{1,5}$=0.034, p=0.861; **Fig. 30C**), or extensor digitorum longus ($F_{1,11}$=2.293, p=0.158; **Fig. 30D**) muscles. No significant exercise effects were observed in p16^{INK4a} gene expression in the GG ($F_{1,11}$=0.001, p=0.158), combined SG and HG ($F_{1,10}$<0.001, p=0.989), IT ($F_{1,5}$=1.738, p=0.245), or EDL ($F_{1,11}$=1.359, p=0.268) muscles.

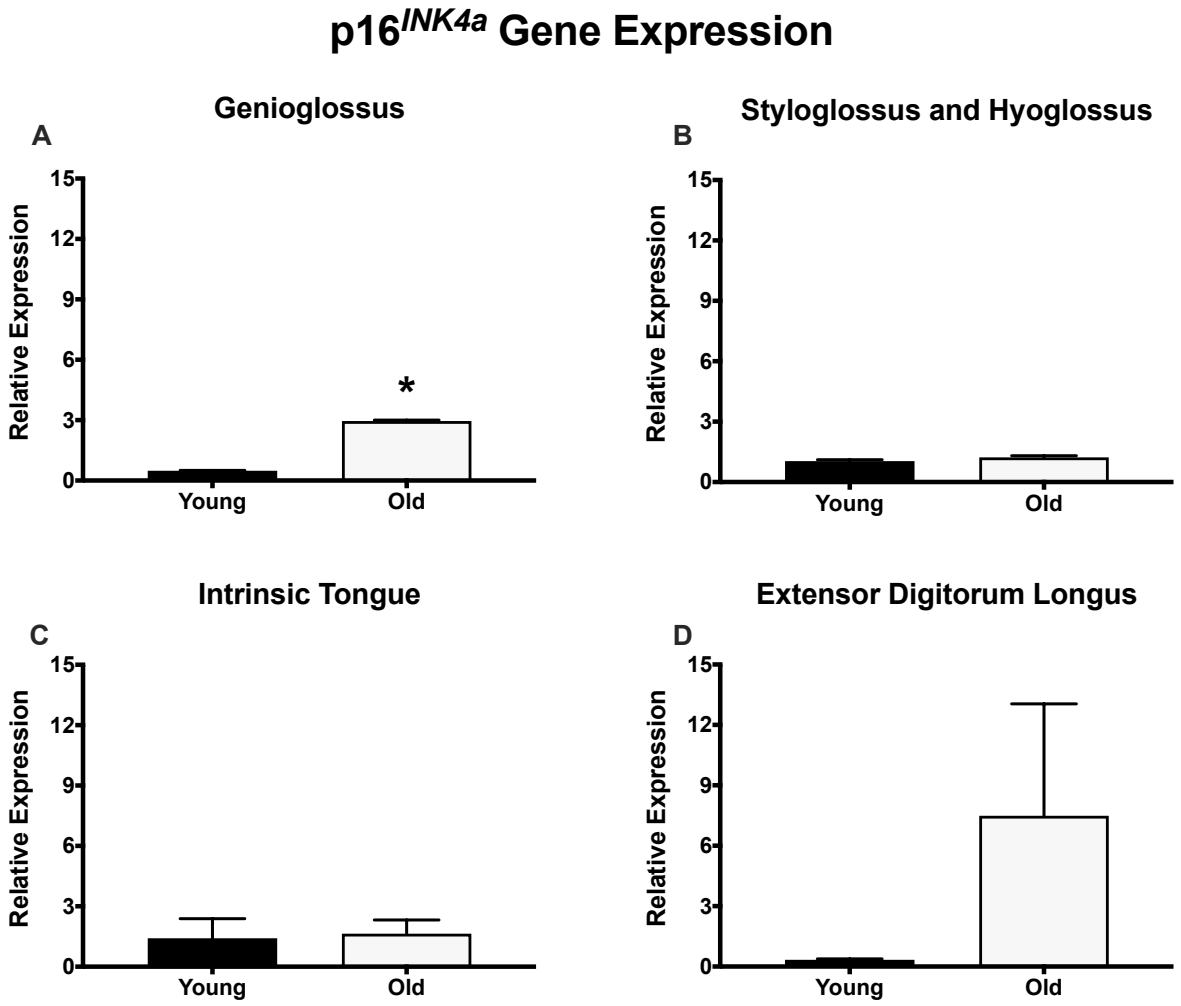

Fig. 30. The Effect of Age on p16^{INK4a} Gene Expression. With age, p16^{INK4a} gene expression was significantly elevated in the genioglossus muscle in the old group (A). No significant age-related changes in p16^{INK4a} gene expression were observed in the combined styloglossus and hyoglossus (B), intrinsic tongue (C), or extensor digitorum longus (D) muscles. * = $p<0.05$

3.3.1.2 Whole Muscle p16^{INK4a} Protein Expression

A significant interaction effect for p16^{INK4a} protein expression was observed in the combined SG and HG muscles (Time x Age; $F_{2,36}$=3.743, p=0.033; **Fig. 31B**). Specifically, p16^{INK4a} protein expression was significantly increased in the young adult group at the 8-week time point in the combined SG and HG muscles. At the 2-week time point, p16^{INK4a} protein expression was significantly increased in the IT ($F_{2,36}$=4.471, p=0.018; **Fig. 32C**) and EDL ($F_{1,24}$=42.38, p<0.001; **Fig. 32D**) muscles. In the GG, no significant effects of age ($F_{1,36}$=2.124, p=0.154), exercise ($F_{1,36}$<0.001, p=0.992), or time ($F_{2,36}$=0.408, p=0.668; **Fig. 32A**) were observed in p16^{INK4a} protein expression.

Significant differences in p16^{INK4a} protein expression were observed among the GG, combined SG and HG, IT, and EDL muscles in the no exercise group at the 2-week time point. p16^{INK4a} protein expression was significantly greater in the GG (Muscle; $F_{3,24}$=8.305, p<0.001; **Fig. 31**). No interaction effects were observed (Muscle x Age; $F_{3,24}$=0.276, p=0.843).

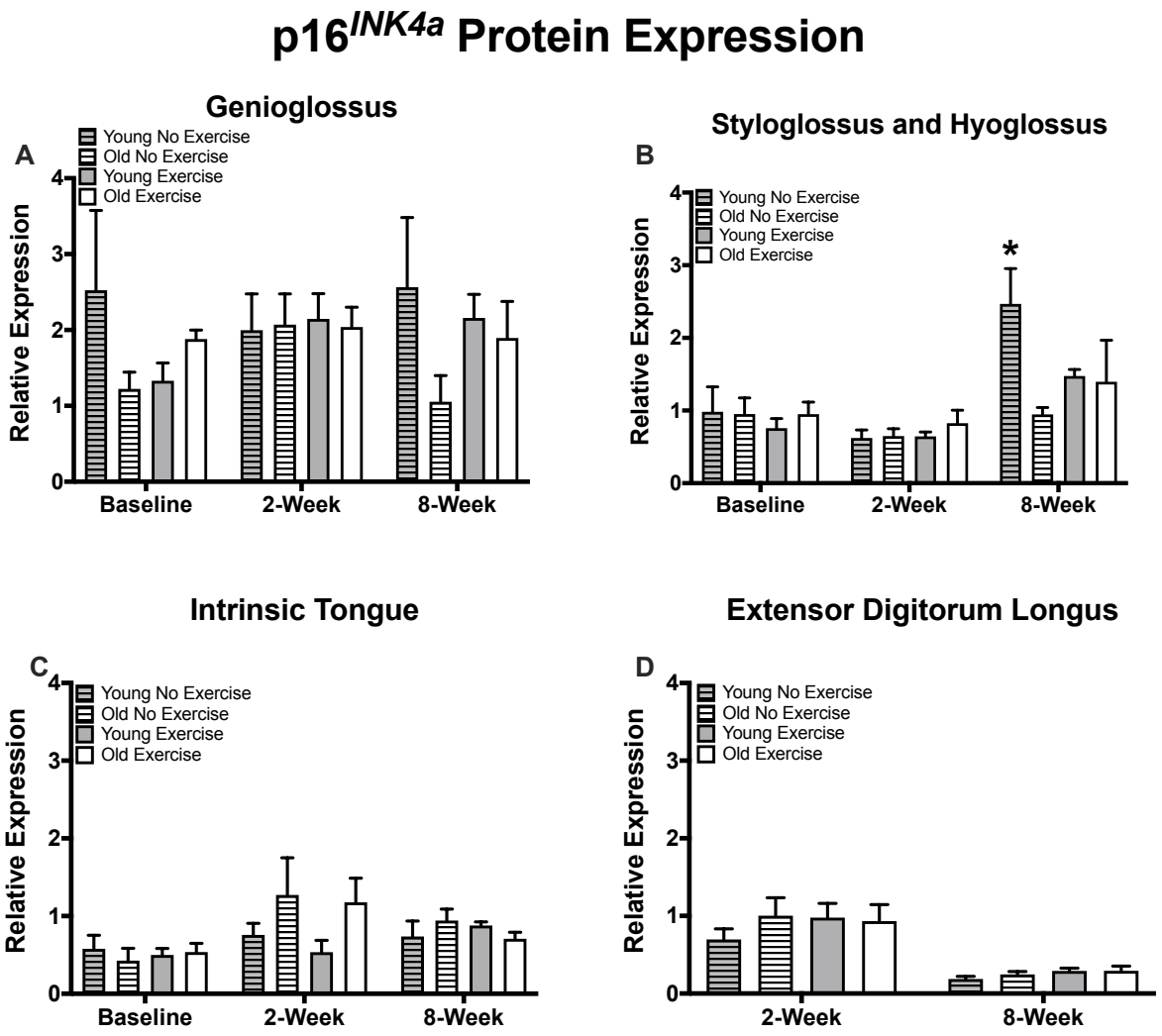

Fig. 31. p16*INK4a* Protein Expression. p16*INK4a* protein expression was elevated in the combined styloglossus and hyoglossus muscles of the young adult, no exercise group at the 8-week time point (B). No other significant interaction effects were observed. * = $p<0.05$

