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UNIVERSITY OF CALIFORNIA,
IRVINE

Interrogating Stem Cell Niche Interactions in Transplanted Skeletal Muscle Stem Cells

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Benjamin Boyd Clock

Dissertation Committee:
Assistant Professor Michael Hicks, PhD, Chair
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2026

DEDICATION

To

my friends and family

for unconsciously and undoubtedly supporting my endeavors, I extend my love and gratitude.

of virtue

“Ignorance is the root of many ills. Knowledge must be the fundamental ally of nations that aspire, desperate all their tragedies and problems, to become truly emancipated.”

- *Fidel Castro*

of equity

“A man who stands for nothing will fall for anything.”

- *Malcolm X*

and for a better tomorrow

“The philosophers have only *interpreted* the world, the point, however, is to *change* it.”

- *Karl Marx*

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It is a true pleasure to get to write both my PhD dissertation and this section of it specifically. I joined UCI as a wide-eyed, curious, passionate, and oftentimes naïve young scientist at twenty-three years old. I had no idea what I wanted to do, but I knew that my academic growth was critical for my career trajectory, so I walked the conservative path and furthered my education (you can never go wrong with that... right?). In hindsight, I am bamboozled how I got into this program – I had no papers, I am a subpar writer, and I cried in one of my interviews. I have so much gratitude to the UCI cell and molecular biosciences gateway program and to Peter Donovan specifically for taking a chance and gambling on me. Against all odds, here I am – the guy who can't answer a single trivia question, has made every mistake possible in the laboratory setting, went 1 for 20 on grants and fellowships in graduate school – writing up my PhD dissertation. This part is sentimental – they say it takes a village to progress and to succeed, but I never realized how good it feels to be a cohesive part of said village. And when you take a step back outside the village, the beauty of seeing all the parts in motion can bring you to tears – the smoke drifting lazily out of the chimney, the birds chirping through the lush greenery, the smell of baked goods wafting through the early morning air, and children giggling and prancing through the streets. It can be easy to lose yourself in the madness of the mundane, and even easier to forget to admire the simplistic features of your own village, only realizing how much you miss those small details once you've left. I wonder how corny people may think my writing style is, and I wonder how myself in the future will perceive re-reading my dissertation.

I feel incredibly proud of the development I've made throughout my life. I've matured from a naive kid to a capable, intelligent, and industrious scientist eager to contribute to a progressive and translational research field that advances contributions to human health. I have so

much love in my life that I proudly attribute the successes of this degree to. My high school friends, Neil, Terry, Vick, and Griffin – the oldest friends I have, the most reliable. I cannot emphasize how much you all mean to me and how deeply important you are to me and my stability. While distance has kept us temporarily separated for the past ten years since we left to different colleges, we've always made time for each other. Our international Boys Trips have been the highlights of my years, as we've figured out how to maximize our paid time off! We've been to Puerto Rico, Hawaii, Vancouver, the Netherlands, and Japan – who knows what's next. Griffin – you're a wild child in the best way possible. You break the mold set forth by societal norms and those around us and set your own stride so casually it makes me envious at times. I love that you have broken free from the shackles of the systemic jail that imprisons the rest of the proletariat. If more folks were as conscious as you, and as eager and willing to question everything and make their own decisions, the world would surely be a better place. I sincerely hope that you figure out a way to influence others and open their minds to spread this consciousness, and to help those who cannot break free even if they wanted to.

To my collegiate and UCI crew, the rag-tag team of questionable persons with even more questionable motives – David, Hannah, Cody, Matthew, Sarah, Temoc, Angel, Whitney, Sarah, Zach, Alyssa, Jacob, Sean, Mara, Yas, Grace, Abbey – where would I be without you all. We joined in the middle of COVID, and it became clear that there were some cool kids who wanted to hang out (safely) and were keen on building relationships with the emerging cohort. It took me a while to feel comfortable around a new group of people, and you did a fabulous job at fostering such a chilled, supportive, inclusive environment. I think one of the highlights of my time here at UCI has been ditching soccer and jumping into a whole new sport, volleyball. We (and when I say we I think I mean I) have become obsessed with the sport since beginning our PhD, playing beach

and indoor. We fought hard to win a trophy in intramurals against those darn undergrad kids playing in their wife-beaters and pajamas and crocs. We came ever-so-close, making it into the final match twice and winning two second-place trophies. The physical activity combined with social hangouts have been a very healthy element of my graduate school life and have kept me sane. I love you all very much, I know I don't say it enough – I am dreading moving to a new place and having to start anew, building a cohesive community from scratch. It is so sad to think that to community we've built together may never come together permanently like this again, and while I am so excited to see everybody move onto the next steps of their careers, I will always cherish the memories we've created together. Sometimes I like to daydream about living on a commune with all of you, where we each have our own house and we share some common spaces. Maybe we will cook shared dinners, sing karaoke, build a sand volleyball court – it's entirely up to us! But in these daydreams, our village looks surreal, and it feels just how life is supposed to be lived...

To my family – I want to share this doctoral degree with you. You have shaped me into who I am today. Mom, Dad, Nate, Peter, and my wonderful nieces Iris and Emilia – I could not have done this without your everlasting support. To my beautiful girlfriend, Ria – you have been my support network for the past few years. I cannot wait to move to a cool new place with you that meets all our requirements, and to move in with you, to start a life with you, founded by the pillars of our love. I love daydreaming about a future with you, and I eagerly anticipate what stories are just around the corner for us. In my village, we have a house. It's not too big, and not too small, its cozy and warm and the natural light illuminates every piece of art perfectly. There's a cat cuddled up on the windowsill, lazily watching the squirrels chatter about in the trees outside. Christmas time is beautiful and sentimental, with decorative festivities dazzling the living spaces. On Thanksgiving, lucrative and vivacious aromas flutter through the air as we host a gaggle of

friends and family. On Halloween, a whimsical spooky vibe is curated from our space as the neighborhood kids flock to collect a haul of candy we generously dole out. I am always eager and comfortable to come home, to you, and to us, and what we've built. And yet one day, everything will change – because there will be somebody new. Somebody who brings a thrilling new light and dynamic to our space, and somebody who expands our happy family, making our cozy home just a bit cozier. I cannot wait for that day to come, and I cannot wait to embark on this journey through life with you.

Thank you specifically to the co-authors of my first-author publication for their tremendous efforts in supporting this work. I will highlight the work conducted by my three undergraduate mentees, Sebastien Colobong, Nikitha Pavar, and Skyler Lu. They have made impressive contributions that have pushed this story forward into uncharted territories that have allowed for the success we are seeing today. I came into graduate school with extensive mentorship from fabulous scientists, and I was excited to pay it forward and impact the next generation of young scientists. I scouted out these students myself, parsing through several hundred undergraduate students to find the ones that ask ample questions because they are genuinely intrigued by science, and those whose passion for foundational biology is vividly detectible. It is their inquisitive nature that makes me confident that these students will continue to succeed in their lives – and while I hope they all pursue graduate school, they should know that I hope for this because their scientific potential is limitless, and any true humanitarian would be aching to see their robust accomplishments and industrious tenacity applied in a graduate school setting. I am proud of the researchers that you have become, and I am proud of the role I have played in supporting and cultivating your growth and maturity as an inquisitive scientist. I hope one day we can all be reunited in the same lab, the dream team reconnected and reborn! Additional thanks are due to

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Dr. Michael Hicks thoroughly deserves his own paragraph, as he has been a wonderfully supportive mentor through my time in his lab at UCI. Mike took a chance on me, and I am pleased to say it paid off quite well. He helped cultivate my tenacity and scientific rigor and curiosity into productivity and the ability to think critically to design experiments and address complex scientific questions. We have an exciting paper in revision, tons of posters presented, a big T32 fellowship obtained, and several national conferences attended. Mike has always been the driver behind my scientific ambition, as every experimental adventure we came up with has been such a fun and wild ride. I would not have been able to reach the pinnacle of my successes without the support and guidance of Mike, and I am excited to see the directions that his lab proceeds in in my absence. And so, I thank you Mike! And I wish nothing but the best to you and the next wave of graduate students... I have high expectations of y'all!

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Well, whoever you are – enjoy my dissertation! You may always reach out to me directly if you have questions about anything: the story discussed here, my trajectory, or just to connect and gather advice or whatnot. Part of being a communist is having a deep sense of humanitarianism and empathy, hence why I keep yapping on about this village I dream about where everything is perfection and harmony. I am always here as a resource for those in need. Power to the people, and may you always take the time to help those in need.

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FIELD OF STUDY

Skeletal Muscle Stem Cell Biology

PUBLICATIONS

- 1) Grace Bower, Ethan Hollingsworth, Sandra Jacinto, Joshua Alcantara, **Benjamin Clock**, Kaitlyn Cao, Mandy Liu, Adam Dziulko, Ana Alcaina-Caro, Qianlan Xu, Dorota Skowronska-Krawczyk, Javier Lopez-Rios, Diane Dickel, Anaïs Bardet, Lena Pennacchio, Axel Visel, Evgeny Kvon. Range Extender Mediates Long Distance Enhancer Activity. *Nature*, 643, 830–838 (2025). doi: 10.1038/s41586-025-09221-6. PMID: 40604280.
- 2) **Benjamin Clock**, Michael Hicks. Human Skeletal Muscle Niche Formation and Analysis with Spatial RNA Sequencing. *Methods in Molecular Biology*, 2939, 1-22 (2025). doi: 10.1007/7651_2025_619. PMID: 40183842.
- 3) Zhuoxin Chen, Valentina Snetkova, Grace Bower, Sandra Jacinto, **Benjamin Clock**, Atrin Dizehchi, Iros Barozzi, Brandon J. Mannion, Ana Alcaina-Caro, Javier Lopez-Rios, Diane E. Dickel, Axel Visel, Len A. Pennacchio, Evgeny Z. Kvon. Increased enhancer–promoter interactions during developmental enhancer activation in mammals. *Nature Genetics* 56, 675–685 (2024). <https://doi.org/10.1038/s41588-024-01681-2>. PMID: 38509385.
- 4) Michael R. Hicks, Kholoud K. Saleh, **Benjamin Clock**, Devin E. Gibbs, Mandee Yang, Shahab Younesi, Lily Gane, Victor Gutierrez-Garcia, Haibin Xi, April D. Pyle. Regenerating human skeletal muscle forms an emerging niche in vivo to support PAX7 cells. *Nature Cell Biology* 25, 1758–1773 (2023). <https://doi.org/10.1038/s41556-023-01271-0>. PMID: 37919520.
- 5) Raven Shah, Christian M. Gallardo, Yoonhee H. Jung, **Ben Clock**, Jesse R. Dixon, William M. McFadden, Kinjal Majumder, David J. Pintel, Victor G. Corces, Bruce E. Torbett, Philip R. Tedbury, Stefan G. Sarafianos. Activation of HIV-1 proviruses increases downstream chromatin accessibility. *iScience* 25(12), 105490 (2022). <https://doi.org/10.1016/j.isci.2022.105490>. PMID: 36505924.
- 6) Zhichao Xu, Dong-Sung Lee, Sahaana Chandran, Victoria T. Le, Rosalind Bump, Jean Yasis, Sofia Dallarda, Samantha Marcotte, **Benjamin Clock**, Nicholas Haghani, Chae Yun Cho, Kadir C. Akdemir, Selene Tyndale, P. Andrew Futreal, Graham McVicker, Geoffrey M. Wahl, Jesse R. Dixon. *Structural Variants Drive Context Dependent Oncogene Activation in Cancer*.

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- 7) Hanqing Liu, Jingtian Zhou, Wei Tian, Chongyuan Luo, Anna Bartlett, Andrew Aldridge, Jacinta Lucero, Julia Osteen, Joseph Nery, Huaming Chen, Angeline Rivkin, Rosa Castanon, **Benjamin Clock**, Yang Eric Li, Xiaomeng Hou, Olivier Poirion, Sebastien Priessl, Antonio Pinto-Duarte, Carolyn O'Connor, Lara Boggeman, Conor Fitzpatrick, Michael Nunn, Eran Mukamel, Zhuzhu Zhang, Edward Callaway, Bing Ren, Jesse Dixon, Margarita Behrens, Joseph Ecker. DNA Methylation Atlas of the Mouse Brain at Single-Cell Resolution. *Nature*, 598, 120-128 (2021). doi: 10.1038/s41586-020-03182-8. PMID: 34616061.
- 8) Jingtian Zhou, Yue Wu, Hanqing Liu, Wei Tian, Rosa G. Castanon, Anna Bartlett, Zuolong Zhang, Guocong Yao, Dengxiaoyu Shi, **Benjamin Clock**, Samantha Marcotte, Joseph R. Nery, Michelle Liem, Naomi Claffey, Lara Boggeman, Cesar Barragan, Rafael Arrojo E. Drigo, Annika K. Weimer, Minyi Shi, Johnathan Cooper-Knock, Sai Zhang, Michael P. Snyder, Sebastian Preissl, Bing Ren, Carolyn O'Connor, Shengbo Chen, Chongyuan Luo, Jesse R. Dixon, Joseph R. Ecker. *Human Body Single-Cell Atlas of 3D Genome Organization and DNA Methylation*. In revision at *Science*, March 2026. bioRxiv; <https://doi.org/10.1101/2025.03.23.644697>
- 9) Jeessoo Chae, Jin Seok Lee, Jongkeun Park, Dong-Sung Lee, Weon Seo Park, **Benjamin Clock**, Jesse R. Dixon, Yuh-Seog Jung, Dongwan Hong. *Deciphering the Evolutionary History of Complex Rearrangements in Head and Neck Cancer Patients Using Multi-Omic Approach*. In revision at *Cancer*. bioRxiv; <https://doi.org/10.1101/2022.08.19.504509>.
- 10) **Benjamin Clock**, Sebastien Colobong, Nikitha Pavar, Xinyu Lu, Nate Addonizio, Manuel Michel, Anita Lakatos, Joanne Kim, Michael Hicks. The developing niche regulates engrafted muscle stem cell retention in dystrophic and regenerating skeletal muscle. In revision at *Nature Communications*, April 2026. Link to preprint: <https://www.researchsquare.com/article/rs-9408825/v1>
- 11) **Benjamin Clock**, Andrea Watson, Cord Brundage. Laser photomodulation decreases the likelihood of wound dehiscence in laboratory mice. March 2018. *California State Polytechnic University, Pomona Scholarly Journal*.

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Awards

2024	School of Medicine Outstanding Student
2020	Awards for the Promotion of Diversity and Student Inclusion

Funding

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------	--

SELECTED PRESENTATIONS

Oral Presentations

- 1) Benjamin Clock, Sebastien Colobong, Nikitha Pavar, Xinyu Lu. The Developing Niche Regulates Engrafted Muscle Stem Cell Retention in Dystrophic and Regenerating Skeletal Muscle. *UCI Muscle Center for Research and Disease Maximus T32 Retreat*. Irvine, April 2026.
- 2) **B Clock**, S Colobong, N Pavar, S Pavani, J Kim, M Hicks. MEGF10 Supports Muscle Stem Cell Retention and Repopulation. *UCI Muscle Center for Research and Disease Maximus T32 Retreat*. Irvine, April 2025. Won best overall presentation.

Poster Presentations

- 3) **Benjamin Clock**, Sebastien Colobong, Nikitha Pavar, Shreya Pavani, Joanne Kim, Michael Hicks. MEGF10 Supports Muscle Stem Cell Retention and Repopulation. *International Society for Stem Cell Research*. Hong Kong, June 2025.
- 4) **Benjamin Clock**, Sebastien Colobong, Nikitha Pavar, Shreya Pavani, Michael Hicks. MEGF10 mediates skeletal muscle stem cell support and muscle regeneration. Data blitz and poster presentation at *New Directions in Skeletal Muscle Biology*. Fort Lauderdale, Florida, 2024.
- 5) **Benjamin Clock**, Sebastien Colobong, Shreya Pavani, M Hicks. Improving Skeletal Muscle Stem Cell Niche Colonization Through MEGF10 Overexpression. *UC Irvine Stem Cell Science Symposium*. Irvine, January 2024. Won best overall poster.
- 6) **Benjamin Clock**, Michael Hicks. Building Skeletal Muscle Niches Through Ligand-Receptor Interactions from Human Pluripotent Cells. *The Skeletal Muscle Stem Cells and Regeneration Conference*. New Orleans, Louisiana, July 2022.
- 7) **Benjamin Clock**, Andrea Watson, Cord Brundage. Laser Photomodulation Decreases the Likelihood of Wound Dehiscence in Laboratory Mice. *Research, Scholarship, and Creative Activities*. Cal Poly Pomona, 2018.
- 8) **Benjamin Clock**, Andrea Watson, Cord Brundage. Assessing the Efficacy of Laser Photomodulation on Laboratory Mice Wound Healing. *Southern California Conference for Undergraduate Research*. Cal Poly Pomona, 2017.

SERVICE AND OUTREACH

- | | |
|-----------|--|
| 2022-2026 | Undergraduate Bio199 Student Mentor |
| 2023-2026 | President and Founder of the School of Medicine Graduate Sports Club |
| 2025 | Volunteer, Muscular Dystrophy Association Summer Camp, Apple Valley |
| 2021-2023 | ReachOut TeachOut Student Mentor and Science Fair Committee Member |

ABSTRACT OF THE DISSERTATION

Interrogating Stem Cell Niche Interactions in Transplanted Skeletal Muscle Stem Cells

by

Benjamin Boyd Clock

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2026

Assistant Professor Michael Hicks, PhD, Chair

Skeletal muscle is a highly regenerative tissue that is responsible for voluntary, contractile movement of the body. Endogenous muscle stem cells are responsible for regenerating skeletal muscle upon injury, although in diseased contexts this regeneration becomes impaired to the deficit of the patient. There remains a pressing need for the discovery of viable, long-lasting therapeutic treatments for patients with genetic congenital neuromuscular diseases as current standard of care fails to improve patient quality of life. Thus, the introduction of induced pluripotent stem cell (iPSC) derived muscle stem cell therapies offers an exciting avenue to introducing an autologous healthy donor stem cell into a diseased patient. While this platform is promising, current research has found that iPSC derivations generate immature skeletal muscle stem cells, transcriptomically on par with embryonic human muscle stem cells. One major limitation of these stem cells is the ineffective retention of the stem cell population upon engraftment, an objective highlighted in this dissertation.

We describe the heterogenous myogenic populations generated upon engraftment *in vivo*, importantly finding distinctly different myofibers that resemble either an immature, regenerative state (marked by fetal cardiac actin, ACTC1) or a mature, developed state that arises through fusion with host mouse myofibers. We found that human PAX7+ muscle stem cells are better retained in

regions of regenerating ACTC1+ myofibers rather than the larger, chimeric myofibers. We then harnessed spatial transcriptomic approaches to study the early stages of human myogenic regeneration upon engraftment, discovering niche factors expressed by regenerating myofibers that likely support stem cell retention and self-renewal. In line with these findings, after rapid cell loss upon transplantation, MYOG+ and PAX7+ myogenic populations stabilized upon the formation of myofibers. With an understanding of the importance of regenerative myofiber population in the retention of muscle stem cells upon engraftment, we then applied this workflow to a dystrophic microenvironment. We identified that the dystrophic microenvironment induces a massive change in the interactome of myogenic populations, and these changes are detrimental towards the regenerative program after subsequent injuries.

Multiple epidermal growth factor like domains 10 (MEGF10) is a poorly understood stem cell niche receptor that appeared as both a candidate niche factor on regenerative myofibers and downregulated in dystrophic microenvironments. Therefore, we postulated that overexpression of MEGF10 may be sufficient to improve stem cell retention in transplantation models. We leveraged CRISPR genomic engineering to generate a TET-On inducible MEGF10 expression iPSC line which can robustly be differentiated to skeletal muscle and engrafted *in vivo*. We found that overexpression of MEGF10 during the first ten days following engraftments significantly supported more PAX7+ muscle stem cells and myofiber regeneration. Further, spatial transcriptomic analysis reveals that MEGF10 accelerates myofiber regeneration but stalls further maturation, arresting myofibers in a regenerative ACTC1+ state, marked by potential downstream MEGF10 signaling candidates LRRN1, CDH15, DLK1, and JAM2. Our findings are the first that describe advantageous heterogeneity of iPSC muscle stem cells upon engraftment, how they regenerate in real time and in a dystrophic host environment, and the temporal significance of stem

cell niche receptors in their roles of transplanted niche formation. These breakthroughs may shed light on how to effectively generate novel stem cell therapy treatments for patients with neuromuscular disorders.

Chapter 1: Introduction

1.1: Terminology and Abbreviations

PAX7: key stem cell marker (myogenic transcription factor)

PAX3: key developmental stem cell marker (myogenic transcription factor)

MYOD1: myogenic differentiation 1. Myoblast marker important for myogenic commitment and differentiation

MYOG: myogenin. Myocyte marker important for myogenic differentiation and fusion

MYF5: myogenic factor 5. Satellite cell activation marker.

Lamin AC: nuclear membrane structural protein – antibody used is human specific

Spectrin: myofiber membrane structural component – antibody used is human specific

Myofiber: multinucleated skeletal muscle cell, capable of contraction

SC: satellite cell (adult muscle stem cell)

SMPC: Skeletal muscle progenitor cell (immature / developmental muscle stem cell)

ECM: extracellular matrix, also referred to as the basal lamina

MEGF10: Multiple epidermal growth factors (EGF) - like domains 10

iPSC: induced pluripotent stem cells

hPSC: human pluripotent stem cells

DMD: Duchenne's Muscular Dystrophy

BMD: Becker's Muscular Dystrophy

Myoblast: terminally differentiated myogenic stem cell. PAX7- and MYOD1+

VML: Volumetric Muscle Loss

EMARDD: Early-onset Myopathy, Areflexia, Respiratory Distress, and Dysphagia syndrome

1.2: Muscle Function and Contraction

Muscles of the Body

There are several kinds of muscle that play a pivotal role in many of our essential bodily functions. Cardiac, smooth, and skeletal muscles are highly specialized for unique physiological purposes. Cardiac muscle is found in the heart and is involved in providing the force, through rhythmic heartbeats, to move blood from the heart through the entire body, enabling oxygenation of all tissues for cellular respiration [1]. Smooth muscle is found in and around many centralized organs through the body, playing a key role in the movement of material through the body [2]. Smooth muscle wraps around the colon and small intestines, where its contractions aid in digestion. It plays a similar supportive role in esophageal peristalsis of food in deglutition. Skeletal muscle is found throughout the body and its contractions allow for precise, robust physical movements that are critical for daily function, metabolism, and posture [3]. The ends of skeletal muscles merge with tendons to attach to bones, which help to anchor the muscle to allow for contractile forces to result in movement (**Figure 1.1**) [4]. The structure of skeletal muscle includes antagonistic muscle groups, which push bones in opposite directions – for example, the biceps pull the arm towards the body, while the triceps push the arm away from the body. This gives rise to an established, capable body system that can move in complex, dynamic, and explosive actions.

Skeletal Muscle Innervation

Skeletal muscle is intricately connected to the central nervous system through robust innervation which allows for precise controlled motor skills that differentiates it from cardiac and smooth muscle [5]. This innervation consists of somatic pathway motor neurons which are part of the “voluntary” division of the central nervous system, contrary to the autonomic pathway which controls the “involuntary” division [6]. The somatic pathway regulates skeletal muscle through

innervation, which we have precise control over (voluntary), while the autonomic pathway regulates smooth muscle, cardiac muscle, adipose tissue, and hormone-secreting glands through the body, with which we have less precise control (involuntary). The autonomic pathway regulates its tissues through the sympathetic and parasympathetic systemic responses, or the famous “fight or flight” and “feed and breed” pathways respectively [7]. One important distinction between autonomic and somatic pathways is the neurons that connect the central nervous system to their target tissue. In autonomic pathways, two neurons in series give rise to this connection, while in somatic pathways, just one neuron makes this connection. Somatic neurons that innervate skeletal muscle myofibrils connect through extensively branched ends that bind to a motor end plate, forming the neuromuscular junction. The neuromuscular junction serves as the liaison between brain stimulus and muscle contraction, allowing for voluntary controlled muscle activity. The neuromuscular junction operates through a series of chronological events to trigger muscle contraction [8].

- 1) Action potential arrives at the axon terminal, triggering Calcium channels to open.
- 2) Calcium enters the synaptic cleft, inducing synaptic vesicles to fuse with the presynaptic membrane and release Acetylcholine into the synaptic cleft.
- 3) Acetylcholine then binds to the nicotinic cholinergic receptor, opening a cation channel.
- 4) Sodium and Potassium pass freely through the cation channel, which leads to the depolarization of the skeletal muscle myofiber.
- 5) An action potential is initiated due to the sodium influx finally induces contraction of the skeletal muscle myofiber.

Skeletal Muscle Contraction

The individual cells of skeletal muscle are multinucleated myofibers that span the entire length of the muscle (**Figure 1.1**) [9]. These myofibers are bundled together into fascicles, packaged tightly with connective tissue, or fascia. The cell membrane of the myofiber is called the sarcolemma, while the myofiber cytoplasm is known as the sarcoplasm. Importantly, the myofibrils are the intracellular structural components in striated muscles that are responsible for contraction. Myofibrils are comprised of myosin, actin, tropomyosin, troponin, titin, and nebulin which play a role in the development of thick and thin filaments.

Skeletal muscle myofibers contract through a complex and rigorous process mediated by myosin proteins and adenosine triphosphate (ATP – the energy currency of the cellular dynamics) [10]. One myosin molecule consists of two identical protein chains, including a heavy and a light chain. Myosin proteins change through development, with myosin 8 (MYH8 - embryonic myosin) developing to myosin 3 (MYH3 - fetal myosin), and further maturing into myosin 1 (MYH1 - adult myosin), 4 and 7 (MYH4, MYH7 - fast and slow twitch myosins) [11]. These different myosins all play a similar role in contraction, but their functional potential changes dramatically through development and regeneration. The visible striations of skeletal muscle that appear as stacked parallel lines are called sarcomeres, and these are the basis of the structural component allowing for the contractile dynamics of myofibers. To describe the intricate process that produces muscle contraction, we must introduce the different elements of the sarcomere that comprise of the position of the myosin and actin filaments during stages of muscle contraction (**Figure 1.1**) [12, 13].

- 1) Z disks: Protein structures that serve as the attachment site for thin filaments.
- 2) I Bands: Light color band of sarcomere, only occupied by thin filaments.

- 3) A band: Darkest band of the sarcomere, mainly encompasses thick filament.
- 4) H Zone: Central region of the A band – lighter than the outer edges because it's only occupied by thick filaments.
- 5) M line: Proteins structures that serve as the attachment site for thick filaments.

The sliding filament theory was proposed in 1954 and postulated that in contraction, actin slides past myosin to generate muscle tension due to the shortening of the sarcomere and thus the muscle [14]. In this process, the H Zone shrinks, the I band shortens, and the A band stays constant, producing a contracted muscle state. The dynamics of myosin pulling on actin to shorten the sarcomere in the sliding filament theory have been refined since the proposition of this theoretical model. Breakthroughs in muscle research found that the ends of each myosin nearest to actin has multiple hinged segments, which bend and regulate contraction [12, 15]. The bending of the myosin interactive region details the way that myosin moves along actin, as it reaches forward, binds to actin, contracts, releases actin, and then reaches forward again to bind actin in a new cycle. As myosin binds and releases actin, it forms cross bridges, which connect thick myosin filaments to thin actin filaments. The contraction of myosin requires the hydrolysis of ATP, breaking a high-energy phosphate bond to release energy to catalyze the reaction. In addition to ATP, calcium is a crucial element in muscle contraction as it regulates both troponin and tropomyosin, which are important for facilitating the process of contraction [16]. Therefore, muscle contraction is highly energy expensive and hinges on ample ATP and calcium influx generation and usage.

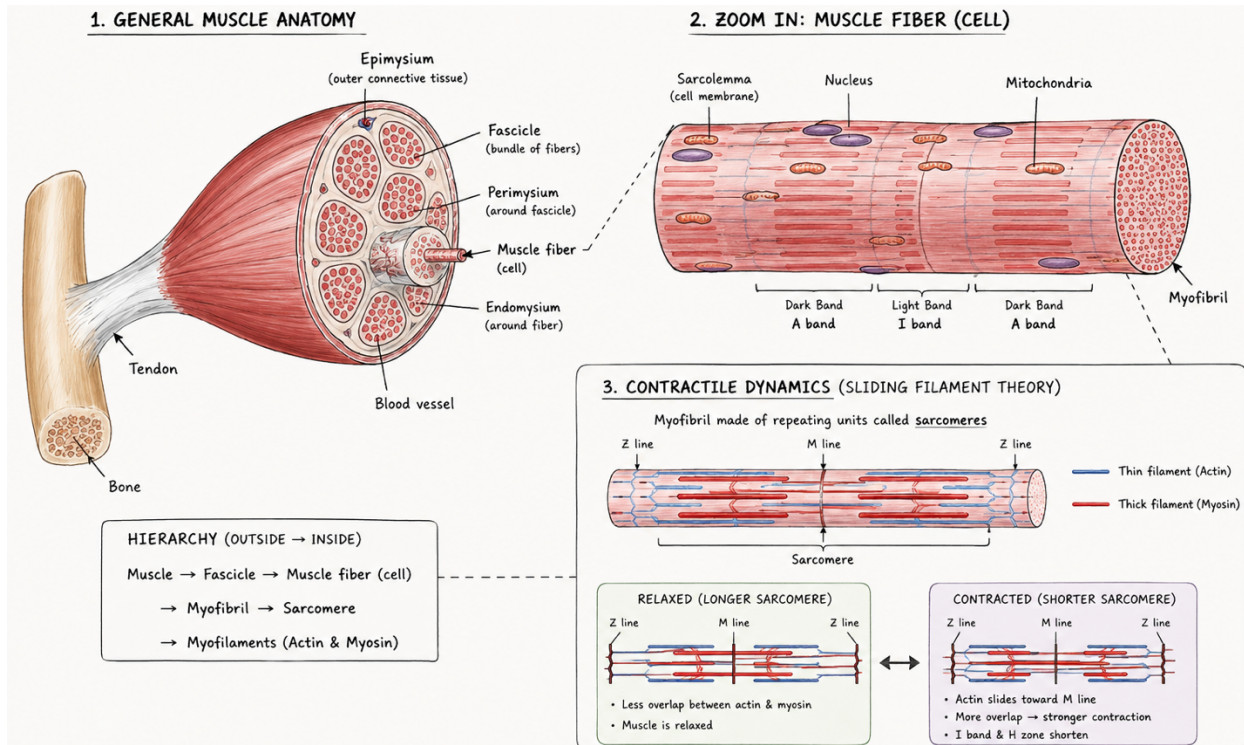


Figure 1.1: Muscle Physiology and Contractile Dynamics

Left (1): General Muscle Anatomy – indicating the structural composition of skeletal muscle, highlighting a general muscle. Skeletal muscle is connected to the bone through tendons, and skeletal muscle itself is comprised of myofiber bundles into fascicles, which are grouped together to form the total structure. Right (2): Myofiber Zoomed In – the myofibers are the individual muscle cells that provide the contractile dynamics of skeletal muscle, enabling voluntary movement. Included are the sarcolemma (myofiber membrane), and multinucleation of the myofiber with myonuclei located at the peripheries to enable space for contractile motor dynamics in the central area, driven by mitochondria and contractile dynamic elements, A and I bands. Right bottom (3): Schematic of the contractile dynamics of myofibers, illustrated by the relaxed and contracted sarcomere and

how the actin/myosin binding and sliding gives rise to myofiber contraction. This depicts the sliding filament theory.

1.3: Skeletal Muscle Stem Cells and Regeneration

Self-Renewal

The dynamic usage of skeletal muscle through action dependent contraction results in a force that allows for precise movements. However, this potential of the tissue is a double-edged sword, as some skeletal muscle myofibers perish in the process of basic exercise [17]. The cellular turnover rate of skeletal muscle tissue is one of the highest in the body, likely due to the explosive potential of its contractile cells. Regeneration, which is defined as the process of replenishing new viable cells following tissue damage, is essential to maintain tissue homeostasis in skeletal muscle [18]. The regenerative process is seen in most bodily tissues, and is regulated by populations of tissue-specific stem cells, with minimal physiological function in resting state – rather, their role in regulating tissue homeostasis is to, upon injury-induced regeneration, undergo mitosis to proliferate and many of their progeny will subsequently differentiate to a specified somatic cell that serve a physiological function (i.e. muscle cells contract; neurons fire; kidney cells uptake molecules) [19-21].

Stem cells are the only cells in the adult body that can undergo mitosis and produce progeny, while somatic cells serve a physiological role until they eventually die, with no ability to divide to produce more of themselves. In regeneration, it is the stem cells that rapidly proliferate and differentiate to produce more somatic cells in tissue repair. The balance of stem cell proliferation and commitment to differentiative programs is pivotal in regenerative dynamics through the body. It is the responsibility of the stem cells to maintain a sufficient level of stem cell pool through regeneration, a phenomenon known as self-renewal [22]. This balance between

differentiation and division, as well as asymmetric versus symmetric stem cell division in the context of regeneration is a widely studied field that researchers continue to uncover new information about.

Stem Cells

The first stem cells that give rise to the tissues in our bodies are totipotent stem cells which arise in the initial cellular divisions after fertilization of oocyte and sperm (**Figure 1.2**) [23]. These stem cells have the potency to give rise not only to any cell type of the body, but also cells outside of the body, like the placenta. As these cells continue to divide and enter the blastocyst stage of development, they enter a bifurcation event, differentiating into either stem cells that give rise solely to the placenta (trophoblast) or pluripotent stem cells (inner cell mass) that can give rise to any cell type of the body. Pluripotent stem cells continue to undergo embryonic and fetal development where they subsequently differentiate to multipotent germ lineage stem cells; those that can give rise to mesoderm (middle layer – muscle, bone, tendon), endoderm (inner layer – pancreas, stomach), and ectoderm (outer layer – skin, hair). Germ line stem cells further commit to bipotent or unipotent stem cells which have the regenerative potential to differentiate to a specific somatic cell type – i.e. skeletal muscle stem cells give rise to skeletal muscle, neuronal stem cells give rise to neurons, skin stem cells give rise to skin, fibroadipogenic progenitor cells give rise to adipose cells or fibroblasts [24]. With the emergence of early developmental precursors and progenitors, the developing tissue begins to take shape. It is important to note that the process of tissue formation in development contains many parallels with the process of regenerative differentiation, and this will be a consistent trend noted in this dissertation [25, 26].

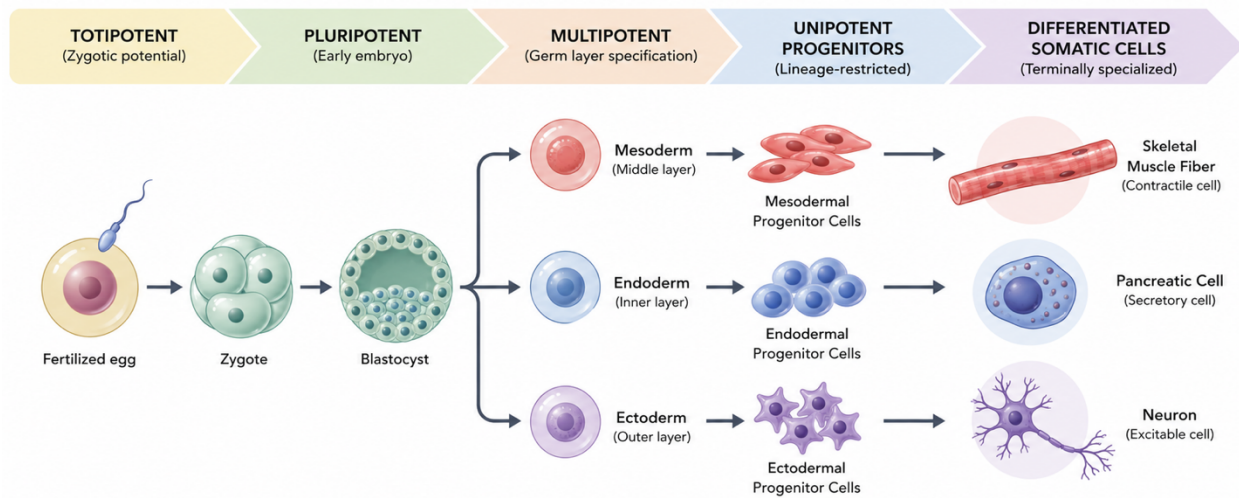


Figure 1.2: Stem Cell Hierarchy

A) Schematic illustrating the developmental trajectory of stem cell potency. Beginning with a fertilized zygote (left-most), which represents the first totipotent cell that can give rise to any cell type of the body or placenta. Subsequently, pluripotency follows at the blastocyst stage, where inner cell mass cells can give rise to any cell type of the body, while cells of the external trophectoderm can give rise to any cell type of the placenta/outside of the body. These pluripotent cells can then further specify to multipotent stem cells, such as mesoderm or endoderm or ectoderm (lineage specific) lineages. Further, each lineage specific stem cell commits to a progenitor which is unipotent, with only the potential to terminally differentiate to a specific cell type. Highlighted are muscle stem cells of the mesodermal lineage, pancreatic stem cells of the endodermal lineage, and neural stem cells of the ectodermal lineage.

Skeletal Muscle Development

The development of tissues and their specified stem cell populations is regulated by spatiotemporal organization of molecular signaling and cellular migration. Skeletal muscle tissue and its resident stem cells develop through the formation of the presomitic mesodermal lineage which gives rise to all musculoskeletal lineages [25]. Researchers have studied the trajectory of somite development in mice, including detailed gene expression profiles of the presomitic mesoderm, which is highly dynamic [27]. Early mesenchymal commitment to the presomitic mesoderm is regulated by wingless-type MMTV interaction site (WNT) signaling [28, 29], and bone morphogenetic protein (BMP) signaling [30, 31]. However, there is discrepancy between these early signaling pathways in the formation of early mesoderm lineages both in development and in models *in vitro*. Historically, it was thought that BMP signaling was essential for promoting early skeletal muscle lineage commitment [31], but recent studies indicate that inhibition, rather than activation, of BMP signaling in addition to WNT activation enhances musculoskeletal commitment [27, 29]. The somites form after early mesenchymal commitment – somites give rise to the axial skeleton and skeletal muscles. From these somites, the dermomyotome and eventually the myotome itself develops, representing some of the earliest forms of skeletal muscle seen through develop.

Of significance, both the stem cells and the myofibers through development dramatically change across time. Myosin heavy chains change from embryonic to fetal to adult through the developmental process, and the muscle stem cell population develops from a progenitor cell to an adult stem cell [11]. Paired homeobox 3 (PAX3) and 7 (PAX7) are crucial skeletal muscle stem cell markers that depict an earlier developmental precursor and a mature stem cell respectively [32]. As PAX3⁺ cells transition and commit to PAX7⁺ cells, they become more functionally

regenerative, but this potency does not stop in utero. The developmental maturation of PAX7 cells continues into adolescence, as PAX7+ stem cells at different stages of development differ in their maturity and therefore their regenerative potency [33]. PAX3+ stem cells give rise to primary (embryonic) myogenesis around 6-9 weeks in humans [33], yielding slow twitch myofibers marked by embryonic myosin heavy chain (MYH3). PAX3+ cells then transition to PAX7+ cells likely through the supportive interactions of primary myofibers which advance the maturation of these progenitors. PAX7+ cells then actively contribute to the secondary wave of myogenesis (fetal) 9-11 weeks gestation, also furthering the development of myofibers to fetal myosin heavy chain. Motor neurons begin to innervate myofibers after secondary differentiation [34]. Secondary myofibers are marked by fetal myosin heavy chain (MYH8) and fetal cardiac actin (ACTC1), representing a slow to fast twitch myofiber switch [35, 36]. Tertiary differentiation is the final element of myofiber maturation that further support subsequent maturation of PAX7+ cells, which consists of adult myosin heavy chains (MYH1, MYH2). Excitingly, myofibers undergoing regeneration re-express developmental myosin isoforms, further highlighting the crossover between regeneration and development [37].

Skeletal Muscle Regeneration by Myogenic Regulator Factors

Skeletal muscle stem cells are the drivers of skeletal muscle tissue development and regeneration. The specific stepwise differentiation process that muscle stem cells undergo is well described from PAX7+ muscle stem cells to multinucleated myofibers (**Figure 1.3**). PAX7+ muscle stem cells in healthy muscle respond to external molecular signaling stemming from injury events to leave quiescence and begin proliferation. Muscle stem cell activation is marked by MYF5 positivity and proliferation markers like mKi67 [38]. The exact signals that can trigger stem cell activation, and what cell types secrete them remain unclear, and is an exciting topic of current

research. Satellite cell activation can be tracked through MYF5 luciferase reporter expression to track the proliferative and regenerative dynamics after injury, detailing the proliferative abundance of an emerging, transient regenerative stem cell population after injury [39]. Activated PAX7 cells can further commit to the differentiation lineage by activating MYOD1 expression, a master transcription factor regulator of the myogenic program [40]. While there is overlap between PAX7 and MYOD1 expression, when PAX7 is turned off, the stem cell commits to the differentiation trajectory, and it can no longer revert back to a PAX7+ muscle stem cell – this stage of terminal commitment to the lineage is defined as a myoblast. In adult muscle stem cells, the activation and commitment pathway are controlled more stringently than developmentally immature counterparts – PAX7 and MYOD1 do not overlap, and thus once MYOD1 is expressed the cell has committed to the differentiation lineage [41, 42]. Committed myoblasts activate myogenin (MYOG), another master regulator of the myogenic differentiation program, transitioning to a myocyte, which are less proliferative and highly prone for fusion. Myocytes fuse to one another, forming the foundational basis of multinucleated myotubes. Myotubes are a developmentally immature myofiber, expressing a host of immature myofiber markers such as embryonic myosin (MYH3) and fetal cardiac actin (ACTC1) [37]. They continue to mature across time, and their maturational progress is demarcated not only by visible structural striations but also by adult myosins MYH1, MYH4, and MYH7.