Time-Related Changes in p16^{INK4a} Protein Expression

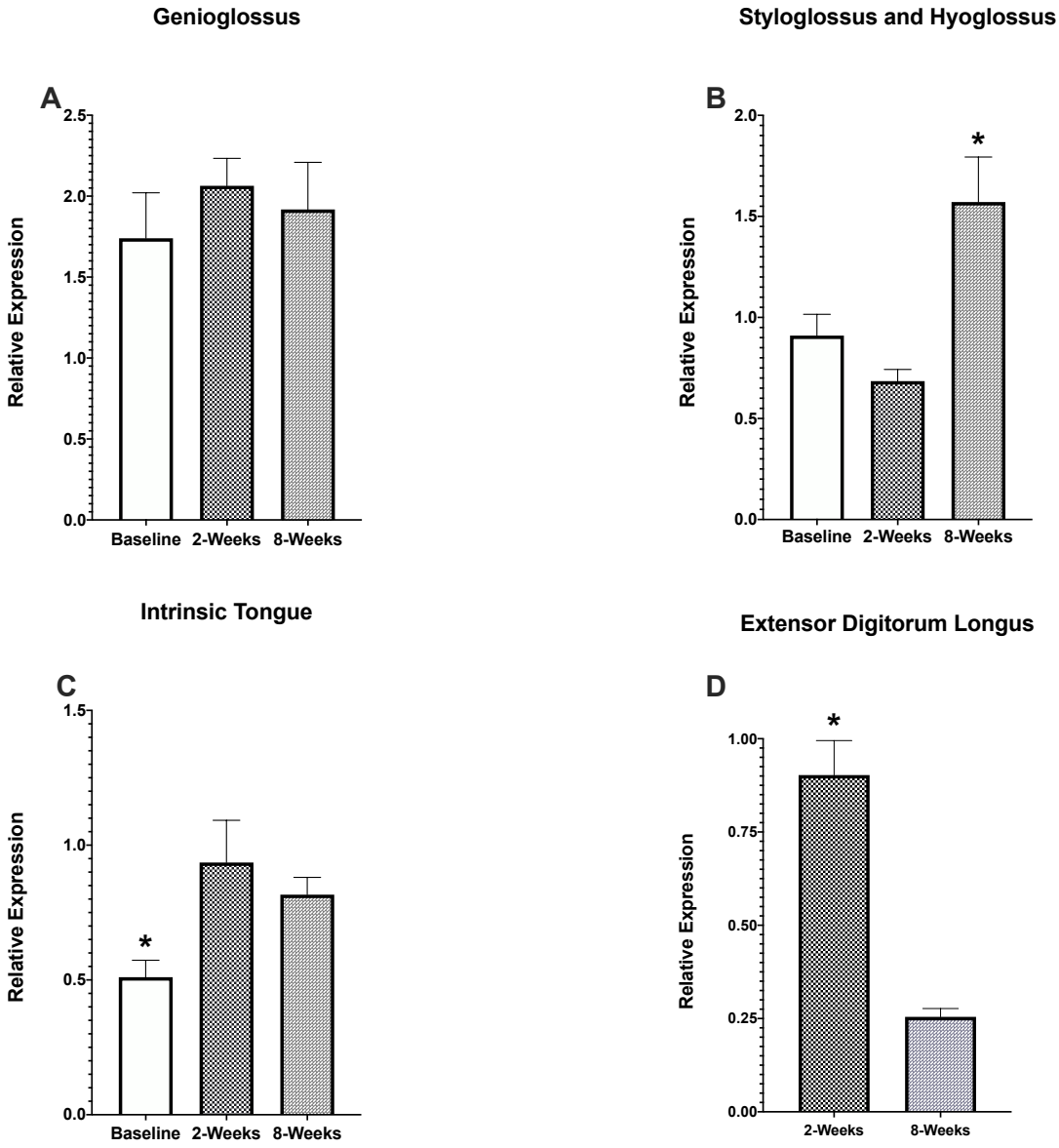

Fig. 32. The Effect of Time on p16^{INK4a} Protein Expression. p16^{INK4a}protein expression was significantly elevated in the combined styloglossus and hyoglossus at the 8-week time point (B) and in the extensor digitorum longus at the 2-week time point (D). p16^{INK4a} protein expression was significantly reduced in the intrinsic tongue at the baseline time point (C). * = $p<0.05$

3.3.1.3 p16^{INK4a} Expression in Isolated Pax7-positive (Pax7+) Satellite Cells (SCs)

Differences in the percentage and expression of p16^{INK4a} in Pax7+ SCs isolated from the tongue and limb musculature were observed. A significant interaction effect was observed in the percentage of p16^{INK4a}-positive/ Pax7-positive (p16^{INK4a}+/ Pax7+) SCs for the GG muscle (Time point x Age; $F_{2,36}$=4.710, p=0.015; **Fig. 33B**). Specifically, a significant increase in GG SCs isolated from the young adult and old groups expressing both Pax7 and p16^{INK4a} proteins was observed at the 2-week time point compared to baseline and the 8-week time point. The percentage of p16^{INK4a}+/Pax7+ SCs also significantly increased in the combined SG and HG muscles at the 2-week timepoint ($F_{2,36}$=3.50, p=0.041; **Fig. 34B**). Significant time point ($F_{2,36}$=11.12, p<0.001; **Fig. 34C**) and age ($F_{1,36}$=6.075, p=0.019; **Fig. 35C**) effects were observed in the percentage of p16^{INK4a}+/Pax7+ SCs isolated from the IT muscle. Specifically, the percentage of p16^{INK4a}+ and Pax7+ SCs isolated from the IT was significantly greater at the 2-week time point, and significantly greater in young adult compared to old groups. The percentage of p16^{INK4a}+ and Pax7+ SCs isolated from the EDL muscle was also significantly greater at the 2-week time point compared to baseline and 8-weeks ($F_{2,36}$=12.55, p<0.001; **Fig. 34D**).

A significant interaction effect was observed in the expression (intensity) of p16^{INK4a} in Pax7+ SCs isolated from the GG muscle (Time point x Age, $F_{2,36}$=5.051, p=0.012; **Fig. 36A**). p16^{INK4a} protein expression was greatest at the 2-week time point in Pax7+ SCs of the old group compared to baseline in the GG. The expression of the p16^{INK4a} protein in Pax7 SCs was also significantly increased at the 2-week time point in SCs from the combined SG and HG ($F_{2,36}$=4.733, p=0.015; **Fig. 37B**), IT ($F_{2,36}$=9.406,

$p<0.001$; **Fig. 37C**), and the EDL (($F_{2,36}=63.77$, $p<0.001$; **Fig. 37D**) muscles. No significant exercise effects were observed in the tongue musculature.

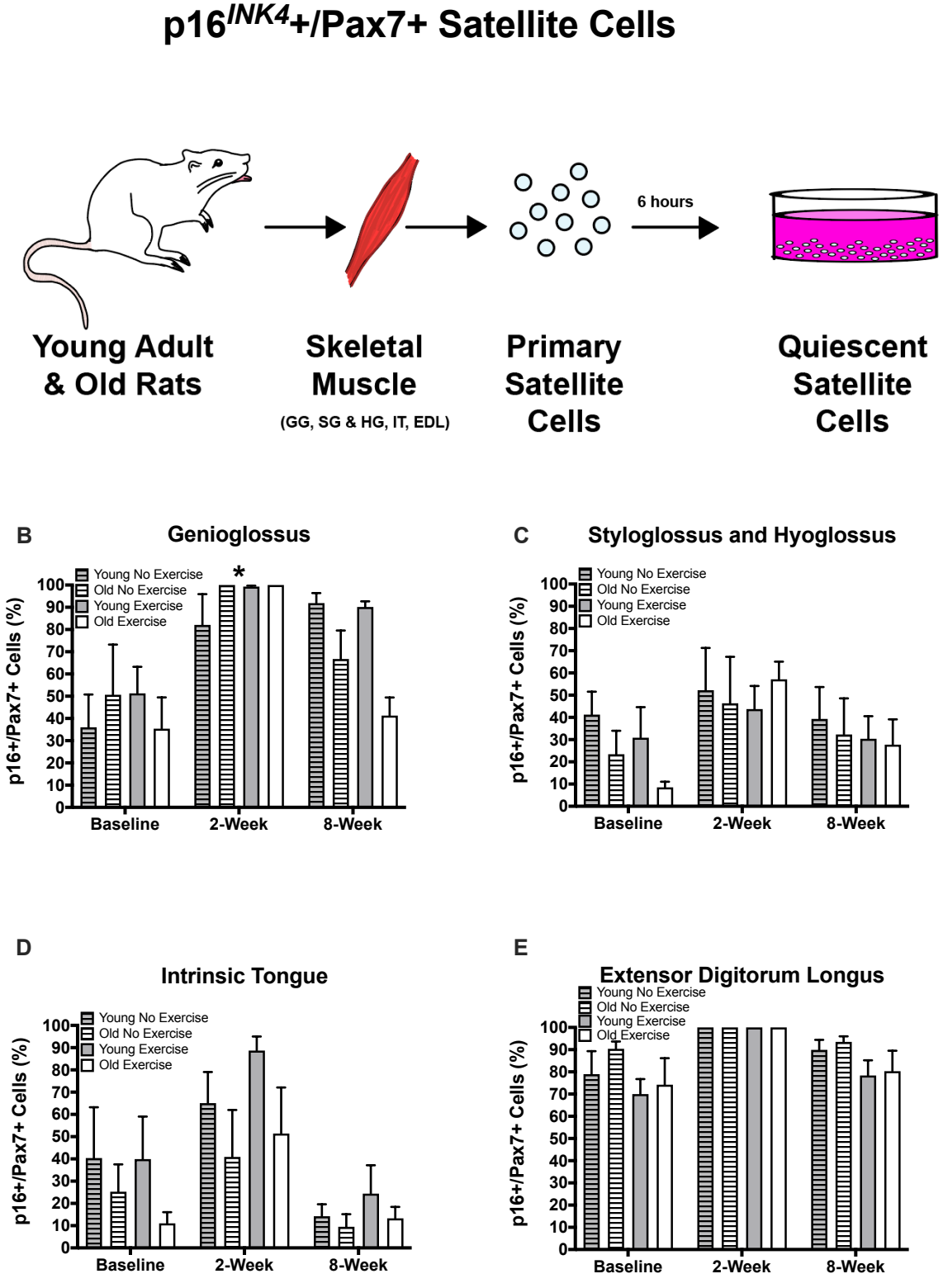

Fig. 33. p16^{INK4a} -positive/Pax7-positive (p16^{INK4a} +/Pax7+) Satellite Cells (SCs). (A) Schematic diagram of *ex*-vivo primary MSC culture from young adult and old F344/BN rats. A significant increase in the percentage off SCs isolated from the young adult and old groups expressing both Pax7 and p16^{INK4a} proteins was observed at the 2-week time point (A). No other significant interaction effects were observed. * = $p<0.05$