A critical piece of this regenerative trajectory of skeletal muscle is the self-renewal of the PAX7+ stem cell pool. A subset of activated stem cells (PAX7+ MYF5+ Ki67+) revert back to quiescence, deactivating MYF5 and Ki67, thus stabilizing and maintaining the stem cell pool [39]. This is significant because self-renewal ensures that the tissue can respond to future injuries and regenerative events through the maintenance of a stem cell population. The impact of a stem cell

pool that cannot self-renew is demonstrated by massively impaired tissue regeneration, highlighting the clinical significance of revealing the factors that support this process (to be discussed in the next section). The molecular signals that support, trigger, or induce self-renewal are not clearly understood and thus remain a hot topic of contemporary stem cell research across many tissues.

Through this dissertation, skeletal muscle stem cells will be used as a general term to include muscle stem cells across all stages of development and maturation. Fully mature and functional adult muscle stem cells may be referred to as satellite cells to distinguish from developmental, immature muscle stem cells, which may be referred to as skeletal muscle progenitor cells or precursor cells (SMPCs).

Another crucial element of skeletal muscle that interacts directly with satellite cells is the extracellular matrix (ECM), also known as the basal lamina. This element is an acellular proteinous sheath rich with collagens, laminins, fibronectins, and other extracellular components secreted by several cell types of skeletal muscle, but predominantly the fibroblasts [43]. The ECM is vital for healthy skeletal muscle homeostasis, and several of its components including Collagen Alpha-3 lead to dramatic impairments in muscle stability with rendered obsolete with loss-of-function mutations [44]. The ECM is comprised of three distinct layers, and it is the basal lamina which is the outermost layer that interacts directly with cells in skeletal muscle.

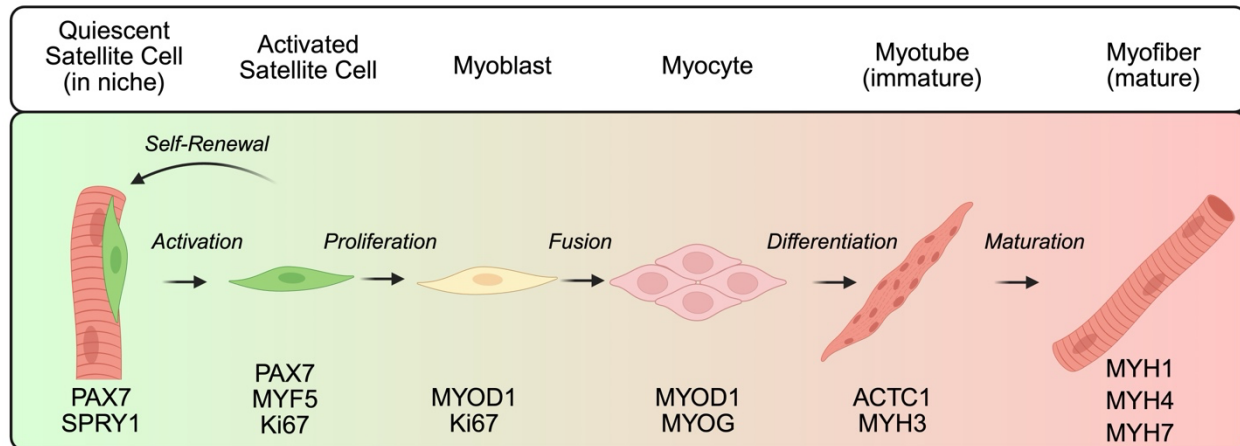


Figure 1.3: Regenerative Trajectory of Skeletal Muscle Stem Cells

A. Schematic illustrating the differentiative trajectory of skeletal muscle stem cells to terminally differentiated multinucleated myofibers. Satellite cells reside in niches, in a state of quiescence in unperturbed skeletal muscle (PAX7⁺ SPRY1⁺) and they activate upon response to muscle injury. Activated satellite cells (PAX7⁺ MYF5⁺ Ki67⁺) mostly commit to the lineage, and when they become myoblasts (MYOD1⁺ Ki67⁺), they are officially committed to the differentiation lineage. A subset of activated satellite cells can revert back to a quiescent state through self-renewal. Myoblasts fuse readily to myocytes (MYOG⁺ MYOG⁺), which lose their proliferative nature and are highly fusive, forming the early basis of skeletal muscle myotubes (ACTC1⁺ MYH3⁺). These immature myotubes further mature to myofibers, marked by fast and slow twitch myosins MYH1, MYH4, and MYH7.

1.4: Skeletal Muscle Diseases and Disorders

Genetic mutations can give rise to disorders and diseases in skeletal muscle if they result in a loss-of-function mutation in a protein that is essential for muscle homeostasis and

regeneration. These mutations can massively stunt muscle regeneration, leading to increased fibrosis and fatty deposition as fibroadipogenic progenitors (FAPs) outcompete the diseased muscle stem cell pool and differentiate to adipose tissue and ECM-secreting fibroblasts [45]. Fibrosis and fatty accumulation are trademark elements of diseased muscle tissue histology and represents a general decrease of muscle function and force because muscle mass has been lost (Figure 1.4) [46, 47].

Skeletal Muscle Deterioration in Healthy Patients

The trademarks of impaired skeletal muscle regeneration in diseased muscle conditions can also be found in healthy individuals. Sarcopenia is one of the most common muscle disorders that describes natural muscle wasting and diminished regenerative potency with age [48]. Patients with sarcopenia are often healthy with no genetic mutations that impair muscle regeneration, but they display extensive fibrosis and fatty disposition that progresses with age [49]. Studies that focused on describing the difference between aged and young muscle stem cells have found extensive evidence that aged muscle stem cells are less regenerative, more senescent, struggle to activate and respond to injury, and are worse at colonizing niches [50, 51]. When transplanted into a young host, geriatric satellite cells are significantly worse at contributing to muscle regeneration, due to their elevated levels of senescence (marked by P16), or irreversible cell cycle arrest [52]. Age-related muscle wasting affects everybody, and age-related detriments and stem cell impairments are a global issue that impact other tissues and their resident stem cell populations in addition to skeletal muscle, including the eye and skin [53, 54]. There is extensive research to uncover the molecular signals of age-related defects through the body. One interesting phenomenon in aging is the loss of primary cilia on satellite cells, which are important for hedgehog signaling dependent locomotion, triggering senescence and rendering loss of stemness [55].

The aging niche microenvironment and interactions with other cells in aging tissue may be a potential cause of deteriorated stem cell potency and decreased numbers with age [56]. To demonstrate the impact of adjacent cell types on satellite cells and their regenerative response in aged settings, researchers made a significant breakthrough leveraging a heterochronic parabiosis murine model. Heterochronic parabiosis surgically connects a young and aged mouse to allow for shared blood cycle and immune response distribution. This yields a cycling immune system that can effectively rejuvenate tissue in the aged mice through global gene expression increases, which is in line with stem cells that are less senescent [57]. In skeletal muscle, aged mice in heterochronic parabiosis systems display significantly improved muscle regeneration [58]. Further, interactions between the stem cells and their ECM, through integrin beta-1 signaling, have been demonstrated to rescue aged satellite cell activity in heterochronic transplantation models [59]. In the context of sarcopenia and aged muscle disease models, the host environment can be a vital tool to influence the stem cell populations. These data shed light on the interplay and the importance of adjacent cell types in skeletal muscle disease settings (such as immune cell populations in heterochronic parabiosis), and suggest that the detrimental effects of muscle wasting in sarcopenia may be reversible through the rejuvenation of satellite cells [60].

Volumetric muscle loss (VML) is another example of increased fibrotic tissue and fatty accumulation due to impaired muscle regeneration in a healthy patient [61]. VML is described as the loss of a large area or chunk of skeletal muscle tissue, as seen in injuries sustained in warfare, automobile accidents, or surgical tumor removal [62-64]. The working hypothesis for the inability to regenerate muscle tissue in VML conditions is the lack of a scaffold (ECM, structural tissue elements) to aid muscle regeneration. This proves too challenging for the satellite cells as they fail to regenerate tissue *de novo* (from scratch), and therefore cannot recover functional skeletal muscle

after VML traumatic injuries [65, 66]. The current standard of care for VML is tissue transfer, or “free functioning muscle transfer”, from a healthy region of muscle to the injured area. While this approach can moderately rescue some muscle recovery, its therapeutic benefits are limited as it cannot completely rescue muscle function in patients, and it renders the healthy site of tissue section mildly impaired [67, 68]. Therefore, there is a pressing need for advanced therapeutic treatments to restore muscle function effectively and completely in patients after VML. Exciting research using reptilian and amphibian models seeks to shed light on the mechanisms that may allow human stem cells (muscle and non-muscle) to regenerate tissue *de novo* [69]. It remains unclear why reptiles can replenish entire new limbs and tails – whether this is because of unique reptilian stem cell populations, or if the stem cells themselves have intrinsic heterogeneity that is absent in mammals – these findings will progress our therapies for VML. However, this work is further away from clinical and translational applications as it seeks to uncover basic foundational biology in *de novo* tissue regeneration.

Alternatively, research teams seek to translate therapies through introduction of artificial scaffolds using compatible biomaterials to aid in the regenerative response to promote *de novo* skeletal muscle regeneration [70]. The objective of this approach is to introduce a physical apparatus to promote cell migration into VML regions and enhance regeneration, yielding biomechanically supported *de novo* tissue reconstruction. Specifically, artificial biomaterials seek to recapitulate the ECM, which is an essential microenvironmental structural component of skeletal muscle and laying the scaffolding of ECM may be beneficial for promoting myogenic programs *in vivo* [71]. Current clinical applications of biomaterials and biocompatibles *in vivo* and *in situ* have yet to yield significant, consistent, functional readouts like muscle recovery in patients [72, 73]. However, the flexibility of this approach is highly attractive as a translational solution

and thus remains a priority of VML translational research – there are a wide array of biomaterials, small molecules, and supportive cell types that require further optimization in preclinical models, and this remains an exciting avenue to develop treatments for VML.

Duchenne's Muscular Dystrophy

Genetic disorders that give rise to muscle diseases include a wide variety of highly fatal, congenital muscular dystrophies. The most common dystrophy is X-linked Duchenne's muscular dystrophy (DMD) which stems from loss-of-function mutations in a hotspot region of exons 45-52 of the human *dystrophin* gene and affects ~1 in 5000 males [74]. DMD and Becker's muscular dystrophy (BMD) both originate from mutations in dystrophin but differ based on the severity of their mutations [75]. DMD stems from a loss-of-function mutation in dystrophin, such as a nonsense mutation leading to an early stop codon, while BMD stems from a missense or frameshift mutation in dystrophin that still leads to its expression, although its function is impaired, leading to a less severe patient phenotype.

Dystrophin, which is the largest gene in our body, is vital for the structural integrity of the dystroglycan complex on the myofiber sarcolemma [76]. This complex mediates the support and stability of myofiber cell membranes and directly interfaces with the ECM externally and the cytoskeleton internally [77]. Disruption of dystrophin impedes the structural composition of the dystroglycan complex, and in turn, the myofiber sarcolemma. Upon contraction, healthy dystroglycan complexes help to absorb the shock between the myofiber and the ECM, and in DMD patients, contraction with mutated or absent dystrophin induces ablation of the myofiber [78]. In DMD, myofibers are constantly perishing – this means that satellite cells are constantly regenerating, and skeletal muscle tissue never reaches healthy homeostasis [79, 80]. As a result, the satellite cell pool becomes exhausted and progressively fails to respond to the consistent

myofiber ablation and muscle injury. This is evident in the late onset of DMD, where young DMD patients show fewer symptoms, but as they age the progression of their disease severity and symptoms exacerbates [78]. As the disease progresses, skeletal muscle quickly begins to atrophy, and patient quality of life takes a turn for the worse with many becoming wheelchair and ventilator bound.

Advances in Treating DMD

Developing a treatment for patients with diseases like DMD to extend their lifespan, improve their quality of life, or cure their disease remains the primary objective of translational muscle biology research. We have made groundbreaking progress in treating muscle disorders, which is exemplified through the extension of DMD patient lifespans well into their 20s and 30s [81]. Furthermore, muscle wasting is no longer the leading death of DMD patients thanks to current standard of care progresses – rather, cardiovascular failures are, and the biology of DMD cardiomyocytes are currently being investigated to continue to expand patient lifespans [82, 83]. One area of standard of care progression includes the application of corticosteroids, like prednisone, to young patients, to build up muscle mass in patients to combat the imminent muscle wasting that will onset in their early teen years [84, 85]. However, steroids have extensive detrimental side effects and thus cannot serve as a permanent treatment, and patients are removed from steroid treatments after ~5-10 years [86]. Corticosteroids have successfully extended patient lifespans, although their side effects after extended usage remain a major limitation in their therapeutic applications [87, 88]. Vamorolone offers an interesting steroid alternative that binds to the same receptors as glucocorticoids but engages in different downstream mechanisms [89]. Vamorolone treatment has fewer side effects compared to typical prednisone treatment, including fewer behavioral issues, less weight gain, and lower risk of osteoporosis, although adrenal gland

and hormone suppression is still detrimentally impacted [90]. Thus, the application of novel therapeutic drugs to replace corticosteroids can extend patient lifespan, but they seem unlikely to serve as a permanent solution for patients with muscle wasting disorders.

Gene editing approaches offer an exciting new direction to treat muscle disorders. The broad umbrella of gene editing includes the therapeutic delivery of healthy dystrophin or CRISPR gene editing components, exon skipping drug treatments, cell therapies to replace the diseased stem cell pool with a healthy one, and drug administration to alleviate the impacts of muscular dystrophies [91]. Viral delivery of mini- or microdystrophin is a promising area of treatment to ameliorate DMD pathophysiology [92, 93]. The viral delivery approach relies on a modified form of a virus, such as adeno-associated virus (AAV), to target host cells and deliver a treatment in the form of engineered genetic material [94]. The size of genetic material that can be packaged in AAV rules out the delivery of dystrophin itself - therefore, microdystrophin, a truncated form of dystrophin focused on retaining the functional elements of the gene, has been implemented to serve as a potential replacement for dystrophin, and recapitulate functional recovery in dystrophic muscle. The elements of dystrophin that should be included or excluded in microdystrophin for the most functional effective rescue effect are still being studied [95, 96]. One obstacle with this approach is the transient delivery of AAV, and thus the virus treatment would have to be repeated with dosages carefully calibrated, rather than a one-time permanent treatment [97]. Myofibers remain the optimal target, as muscle stem cells would rapidly deplete the viral delivery load upon cellular division, and therefore differentiated myofibers would likely be poorly impacted by the viral treatment.

AAVs have also been optimized to carry CRISPR-Cas9 gene editing tools to excise the region of mutation in the dystrophin gene [98]. With this approach, we could carry out *in vivo* gene

editing techniques to render a healthy genotype in edited cells and targeting the self-renewing stem cells would be ideal for this permanent solution. This is a promising approach, although the potential off-target effects of CRISPR-Cas9 gene editing, such as oncogene activation, are incredibly risky and must not be overlooked [99]. Lastly, the delivery of CRISPR gene editing systems using AAV approaches in clinical settings has demonstrated severe immune responses and have resulted in patient fatality, which has slowed the progress of advancing therapeutic solutions for genetic disorders [100]. This remains frustrating for scientists and patients alike, as the tools to treat genetic disorders are highly prevalent and promising, but the delivery systems are lacking.

The implementation of therapeutic drugs remains another appealing and less invasive avenue to combat muscular dystrophies such as DMD. The first FDA approved drug treatment for DMD was Eteplirsen, an exon skipping drug that targets exon 51 of the dystrophin protein, and thus has been applied to patients with mutations in exon 51 (roughly 14% of patients) [101]. This drug was accelerated by the FDA after it showed increased dystrophin transcripts and rescued dystrophic heart defects, but it remains unclear whether it can rescue global clinical muscle function [101]. Further, this drug must be administered once a week, which is a major barrier to patient treatments. Eteplirsen are antisense oligonucleotide therapies that specifically operate through phosphorodiamidate morpholino oligomer technology, which skips exon 51 during mRNA splicing and corrects the reading frame to yield a shorter but still functional dystrophin [102]. Other exon skipping drugs include Viltolarsen, which targets exon 53 of dystrophin – to date, these drugs remain promising but have yet to show a significant rescue effect on patient muscle function and quality of life [103, 104].

Lastly, Givinostat is an exciting drug that targets an entirely different pathway in patients with muscle diseases like DMD. DMD patients display higher levels of histone acetylation, and thus histone deacetylase inhibitors like Givinostat are a promising alternative that operate at the epigenetic level [105]. Clinical trials using Givinostat significantly promoted muscle function in a basic functional test of stair ascension [106]. In summary, Givinostat may slow progression of DMD, but it indicated little change in BMD [107, 108]. Histone deacetylase inhibitors remain a potential solution for combatting DMD and other muscular dystrophies, and while Givinostat may be viable, there are other exciting histone deacetylase inhibitors that have yet to be fully assessed in terms of their potential therapeutic applications such as SR-4370 which ameliorates impaired DMD muscle regeneration in zebrafish models, or Valproic Acid, which restores Notch signaling and cellular proliferation [109-111]. However, a major limitation of histone deacetylase inhibition are off-target responses raising concerns about how other tissues and non-target gene programs may be impacted.

Furthermore, in DMD, chronic cycles of myofiber degeneration, inflammation, fibrosis, and failed repair may disrupt the myogenic niche, reducing the ability of PAX7+ muscle stem cells to remain properly supported, self-renew, and regenerate functional skeletal muscle.

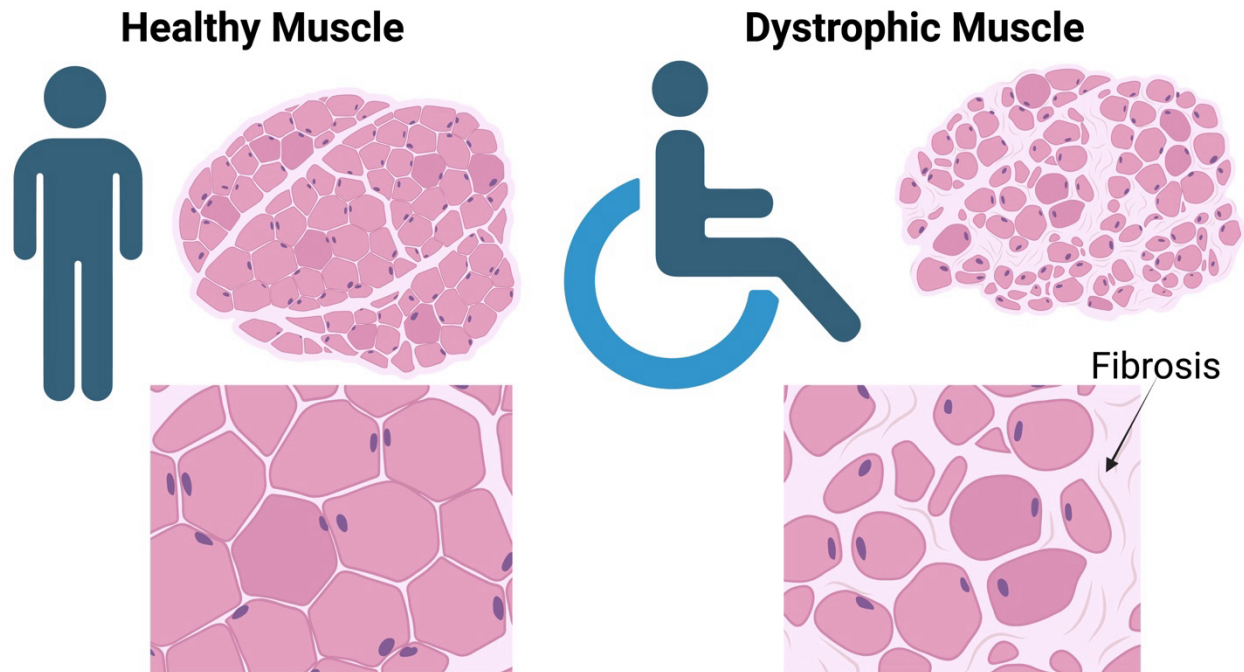


Figure 1.4: Pathophysiology of Neuromuscular Diseases

A. Cartoon illustration of healthy and diseased muscle histology. Left, Healthy Muscle patient muscle section highlights transverse section of extensive myofibers with minimal fibrotic infiltration. Right, Dystrophic Muscle patient sections indicate a significant influx of fibrotic lesions, deteriorated skeletal muscle fiber numbers and size, and a physical detriment to the patient's quality of life.