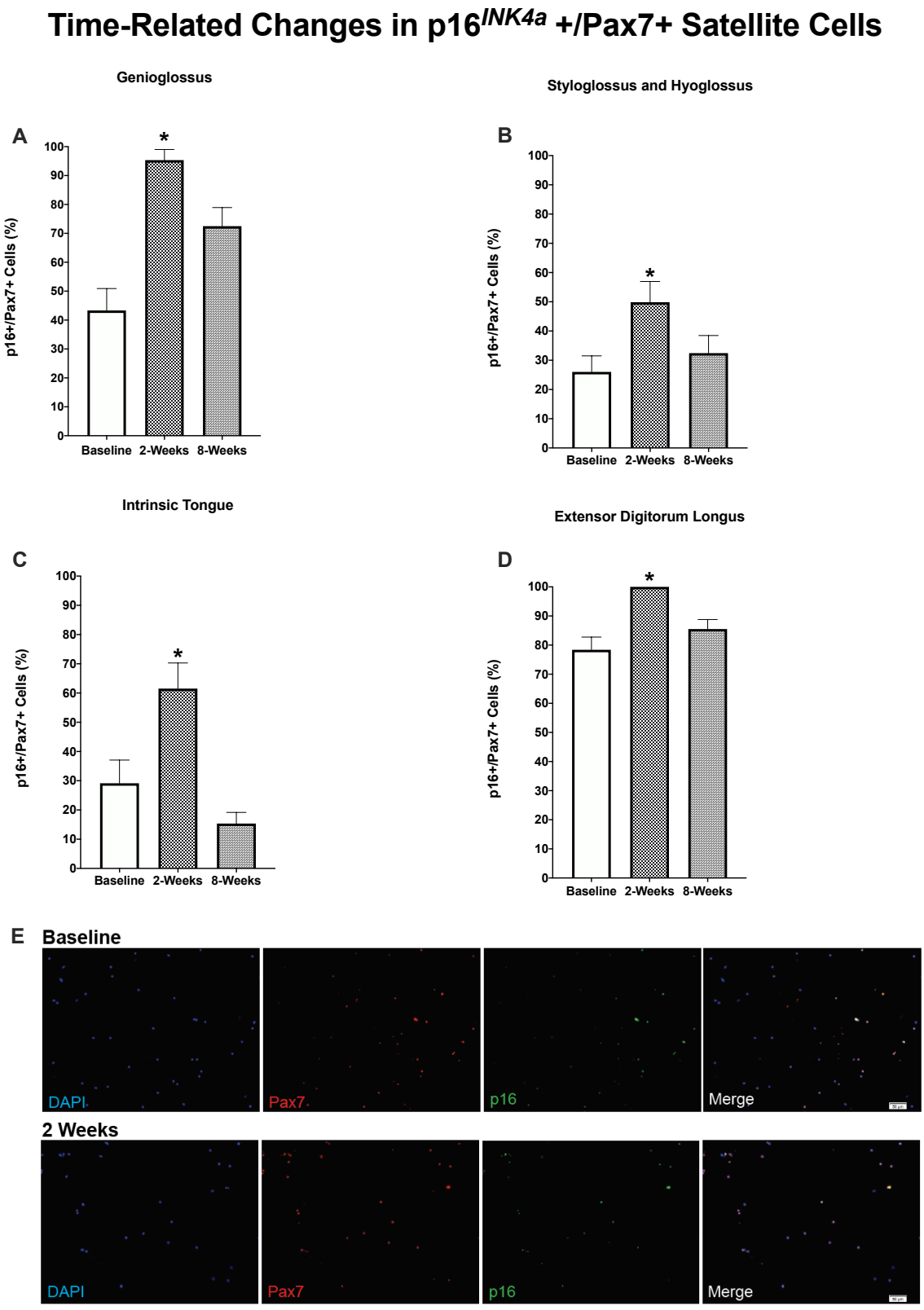

Fig. 34. The Effect of Time on Isolated p16^{INK4a} -positive/Pax7-positive (p16^{INK4a} +/Pax7+) Satellite Cells (SCs). The percentage of p16^{INK4a} +/Pax7+ SCs isolated from the genioglossus (A), styloglossus and hyoglossus (B), intrinsic tongue (C), and extensor digitorum longus (D) muscles significantly increased at the 2-week time point. (E) Representative images of p16^{INK4a} +/Pax7+ SCs isolated from the combined SG and HG at baseline and 2-week time points. * = $p<0.05$

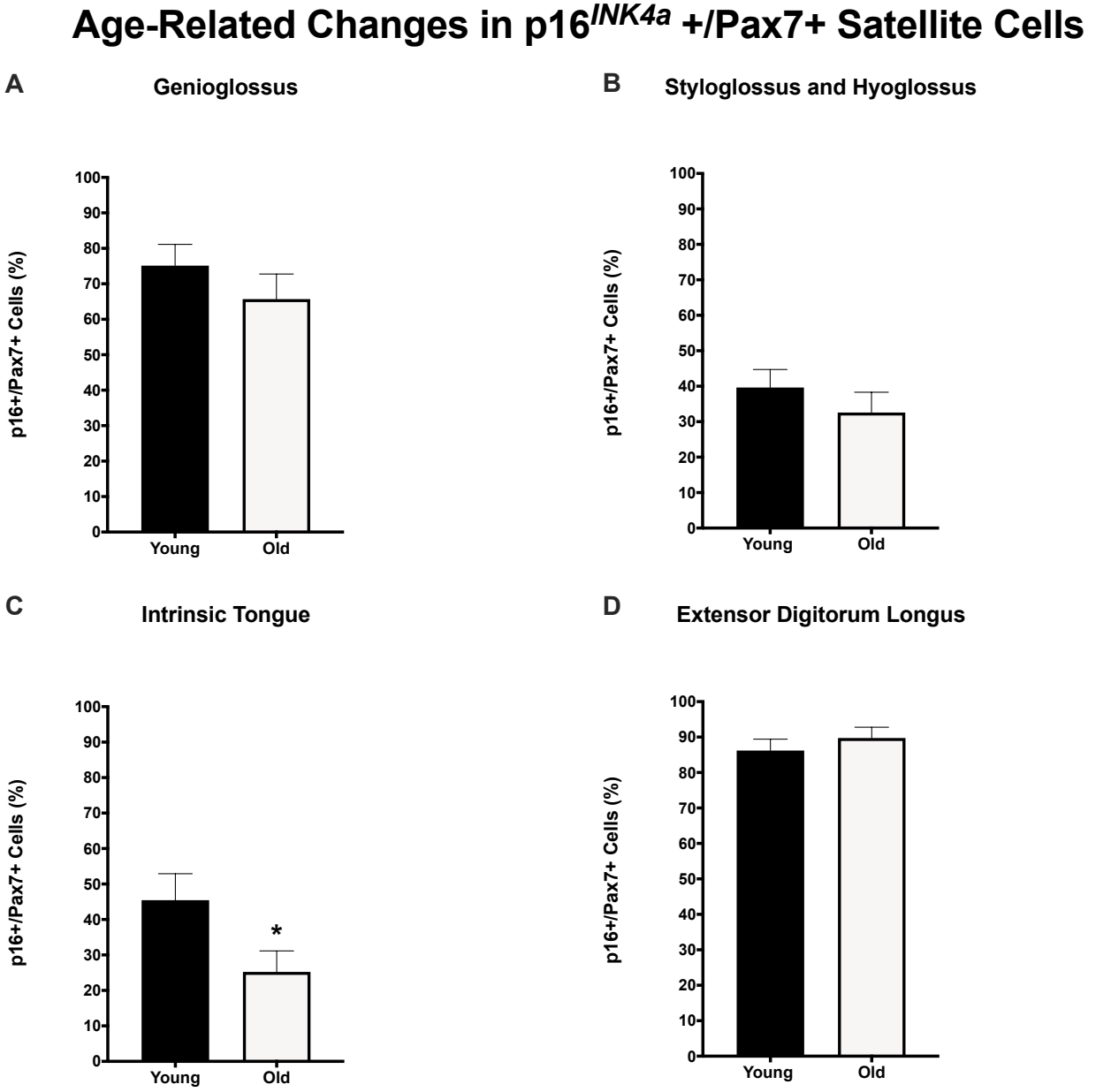

Fig. 35. The Effect of Age on Isolated p16^{INK4a} -positive/Pax7-positive (p16^{INK4a} +/Pax7+) Satellite Cells (SCs). The percentage of p16^{INK4a} +/Pax7+ SCs isolated from the intrinsic tongue (C) significantly decreased in the old group. * = $p<0.05$