1.5: The Muscle Stem Cell Niche

Stem Cell Quiescence

By nature, adult stem cells have no physiological function – rather their role in healthy tissue is to respond to regenerative events to maintain tissue homeostasis. Thus, when regeneration is not required, stem cells reside in a state of quiescence, or reversible cell cycle arrest. While quiescence is a reversible or transient cell cycle arrest seen in adult stem cells, senescence is a

permanent cell cycle arrest often seen in aging or diseased settings. Understanding what supports muscle stem cells in quiescence and what factors are involved in their activation in response to injury or regenerative events is a current topic of investigation. The muscle stem cell quiescent state is composed of two distinct functional phases, a G_0 phase and an alert phase called G_{Alert} [112]. Muscle stem cells can actively and reversibly transition between phases in response to injury-induced systemic signals, and mTORC1 plays an active role in the transition from G_0 into G_{Alert} . Fascinatingly, quiescent stem cells that transition into G_{Alert} possess enhanced tissue regenerative function, supporting the notion that the transition of quiescent stem cells into G_{Alert} functions as an alerting mechanism priming them for cell cycle entry and response to regenerative events. It remains unclear in direct response to an injury event what proportion of stem cells transition to G_{Alert} , and further what proportion of these stem cells transition to an activated cell state and enter the cell cycle.

The Skeletal Muscle Stem Cell Niche

The stem cell niche is defined as the molecular cues and factors from the tissue microenvironment that support a stem cell in a state of quiescence. These niches are highly specialized to both facilitate and support quiescent stem cells. Across different tissues, niches regulate progenitor to adult cell maturation, stem cell self-renewal, and cell state activation and quiescence. The niche's influence on muscle stem cell quiescence and cell state is highlighted by the different in stem cell activation in developing versus adult skeletal muscle niche systems. In healthy tissue, the fetal niche supports high levels of proliferation markers EdU and Ki67 which are not seen in adult tissue [113]. Coinciding with this finding in proliferative difference, the fetal niche also supports 100-fold greater densities of SMPCs.

Through embryonic and fetal organogenesis, the stem cell niche develops and matures along with the tissue and resident cells. This pattern is noted through the emerging niche of hematopoietic stem cells (HSC). Through development, the HSC niche consists of two major waves, with the first wave yielding transient HSCs that are central for the needs of embryonic oxygenation. The second wave then produces a more concrete hematopoiesis, producing HSCs that give rise to all lineages of mature blood cells, and crucially, have long-term repopulation potential through self-renewing characteristics. In HSC development, the niche is a key factor in preventing alternate fate specification and maintaining HSC specificity [114]. An integral part of the HSC niche are adjacent cell types and signals they secrete such as inflammatory cytokines, which regulate the dynamics of the HSC niche across time [115].

The overlapping parallels between development and regeneration is a shared theme relevant to the adult stem cell niche. This is well documented in the brain, where neural stem cells (NSC) reside in niches in the subgranular zone in the hippocampal dentate gyrus, and the subventricular zone around lateral ventricles [116]. Single-cell gene expression profiling and lineage tracing in mice uncovered a phenomenon where dormant NSCs reacquire developmental-adjacent states when activated to regenerate adult neurons [117]. Nearly half of the transcriptomic differences were involved in regulating or sensing the niche microenvironment, which includes cues from neurotransmitters, ligand receptor interactions, and extracellular matrix.

The skeletal muscle stem cell niche is anatomically defined between the multinucleated skeletal muscle myofiber and the basal lamina, an extracellular matrix rich sheath that weaves its way through skeletal muscle (**Figure 1.5**) [18]. Muscle stem cells that form these niches are still their own autonomous cells, though they are sandwiched between the niche components. The adult satellite cell niche differs distinctly from the developing SMPC niche, as satellite cells reside in

quiescence until regeneration is necessary while developmental SMPCs consistently play an active role in skeletal muscle tissue growth [118]. Through development, the first interaction in SMPC niche formation is the initial adhesive interaction between the stem cell and the myofiber sarcolemma, which is mediated through M- and N- cadherins and CD82 [119-121]. Importantly, this interaction occurs prior to basal lamina formation, and thus this may represent a distinct difference between the developing and adult niches. As the regenerating myofibers grow, they begin to fuse to one another, linked through spectrin-positive cross-bridges while the basal lamina forms around the external perimeter of the myobundle [122]. The basal lamina also matures across time, as Laminin-111 isoform demarcates the basal lamina in developing niche systems is replaced with Laminin-211 in adult satellite cell niches [123, 124]. After the initial adhesive interaction with the myofiber, the stem cell then interfaces with the basal lamina, which encompasses both cell types. The basal lamina – stem cell adhesive interactions include integrin mediated signaling pathways, such as integrin alpha-6, integrin alpha-7, integrin beta-1, and Lama2 [125, 126].

Intercellular Interactions in the Muscle Stem Cell Niche

The complexity of the muscle stem cell niche arises from the sources of potential interactions with quiescent stem cells. Adjacent and nearby cell types have been shown to influence stem cell niche dynamics, primarily through the secretion of signaling molecules and growth factors. In the developing niche, preosteogenic SHOX2⁺ progenitors and prechondrogenic SOX9⁺ progenitors are important for early bone and cartilage formation and mediate stem cell niche systems [33, 127]. Multipotent mesenchymal DUSP6⁺ progenitors provide critical morphogens required for the lineage specification of skeletal muscle [128, 129]. These mesenchymal progenitors further commit through development, becoming TWIST2⁺ fibroblasts of fascia, TNMD⁺ tenocyte precursors, and CD73⁺ stromal cells which impact the primary niche

[130]. These fetal stromal cells also express an integral adult fibroadipogenic progenitor (FAP), fibroblast growth factors (FGFs) are essential for healthy muscle stem expansion after muscle injury and are secreted by FAPs in adult niche microenvironment settings [131-134]. It is important to note that fetal stromal cells and adult FAPs are remarkably different cells, marked by higher levels of hepatocyte growth factor and less interleukin-6 secretion – these secreted factors mediate the expansion and migration of SMPCs [135]. FAPs, which are multipotent stem cells that can give rise to adipogenic or fibroblast cells, play an integral role in the regulation of muscle stem cell dynamics in regeneration [136]. FAP populations are dynamic, with extensive heterogeneity in healthy skeletal muscle including an ECM gene signature rich population and a cell signaling enriched population [137]. After injury, both satellite cell and FAPs exit quiescence and enter the cell cycle, where FAPs secrete trophic factors that support myogenic differentiation of muscle stem cells [138]. Further, these FAPs secrete chemokines which recruit monocyte and neutrophils to the scene of muscle damage, which in aid in the removal of debris and waste [139]. On the contrary, inhibition of FAP activity is associated with accumulation of fibrosis and fatty infiltration, often seen in diseased settings.

Muscle stem cells express fibroblast growth factor receptors (FGFRs) that bind to FGFs, thus demonstrating a potent interactome pathway that connects secreted FAP signaling to the regulation of muscle stem cell dynamics [140]. Without these FGFRs, muscle stem cells fail to enter quiescence and muscle regeneration is massively impaired. FAPs themselves are found throughout the basal lamina and interstitial space in skeletal muscle, and their primary responsibility is to promote the maintenance and secretion of extracellular matrix components. Thus, FAPs are critical for both tissue regeneration, homeostasis, and satellite cell self-renewal,

and the direct roles that FAP subpopulations play in influencing satellite cell niche support dynamics require further intensive studies [138, 141].

Macrophage subtypes in developing skeletal muscle tissue regulate clearance of cellular debris and inflammation, and in tangent they also regulate SMPC activation [142]. During muscle regeneration, there is a definitive switch in macrophage phenotype from pro-inflammatory macrophages positive for Ly6C, which secrete cytokines that promote muscle stem cell and myoblast proliferation, to anti-inflammatory macrophages negative for Ly6C, which release factors that stimulate myoblast differentiation and fusion [142, 143]. Thus, after an injury event, the distinction between Ly6C⁺ and Ly6C⁻ macrophages is vital because they conduct opposite functions at the site of muscle injury, with Ly6C marker indicating the difference between the two populations and how they influence muscle stem cell regenerative behavior [144]. LyC⁺ macrophages help regenerative response by secreting ADAMTS1, which promotes muscle stem cell activation [145]. LyC⁺ macrophages not only impact the activated and proliferative muscle stem cell pool, but also the FAP pool, as they can induce apoptosis in proliferative FAPs to regulate the fibrotic response in injury [146]. To further the proof of macrophage importance, they have been demonstrated to provide a transient satellite cell niche through regeneration [147]. In dystrophic settings, heterogeneity of SPP1⁺ and GAL3⁺ subpopulations of macrophages suggested that SPP1⁺ macrophages regulated FAP differentiation in muscle injury, while GAL3⁺ macrophages are chronically activated [148]. Thus, macrophages play a definitive role in muscle regeneration, although it remains unclear how circulating immune cell populations influence healthy satellite cell niche dynamics in homeostasis [149].

Stem Cell – Myofiber Niche Interactions

Adhesive myofiber – muscle stem cell interactions regulate downstream quiescence, differentiation, and self-renewal of the stem cell pool. These supportive interactions in turn help to promote the emergence of stem cell polarity, which arises through development. Calcitonin receptor (CALCR) is expressed specifically by adult satellite cells and is a marker of a mature quiescent stem cell – when removed from niches, satellite cells rapidly lose CALCR expression [150]. CALCR is important for preventing satellite cells from escaping their niches by acting through intracellular cAMP signaling, and thus is critical for healthy self-renewal of the stem cell pool. When CALCR is knocked out through a lost-of-function mutation, satellite cells fail to colonize niches and self-renew [151]. Melanoma cell adhesion molecule (MCAM) is a muscle stem cell surface protein that is highly expressed on proliferative fetal SMPCs and lost during differentiation and can be applied to enrich a more myogenic population of progenitor cells [152]. Multiple EGF-like domains 10 (MEGF10) is a transmembrane protein that is found at the interface between embryonic PAX3⁺ SMPCs and myofibers and is critical for Notch-dependent niche colonization, although it remains unclear which cell, or both, express it [124]. MEGF10 knockouts mirror conventional muscle disorders, with impaired muscle regeneration and extensive fibrosis [153].

N- and M- Cadherins are another class of transmembrane protein that are integral for adhesive junction interactions in niche localization and quiescence [18]. Cadherins anchor through interactions with alpha- and beta-catenins inside the cell and myofiber cytoplasm, and when these are removed through loss-of-function mutations, cadherins fail to support stem cell niche formation highlighting the co-dependence of cadherin and catenin interactions [119]. Removal of cadherins through loss-of-functional mutations in mice results in failed niche localization as

satellite cells hone to the interstitial space, therefore leading to impaired regeneration of skeletal muscle and worsened self-renewal [120].

Stem Cell – ECM Niche Interactions

The connectivity and downstream signaling cascades from cell – ECM interactions was first described in 1977, where fibronectin induced a change in intracellular actin filaments, providing exciting evidence of the first cell – ECM interaction [154]. Satellite cell – ECM interactome candidates are critical for the polarization of quiescent stem cells and healthy niche formation. These candidates are involved in bidirectional signaling pathways, including internal cues that regulate external adhesive interactions and relaying cues from the outside environment to regulate intrinsic internal cellular pathways [155]. Integrin signaling is at the forefront of defined pathways that mediate satellite cell – ECM interaction dynamics. In fetal muscle, integrin alpha-6 regulates SMPC commitment by triggering YAP/TAZ signaling cascades [156]. In adult satellite cell interactions, integrin alpha 7 (ITGA7) is pivotal for healthy stem cell self-renewal and contribution to regeneration [157, 158]. ITGA7 is important for healthy polarity of the stem cell for niche stabilization and quiescence to proceed, and the splicing pattern, which is mediated by factors like QKI, and isoform of ITGA7 are definitive in this process [159]. Oncostatin M receptor (OSMR) is a member of the interleukin-6 signaling family and is another niche supportive factor in adult satellite cell niche microenvironments.

OSM is expressed by myofibers after injury events and is a potent molecule for reintroduction of satellite cells to their niches and reentrance into quiescence [160]. OSMR, which is likely not relevant in fetal niche systems, operates via the JAK/STAT signaling pathway, more specifically through Stat3, which is an integral regulator in satellite cell quiescence and support [161].

Recent findings uncovered Tenascin-C (TNC) as a novel candidate in satellite cell – ECM interactome biology, mediated by stem cell surface protein Annexin A2 [162]. In mice that were engineered with a TNC loss-of-function mutation, their satellite cells were unable to self-renew, greatly impairing efficient regeneration, although this effect can be rescued with soluble TNC.

Secreted Signaling in the Muscle Stem Cell Niche

Signaling cascades that regulate the resulting interactions of the stem cell niche are essential elements of self-renewal of the stem cell pool. Notch signaling is a well-defined pathway that consists of adjacent cell-cell signaling, in line with myofiber – stem cell interactions. Through ligand/receptor binding and interactions, like Delta and Jagged-1, Notch signaling can impact gene expression to alter stem cell behavior in their niches [163]. The role that different Notch signaling candidates play, and how these change through development, is still being defined – for example, Notch 3 is critical for adult satellite cell quiescent support, while *Rbpj* is necessary for the initial colonization of SMPCs in development [124]. Of note, SMPCs that fail to locate to their niches do not contribute to myogenesis, thus exemplifying the significance of the stem cell niche [124].

Epidermal growth factor receptor (EGFR) and Aurora Kinase were identified through an *in vitro* screening assay in skeletal muscle, identifying it as a determining factor of satellite cell polarity through CARM1 interactions [164]. Further, EGFR expression is rapidly reduced in satellite cells that fail to colonize niches. Early fetal SMPCs do not express EGFR but rather they do highly express other candidate members of the EGFR pathway such as ERBB3, which is not expressed on quiescent satellite cells but rather upon activation [165]. Lastly, the JAK/STAT pathway plays a crucial role in stem cell maintenance and self-renewal, and STAT3 is central to these processes [161]. STAT3, which is a downstream target of the IL-6 signaling cascade, aids in

muscle regeneration by triggering symmetric muscle stem cell divisions [166]. Blocking STAT3 through genetic knockouts leads to improved niche colonization, although the regenerative response is stunted [167].

The Muscle Stem Cell Niche Set Point

Regulating the stem cell niche set point, or the number of stem cells supported within an *in vivo* niche, is an exciting topic of research that requires further examination [168]. Skin and hair follicle contain densely packed stem cells, while liver and skeletal muscle are relatively sparsely populated [169]. Manipulating the molecular signaling that controls the intrinsic stem cell niche set point would open new possibilities to deliver promising therapies for patients with muscle disorders. Myofiber driven WNT4 signaling has been shown to control the stem cell niche set point through Rho Kinase activity, which leads to suppression of YAP induced stem cell activation [170]. Through skeletal muscle injury, WNT4 knockouts display accelerated stem cell activation and rapidly contribute to regeneration, while WNT4 overexpression leads to deeper quiescence of muscle stem cells, delaying their activation and contribution to regeneration.

Disruption of the Stem Cell Niche in Neuromuscular Diseases

Niche disruption is a key element of perturbation of self-renewal and failure to support satellite cell populations and is frequently documented in cases of genetic mutations of niche supportive factors. Therefore, cases of niche disruption are often the primary cause of myopathies or muscle disorders, which manifest as severe muscle wasting and other physiological defects. Without integral elements of the stem cell niche, either through the removal or loss-of-function of niche factors described through this section, the muscle stem cell populations are unable to sufficiently form niches and self-renew, thus leading to a deficit in their regenerative potency that progresses over time. Documented disruptive niche events include mutations in laminin-2 or

collagen-6 genes, which result in congenital myopathies and lead to loss of satellite cells and muscle atrophy [171]. The loss of these proteins significantly impairs satellite cell – ECM interactions, which are critical for niche polarity and stabilization. In DMD, satellite cells lose polarity and thus fail to form niches, significantly impairing self-renewal and tissue regeneration [76]. Thus, congenital myopathies can be broken down into several realms, depending on the root cause of disease: niche disruption, myofiber instability, ECM composition, neuromuscular junction failure, or mutations that effect other cell types such as FAPs or macrophages which play a key role in muscle regeneration (**Figure 1.5**). The focus of this dissertation will be on congenital myopathies that result from niche disruption.

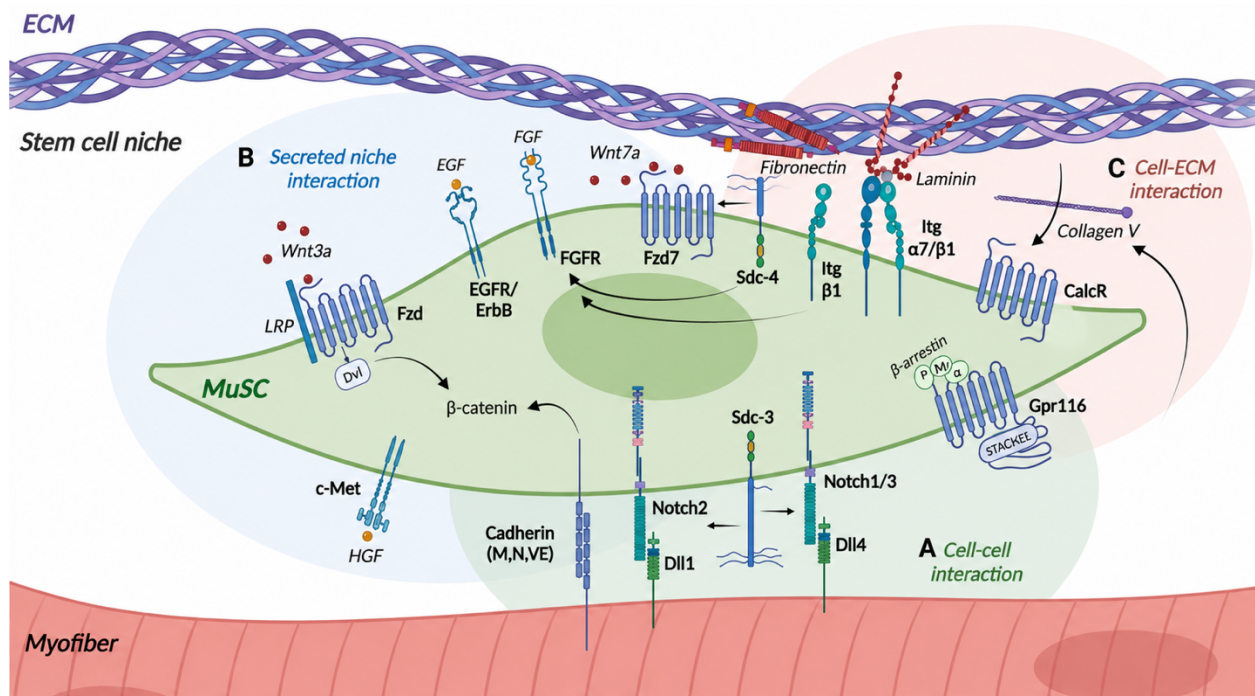


Figure 1.5: The Muscle Stem Cell Niche

A. Diagram of the Muscle Stem Cell Niche and microenvironmental interactions that support the niche. Highlights the Muscle stem cell, myofiber, and extracellular matrix,

along with interactions between cell types and components. A) Cell-cell interaction between the muscle stem cell and the myofiber are highlighted by Cadherin (CDH15, Alpha/Beta Cadherin) and Notch (Notch2/DLL1, NOTCH1/3/DLL4) interactions. B) Secreted niche interactome components can help stabilize niche interactions and self-renewal, such as WNT (WNT3A), EGF, and FGF. C) Muscle stem cell to extracellular matrix interactions can include fibronectin, integrin (ITGA7/ITGB1), and collagens (COL6A1/CALCR).

1.6: Stem Cell Therapies

Past Clinical Trials for Skeletal Muscle Therapies

Cell transplantation therapies offer a particularly exciting avenue to introduce a healthy stem cell pool into a dystrophic patient. Some of the first approaches to treat patients with muscular dystrophy, like DMD, focused on using healthy stem cells from immune matched sources. They sought to isolate biopsies from siblings or parents, isolate myoblast populations, expand them *in vitro*, and transplant them. These early studies focused on myoblast populations, which are already committed to the differentiation lineage, and thus, although healthy dystrophin⁺ myofibers were detected in the patient, the retention of a functional stem cell population failed [172, 173]. This work was a breakthrough in stem cell therapies because it proved that cell therapies could deliver healthy cells and transcript expression inside of a diseased patient environment. However, the functional rescue of skeletal muscle dynamics in dystrophic patients showed little to no improvement, highlighting a major area for improvement.

Advances in the field of muscle stem cell biology then highlighted that the satellite cell is the stem cell population capable of undergoing self-renewal, contrary to prior dogma that the

myoblast population could do so [39]. These findings were established in part by the transplantation of a single mouse donor satellite cell into a new host, where it was able to regenerate to form new myofibers and persist as a pool of PAX7⁺ satellite cells. Serial transplantations and injuries of these donor satellite cells showed consistent results of healthy contribution of muscle regeneration [174]. The ability of an adult stem cell to contribute to self-renewal and regeneration upon transplantation is a crucial element of stem cell biology and is the gold standard of cell therapies, where the transplanted stem cells can respond to infinite injury events. This phenomenon has also been demonstrated in both the intestine using LRG5⁺ stem cells [175], and in the skin using epithelial progenitor cells [176].

Induced Pluripotent Stem Cells

In 1957, Waddington et. al. described developmental patterns in cellular gene expression as a ball rolling down a hill – its gravitational potential energy prevents it from spontaneously proceeding backwards. However, John Gurdon challenged this theory through somatic cell nuclear transfer, where he replaced *Xenopus* blastocyst nuclei with that of a somatic cell, giving rise to a clonal individual and actively giving rise to healthy development [177, 178]. His findings revealed that a somatic cell could reprogram to an earlier developmental stem cell state – however, the genetic mechanisms that regulated these changes remained elusive. Further, directed transdifferentiation of fibroblasts to myoblasts (somatic cell 1 to somatic cell 2) is possible through transfection of single copy of cDNA [179]. These seminal findings paved the way for understanding the complexity of gene expression in early development and shed light on the plasticity of cell fate determination, which can be leveraged for scientific advances. Shinya Yamanaka's research team used this as a foundation to make a revolutionary discovery, reverse engineering fibroblasts to a cell that demonstrated a pluripotent-like state through the forced

overexpression of pluripotent cell factors (dubbed Yamanaka factors) OCT3/4, SOX2, KLF4, and c-MYC [180]. These induced pluripotent stem cells (iPSC) displayed morphology, behavior, and growth properties in line with pluripotent stem cell markers and expressed a wide array of pluripotent stem cell markers including those overexpressed and those downstream, like Nanog. Critically, mouse iPSCs injected into a developing blastocyst successfully contributed to mouse embryonic development, demonstrating their ability to functionally act as a true pluripotent stem cell which can give rise to any cell type of the body [181].

Induced pluripotent stem cells (iPSCs) offer an exciting therapeutic pathway to combat muscle disorders through autologous (patient-specific) stem cell transplantations. The primary objective of this approach is to replace the disease muscle stem cell pool with a healthy one through this autologous cell therapy (**Figure 1.6**). iPSC biology work for translational applications has exploded over the past two decades, with impressive advances in several fields. To date, there are 115 clinical trials using over 82 pluripotent stem cell products across 1200 patients total, with most focusing on the eye, central nervous system, and cancer [182]. There is a current ongoing clinical trial of iPSC skeletal muscle progenitor cell transplantations associated with the University of Minnesota as of 2025 [183]. Good manufacturing practices have been applied and preclinical modeling in non-human primates of ~50 million engrafted cells identify healthy dystrophin transcripts and functional contribution of healthy stem cells to muscle regeneration. The field of skeletal muscle and neuromuscular disease research remains optimistic that these cell therapy approaches will yield successful results that rescue patient muscle function and improve quality of life.

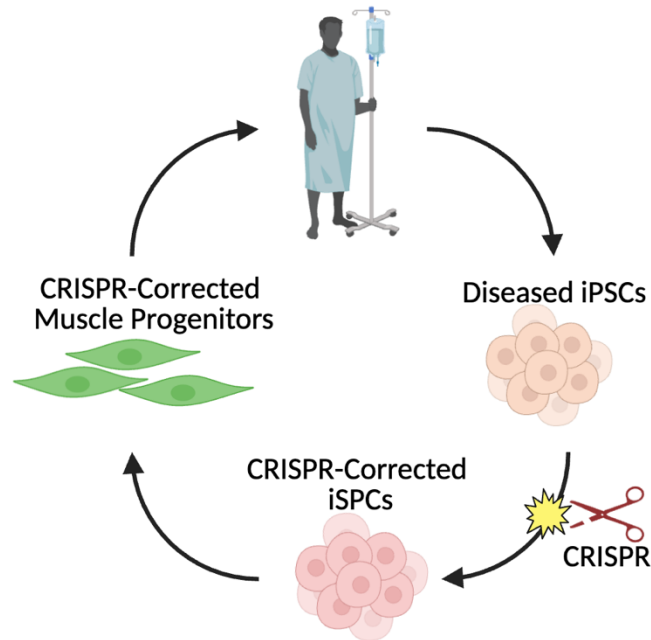


Figure 1.6: Translational Objective of Muscle Stem Cell Therapies

A. Schematic of the translational goal of muscle stem cell therapies, as highlighted through this dissertation. Dystrophic patient specific induced pluripotent stem cell lines can be generated through transcriptional reprogramming approaches. These iPSCs are then readily expanded *in vitro*, to allow for CRISPR correction of the patient's genetic mutation. CRISPR corrected iPSCs are differentiated to skeletal muscle progenitor cells, prior to engraftment back into the dystrophic patient for an effective, long-lasting treatment.

Differentiation of iPSC to Skeletal Muscle in a Dish

With the breakthrough of iPSC model systems, researchers have shifted their focus towards establishing *in vitro* differentiation pipelines to generate specific cell types of interest for cell therapies, *in vitro* cellular modeling, or organoid systems. However, a limitation in the directed differentiation of iPSC/hPSC to specific somatic cells or unipotent stem cells is that the product is immature compared to adult counterparts. This immature product results in muscle stem cells that

regenerate, fuse, and activate less efficiently when compared to adult satellite cells, and generally show reduced regenerative potency both *in vitro* and upon *in vivo* engraftment [184]. Therefore, groundbreaking work identified that muscle stem cells generated from iPSC *in vitro* show transcriptomic profiles that closely resemble late embryonic human muscle progenitor cells [33]. This work also found novel transcription factors and ligands/receptors expressed across the developmental trajectory of human skeletal muscle stem cells, which was then used to cross-compared to several protocols of iPSC derived SMPCs. This immaturity phenomenon is applicable but not restricted to skeletal muscle differentiations *in vitro*, which closely model development but lack the true *in vivo* signals of support that enable developmental maturation [25]. Therefore, further research is focused on understanding the biological cues in development and recapitulating them in a dish to support a more mature and functional stem cell (for skeletal muscle, cardiomyocytes, neurons, skin, etc.) through directed differentiation approaches.

In skeletal muscle, there are many published directed differentiation workflows from pluripotent stem cells that can yield skeletal muscle myotubes and progenitors for cell therapy approaches – they differ in their duration, their stepwise signaling and growth factors, and their constructive elements [27, 29, 32, 185]. Excitingly, these approaches yield a functional, myogenic muscle progenitor cell that is capable of differentiation and regeneration both *in vitro* and *in vivo* and can be used reliably to model neuromuscular diseases in a dish. In skeletal muscle directed differentiation cultures, PAX7 expression increases over 1000-fold, one of the few *in vitro* systems demonstrating an influx of muscle stem cell potency [185]. Thus, we postulate that these systems produce an *in vitro* niche (of ECM, myotubes and other cell types) that supports the development of PAX3 progenitors and PAX7 SMPCs. We now understand that maximizing the yield of PAX3+SIX1+ cells represents an early developmental stage of PAX7+ muscle stem cells which

can be effectively screened at earlier time points of differentiation, thus paving the way for minimized cell line variability and maximized yield [185]. Interestingly, many of these protocols yield a heterogeneous culture, and work from our group has shown that enrichment for muscle progenitors using ERBB3+ NGFR+ HNK1- FACS markers results in a more functional and regenerative cell population upon engraftment [184]. It is important to note that this enrichment strategy yields an SMPC population that is purer, rather than more functionally myogenic. Excitingly, these SMPCs can be engrafted into murine skeletal muscle to trigger and study human iPSC skeletal muscle regeneration *in vivo*.

Recent work illustrated the heterogeneity of these cultures as advantageous for supporting PAX7+ SMPC development, including the supportive interactions of an intermediate mesoderm lineage marked by PAX8 positivity that may be integral for *in vitro* myogenesis [186]. However, this work also highlighted that we are only FACS enriching a fraction of the total PAX7+ SMPC population, so more efficient and inclusive enrichment markers are highly sought after to improve the yield of directed differentiations. Excitingly, these approaches have been utilized to generate a patient-specific DMD muscle stem cell, use CRISPR gene editing to remove the DMD mutation from the dystrophin coding region, and engraft into a DMD disease modeling mouse to showcase their viability, functionality, and healthy dystrophin expression [187]. This approach is precisely the translational goal of current and future clinical cell therapy trials for patients with neuromuscular disorders like DMD.

Recapitulating the Stem Cell Niche in a Dish

There is also a lot of promising research in recapitulating the stem cell niche in a dish to progress the maturation of iPSC derived skeletal muscle stem cells. Generally, expansion of freshly isolated muscle stem cells either from iPSC differentiations [188] or from primary sources like

human or mouse skeletal muscle [39] rapidly lose PAX7 expression due to precocious activation and differentiation. Quarta et. al. demonstrated that freshly isolated muscle stem cells could be maintained in a quiescent-mimicking state through growth in media that contains extensive growth factors and signaling molecules supporting quiescence [189]. These additions include calcitonin receptor ligand elcatonin [190], cAMP activator forskolin [191], p38 inhibitor SB203580, FGF receptor inhibitor and TGF-Beta. Further, they showcased the importance of the myofiber for extended niche support of the muscle stem cell population, and engineered myofibers with ECM secretion to enable a system of extended muscle stem cell growth without PAX7 loss *in vitro*. Other work has demonstrated the impact not only of the myofiber, but of the substrate that they grow and adhere to regarding the establishment of an *in vitro* niche to study muscle stem cell dynamics. Jacques et. al. designed a system that promoted the attachment of myotubes *in vitro* through the implementation of thrombin, fibrinogen, and geltrex to cell culture wells, expanding myotube attachment to ~15 days [192]. The extended myotube support enabled more rigorous co-culture experiments with satellite cells, where they found a more quiescent-like status upon grown in their model systems, marked by the maintained expression of calcitonin receptor, an adult satellite cell specific quiescent marker. Despite advances in cell culture systems that model essential elements of the *in vivo* niche system, there still are no protocols that fully support or mature the adult satellite cell niche.

The Microenvironment Impact on Transplanted Muscle Stem Cells

Considering the inability of an *in vitro* niche to recapitulate the true adult *in vivo* niche, the question that follows is what elements of the *in vivo* niche are essential towards the maintenance of a quiescent, regenerative stem cell through development and regeneration? How the stem cell niche modulates satellite cell behavior and dynamics remains unclear and is a pivotal question

guiding current muscle engraftment research to develop efficient cell therapies for patients with neuromuscular disorders. Lazure et. al. demonstrated the impact of the *in vivo* niche through restorative heterochronic transplantations of aged muscle stem cells into a young host [56]. This work highlighted that aged environments support more FAPs at the expense of a less regenerative adult satellite cell population, but engraftment of these satellite cells into a young mouse and inducing a regenerative event supports the rejuvenation of aged donor satellite cells. These findings highlight that the stem cell niche, along with the interactions with other cell types and signaling interactions, develops through aging and can rescue diseased or senescent phenotypes in satellite cells.

The effect of the *in vivo* stem cell niche has also been described on engrafted iPSC skeletal muscle progenitor cells, shedding further light on therapeutic advances for muscle diseases (**Figure 1.7**). However, it remains unclear how transplanted muscle stem cells form niches for the first time *in vivo*, and what overlaps are found between self-renewal in homeostasis and niche colonization upon engraftment. Inducible PAX7 expression yields a rapid directed differentiation from pluripotent stem cell to skeletal muscle, although differentiation of these enriched PAX7+ progenitors *in vitro* yield an immature product [193]. Engraftment of these progenitor cells into an adult mouse microenvironment supports regeneration of a more mature product, supporting the notion that the adult stem cell niche microenvironment enables further maturation of myogenesis. However, this study crudely combined all engrafted cell samples together, including muscle stem cell and myofibers, decreasing the precision of their RNA sequencing readouts. Finally, a particularly exciting study published in Cell Stem Cell focused on human iPSC derived muscle stem cells, and how they specifically respond to engraftment into the *in vivo* microenvironment [188]. They leveraged a PAX7-GFP reporter cell line to FACS re-isolate engrafted stem cells after

engraftment, turning to single cell RNA-sequencing techniques to map and project heterogeneity and transcriptomic changes upon transplantation. Fascinatingly, they found that engraftment and the support of the *in vivo* microenvironment subjects muscle stem cells to a transcriptomic shift that matches a more mature, quiescent stem cell – specifically, stem cells mature from embryonic-like to adult-like upon transplantation. They also found that serial transplantation of these muscle stem cells dramatically improved the stem cell niche formation and regenerative potency of engraftments, demonstrating that these *in vivo* supported stem cells are more functional. These data together highlight the importance of the *in vivo* microenvironment, both understanding its role in generating more efficient engraftment strategies and harnessing its elements in a dish to better support the expansion of myogenic progenitors. Therefore, work has progressed to leverage the *in vivo* ECM, which has extensive roles in satellite cell maintenance and self-renewal [155, 162, 194], as an element to support an *in vitro* niche system. Decellularized human skeletal muscle tissue sections left behind hollowed-out ECM matrices (Myoscaffolds) to grow muscle stem cells upon [195]. The stem cells could remodel these ECM matrices as a scaffold to support their growth and behavior. Critically, this study identified extensive differences between the proteomic contents of healthy vs dystrophic ECMs, such as less integrin signaling in dystrophic scaffolds. SMPCs cultured on dystrophic myoscaffolds struggled to remodel and interact with their matrices and had stunted regeneration *in vitro*.

Reserve Muscle Stem Cells

The heterogeneity of muscle stem cells *in vitro* cultures is also clearly defined by their differentiation to myotubes in serum-free media. Specifically, *in vitro* self-renewal is present in these differentiations, as myotubes generate but there are still extensive populations of PAX7+ cells that persist. Thus, the PAX7+ muscle stem cells that persist through differentiation *in vitro*

raised questions about muscle stem cell heterogeneity, asymmetric cell division, and whether these stem cells that self-renew in a dish were more functionally potent. Human myoblasts were differentiated and the PAX7⁺ self-renewing stem cells were enriched for and engrafted in comparison to the bulk myoblast culture, and the reserve population yielded significantly better engraftments in terms of niche formation, myofiber regeneration, and PAX7 persistence [196]. This work was repeated with iSPC skeletal muscle progenitor cells to a similar but less dramatic effect, suggesting that the immaturity of these cells is a limitation in their reserve cell potential [197].

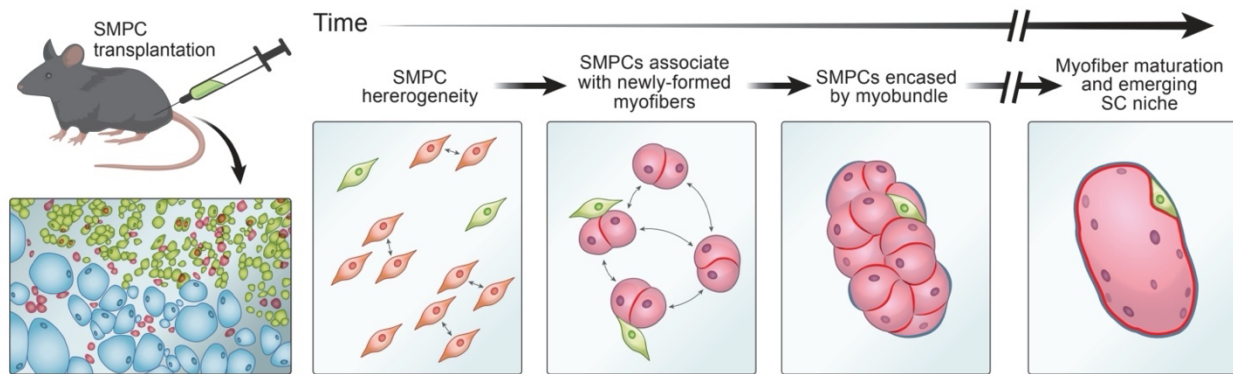


Figure 1.7: Muscle Stem Cell Niche Formation Upon Transplantation

A. Schematic illustrating the niche formation of engrafted muscle stem cells *in vivo*. The initial pool of engrafted stem cells (left) are relatively heterogenous, with PAX7⁺ cells (green) and committed myoblasts (orange). Across time, the first phenomenon that occurs is the adhesion of the PAX7⁺ stem cell with the newly formed, regenerative myofiber. Then, as myofibers continue to regenerate and develop, they readily fuse together into myobundles. The PAX7⁺ stem cell then becomes encompassed by the basal lamina. Finally, the PAX7⁺ cell is supported in downstream and long-term quiescence as the myofiber fully matures (right).

1.7: The Biological Role and Function of MEGF10

MEGF10 in Muscle Disease

Multiple EGF-like domains 10 (MEGF10) is a transmembrane receptor that plays a role in skeletal muscle myogenesis, regeneration, and homeostasis. Mutation of MEGF10 renders a loss-of-function, triggering congenital recessive myopathy early onset myopathy, areflexia, respiratory distress and dysphagia (EMARDD). EMARDD was first discovered and characterized by Logan et. al. in 2011, which conducted a systematic analysis on several patient biopsies with MEGF10 mutations [198]. This study identified chronic regenerative impairments, increased fibrosis with age, centralized nucleation in myofibers after regeneration, and limited PAX7+ muscle stem cells. Further, they identified that MEGF10 predominantly stains the myofiber in healthy control adult skeletal muscle biopsies. The pathophysiology, while variable across disease severity (which is dependent on mutation location), is typically defined by diaphragm weakness, difficulty swallowing, distal limb weakness, and foot deformity. Since the establishment of EMARDD as a muscular dystrophy, other clinical studies have published patient mutations through whole exon sequencing and described their pathophysiology to expand the EMARDD literature base [199-201]. It remains unclear how loss of MEGF10 gives rise to such a detrimental and lethal myopathy. Which cell types in skeletal muscle express MEGF10 and what its roles are on those cell types, whether the same or different, and what its binding partner in skeletal muscle is, remain unknown and is central to the questions proposed in this dissertation.

MEGF10 Structure

MEGF10 contains a large extracellular region composed of an N-terminal EMI domain followed by 17 epidermal growth factor (EGF)-like domains (**Figure 1.8**). The EMI domain was

originally identified in members of the EMILIN glycoprotein family and has been implicated in protein multimerization [202]. Although the MEGF10 EMI domain retains the conserved C-terminal consensus sequence, it contains six cysteine residues rather than the seven typically found in canonical EMI domains, making it more like the EMI domain of multimerin proteins. This domain has been predicted to bind the membrane phospholipid phosphatidylserine, which becomes exposed on the surface of apoptotic cells and serves as an “eat-me” signal during phagocytic clearance via TTR-52 [203], supporting a potential role for MEGF10 in engulfment pathways. phospholipid phosphatidylserine exposure also occurs transiently during skeletal muscle myoblast fusion [204], and the phospholipid phosphatidylserine receptor BAI1 has been shown to promote myoblast fusion [205]. Together, these findings suggest that the EMI domain of MEGF10 may additionally contribute to myoblast–myoblast adhesion and fusion through interactions with phospholipid phosphatidylserine. The extracellular EGF-like repeats of MEGF10 consist of two distinct subclasses. Twelve are canonical EGF-like domains containing seven conserved cysteine residues, whereas five are laminin-type EGF domains characterized by eight conserved cysteines capable of forming four disulfide bonds. EGF-like domains are widely implicated in receptor–ligand interactions and intercellular signaling processes [206], suggesting that these extracellular motifs contribute to the adhesive and signaling functions of MEGF10. Consistent with this, MEGF10 has been proposed to participate in cell–cell adhesion and communication pathways [207]. The intracellular domain of MEGF10 may be dispensable, as myoblasts overexpressing a modified variant of MEGF10 of just the emilin-binding domain and EGF-repeats displayed similar functional characteristics as the full length MEGF10 transduction [208].

The Role of MEGF10 Across Tissues

MEGF10 is better established in the brain, where it serves as a key receptor mediating phagocytosis of debris by astrocytes [209]. Indeed, transient overexpression of MEGF10 on HEK293 cells induced phagocytotic engulfment of debris, matching this finding. In astrocytes, MEGF10 mediates this process through binding to C1Q receptor, establishing the first proof-of-concept biological finding that MEGF10 binds directly to another protein to regulate a functional role. Further supporting its role as a regulator of engulfment across tissues, in *C. Elegans* MEGF10 directly modulates engulfment, established as the mammalian ortholog of CED-1 protein in *C. Elegans* [207]. Lastly, in Hela cells, direct and transient overexpression of MEGF10 also induced phagocytosis through interactions with ABCA1 [210]. These findings suggest that MEGF10 may play a role in the engulfment of the basal lamina, supporting stem cell niche formation.