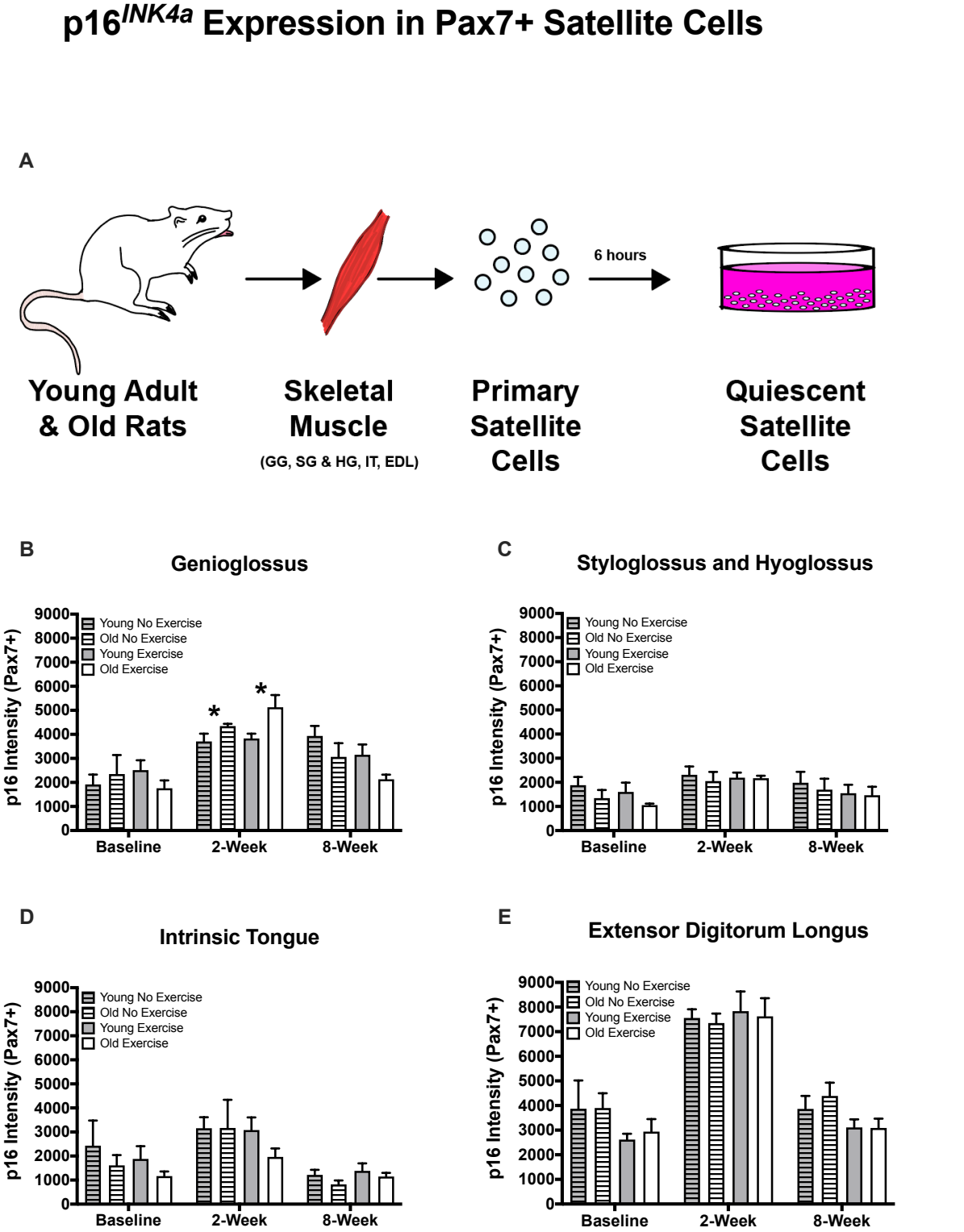

Fig. 36. p16^{INK4a} Intensity in Pax7-positive (Pax7+) Satellite Cells (SCs). (A) Schematic diagram of *ex*-vivo primary SC culture from young adult and old F344/BN rats. p16^{INK4a} protein expression was significantly elevated at the 2-week time point in Pax7+ SCs isolated from the genioglossus muscle of the old rat group compared to the baseline time point (A). No other significant interaction effects were observed.

Time-Related Changes in p16^{INK4a} Intensity in Pax7+ Satellite Cells

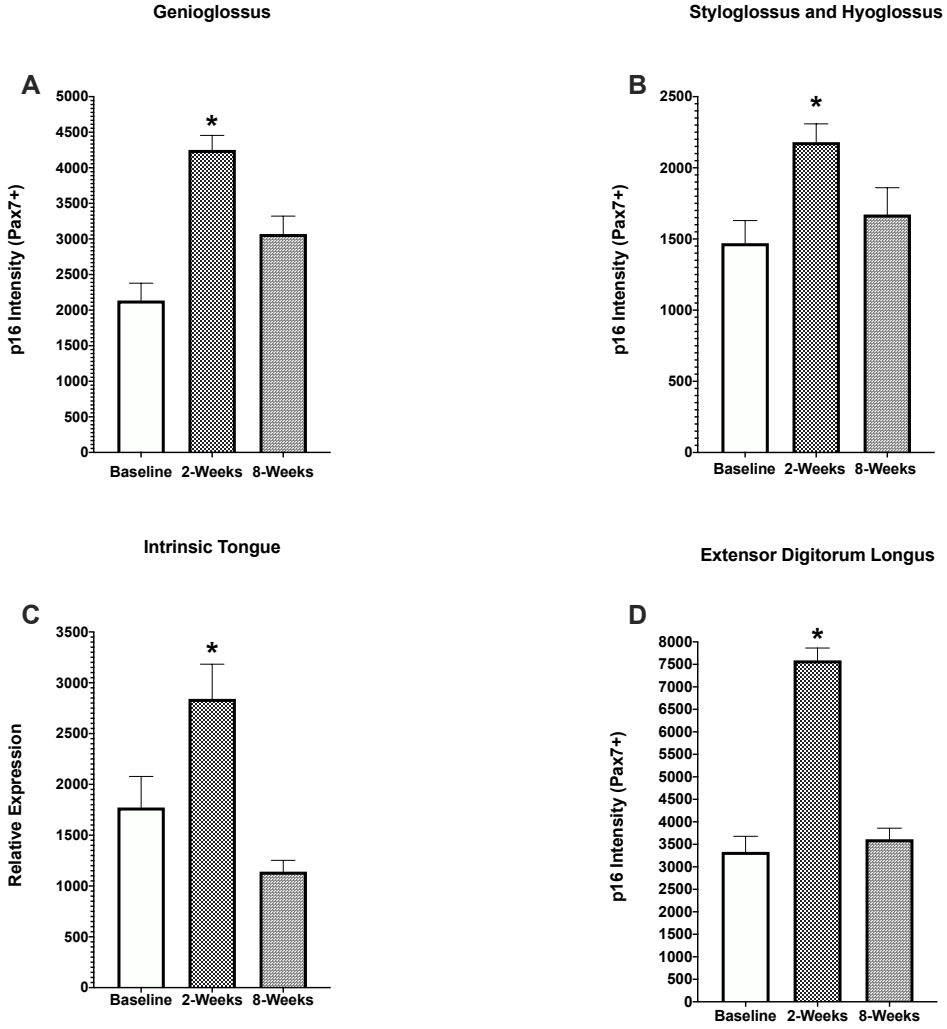

Fig. 37. The Effect of Time on p16^{INK4a} Intensity in Pax7-positive (Pax7+) Satellite Cells (SCs). The intensity of p16^{INK4a} protein expression in SCs isolated from the genioglossus (A), combined styloglossus and hyoglossus (B), intrinsic tongue (C), and extensor digitorum longus (D) muscles was significantly elevated at the 2-week time point. * = $p<0.05$

3.4 Relationship between Tongue Strength and Satellite Cell Regenerative Capacity

As shown in Figure 38, a strong, significant relationship was observed between MVTF and protein markers of SC regenerative capacity (R^2=0.953; $F_{14,9}$=12.96, p<0.001; y= 18.57 + 0.462*GGPax7 + 5.170*GGMyoD + 0.851*GGMyogenin + 10.70*GGp16 + 1.719*SGHGPax7 – 4.389*SGHGMyoD – 9.064*SGHGMyogenin + 12.39*SGHGp16 + 2.903*ITPax7 + 11.40*ITMyoD - 10.59*ITMyogenin + 6.628*ITp16 – 9.077*Old + 28.63*Time) in the tongue muscles. Specifically, time (p<0.001), and MyoD (p=0.019), myogenin (p=0.030), and p16^{INK4a} (p=0.036) protein expression in the combined styloglossus and hyoglossus muscles were significant predictors of Maximal Voluntary Tongue Force following tongue exercise.

A strong, significant relationship in the combined SG and HG was observed between MVTF and protein markers of SC regenerative capacity (R^2=0.877; $F_{6,17}$=20.16, p<0.001; y= 53.11 + 4.958*SGHGPax7 – 3.751*SGHGMyoD – 9.514*SGHGMyogenin + 16.47*SGHGp16 – 8.834*Old + 21.78*Time; **Fig. 39B**). Following tongue exercise, time (p<0.001), and Pax7 (p=0.048), MyoD (p=0.013), myogenin (p=0.010), and p16^{INK4a} (p=0.003) protein expression were significant predictors of MVTF in the SG and HG muscles.

A moderate, significant relationship was observed between MVTF and protein markers of SC regenerative capacity in the GG (R^2=0.673; $F_{6,17}$=5.818, p=0.002; y= 23.65 – 0.027*GGPax7 + 4.060*GGMyoD + 0.612*GGMyogenin + 5.881*GGp16 – 10.70*Old + 24.12*Time; **Fig. 39A**) and IT (R^2=0.763; $F_{6,17}$=9.098, p<0.001; y= 13.91 + 8.750*ITPax7 + 9.481*ITMyoD – 3.453*ITMyogenin + 14.99*ITp16 – 19.72*Old +

25.31*Time; **Fig. 39C**). Specifically following exercise, in the GG, time ($p<0.001$) was a significant predictor of MVTF, and in the IT, time ($p<0.001$) and old age (0.017) were significant predictors of MVTF.

The Relationship Between Tongue Strength and Satellite Cell Regenerative Capacity in the Tongue

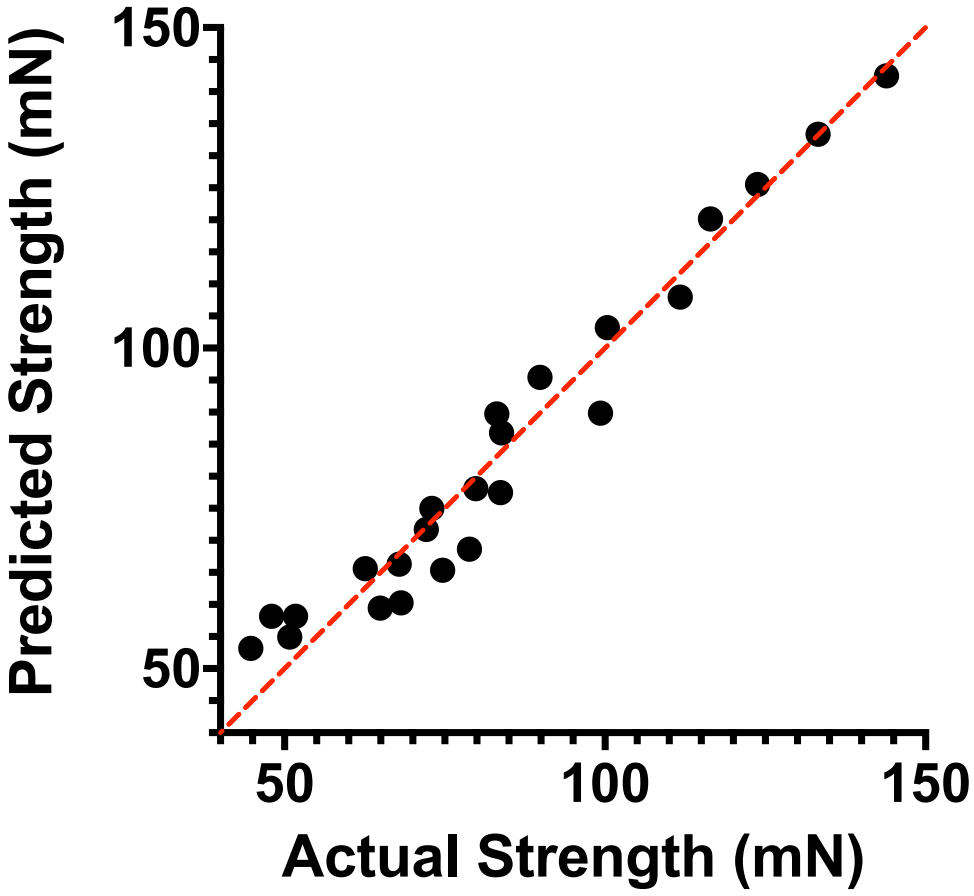

Fig. 38. The Relationship Between Tongue Strength and Satellite Cell Regenerative Capacity in the Tongue. A strong, significant relationship was observed between tongue strength (MVTF) and protein expression of MyoD, myogenin, and $p16^{INK4a}$ in the combined styloglossus and hyoglossus muscles following tongue exercise at all 3 time points (R^2=0.953; p<0.001).
MVTF = Maximal voluntary tongue force.

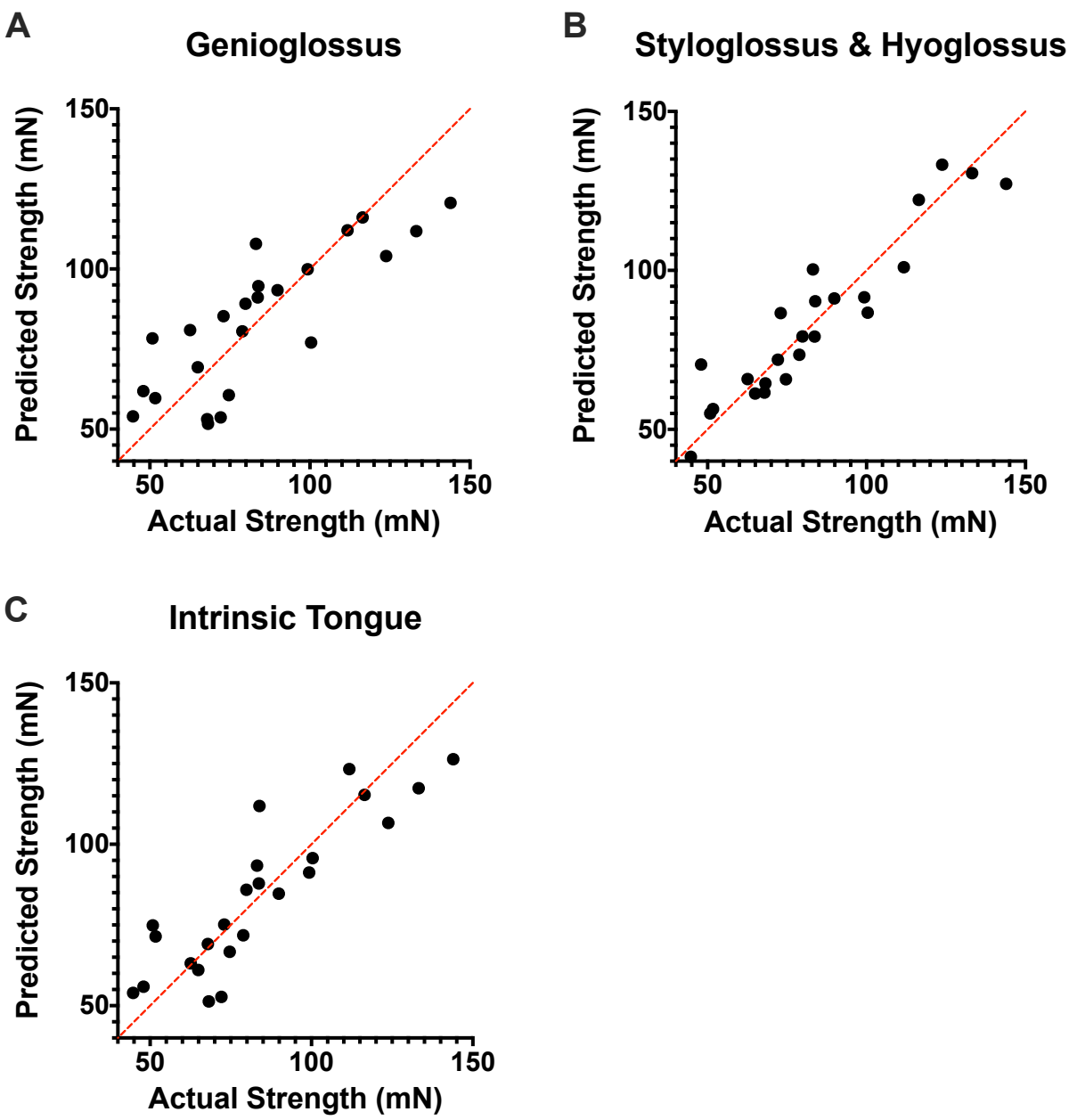

Fig. 39. The Relationship Between Tongue Strength and Satellite Cell Regenerative Capacity in the Tongue Muscles. A moderate, significant relationship was observed between tongue strength (MVTF) and protein expression in the genioglossus (A; R^2=0.673; p=0.002), combined styloglossus and hyoglossus (B; R^2=0.877; p<0.001), and intrinsic tongue (C; R^2=0.763; p<0.001) muscles. MVTF = Maximal voluntary tongue force.

3.5 Summary of Significant Results

Table 2 contains a summary of all significant main effects for time point, age, and treatment, and interaction effects.

Summary of Major Significant Results				
Marker	**Muscle**			
Gene Expression	**GG**	**SG & HG Combined**	**IT**	**EDL**
Pax7	*Age:*↓ Old	NS	NS	*Age:* ↓ Old
MyoD	NS	NS	NS	*Age:* ↑ Old
Myogenin	NS	NS	NS	*Age:* ↑ Old
p16^{INK4a}	*Age:* ↑ Old	NS	NS	NS
Protein Expression	**GG**	**SG & HG Combined**	**IT**	**EDL**
Pax7	***Muscle:*** *Time:* ↑ 2-Wks *Age:* ↓ Old ***SC:*** NS	***Muscle:*** NS ***SC:*** **Time:** ↓ 2-Wks	***Muscle:*** *Time:* ↑ 8-Wks ***SC:*** ↑ 2-Wks, Young Ex ↓ 2-Wks, Old Ex	***Muscle:*** *Time:* ↑ 8-Wks *Age:* ↑ Old ***SC:*** ↓ 2-Wks, Old
MyoD	***Muscle:*** NS	***Muscle:*** ↑ Young Ex, Baseline	***Muscle:*** *Time:* ↑ Baseline	***Muscle:*** *Time:* ↑ 8-Wks
Myogenin	***Muscle:*** NS	***Muscle:*** *Tx:* ↑ Ex	***Muscle:*** *Time:* ↑ 2- & 8-Wks	***Muscle:*** NS
p16^{INK4a}	***Muscle:*** NS ***SC:*** *Positive Cells:* ↑ 2-Wks, Young & Old *Expression:* ↑ 2-Wks, Old	***Muscle:*** ↑ Young, No Ex, 8-Wks ***SC:*** *Positive Cells:* *Time:* ↑ 2-Wks *Expression:* *Time:* ↑ 2-Wks	***Muscle:*** *Time:* ↑ 2-Wks ***SC:*** *Positive Cells:* ↑ 2-Wks, Young *Expression:* *Time:* ↑ 2-Wks	***Muscle:*** *Time:* ↑ 2-Wks ***SC:*** *Positive Cells:* *Time:* ↑ 2-Wks *Expression:* *Time:* ↑ 2-Wks
Myofiber	**GG**	**SG**	**HG & IT**	**EDL**
Pax7	↑ Exercise, Young ↑ No Exercise, Old	*Tx:* ↑ Exercise	NA	NS
Type IIb, IIx	NS	*Tx:* ↑ Exercise	NA	NS
Type IIb, IIx, IIa	NS	*Tx:* ↓ Exercise	NA	NS
Type IIa	↓ Exercise, Young & Old	NS	NA	NS
Type I	NS	No MyHC Type I	NA	*Age:* ↑ Old
Non-Myofiber	*Tx:* ↑ Exercise	↓ Young, No Exercise	NA	NS
Myofiber Repair	**GG**	**SG**	**HG & IT**	**EDL**
Centralized Nuclei	*Age:* ↑ Old *Tx:* ↑ Exercise	*Age:* ↑ Old *Tx:* ↑ Exercise °	NA	*Age:* ↑ Old
Function	**Tongue**			**EDL**
MVTF	*Tx:* *Time:* ↑ 2- & 8-Wks, ↓ Variability ° *Age:* ↑ Variability ° Related to MyoD, myogenin, & p16^{INK4a} Protein Expression in SG & HG			NA