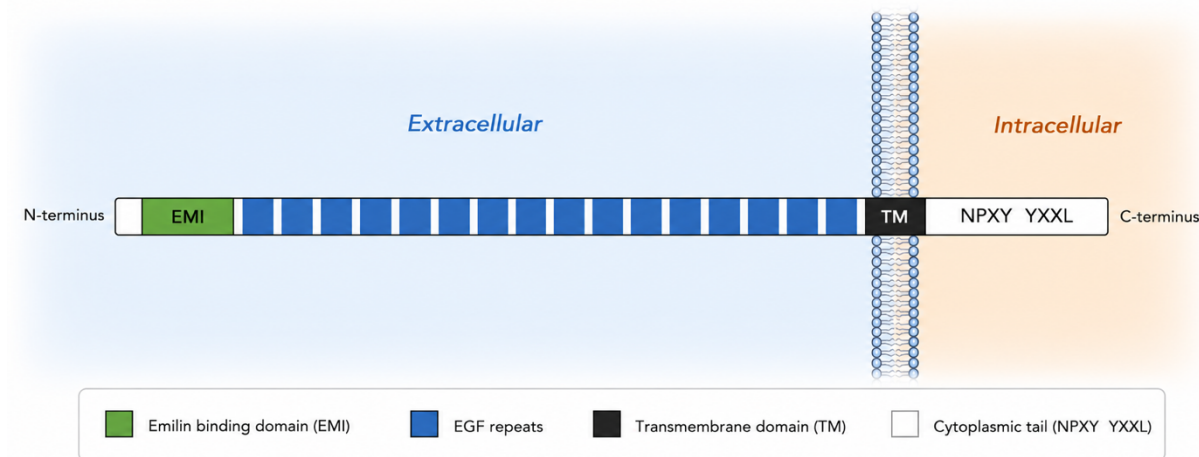


Figure 1.8: The Structure of MEGF10

A. Cartoon of the molecular structure and integrity of Multiple EGF-Like Domains 10 (MEGF10). Extracellular components (left) include Emilin binding domain and EGF repeats. The transmembrane element sits within the cellular plasma membrane. Intracellular components (right) are not well understood, but are much smaller in size and are summarized by a cytoplasmic tail.

MEGF10 Signaling in Skeletal Muscle

MEGF10 in skeletal muscle seems to wear many hats across cell types. It is highly expressed in activated satellite cells and myoblasts that are contributing to the regenerative program [211]. MEGF10 also seems to be expressed on satellite cells, although at a much lower level suggesting its involvement in early regenerative dynamics, and its overexpression enhances proliferation in myoblasts. This study also found that MEGF10 overexpression inhibited differentiation, while silencing of MEGF10 impaired self-renewal dynamics and led to precocious differentiation. Interestingly, MEGF10 silencing also led to a reduction in Notch signaling molecules like NOTCH1 and NOTCH3.

Recent literature has shed further light on the mechanisms of MEGF10 biology and how its mutations may give rise to EMARDD pathology. Recapitulating an EMARDD phenotype in C2C12 immortalized human cells through a p.C774R mutation yielded impairments in myoblast proliferation and migration [212]. Further, they generated a mouse model for EMARDD with a MEGF10^{-/-} model system, finding similar changes in cellular behavior suggesting MEGF10's involvement in activation status and early differentiation dynamics of muscle stem cells. MEGF10^{-/-} in an MDX model system (double knockout) yielded severely impacted mice with abnormal posture and spinal arrangement, and their histology displayed fibrosis and centralized nucleation in line with stunted regeneration. The fibrosis noted in this mouse model accumulates rapidly upon each injury event [153]. This study also suggests that NOTCH1 may directly interact with MEGF10 in skeletal muscle, although these data have yet to be confirmed through mechanism of action studies. Lastly, MEGF10^{-/-} mice were identified to have higher rates of death through development and early adolescence, likely due to a weakened diaphragm, which is also seen in patients with EMARDD [208]. Subsequent overloading of the EDL muscle by removal of the

adjacent tibialis anterior muscle and comparison to contralateral control revealed that MEGF10^{-/-} mice fail to display muscle hypertrophy and indicate a massive reduction of satellite cells.

Subsequent attempts to ameliorate EMARDD pathophysiology are in the works, although a final study identified that selective serotonin reuptake inhibitors may help to rescue the detrimental effects of EMARDD on skeletal muscle regenerative dynamics [213]. A thorough screening pipeline to generate candidates of interest revealed sertraline as an appealing molecule to rescue proliferative effects of EMARDD. This study's strength was through the CRISPR disease modeling of EMARDD patient derived iPSCs, which were then differentiated to skeletal muscle myoblasts for testing with sertraline and defining the EMARDD pathology in a dish. While they fail to showcase any myogenic readouts of their myoblasts, they showcase that EMARDD patient iPSC myoblasts have significantly reduced MEGF10, PAX7, MYOD1, ITGA7, and Notch signaling factors HEY1, and NOTCH1. Their studies on patient derived EMARDD iPSC models are incomplete, although they apply sertraline to a zebrafish model and showcase rescued survival – however, they never applied this drug to their mouse model of EMARDD.

To date, current literature has defined clear involvement of MEGF10 in skeletal muscle and its regeneration but there are several discrepancies about the role it plays, and the signaling mechanisms and binding partners it assumes are unclear. We have revealed some association with Notch signaling as a transmembrane protein, likely receiving signaling from external ligands and triggering a downstream signaling cascade within the cell [214]. Canonical Notch signaling is well described, with cell-cell signaling being the main initiation of this signaling pathway (**Figure 1.9**). JAG1 and JAG2 are described Notch signaling ligands that bind to extracellular domains of NOTCH1-4 [215-217]. Other ligands that bind to NOTCH1-4 include the Delta-Like Ligand family (DLL), which are expressed on skeletal muscle myofibers and represent an axis of

interaction between stem cell and myofiber, and when conditionally removed, self-renewal is significantly impaired [218, 219]. The intracellular domain of NOTCH1-4 is then cleaved by gamma-secretase to free the Notch intracellular domain (NICD) which then enters the cytoplasm enroute to the nucleus – this cleavage step can be blocked through administration of DAPT, a gamma-secretase inhibitor [220]. ADAM10 has also been demonstrated to play a role in assisting the gamma-secretase dependent cleavage of NICD [221]. NICD enters the nucleus and triggers downstream transcription and gene expression including the expression of HEY1 and HES1 [222, 223]. Notch gene expression is co-regulated by MAML and RBPJ, transcription factors that are integral for Notch signaling and its role in tissue homeostasis [124, 224]. With the establishment of canonical Notch signaling comes the postulation of MEGF10's role in noncanonical Notch signaling – what role it has in mediating or regulating this process is central to its mechanism in skeletal muscle regeneration. It is unknown whether MEGF10, which is a transmembrane receptor similar to NOTCH1-4, operates in a similar fashion to NOTCH 1-4 – it receives a stimulus from a ligand binding partner on an adjacent cell and this triggers cleavage of the intracellular element of MEGF10 which traverses to the nucleus for gene expression initiation. Or MEGF10 once bound by ligand partner may recruit NICD to trigger its downstream activation of Notch signaling candidates. Further, MEGF10 may directly interact with other transmembrane receptors, like NOTCH1-4, to initiate their signaling cascade. Thus, while Notch signaling is well described, MEGF10's role in this process is an exciting element to uncover, especially in the context of skeletal muscle niche support and tissue regeneration. The importance of Notch signaling is unprecedented in skeletal muscle – in Golden Retrievers with a novel mild phenotype for DMD, endogenous upregulation of Jagged-1 was observed, highlighting both JAG1 and Notch signaling globally as a pathway to target to ameliorate the pathophysiology of DMD [217].

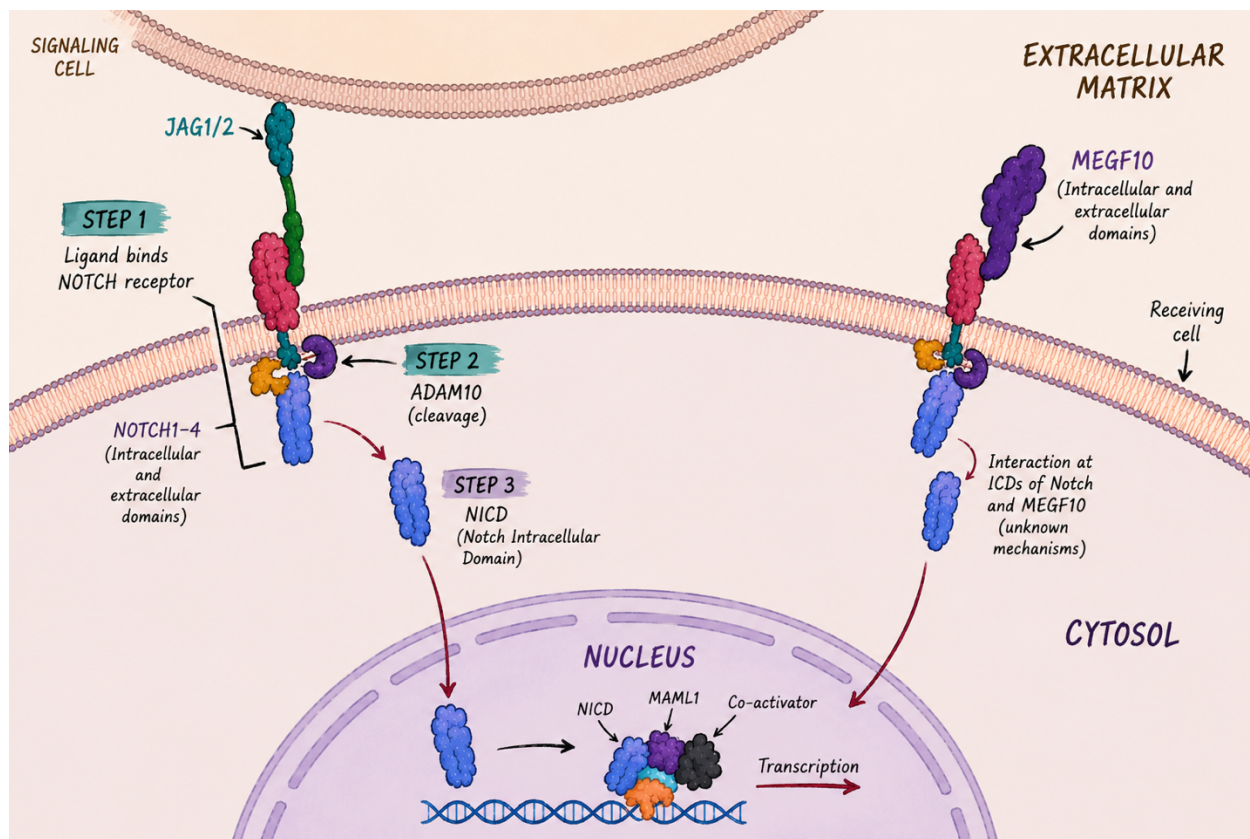


Figure 1.9: The Role of MEGF10 in Canonical Notch Signaling

A. Schematic portraying the proposed role of MEGF10 in Notch signaling. The right side indicates canonical Notch signaling cascades, initiated by cell-cell interactions and ligand/receptor binding that mediates this interaction, such as JAG-NOTCH. Intracellular domains of NOTCH are cleaved by ADAM10, releasing NICD (Notch intracellular domain). NICD enters the nucleus and triggers downstream transcription with support of co-activator MAML1. The left side illustrates a proposed role of MEGF10 in this pathway to trigger NICD cleavage, where MEGF10 interacts with NOTCH binding to trigger a similar NOTCH-dependent cascade of transcriptional activity.

1.8: An in Depth Look at Contemporary Spatial Transcriptomic Approaches to Study Engrafted Stem Cell Dynamics

Taken from my manuscript: Ben Clock & Michael Hicks. Human Skeletal Muscle Niche

Formation and Analysis with Spatial RNA Sequencing. Methods Mol Biol. 2025;2939:1-22

Skeletal muscle is responsible for voluntary contractile movements, and to meet the functional demands of exertion, it is highly regenerative. To maintain its mass, skeletal muscle contains endogenous stem cells, known as satellite cells, that proliferate, differentiate, and fuse to form multinucleated myofibers. Satellite cells transition through dynamic states of quiescence and regeneration by physical contact and interactions with their niche, which is primarily comprised of myofibers and the basal lamina, an extracellular matrix that weaves its way throughout skeletal muscle.

While the dynamics of the muscle regeneration and niches can be studied in model organisms such as in mice, less is known about how the human satellite cell niche, including human ligand-receptor binding, regulates stem cell states. A major gap in knowledge of the field of human muscle stem cell biology is understanding how the niche regulates satellite cell behavior and support through regeneration and development. Satellite cell niche formation is highly significant because in cases of muscle aging and disease, the niche often becomes dysregulated and leads to improper stem cell niche formation.

Transplantation studies are an appealing model for niche formation and colonization of satellite cells. Contemporary practices can engraft human muscle stem and progenitor cells from post-natal, fetal, or human pluripotent stem cell (hPSCs) sources into animal models to assess and study stem cell niche colonization and myofiber regeneration of the transplanted human stem cells. Importantly, just as a subset of satellite cells return to their niches to maintain the stem cell pool

to respond to future injuries as regeneration completes, engrafted muscle cells also need to form a niche to support long-term cell therapies. However, minimal research has been conducted on the transcriptomic changes of engrafted human stem cells to describe how niche colonization occurs, largely due to the technical and biological complexities that exist to address that question in studies.

Spatial RNA-Sequencing (Spatial RNAseq) remains among the most promising approaches to studying engrafted human muscle stem cell niche formation and myofiber development. Spatial RNAseq provides additional spatial information about muscle stem cell niche colonization that is lost upon disruption and digestion of skeletal muscle needed for other transcriptomic approaches such as single cell or single nucleus RNA sequencing. In a recent study, we used spatial RNAseq to distinguish several developmental states of engrafted human myofibers [37]. We were also successfully able to capture and analyze populations of engrafted PAX7+ human muscle stem cells near subsets of myofibers and could distinguish unique ligand-receptor interactions that we demonstrated better support PAX7+ cell survival.

To date, there are several spatial RNA sequencing platforms, and the number of new technologies is rapidly growing. Sequencing platforms include 10X genomics (Visium and Xenium), Nanostring/Bruker (GeoMx and CosMx), Visgen (MERFISH), Spatial Genomics (SeqFish), STOmics (Stereo-seq), and others. These spatial RNAseq technologies are typically probe-based or barcoded spot-based. These platforms enable transcriptomic interrogation of cells within their spatial environment such as engrafted human PAX7 cells undergoing niche formation. In **Table 1.1**, we highlight two systems, including the Nanostring/Bruker GeoMx and Visium with and without high definition (HD), which allow for whole transcriptome analysis. Many of the higher-resolution systems, such as Xenium, CosMx, and MERFISH, currently require predefined

probe sets limited to several hundred genes, although these probe sets are continuing to improve each year.

For this chapter, we discuss and outline a protocol for the Nanostring/Bruker GeoMx system as we have optimized this for precise detection of PAX7⁺ human muscle cells following transplantation per our publication [37]. Thus, we describe the process of human muscle stem cell engraftment and spatial RNAseq analysis of the tissue to study human PAX7 cells and myofibers. This procedure is broken down into (1) engraftment of human muscle stem cells, (2) analysis of engrafted efficiency, (3) slide preparation for spatial RNAseq, and (4) RNA oligo collection and spatial RNAseq data analysis.

Materials

Human Muscle Stem Cell Culturing

1. SkGM-2 Skeletal Muscle Cell Growth Medium-2 BulletKit (CC-3245 Lonza).
2. bFGF.
3. TrypLE Dissociation Buffer (12604013, Gibco).
4. Tissue Culture Treated 6-well plates (Corning).

Human Muscle Stem Cell Engraftment

1. Ice bucket.
2. Centrifuge.
3. Hanks Balanced Buffer Sulfate (Gibco).
4. Barium Chloride (Sigma).
5. 0.9% NaCl Saline Solution.
6. Mouse Fur Trimmers.
7. 26 gage Hamilton Micro-Syringe (Hamilton).

8. 50 mg/mL Carprofen/Rimadyl.

9. Insulin Injection Needles.

10. Saline Solution.

11. Isoflourane.

Tibialis Dissection and Cryo-Sectioning

1. Straight Sharp Scissors (Fine Science Tools).

2. Fine Forceps (Fine Science Tools).

3. Liquid Nitrogen & Container for Liquid Nitrogen.

4. Isopentane.

5. Wood pieces wrapped in parafilm.

6. 70% Ethanol

7. Tin Can.

8. Scintillation Vials.

9. OCT

Immunofluorescent Staining

1. Hydrophobic Barrier Pen (Thermo Fisher).

2. 16% Paraformaldehyde (Thermo Fisher).

3. 1X Dulbecco's Phosphate Buffer Saline (Gibco).

4. Tween-20 (Sigma).

5. Triton-X (Sigma).

6. Goat Serum.

7. Secondary Antibodies, Examples:

Goat anti-Mouse IgG1 (Thermo Fisher)

Goat anti-Rabbit IgG (Thermo Fisher)

Goat anti-Mouse IgG2b (Thermo Fisher)

*For Nanostring Alexafluor-532, Alexafluor-594, and Alexafluor-647 are required

8. Primary Antibodies, Examples:

Lamin AC (Thermo Fisher)

Spectrin, Laminin (Thermo Fisher)

PAX7 (PAX7, DSHB)

Dystrophin (MANDYS106, DSHB)

MYOD1 (D8G3, Cell Signaling Technologies)

MYOG (5FD, DSHB)

9. Blocking Buffer; 3% Bovine Serum Albumin, 0.5% Triton, in DPBS.

10. Hoechst, 1:1000 (Sigma).

11. Flouromount (Sigma).

Nanostring GeoMX Slide Preparation and Data Collection

1. 16% Paraformaldehyde (Thermo Fisher).

2. RNase-free DEPC-treated water:

200 μ L of diethylpyrrocarbonate (DEPC) to 200 mL dH₂O for a final concentration of 0.1% DEPC

in a glass container

3. RNase-free DEPC-treated PBS.

4. 20X SSC. Sigma-Aldrich (S6639).

5. 100% deionized formamide. VWR (VWRV0606).

6. 10% Tween- 20 Nanostring kit.

Buffer R

Buffer W

RNA Probes

Buffer S + Proclin

7. Cyto13, Alexafluor-488.

8. Proteinase K (New England Biolabs, P8107S).

9. Superfrost Plus slides.

10. Grace Biolabs HybriSlip (Grace BioLabs).

11. Grace Biolabs Slide Barrier (Grace BioLabs).

12. Glass Coplin Jars.

13. RNase Away.

14. Hybridization Oven.

Workflow

Inducing Localized Muscle Injury with Barium Chloride (BaCl₂)

(Please see Note 1 on mouse aging and genotyping.)

(Please see Note 2 on use of BaCl₂ compared to other muscle injury methods.)

*To be performed about ~24 h prior to cell transplantations

1. Place mice under a controlled 1.8–2.2% isoflurane gas to anesthetize supplied with 2% Oxygen flow. Fit the tubing with a nose cone to sustain isoflurane and oxygen flow throughout the procedure.

2. Thoroughly shave the lower leg that will be injected with hair clippers.

(a) Use ethanol wipes to clean off hair and better visualize the shape of the TA muscle through the skin.

3. Subcutaneously inject 200 μ L of 1.0% Carprofen/Rimadyl solution using a 27–29 gauge insulin syringe Gently lifting the skin of the mouse abdomen,

(a) Make a working stock add 100 μ L of 50 mg/mL Carprofen/Rimadyl to 10 mL of 0.9% NaCl physiological saline solution, and store at 4 °C.

4. Intramuscularly inject 50 μ L of 1.2% barium chloride into the TA muscle.

(a) Make a working stock by adding 120 mg BaCl₂ powder to a 10 mL vial of 0.9% NaCl physiological saline solution, and store at 4 °C.

(b) Angle the needle distally, towards the foot, and insert near the knee, continue to insert into the central belly of TA.

(c) Tip. Slowly and carefully inject. Successful injection should result in an immediate ballooning of the TA that will transiently subside. Slowly pull the needle out as to not draw solution back through the needle prick and/or to prevent bleeding.

Transplantation of Human Pluripotent Stem Cell-Derived Skeletal Muscle Progenitor Cells

(Please see Note 3 on use of other cell types for transplantation.)

1. Prepare hPSC SMPCs for transplantation accordingly: between days 35 and 50 of directed differentiation, dissociate hPSC SMPCs from culture using TrypLE buffer and titrate several times to break apart 3-dimensional structures.

2. Filtered hPSC SMPC through 70 μ M mesh filter attached to a 15 mL conical tube, pelleted at 300 $\times$ g, and stained for flow cytometry markers such as HNK1, ERBB3, and NGFR (**Figure 1.10 A**).

3. Allow for FACS-sorted ERBB3+NGFR+ hPSC SMPCs to recover in SkGM2 + 10 ng/mL bFGF for 2–4 days. This allowed us to obtain at least one million cells per mouse for engraftment (**Figure 1.10 B**).

4. On the day of engraftments, collect cells using TrypLE for dissociation, and pellet at 300–500× g. Resuspend cells in 1 mL Hanks Buffer Saline Solution (HBSS) and perform cell count. Centrifuge a second time in a 1.5 mL tube and pellet at 300–500× g.
5. Resuspend hPSC SMPCs at a concentration of 1×10^6 cells per 5–10 µL in HBSS.
6. Keep cells on ice while transporting to the vivarium to prevent cell clumping which can be obvious due to bubble formation in the cell solution. This will help with reproducibility.
7. Anesthetize mice using 2% isoflurane, and inject 5–10 µL of cells in solution (1–1.2 million cells total) into the tibialis anterior (TA) muscle using Hamilton micro-syringes. Angle the needle distally, towards the foot, with the bevel side down, and insert near the knee; continue to insert into the central belly of TA.

(a) Slowly inject the cells, gently rotating the needle 15° clockwise and counterclockwise as cells are injected to open up space and allow for improved biodistribution.

(b) Once finished, leave the needle in while continuing to slowly rotate the needle for 10–20 s before slowly pulling the needle out as to not draw cell solution back through the needle prick and/or to prevent bleeding.

8. Titrate Hamilton syringe with 70% EtOH and milliQ H₂O in between mouse injections, which will prevent needle clogging and cross contamination.
9. Titrate Hamilton syringe using 10% bleach, 70% EtOH, and milliQ H₂O when completed for reuse of needle.

Dissecting and Flash Freezing the Tibialis Anterior (TA)

(Please see Note 4 on timing of dissection.)

1. Before starting or while mice are being euthanized, prepare your freezing apparatus. This consists of a metal/tin can 1/3 full of isopentane placed inside a bucket of liquid nitrogen.

(a) Tip: The isopentane within the tin will eventually freeze solid if left in liquid nitrogen too long. Circumnavigate this by keeping a watchful eye on the level of liquid isopentane and frozen ring around the periphery of the tin can. Additional isopentane may be added, or the can may be removed from the liquid nitrogen bath while waiting for the mouse to be euthanized. A small frozen ring around the periphery of the tin can indicates the isopentane is chilled enough to begin tissue collection. Isopentane that is insufficiently cooled can result in freeze damage of the muscle tissue.

2. Before starting, pre-label scintillation vial caps with ethanol-proof cryogenic marker. Fill each scintillation vial 50–70% with isopentane and place on dry ice to chill for later tissue collection.

3. Euthanize mice following ethical guidelines using CO₂ and/or cervical dislocation.

4. Thoroughly spray the engrafted limb of the mouse body with 70% ethanol.

(a) Tip: This helps to prevent the fur from sticking to the scissors during dissection, when removing the skin around the leg.

5. Using tweezers, gently lift up on the skin above the mouse TA. Then, using dissection scissors, slice open the skin and begin to remove the skin to expose the TA. Continue until all skin is removed from the knee to the ankle of the mouse, exposing the TA muscle and tendons that connect it to the ankle.

6. When the outline of the TA is visible, take the dissection scissors and gently slice along up and down the shin bone of the mouse. Then, slice along the lateral of the TA muscle. Finally, slide the scissors deep underneath the TA muscle and gently slice it lengthwise to separate it from the leg.

(a) Tip: When in doubt, be more inclusive than exclusive. It is better to include additional tendon and muscles like the EDL rather than being too conservative and risk losing the region your cells have engrafted to. We often find donor cells in both the TA and EDL.

Collection of one or more muscles may also depend on factors such as weight measurements.

7. Carefully place the dissected TA onto a thick dab of OCT on a wood chip wrapped in parafilm. Swish the TA to evenly mix in OCT.
8. Using a longer pair of tweezers, grab the woodchip with the muscle sitting on top.
9. Submerge the tissue with more OCT so that all sides of tissue are covered and immediately place into the tin can with isopentane before the OCT has time to drip off the wood chip. Hold here for ~20 s to flash freeze the TA thoroughly.
10. Use long forceps to transfer the pre-labeled scintillation vial containing isopentane from the dry ice into the liquid nitrogen for 10–20 s to assimilate to the same temperature as the flash-frozen tissue.
11. Transfer your flash-frozen tissue into the scintillation vial by gently tapping it with your tweezers until it falls off the wooden chip and into the vial.
12. Store scintillation vial and tissue on dry ice temporarily until all tissues are collected, and transfer to –80 °C freezer for long-term storage, until ready to section.

(a) Tip: For spatial RNA seq, tissues can remain in isopentane-filled scintillation vials for several months without noticeable loss of RNA quality. However, once sectioned, tissues need to be processed within the same week, or RNA quality will be quickly degraded.

Labeling Strategy for Sectioning the Tibialis Anterior

The Hamilton needle injection site will vary for every cell transplantation. In order to find the epicenter and biodistribution of engraftment throughout the TA, we describe a Sampler/Quadrant sectioning strategy below. This approach allows for the user to quickly survey for the epicenter of

muscle engraftment on sample slides and then perform more in-depth analysis on regions of interest in the quadrant slides.

1. Come up with a tissue sectioning strategy such as the one described below and in Fig. 2a. Be sure to record sectioning strategy in a lab notebook for later analysis.

(a) Section all tissues at 10 μm .

(b) Collect six sampler slides per tissue and four quadrant slides corresponding to tissues on the sampler slide for a total of 36–48 microscope slides per tissue.

(c) First, collect a tissue along the first row for each sampler slide. Skip 40 μm between slide collections so that each tissue is 50 μm apart.

(d) Second, collect two tissues on four total quadrant slides.

(e) Tip: Mount the two tissues on quadrant slides within 1 cm as these will be used for GeoMx analysis. The GeoMx kit designates 250 μL reagent to each microscope slide for a total of 4 slides. These reagents are designed to cover an entire microscope slide; however, if the tissue covers only $1/3$ – $1/2$ of the slide, the volume of reagents can be reduced accordingly. This can result in cost savings of \$10,000+ by increasing the number of slides per spatial RNA seq kit to 8–12.

(f) After tissue is collected, place the quadrant slides into a microscope slide box sitting on top of dry ice to keep the tissues cool. This minimizes freeze fracture of the tissues and will preserve the RNA better for sequencing, as opposed to leaving quadrant slides at room temperature.

(g) Third, before starting on the second row of the sampler slides, discard 500 μm of tissue.

- (h) This approach will result in 1200 μm tissue depth between sampler row 1 and 2 and so on. 6–8 tissues can be collected onto a sampler slide, which would allow for a surveying depth of 1 cm, which is approximately the length of a mature TA.
2. Pre-cool the cryostat to $-22\text{ }^{\circ}\text{C}$. Then, pre-cool the paint brush and tweezers inside it.
 3. Using pre-cooled tweezers, transfer the tissue block to a thin layer of OCT atop a sectioning puck. Angle the tissue perpendicularly so you can section it transversely.
 4. Load the puck with the tissue into the puck holder of the cryostat and load the sectioning blade carefully into the blade holder.
 5. Trim off the first 1000 μm of tissue. This is the very end of the TA and rarely holds high numbers of transplanted cells.
 - (a) For successful downstream spatial RNAseq probe collection from sectioned tissues, sections must be placed in the scan area (the green area) in the center of the slide and be no larger than 36.2 mm long by 14.6 mm wide. They should not overlap the slide gasket or the tip calibration area (this is the triangular region to the left of the green scan area), so they do not impede with downstream spatial RNAseq oligo collection.
 6. Store sectioned tissue slides at $-20\text{ }^{\circ}\text{C}$ long-term in a labeled slide box wrapped in tin foil.
 7. Store the slides to conduct spatial RNAseq at $4\text{ }^{\circ}\text{C}$ in a dry box to avoid any liquid accumulating on the tissue and slide, as this will decrease RNA quality.
 - (a) Plan to perform spatial RNA seq on sectioned slides stored at $4\text{ }^{\circ}\text{C}$ as soon as possible, preferably within 72 h for maximum RNA integrity.
 - (b) Many things can serve as a “dry box,” for example, an empty pipette tip box layered with clean, dry Kim wipes.

Assessment of Engraftment Efficiency Prior to Spatial RNA Seq

(Please see Note 5 on timing for immunostaining prior to spatial RNAseq.)

1. Bring two sampler slides from storage to room temperature. Allow them to thaw for 5–10 min before drawing a hydrophobic barrier around the tissue.
2. Let slides acclimate at room temperature for 20–30 min to help with antigen presentation.
3. Fix tissues with 2% PFA for 15 min. Then, wash off 3 times with DBPS-T.
4. Permeabilize tissues with 0.5% Triton-X for 10 min.
5. Block tissues with blocking buffer (see Materials section) + 10% fresh goat serum for 1 h.
6. Stain tissues overnight at 4 °C with primary antibodies of interest.
 - (a) For quick analyses, use a combination of PAX7 (1:30) to mark muscle stem cells, Laminin (1:200) to mark basal lamina, Spectrin (1:50) to outline human myofibers, and Lamin A/C (1:200) for transplanted human nuclei.
7. Wash off the primary antibody 3 times with DBPS-T.
8. Stain tissues with secondary antibody (1:250) for 1 h, covered from light. From here on, avoid prolonged exposure to light as it may photo bleach the antibody staining.
9. Wash off secondary antibody 3 times with DPBS-T.
10. Stain tissues with DAPI or Hoechst (1:1000) for 10 min covered from light.
11. Apply drops of Flouromount or Vectashield and mount the slide with a coverslip.
12. Image the stained sampler slides.

Spatial RNA Seq Protocol: GeoMX Spatial RNA Sequencing Slide Preparation

1. Take out the quadrant slide(s) you want to move forward with from the 4 °C dry box. Place them into a Coplin jar with 1X DPBS for 5 min.

(a) Tip: The following steps until the blocking step (step 9) should be conducted in clean, RNase-free Coplin jars. Use RNase Away to clean all surfaces and container slides may come in contact with.

2. Fix muscle sections with 4% PFA for 30 min.

3. Wash off the muscle sections with 1X DPBS twice for 5 min each.

4. To expose RNA targets, we empirically determined that tissues needed to be treated with 0.025 µg/mL proteinase K for 5 min at 37 °C as higher concentrations cleaved antigen binding sites.

(a) Tip: PAX7 protein epitopes are lost at higher concentrations; thus, use a lower concentration than recommended by Nanostring. Please note that if you have antibodies untested with this protocol, you may need to optimize this step.

5. Wash off the muscle sections with 1X DPBS twice for 5 min each.

6. For in-situ hybridization, apply the human whole transcriptome atlas (WTA HS) RNA Probes to the tissues and cover the tissue and probes with a Grace Bio-Labs HybriSlip.

(a) Tip: The original Nanostring protocol calls for 200 µL Buffer R, 25 µL RNA probes, 25 µL DEPC water to cover one slide. However, we found that when muscle sections were placed in a smaller area, the volume of probes needed to completely coat the tissues could be reduced, respectively. Thus, to minimize the use of the reagents, save costs, and increase the number of runs per kit, we cut the recommended probe volume by a third or half. This can be accomplished by cutting the HybriSlip respectively.

(b) Tip: Add the probe solution dropwise atop the tissue to avoid bubbles when adding it. Also seek to avoid bubbles when placing the HybriSlip atop your slide. Bubbles may interfere with the probes binding to the RNA upon the tissue.

7. Incubate in a hybridization oven at 37 °C overnight.

8. The following day, HybriSlips were removed in 2X SSC-T buffer, and tissue slides were washed twice for 25 min with a solution made of 4X SSC and deionized formamide.

(a) Tip: Warm the formamide to room temperature before using it.

9. Wash the slides two more times in 2X SSC for 5 min each.

10. To add morphology markers, block with Buffer W for 30 min, then add primary antibodies in Buffer W for 90 min.

(a) Muscle stem cell and activation cocktail. PAX7 (DSHB, 1:30), MYOD1 (CST, 1:100) Spectrin (1:50), Lamin A/C (ThermoFisher, 1:200).

(b) Myoblast-myocyte cocktail. MYOD1 (CST, 1:100), MYOG (DSHB, 1:100), Spectrin (1:50), Lamin A/C (ThermoFisher, 1:200).

(c) Tip: Wash and stain slides using a Grace Bio-Labs silicon coverslips to avoid hydrophobic pen usage. We found that hydrophobic pen usage caused the GeoMx Gasket to leak when loading the samples onto the machine for ROI selection.

(d) Tip: This primary antibody incubation step can also be performed overnight at 4 °C.

This may allow more time for ROI selection, which can be a tedious process.

11. Wash tissues three times with DPBS, 5 min each.

12. Add secondary antibodies conjugated to Alexa Fluor 532, 594, and 647 (at 1:250) alongside SYTO-13 Nuclear Dye (Nanostring, 1:50) in DPBS for 60 min.

(a) Tip: From this step forward, keep slides covered from light to protect secondary antibody fluorescence.

13. Wash tissues in DPBS three times, 5 min each.

14. Transport the tissues in a Coplin jar of 2X-SSC, shielded from light, to the GeoMx machine for scanning and ROI selection (Fig. 3).

RNA Probe Collection

(Please see Note 6 on the Nanostring GeoMx user interface and Note 7 on spatial transcriptomic sensitivity.)

1. Log into the Nanostring GeoMx Machine. Select “New Data Collection.” The machine will take a moment to calibrate at this point.

2. The machine has an easy step-by-step walk through and visualization on how to load your samples and collection plate into the GeoMx machine.

- (a) Tip: Collection plates can be reused if they have not been fully filled up with 96 different select ROI samples. This will allow for multiplexing of spatial transcriptomic data collections from different runs onto the same plate, which can save costs at the library construction step.

3. Take the tissues out of the Coplin jar, tap dry the slides with a Kimwipe, and set them down on the GeoMx slide chamber. Use a new clean Kimwipe to dry the edges of the slide where the magnetic sealing gasket will clamp down on the slide to avoid any possible leakage. Close the gasket and add 6 mL Buffer S + Proclin (Nanostring) dropwise to the slides in chamber.

- (a) Tip: Data collection on the GeoMx Platform is limited to four slides at a time and a total of 95 regions of interest (ROI's). The first well of the 96-well collection plate is always going to be a negative loading control conducted by the GeoMx machine.

4. After loading the plate and slides into the machine, the software will automatically hydrate slides, conduct a brightfield scan, and image the entire slide with 20X objective so the user can determine where the tissues are. This process can take 5–10 min per slide. Once the quick scan is complete, check that the regions of tissue are being included as input for the visualization/in-depth scan.

(a) Tip: The quick scan often picks up debris or other non-specific pieces on the slide. This can be adjusted by sliding the green levers either lengthwise or widthwise to be more selective on the regions to be imaged in depth. If the machine has left out a tissue that needs to be imaged, please repeat this quick scan step.

5. Next, select the parameters to scan the slides and where to save the experiments. For these imaging parameters based on antibody fluorescence, we recommend using the LaminAC/Spectrin channel to focus on and exposure times of 250–500 ms for all channels except PAX7, which we scan at 600–800 ms.

(a) Tip: These values will likely need to change on a machine-to-machine basis. An imaging scan can always be repeated as needed, but after it is completed, fluorescence intensity can be manipulated to display a clearer stain. It is much easier to increase intensity to pick up lower stains than it is to reduce intensity if the parameter exposures are too high.

(b) Tip: This step is very long. Depending on how many antibody channels are present, the exposure time, and the area to be imaged (defined by the quick scan), this step can take upwards of 30 min per slide. Importantly, once one slide is finished, the next step of ROI selection can begin immediately on that slide while the other slides are still being imaged.

6. Begin ROI selection and segmentation based on fluorescence intensity for the targets of interests, such as human cells, human muscle stem cells, and human myofiber. ROIs will be entirely dependent on the user's experiment. Several relevant notes are included below by category.

(a) We have used two slides for morphology markers per treatment to generate myogenic lineage trajectories of engrafted human cells as follows:

(b) Pristine human muscle stem cells PAX7+ MYOD- LaminAC+ Syto13+

(c) Activated human muscle stem cells: PAX7+ MYOD+LaminAC+Syto13+

(d) Activated human myoblasts: MYOD+ MYOG- LaminAC+ Syto13+

(e) Differentiated human myocytes: MYOD- MYOG+ LaminAC+ Syto13

(f) Human Myofibers: Spectrin+ LaminAC+ Syto13+

7. Statistical Data Collection: Since each ROI can be treated as a Bulk RNAseq dataset, we highly recommend the collection of at least three ROIs for each group to be analyzed. For example, PAX7 cells are desired, collect at least three ROIs of PAX7 cells.

(a) Tip: Limitations: Only collect data from 95 ROIs, as the machine calibrates one loading control.

8. Negative Controls: While the RNA probes are human-specific, there is always going to be some level of background noise from data collection upon a mouse skeletal muscle tissue. Therefore, collecting negative control ROIs from regions of mouse tissue with no human stem cell engraftment will allow for the determination of background level noise at the data analysis steps.

9. Segmentation: One way to collect data is on the entire region selected by an ROI, but we have found that this is not as precise as segmenting your ROIs and collecting data on the most fluorescent regions of your morphology markers of choice. Therefore, drawing ROIs and segmenting off PAX7+LaminAC+Syto13+ triple positive regions is the ideal way of collecting engrafted human PAX7 cells. Segmentation strategies should be optimized from person to person, including (but not limited to) hole size, particle size, and fluorescent threshold.

(a) Tip: For data collection of a rare nuclei marker like PAX7, one cell will not be enough for analysis. We have found that collecting data on 5–7 cells is the minimum required input for sufficient collection.

10. Approve the ROIs and their segmentation selection strategies. The GeoMx platform will begin the process of photocleaving and collecting RNA probes from these ROIs into the loaded 96-well collection plate.

(a) Tip: Let this process run overnight. It will take at least a few hours depending on how many ROIs have been selected. Additionally, the evaporation of the liquid in each well leaves behind dried barcodes, which makes the first step of library construction more streamlined.

11. Use the collection plate to construct libraries with the GeoMx Seq Code Pack A-D, allowing for barcoding of each well collected and subsequent pooling of the libraries for Illumina sequencing.

12. We sequence 2X the recommended coverage (2000 reads/ μm^2).

Spatial RNA Seq Analysis

1. FastQ files generated from the Illumina sequencing run must first be converted to DCC files which can then be uploaded into the GeoMx machine for analysis. DCC files identify the human transcriptome probes present in each sample and maintain the barcoding integrity of each sample with the well ID and plate layout. There are several ways to generate DCC files that are well described in the Nanostring University Manual (<https://university.nanostring.com/>). We recommend the command line interface method (CLI) as it is the most versatile and cost-effective strategy available on either Linux or Windows platforms.

(a) In addition to the fastq files as an input, the configuration.ini file generated from the illumine sequencing run is also required as an input to match the fastq files to their respective well ID's.

2. Upon generation of DCC files for each collection plate, upload the DCC zip files into the GeoMx hardware using the Data Upload option. The machine can cross-reference the collection plate and well IDs from the DCC file and automatically pair the raw reads into the experiment that was created and saved with the sample images and scans.
3. Begin by running a general QC and confirm all ROIs passed QC analysis.
 - (a) The platform may flag smaller ROIs, especially the segmented PAX7 cells, as being low area or low nuclei or both. This flag can be manually ignored as the data can still be normalized. However, ROIs can be excluded from downstream analysis by deselecting them at this step.
4. For filtration, we filtered at 1–5% of limit of quantification and set the threshold at Higher of LogQ and User Defined Value (Fig. 4).
 - (a) As shown in **Figure 1.13**, after filtration, the whole transcriptome gene number of ~18,700 genes are reduced to ~4900 genes, which are then used for downstream analysis. This filtration percentage will need to be optimized based on upstream parameters.
 - (b) Tip: Compare the raw read values of the negative control of mouse tissue collected to those of human regions of engraftment to help establish the filtration cutoff.
5. Normalize gene count data using the Quartile-3 (Q3) method.
 - (a) GeoMx spatial RNA seq data needs to be normalized to account for unequal ROI sizes and unequal numbers of cells collected within each ROI.
 - (b) Q3 normalization works by making a bell-curve Gaussian distribution of gene expression from all ROIs and then normalizing based on the 3rd quartile, genes expressed between 50% and 75% of each ROI.

(c) Tip: We found other normalization approaches, such as by ROI size or # of nuclei, were not effective for skeletal muscle because myofibers unequally distribute RNA based on size and have less dense nuclei than other cell types in muscle. Thus, Q3 normalization worked best.

6. Following normalization PCA plots of the data can be generated in the analyses tab, and examples are shown in **Figure 1.13B, C**.

(a) This is another good check point for quality control to identify outliers. By clicking on the individual points on the PCA plot within the GeoMx user interface these can be quickly mapped to the segmented ROI on the imaged tissue. If issues with segmentation were missed during oligo capture, such as over-segmentation of myofibers in the PAX7 cell ROIs, this is a good point for technical replicate exclusion.

7. For comparing different cell types or treatments, perform Linear Mixed Model (LMM) statistical tests with Benjamini-Hochberg (BH) correction.

8. Several visualizations, such as volcano plots and bar plots can be directly generated within the GeoMx user interface.

9. GeoMx analysis also provides .csv files, which can be input into third-party software packages such as R Studio for additional analysis.

R-Studio Analysis for GeoMX Nanostring Spatial RNAseq Data

(Please see Note 7 on Analysis of Spatial Transcriptomic data in R studio.)

1. Analysis of collected Spatial RNAseq data can be performed on the GeoMX platform itself, or it can be outsourced to R Studio. In R Studio, run DESeq2 for differential gene expression analysis and PCA plot generation, followed by the generation of volcano plots using the EnhancedVolcano package. Finally, data can be input into CellChat for accurate prediction of intercellular

interactions from the spatial RNAseq data. We have included links to stepwise interactive tutorials for these three packages below in this section.