Table 2. Summary of Significant findings. NS = not significant; ° = approach significance; Tx = treatment effect; Wks = weeks; SC = satellite cell; Ex = Exercise; NA = not available; GG = genioglossus; SG = styloglossus; HG = hyoglossus; IT = intrinsic tongue; EDL = extensor digitorum longus; MVTF = maximal voluntary tongue force

Chapter 4. Discussion.

Cellular mechanisms of age-related changes in lingual muscle structure and function are unknown. A putative mechanism is decline in the muscle satellite cell (SC) regenerative capacity, or the ability of skeletal muscle SCs to repair and remodel damaged myofibers.[61,63,64] Accordingly, the purpose of this study was to examine the manner in which aging contributes to the decline in SC regenerative capacity and to examine the potential for a clinically relevant tongue exercise-program to improve the regenerative function of SCs in aging tongue musculature. The hypotheses were: (1) SC regenerative capacity and maximal voluntary tongue force (MVTF) will be reduced as a function of aging; (2) exercise will enhance the regenerative function of young and aged SCs as manifested by upregulation of SC myogenic transcription factors and increased MVTF at the 2-week and 8-week time points; (3) $p16^{INK4a}$ expression will be up-regulated as a function of aging and down-regulated as a function of exercise in whole muscle and isolated muscle SCs; and (4) expression of SC myogenic transcription factors will be differentially expressed in the tongue versus limb muscles.

The results of this study supported our hypotheses, in part. Although age-related deficits in tongue strength, as reflected by MVTF, were not observed, SC regenerative capacity was impaired with age. Age-related reductions in SC regenerative capacity may be related to increased expression of the senescent marker $p16^{INK4a}$. With age, the gene expression of $p16^{INK4a}$ was also upregulated in the GG. Following tongue exercise, tongue strength (MVTF) significantly increased at the 2-week and 8-week time points. With tongue exercise, the regenerative potential of SCs increased in both the acute and chronic stages of the tongue exercise program, and was significantly related to the

protein expression of SC myogenic transcription factors and p16^{INK4a} in the muscles of the tongue. The location of Pax7-positive SCs according to myofiber type also changed following 2-weeks of exercise in the tongue. The expression of SC myogenic transcription factors were also different between the tongue and limb muscles (no exercise group), suggesting that the SC regenerative capacity of the tongue muscles is increased in tongue compared to limb.

4.1 Tongue Strength

Age-related reductions in tongue strength were not observed at any time point in this study. Because the tongue exercise condition was a behavioral, voluntary task there is a possibility that rats in the young adult and old exercise groups did not achieve maximal tongue forces over the 3 day testing period. This finding is consistent with previous studies from our laboratory in the aging rat model and with the reports from the human population, where deficits in measures of voluntary tongue strength with age have not been observed.[131,132,148,154,164,192-194] We did observe slightly increased variability in MVTF in the old exercise group that could be related to alterations in motor variability reported in elderly humans.[45] However, tongue strength significantly increased after both 2-weeks and 8-weeks of tongue exercise in the young adult and old groups that stimulated the activation of SCs, and paralleled gains in strength observed in elderly patients following the completion of tongue strengthening programs.[54] As such, the results of our study suggest that tongue strengthening exercises may activate SCs in elderly people, representing a putative cellular mechanism for enhancement of muscle regenerative capacity with therapy.

4.2 Novel Methodological Approach

This is the first study to relate voluntary tongue strength following an exercise treatment with mechanisms of SC regenerative capacity in tongue muscles and isolated tongue SCs in an aging rodent model. By taking this methodological approach, we examined how the SC-intrinsic expression of genes and proteins of aged SCs are influenced by the external environmental cues from the myofiber, and contribute to increases in voluntary tongue muscle strength following an exercise treatment. Further, including the EDL muscle allowed for the direct comparison of the SC regenerative profiles among muscles of the tongue and limb.

4.3 Satellite Cell Regenerative Capacity in the Tongue Muscles

4.3.1 Age-Related Changes in Satellite Cell Regenerative Capacity

The results of our study suggest that the regenerative capacity of SCs in the tongue are distinct and muscle specific. The gene and protein profiles of the SC myogenic transcription factors mediating SC regenerative capacity were expressed differentially in the GG, SG, HG, and IT muscles with age and following exercise. The GG muscle was most susceptible to age-associated dysfunction in the SC pool, both Pax7 gene and protein expression decreased with age in the GG. Reductions in the gene and protein expression of Pax7 in the GG may be evidence of an age-related loss of the resident, quiescent SC population and an overall, global decline in the regenerative potential of aged SCs.[195-198] However, when we examined SC content in the mid -belly of the GG muscle, no age-related alterations in the percentage of Pax7 SCs were observed. This may indicate that age-related changes in Pax7 SC content may be region dependent, and the distribution of Pax7+ SCs may be instead reduced in

the anterior or posterior regions of the GG muscle. We also observed an age-related upregulation of whole muscle p16^{INK4a} gene expression in the GG, and increased protein expression of p16^{INK4a} in isolated Pax7+ SC from the GGs. Expression of p16^{INK4a} blocks the transition from the G_0 to G_1 phase of the cell cycle and inhibits entry into the S phase, ultimately inducing a state of senescence.[64,106,108-112] Cellular senescence is an active state of cell cycle arrest, and inhibits cell proliferation, and promotes age-related tissue dysfunction and degeneration. The accumulation of senescent cells in post-mitotic tissue, such as skeletal muscle, disrupts tissue structure, impairs the normal biological function of that tissue, and may be a causal factor of aging.[113-116] The upregulation of this cell cycle inhibitor in the aged GG may block cell cycle progression globally in whole muscle and, intrinsically in isolated SCs. Increased p16^{INK4a} expression may contribute to the reduction in Pax7 expression observed in the GG, and may impact the ability of Pax7+ SCs to become activated by an exercise stimulus. This finding is consistent with previous studies that found increased levels of p16^{INK4a} expression in numerous aged tissue types and within aging, cultured cells.[64,106,108-116] The inability of aged adult stem cells to progress through the cell cycle significantly impacts their capacity to initiate a regenerative response in response to injury, exercise, or disease.

Unlike the GG, we did not observe any age-related reductions in the expression of the SC myogenic transcription factors, Pax7, MyoD, or myogenin, or upregulated gene or protein expression of p16INK4a within the SG, HG, or IT muscles. This suggests that age-related deficits in SC regenerative capacity of the SG, HG, and IT muscles may be spared with age. In previous studies from our laboratory, age-related

alterations in the contractile properties and in the MyHC isoform and myofiber type composition have been found in the GG, SG, HG, and IT muscles.[131-133,137,141,142,145-147,149,152,153,155,164] Tongue muscle protrusive and retrusive contraction times are significantly longer in aged rats, tongue muscle fatigue is increased with age, and protrusive twitch and tetanic forces are reduced in old rats.[131,137,141,142,147,149,152,153] With regard to changes in MyHC isoform and myofiber type composition, we have previously found age-related transitions to a slowly contracting, fatigue resistant phenotype in the extrinsic and intrinsic tongue muscles.[131,133,146,147,164] Because the MyHC isoform and myofiber type composition of the tongue muscles change with age, we also examined the location of Pax7+ SCs according to myofiber type and proximity to vessels and nerves in the GG and SG muscles. No age-related alterations in the location of Pax7+ SCs according to myofiber type were found in the GG or SG, contrary to what has been reported in the limb.[199-201] However, these studies in the limb only examined the location of Pax7+ SCs in proximity to either type II or type I myofibers, and did not take into consideration the location of Pax7+ SCs near specific myofiber subtypes as was performed in this current study.[201]

We observed a significant age-related increase in centralized myofiber nuclei in both the GG and SG muscles, which is suggestive of an active state of myofiber repair and remodeling.[202-204] Factors contributing to age-related changes in tongue muscle physiology and composition may include alterations in neuromuscular junction density and morphology, innervation ratio, increased cell death, and the dysregulation of apoptotic pathways.[131-133,137,141,142,145-149,151-153,155,164] Data from the present study suggest that SC regenerative capacity of the SG, HG, and IT does not change with age.

SC content in the mid-belly of the GG and SG did not change with age or by myofiber type. Thus, other mechanisms within the aged systemic or local microenvironment, or intrinsic changes in the gene and protein expression of aged SCs likely contribute to the age-related changes in muscle physiology and composition previously observed in the tongue.

4.3.2 Effects of Tongue Exercise on Satellite Cell Regenerative Capacity

Recent emphasis has been placed on the development of novel therapies that are capable of improving the regenerative potential of SCs and restoring muscle function in the aging population.[90,96,205-208] In this study, we examined the regenerative ability of tongue muscle SCs in young adult and old rats following a tongue strengthening exercise modeled after a current clinical treatment for the rehabilitation of swallowing disorders in the elderly. Our progressive resistance tongue exercise treatment affected the expression of the SC myogenic transcription factors mediating SC differentiation, differentially in the IT, GG, SG, and HG muscles.

In the IT muscle, the only exercise-related change in SC regenerative capacity occurred at the 2-week time point. Following 2 weeks of tongue exercise, the percentage of isolated Pax7+ SCs increased in the young adult group and decreased in the old group.

In the GG, we observed an increase in the Pax7+ SC pool in the muscle mid-belly following 2 weeks of exercise in the young adult group. When we examined the location of Pax7+ SCs by myofiber type following tongue exercise, we observed that the percentage of Pax7+ SCs localizing near type IIa myofibers was reduced, and those localizing near vessels or nerves increased. SCs are localized in close proximity to

vessels, regardless of their regenerative state, and SC number is positively correlated with myofiber capillarization.[209] Skeletal muscle angiogenesis, and the development of new capillaries in response to exercise may contribute to the influx of SCs expressing Pax7 we observed near vessels within the GG muscles following exercise.[210,211] We also observed an increase in centralized myofiber nuclei with 2 weeks of exercise in the GG. The activation and self-renewal of Pax7 SCs may be related to the increase in centralized nuclei, and the repair and remodeling of myofibers in young GG muscles following tongue exercise.[202-204] In the GG, exercise did not affect the expression of $p16^{INK4a}$.

However, the SC regenerative capacity of the SG and HG muscles were the most impacted by tongue exercise as evidenced by alterations in the SC myogenic transcription factors that facilitate muscle repair and regeneration. The upregulation of Pax7, MyoD, and myogenin throughout the 8-week tongue exercise program suggests that tongue strengthening programs have the ability to activate and stimulate the SC population in the tongue muscles. At the baseline time point in the SG and HG muscles of the exercise group, we observed increased protein expression in myogenin. This suggests that performing an unfamiliar exercise task is capable of stimulating the SC pool, and that SC proliferation and differentiation occurred within the acute phase following tongue exercise in the SG and HG muscles.[119-122,169] This is consistent with findings in the limb literature, where SC myogenic factors were upregulated following a single bout of treadmill running within the acute phase following the exercise stimulus.[167-169]

Following 2 weeks of exercise, we observed an increase in the percentage of Pax7+ SCs in the muscle mid-belly of the SG. Because protein expression of myogenin was upregulated at baseline, an increase in Pax7+ SCs at the 2-week time point is likely an indication of SC pool self-renewal and ongoing SC activation. Myogenin protein expression was also upregulated in the SG and HG at the 2-week timepoint in the exercise group. Increased expression of Pax7 and myogenin following 2 weeks of tongue exercise may also be related to the increase in centralized myofiber nuclei, and may be evidence of an activated state of exercise-induced myofiber repair and remodeling.[202-204] This is further supported by exercise-related alterations in the location of Pax7+ SCs in the SG muscle. In the SG muscle, the percentage of Pax7+ SCs near type IIb and IIx myofibers increased following 2 weeks of tongue exercise, and the number colocalizing with type IIb, IIx, and IIa fibers decreased. The colocalization of Pax7+ SCs around or near vessels or nerves also significantly increased following exercise. These alterations in SC location may be related to exercise-induced skeletal muscle angiogenesis and to shifts in the MyHC isoform composition and myofiber type phenotype that we have previously observed following exercise tasks.[131,132,164] An increase in SCs colocalizing around type IIb and IIx myofibers may also be indicative of myofiber hypertrophy and related to increased voluntary tongue strength following 2-weeks of tongue exercise.[212-214]

At the 8-week time point myogenin protein expression was upregulated in the SG and HG relative to the no exercise group, suggesting that SC differentiation persisted throughout the entire 8-week exercise period. This finding may reflect the type of tongue exercise implemented. Because we used a progressive resistance exercise approach to

strengthen the tongue, exercise-induced damage and injury to the SG and HG myofibers likely occurred as tongue forces were increased over the 8 weeks of exercise, manifesting as increased SC regenerative capacity.[120,121,170,171] Further, protein expression of MyoD, myogenin, and p16^{INK4a} in the SG and HG muscles was predictive of maximal voluntary tongue forces obtained at all 3 time points following tongue exercise, suggesting that the activation, proliferation, self-renewal, and differentiation of SCs in the SG and HG are related to gains in tongue strength.

Regenerative medicine strategies to combat age-related deficits in muscle strength and function have taken many forms in the limb musculature. They include endogenous delivery of stem cells, implantation of bioscaffolds, pharmacologic treatment, and exercise.[90,96,205-208] Although exercise has been suggested as a potential intervention to improve muscle strength, performance, and function in elderly individuals, few studies to date have examined the effects of exercise on SC regenerative capacity in the acute and chronic phase. Progressive resistance weight training, or mechanical loading, and high intensity endurance exercises, such as cycling and running (voluntary wheel running or treadmill exercise), have been shown to stimulate SC regenerative processes in the limb musculature through the activation of the SC pool in both humans and mice. [120,121,131,132,164,167,170,171] Similar to the results of our study, these investigators also reported increased SC number and content, upregulation of SC myogenic transcription factors, improved muscle function, and gains in muscle strength following exercise.[120,121,131,132,164,167,170-172] In the present study, we examined the expression of Pax7, MyoD, and myogenin in both an acute (baseline and 2-weeks) and chronic (8-weeks) phase following exercise. Similar to the limb, we

observed upregulation of myogenic transcription factors in the acute phase following an exercise stimulus. Specifically, we observed increased myogenin protein expression at the baseline time point, and increased SC content and upregulated protein expression of myogenin following 2-weeks of exercise. At the 8-week time point (chronic phase), myogenin protein expression was upregulated in the absence of increased Pax7 or MyoD expression, which is a slight deviation from what has been reported in the limb muscles following exercise. [120,121,131,132,164,167,170,171] The results from our study suggest that tongue exercise has the capacity to stimulate and activate the SC pool of head and neck muscles. The upregulation of SC myogenic transcription factors following tongue exercise, may be a mechanism related to observed improvements following rehabilitation using this strategy in elderly people with dysphagia.

4.3.3 Factors Contributing to Differences in Satellite Regenerative Capacity in Tongue

The differences we observed in SC regenerative capacity in the tongue with age and exercise may be related to the specific function of the GG, SG, HG, and IT muscles. The exercise intensity required to elicit a regenerative response in SCs may also vary by tongue muscle. The muscles of the tongue have important roles in swallowing, speech and respiratory tasks. For swallowing actions, the GG, SG, HG, and IT are all active in bolus formation, transport, and propulsion. [20-22,25-28] The extrinsic and intrinsic muscles of the tongue act in concert to perform highly coordinated and precise movements of the tongue for the production of speech.[215-217] The tongue musculature is also active during respiration, regulating upper airway resistance.[22,218-224] Further, data support that the hypoglossal motor nucleus is somatotopically organized, which may

account for the precision and coordination of activated tongue muscles during these critical functions.[225] The GG muscle has been identified as the primary workhorse in swallowing, speech, and respiratory actions. During the swallow the GG is the major force generator.[20] The GG is persistently active and plays a significant role during speech tasks.[215-217] For respiratory actions, the GG is crucial to the dilation and/or narrowing of the pharynx during breathing, and is involved in opening the oropharynx and reducing resistance to breathing.[226-228] Due to the high demand placed on the GG muscle and its cross-system role, the GG may be more susceptible to age-related changes in physiology, structure, and SC regenerative capacity than the SG, HG, and IT muscles.

Our results suggest that tongue exercise had differential effects on SC regenerative capacity in the extrinsic and intrinsic tongue muscles. Most surprising was the diminished SC regenerative response of the GG compared to that of the SG and HG muscles. Following a progressive resistance exercise task that required protrusion of the tongue and activation of the GG muscle, we hypothesized the SC response would be most upregulated in the GG at the 2-week and 8-week time points. Because of the constant activation required of the GG for swallowing, speech, and respiratory actions, a more intense exercise stimulus may be required to activate the SC pool in the GG compared with the other tongue muscles. In an uninjured state, SCs remain in a state of quiescence, expressing the Pax7 protein. Upon activation by skeletal muscle injury or trauma, SCs then proliferate, and either self-renew or enter a terminal state of differentiation, and fuse to the myofiber for repair or remodeling. Three cellular responses have been postulated to occur in skeletal muscles in response to exercise:

(1) low-level sublethal damage, (2) necrosis, (3) hyperplasia and hypertrophy.[229] Sublethal damage to the myofiber following eccentric muscle contractions, characterized by disruption and damage to the myofibrillar structure and membrane, may not be sufficient in facilitating a regenerative response from SCs.[230] This minimal damage to the myofiber may instead be repaired by local immunogenic factors, rather than SCs.[231] With more severe muscle injury following intense or unaccustomed forms of exercise, focal nercrosis of the myofiber results in a rapid immune and regenerative response where SCs become activated and enter the differentiation cascade to repair the damaged skeletal muscle tissue.[232,233] Exercise that induces skeletal muscle hyperplasia, an increase in the number of myonuclei, or hypertrophy, an increase in myofiber size, is also proposed to stimulate SC activation and subsequent regeneration.[171,234-236] The upregulation of myogenic transcription factors following our progressive resistance exercise program suggests that this form of exercise is sufficient in sustaining a more severe skeletal muscle injury and in provoking a SC regenerative response in the SG and HG muscles, due to its intensity and perhaps unfamiliarity. During the protrusive exercise task, the SG and HG muscles likely played a crucial role in posturing the tongue and propelling the water bolus. Specifically, the SG and HG muscles were activated and contributed to the retrusion, depression, and elevation of the tongue during each exercise session.[237] The lack of a pronounced SC regenerative response in the GG and IT muscles, may indicate that the tongue exercise treatment used in this study results in minimal damage to the myofibers.

4.4 Differences in Satellite Regenerative Capacity in Tongue and Limb Muscles

The results of our study also suggest that the regenerative function of SCs in the tongue and limb are distinct and muscle specific. The protein and gene expression profiles of the SC myogenic transcription factors were differentially expressed between the tongue and limb muscles in the no exercise group. Age-related differences in the gene expression profiles of the SC myogenic transcription factors existed between tongue and limb muscle. Specifically, age-related alterations in Pax7, MyoD, and myogenin gene expression, and in Pax7 protein expression were observed in the EDL muscle. The only change that occurred with aging in the tongue muscles was an age-related decrease in Pax7 gene and protein expression in the GG. Interestingly, the SG, HG, and IT muscles were spared from an age-associated functional decline in the SC pool that is consistently observed within aging limb muscles. However, the most striking difference between the muscles of the tongue (GG, SG, HG, IT) and the limb (EDL) was the elevated relative protein expression of Pax7, MyoD, and myogenin in the tongue muscles compared to limb in the no exercise group. For example at the 2-week time point in the no exercise group, in comparison to the EDL, Pax7 protein expression in the GG was increased 19-fold, MyoD protein expression in the SG and HG was increased 3-fold, and myogenin protein expression in the GG was increased 4-fold. The protein expression profiles of the SC myogenic transcription factors between the IT and EDL were most similar. Our data demonstrate unique profiles of myogenic SC markers in the GG, SG, HG, and IT compared to the EDL. The differences we observed in Pax7, MyoD, and myogenin exrpression may be related to the diverse functional requirements and embryological origins of the tongue and limb muscles.

Because the muscles of the tongue are critical for swallowing, speech, and respiratory actions, the functional and regenerative demand on these muscles may be higher than in the limb muscles, which are primarily active for locomotion, balance, and postural tasks. The increased activity of the tongue muscles for these critical life functions throughout a given day may contribute to an elevated regenerative turnover of tongue muscle SCs in comparison to limb SCs. We observed over the 8-week study period, upregulated expression of myogenic SC transcription factors in the GG, SG, HG, and IT compared to the EDL. The elevated protein expression of Pax7, MyoD, and myogenin in the tongue muscles may be indicative of a state of chronic SC activation, similar to the increased state of SC activation observed in extraocular and pharyngeal muscles.[87,238] Alterations in SC regenerative capacity have also been observed in the trunk, diaphragm, intrinsic muscles of the larynx (thyroarytenoid, posterior cricoarytenoid, lateral cricoarytenoid), pharynx, and masseter in comparison to the muscles of the limb (EDL, tibialis anterior, soleus, gastrocnemius, quadriceps, plantaris, biceps, deltoid).[75,83-87] Further, it has been suggested that differences in SC regenerative capacity among muscles are also related to differences in their embryological development. Limb skeletal muscles originate developmentally from the dermomyotome of the paraxial mesoderm, or the somatic mesoderm, while the tongue muscles arise from both the cranial mesoderm (supplied by all 4 pharyngeal arches) and the somatic mesoderm.[126,239-241] The regulatory factors involved in limb and tongue myogenesis also differ drastically.[241] Differences in function, embryologic origin, and myogenic regulatory factors may contribute to the diverse SC regenerative profiles we observed in the GG, SG, HG, IT, and EDL muscles

4.5 Limitations

There are some limitations to consider when interpreting the findings of this study. First, there were no functional swallowing measures. Because we did not include videofluoroscopic swallow studies in this study, we cannot attribute changes in the SC regenerative capacity of the lingual muscles to improvements in the functional swallow or in swallowing biomechanics. Second, the SG and HG were combined for gene and protein expression experiments and SC isolation experiments because they are functionally similar, in that they are both muscles of tongue retrusion. As a result, we were not able to determine the SC regenerative capacity for each individual muscle. Third, immunohistochemical assays of the HG and IT could not be performed due to the complex myofiber orientation of the muscles, and the inability to obtain true myofiber cross sections. Fourth, because the muscles of the IT were pooled for all experimental assays, the SC regenerative potential of the superior and inferior longitudinals, transverse, and verticalis muscles could not be individually determined. Fifth, mechanical and enzymatic digestion of muscle tissue to isolate SCs was used instead of magnetic activated cell sorting (MACS) or fluorescent activated cell sorting (FACS). Because the isolated SC pool was not enriched with MACS or FACS, a low percentage of non-muscle stem cells may have been present in our isolated single cell suspensions. Sixth, due to the low number of SCs isolated from the individual tongue muscles, we were unable to perform western blot or RT-qPCR analyses along side immunocytochemical analyses. Seventh, because Pax7 expression was chosen as a primary outcome variable for immune-histochemical and cytochemical analyses, we were not able to determine the percentage of SCs expressing other myogenic

transcription factors, such as MyoD or myogenin, that also contribute to the regenerative properties of skeletal muscle. Eighth, there is a possibility that we may have missed the initial period of SC activation that occurs following exercise or at later chronic stages of regeneration. Ninth, the exercise regimen chosen may not have been effective in eliciting a strong or sustained SC response in all muscles of the tongue. It would have been advantageous to employ either a cryoinjury or cardiotoxin-induced skeletal muscle injury to study age-related alterations in SC regenerative capacity. Tenth, because of the length of time and the number of rats required to complete the study, animal batch differences may have occurred. Although rats were genetically identical, and rats in each time by age by treatment condition were represented in each experimental cohort, there is a possibility that there were slight differences in animal care or other factors throughout the length of the study.

4.6 Future Studies

The characterization of the regenerative capacity of lingual muscle SCs and isolated tongue muscle SCs with age and following a clinically relevant exercise treatment is a necessary first step to determine the therapeutic potential of SCs as a future treatment for degenerative muscle diseases that affect swallowing function. This study will serve as the foundation of my future work in developing cell- and tissue-based regenerative therapies for the prevention and treatment of swallowing disorders and for other translation studies in humans. Better understanding of the underlying extrinsic (systemic and local) and intrinsic (within the SC) mechanisms of age-related muscular

degeneration and exercise-induced muscular regeneration will guide future clinical studies and treatment choices.

4.7 Summary of Findings

4.7.1 Tongue Strength

1. Age-related impairments in tongue strength as indicated by the MVTF were not observed.
2. MVTF was slightly more variable with increasing age.
3. MVTF increased in young adult and old groups following 2-weeks and 8-weeks of tongue exercise.

4.7.2 Satellite Cell Regenerative Capacity

1. The GG was most susceptible to mechanisms of age-related SC regenerative decline.
    a. Pax7 gene and protein expression were reduced with age in the GG.
    b. $p16^{INK4a}$ gene expression was upregulated in the GG. SCs isolated from the GG also expressed increased levels of the $p16^{INK4a}$ protein.
2. The SC regenerative capacity of the GG, SG, and HG muscles was enhanced following tongue exercise
    a. Alterations in all SC myogenic transcription factors were observed throughout the 8-week exercise treatment.
        i. Myogenin protein expression was upregulated at baseline, 2-weeks, and 8-weeks in the exercise group.

ii. Pax7 SC content increased in both the GG and SG following 2-weeks of exercise.

b. The location of Pax7+ SCs near type IIb, IIx, and IIa myofibers and near vessels was altered following 2-weeks of exercise in the GG and SG muscles.

i. In the GG, the percentage of Pax7+ SCs near type IIa myofibers decreased.

ii. In the SG, the percentage of Pax7+ SCs near type IIB and IIx myofibers increased, and the percentage of Pax7+ SCs near type IIb, IIx, and IIa myofibers decreased.

iii. In both the GG and SG, the percentage of Pax7+ SCs near non-myofibers (vessels and nerves) increased.

3. The SC regenerative capacity of tongue and limb muscles were differentially affected.

a. In the no exercise group, the GG, SG, HG, and IT muscles had elevated expression of SC myogenic transcription factors in comparison to EDL. This may indicate a state of constant SC activation, increased SC turnover, and continual repair and remodeling of myofibers in muscles of the tongue.

4.7.3 Relationship Between SC Regenerative Capacity and Tongue Strength

1. The protein expression MyoD, myogenin, and $p16^{INK4a}$ in the combined SG and HG muscles were predictive of MVTF at baseline, 2-week and 8-week.

4.8 Conclusion

A clear understanding of underlying skeletal muscle biology is necessary for the development of novel therapeutics for the treatment of age-related musculoskeletal disorders that affect critical life functions, such as swallowing. Skeletal muscle has the unique ability to regenerate post-mitotically through the activation, proliferation, self-renewal, and differentiation of SCs (satellite cells; adult muscle stem cells). This research is significant because: 1) SC regenerative capacity of aging tongue muscles was characterized for the first time; 2) the role of extrinsic environmental and SC-intrinsic factors affecting the regenerative potential of SCs were identified; 3) a novel modality to study cellular mechanisms underlying the effects of a clinically relevant exercise model on aging lingual muscles was used; and 4) the results of this study may lead to the development of treatments targeting underlying cellular mechanisms of tongue muscle degeneration and may guide treatment parameters in future clinical studies for the prevention of age-related dysphagia.

4.9 Clinical Implications

Tongue strengthening therapies in current clinical use for the treatment of swallowing disorders in the elderly may improve the regenerative potential of tongue SCs. The activation, proliferation, self-renewal, and differentiation of lingual SCs may contribute to gains in tongue muscle strength following tongue exercise, and may lead to improved swallow function. Because the SC regenerative capacity among the tongue muscles differed following exercise, the results of this study suggest that the type of exercise and exercise intensity should be key considerations in the development and

implementation of exercise-based oropharyngeal therapies. The development of rehabilitation strategies that target SC regenerative process of the tongue musculature may improve tongue and swallowing function with age and disease. However, the most effective treatment strategy may invoke a multidisciplinary approach that includes exercise-based treatments provided in conjunction with of cell- and tissue-based therapies, and pharmacologic manipulation.

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