2. The GeoMX platform includes a “data download” option at each step of the data analysis. We downloaded the pre-normalized dataset for DESeq2 analysis, since one of the first steps of DESeq2 is to normalize the data and thus needs an input of non-normalized counts data.

3. Format the .csv so that the rownames are the sample/ROI names and the column names are the gene names. Match this to a metadata file that matches the rownames exactly, while containing additional metadata in each column. For example, we included Cell_Type column to indicate whether a sample was PAX7 Cells, Human Myofibers, or Human Nuclei. An n = 3 of each sample and column metadata is required for DESeq2 to operate successfully.

(a) This dataset can now be uploaded into R Studio for DESeq2 differential gene expression and other downstream analyses.

4. DESeq2—Differential Gene Expression Analysis and PCA Plot Generation

https://github.com/hbctraining/DGE_workshop_salmon/blob/master/lessons/04_DGE_DESeq2_analysis.md

5. Enhanced Volcano—Volcano Plot Generation

<https://github.com/kevinblighe/EnhancedVolcano>

6. CellChat—CellChat Intercellular Communications Prediction Analysis

<https://github.com/sqjin/CellChat>

Notes

1. We usually keep the engrafted leg consistent to improve reproducibility, i.e., the right leg only, as BaCl₂ injury to both hind limbs affects rodent gait and duress. Human SMPCs readily engraft into immunocompromised NSG mice, including mice crossed to other strains with various levels

of muscular dystrophy. Generation of maximal levels of transplanted PAX7⁺ stem cells can be boosted by using a tamoxifen-inducible Pax7 ablation mouse model, which we hypothesize decreases niche occupancy or decreases donor-host cell competition during the regeneration phases of muscle engraftment.

2. We have attempted to transplant human cells into uninjured immunocompromised mice. However, these studies resulted in minimal donor cell engraftment, potentially due to lack of space or an inability to efficiently fuse with host myofibers without a regenerative stimulus. There are several ways to administer a regenerative stimulus to mouse muscle, and below we describe BaCl₂ as a common and consistent method of damaging myofibers while preserving the endogenous mononucleated cell response to regeneration.

3. The protocol below describes using hPSC SMPCs for engraftments, but primary human muscle stem and progenitor cells can readily be adapted using similar techniques after optimizations for including FACS antibodies and cell numbers for injection.

4. We typically perform tissue collection 30 days after transplanting human muscle stem cells in vivo, as regeneration is finished by this time point. However, based on experimental goals, this time frame can be modified to any time point.

5. The goal of assessing engraftment efficiency prior to spatial RNA seq is to find the quadrant with the most human fibers and PAX7 cells to get the optimal transcriptomics data. Because RNA transcripts rapidly degrade after sectioning i.e., within a week, these experiments should be done immediately prior to Spatial RNAseq Slide Preparation. In contrast, we have found that whole tissues embedded in OCT can remain in the -80 °C for several months and still yield high RNA quality. We typically perform immunostaining on a sample slide, which includes all sectioned quadrants to determine the regions of transplanted cells within 24 h of sectioning. Once the ideal

quadrant is identified, we move forward immediately to the next step for Spatial RNAseq Slide Preparation using serial sectioned quadrant slides of interest that we store for 24–48 h at 4 °C in a dry box while engraftment regions are being identified.

6. The Nanostring GeoMx Machine has an extensive but intuitive software user interface that can also be accessed remotely through wireless networks. General training on the GeoMx can be provided by Bruker technical support, institutional core staff, or through the user manual provided through <https://university.nanostring.com/>. Protocols for maintenance and buffer replacements for Nanostring GeoMx can also be found through the Nanostring university. Make sure all buffers are not expired and at sufficient volume prior to starting.

7. Skeletal muscle stem cells are in direct proximity to myofibers, which complicates the precise spatial capture of RNAs and sample purity. In some skeletal muscle stem cell ROIs, we found low levels of myofiber genes. The Erode function is one tool that can be used to increase stringency for RNA capture by increasing the boundary between myofibers and muscle stem cells. However, too much erosion will prevent sufficient number of oligos needed for sequencing. Other strategies we have employed include differentiation gene expression analysis between myofibers and muscle stem cells, which show the negligible amounts of myofiber genes in muscle stem cell ROIs are compared to myofiber ROIs in this comparison. We have not tried to segment ROIs containing no cells in direct proximity to myofibers as an approach to remove cell free mRNA contamination, analogous to SoupX pipeline in single-cell RNA seq, but such an approach might be used to control for the over-segmentation of contaminating RNA between adjacent cells.

8. While GeoMX platform can conduct differential expression analysis and generate some visualization plots, to have more control over the presentation and visualization of the data at hand, we recommend using R Studio to conduct our analysis.

Table 1.1: Comparison of Spatial Transcriptomic Platforms

Nanostring GeoMx DSP	Visium and Visium HD
Probe based	Barcode based
Tissue sections are placed on any microscope slide which are directly input into the GeoMx DSP	Tissue sections are placed directly onto Visium slides or with purchase of CytAssist RNA probes can be transferred from tissue sections on any microscope slide
Whole transcriptome (mouse or human)	Whole transcriptome (mouse or human)
Requires pre-existing knowledge of morphology markers which is limited to 3 markers plus a nuclear dye per slide	Unbiased sequencing using predetermined spots. Does not require pre-existing knowledge for spatial capture
GeoMx collects probes from >5 cells on user-selected morphology and segmentation	Visium: Captures ~10 cells per 50 μm spot Visium HD: Captures ~1–3 cells per 2–8 μm spot
Up to 95 user-selected regions of interest are sequenced per run	Up to 5000 predetermined barcoded regions are sequenced per run
One kit can sequence 32–40 tissues	One kit can sequence 4 tissues

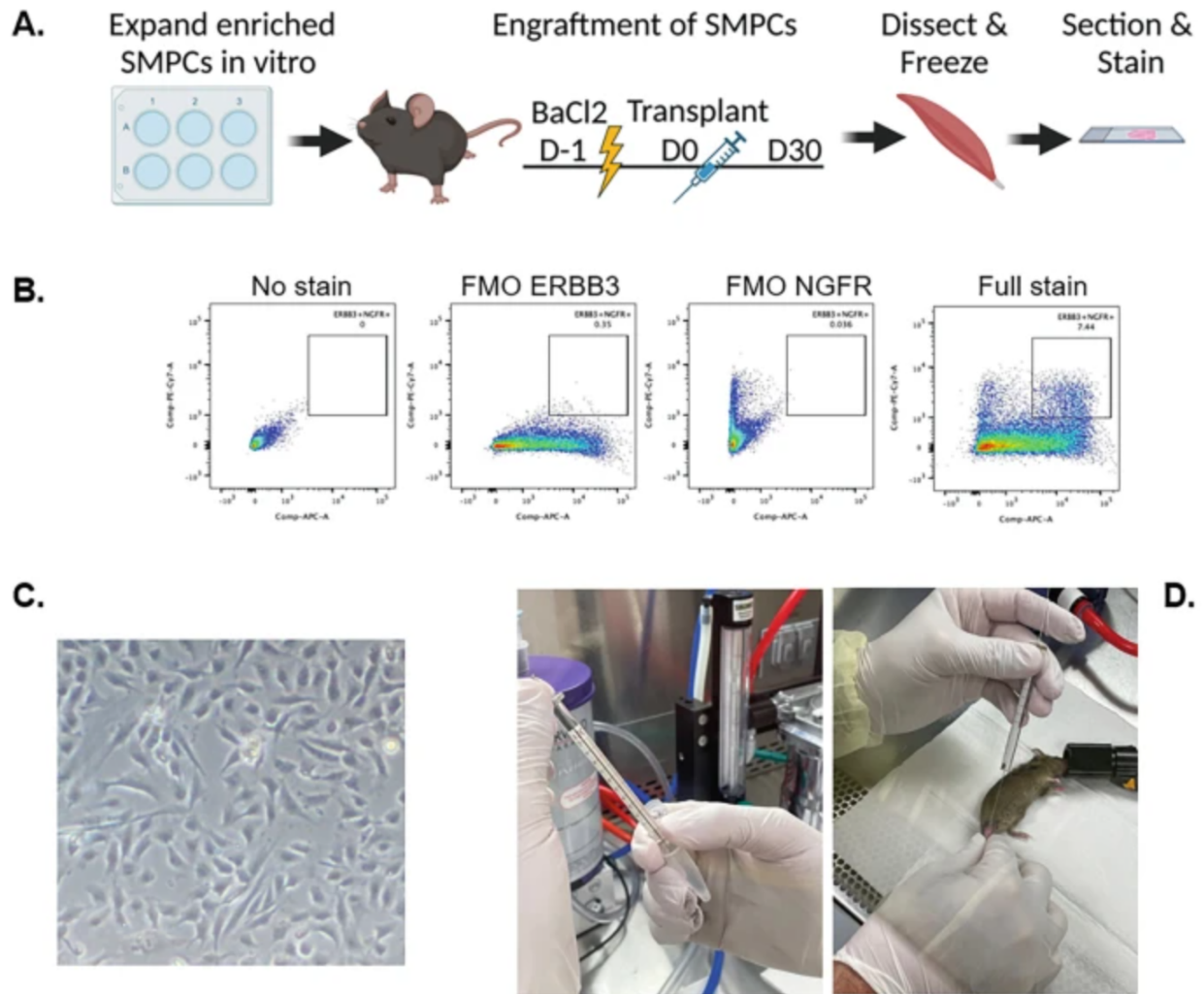


Figure 1.10: Engraftment of Human Skeletal Muscle Stem Cells

(a) Schematic describing the engraftment of human muscle stem cells in vivo. (b) Flow Cytometry enrichment of human iPSC-derived skeletal muscle stem cells. Flow plots shown from left to right are ERBB3 FMO, NGFR FMO, No Stain Sample, and Full Stained Sample. For all plots, the Y-Axis is ERBB3 Pe-Cy7 and the X-Axis is NGFR-APC. ERBB3+NGFR+ Cells shown by the double positive gate. (c) Image of the freshly enriched SMPCs 48 h after sorting. Labeled with tdTomato and Brightfield to visualize the morphology of the SMPCs. (d) Pictures of the Hamilton syringe taking human muscle stem cells (left) and using the loaded syringe to inject these cells into the TA of an anesthetized mouse (right).

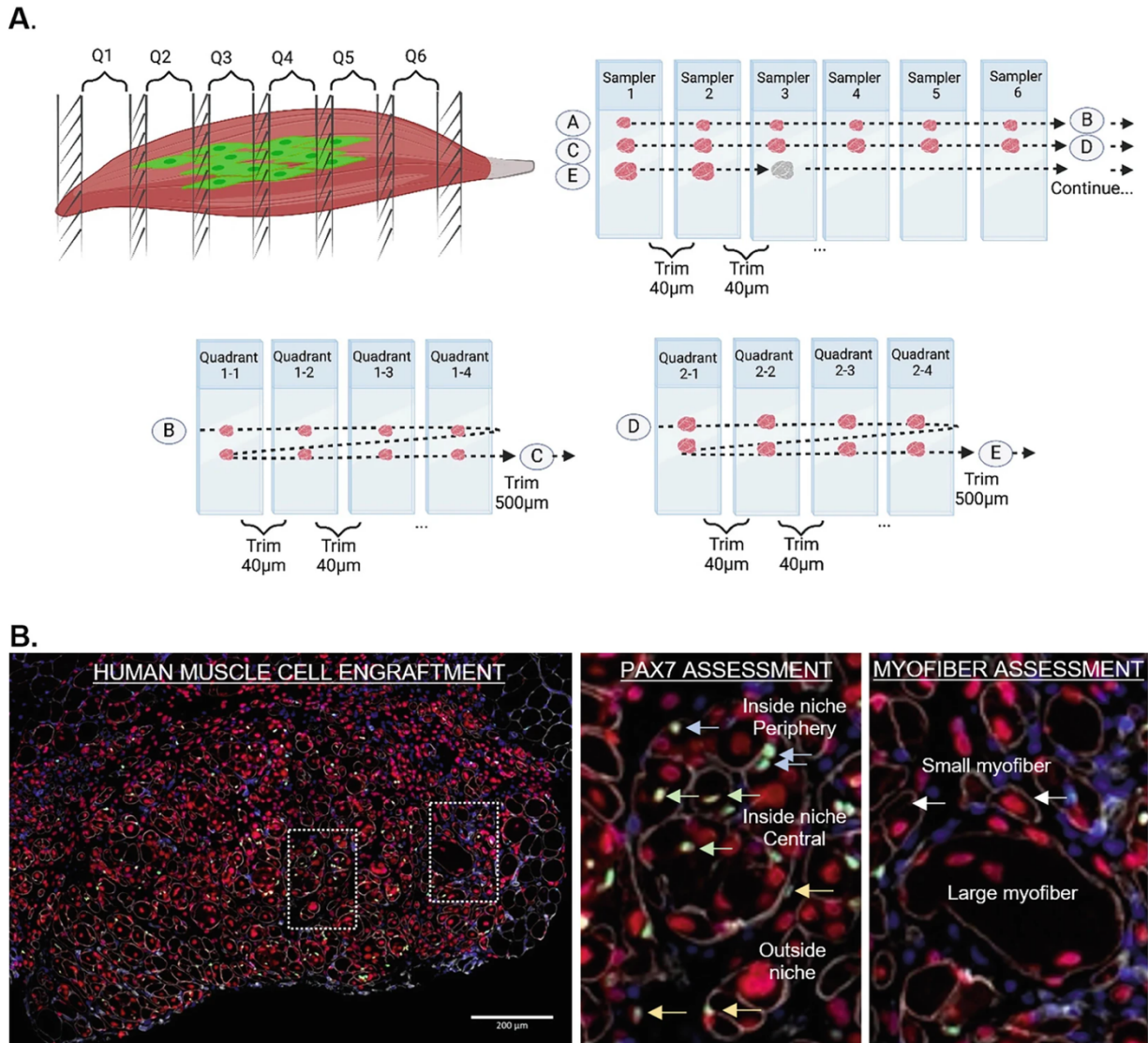


Figure 1.11: Heterogeneity and analysis of transplanted human skeletal muscle cells

(a) Illustration for the sectioning strategy to identify engrafted human skeletal muscle in mouse tibialis anterior (left). Example of what the slides with tissues sectioned on them will look like upon completion of sectioning (right), includes a sampler slide with tissue sections from all quadrants and an individual quadrant slide with only tissues from a specific quadrant. (b) Confocal imaging of engrafted human muscle. Immunofluorescent staining using PAX7 (green), Laminin (grey), LaminAC (red), Spectrin (red), DAPI (blue). Entire region of engraftment shown on the

left, scale bar = 200 μ M. PAX7 cells and their localization can be identified as shown in the middle.

Myofiber heterogeneity can be assessed by large and small fibers (right)

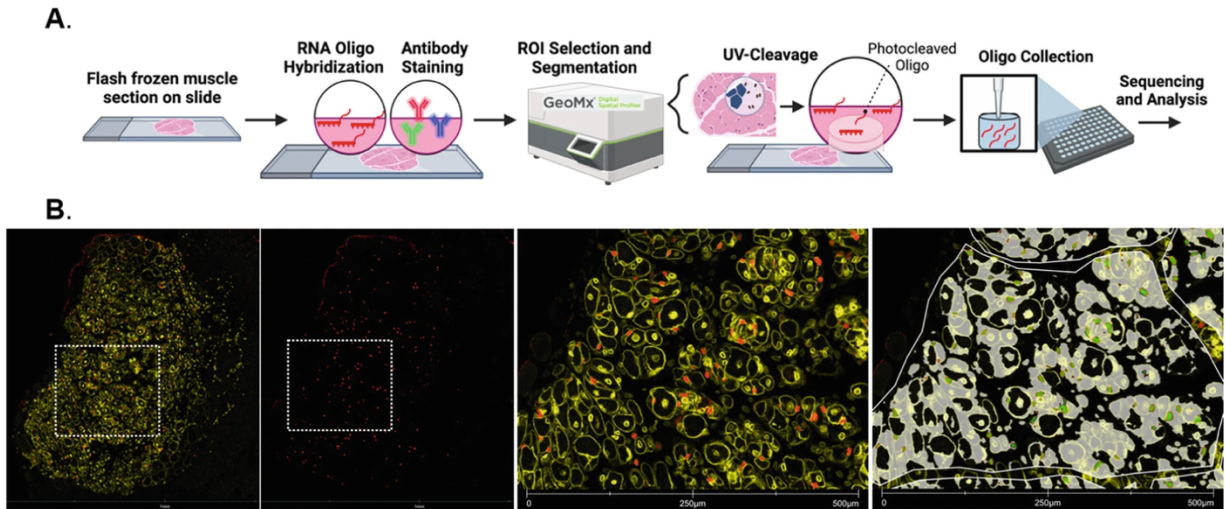


Figure 1.12: Spatial Transcriptomic Workflow Using the Nanostring GeoMx

- (a) Schematic for the Nanostring GeoMx Spatial RNA-Sequencing slide preparation workflow.
- (b) Images of engrafted human skeletal muscle on the GeoMx platform; PAX7 in red, Lamin AC and Spectrin in yellow (Top Left). PAX7 cells single channel (Top Right). Zoomed in region of the full stained sample (Bottom Left). Masks for the zoomed in region of the full stained sample, Spectrin+ Fiber mask in White, PAX7 Mask in Green (Bottom Right). Scale Bars shown are 2 mM for the top images, and 500 μ M for the bottom images

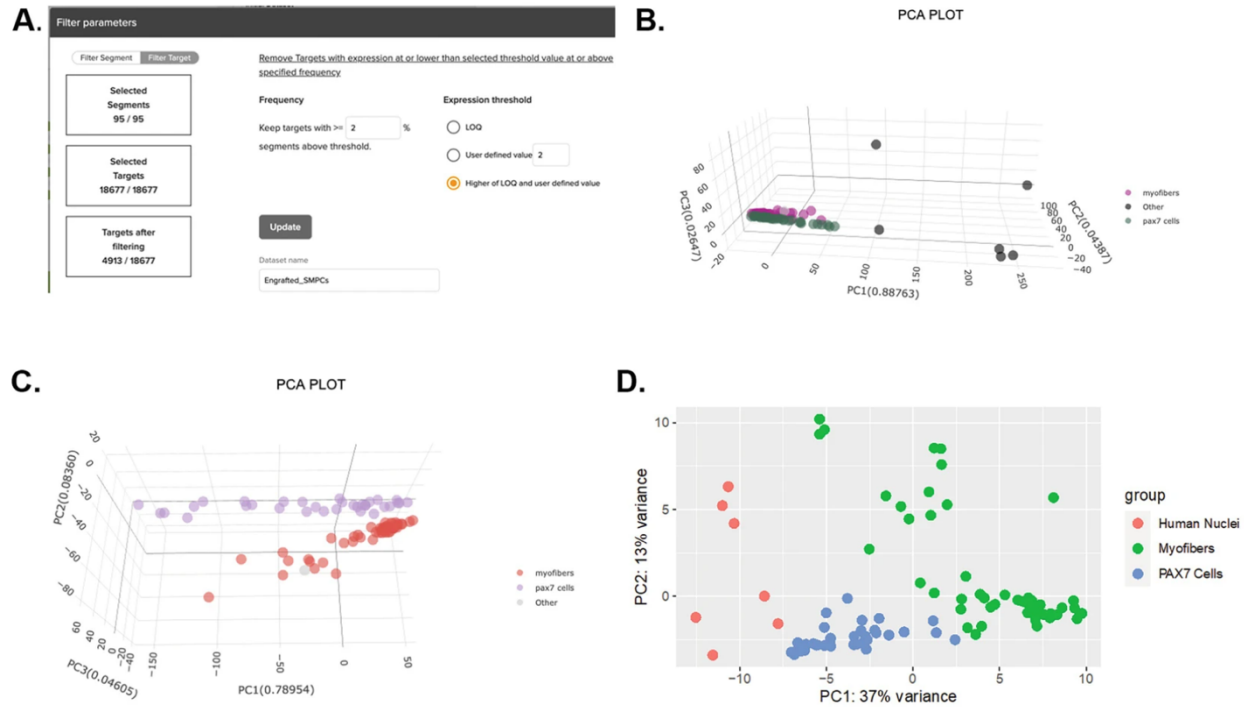


Figure 1.13: Analysis of Spatial Transcriptomic on the GeoMx Platform and R Studio

(a) Filtration of targets on the Nanostring GeoMx platform. (b) PCA Plot generation on the Nanostring GeoMx platform. Magenta; Myofibers, Dark Green; PAX7 Cells, Black; ROIs of Mouse-only tissue regions. (c) PCA Plot generation on the Nanostring GeoMx platform excluding the Mouse-only tissue regions. Myofibers in red, PAX7 cells in lavender. (d) PCA Plot generation with Spatial Transcriptomic data exported for analysis in R Studio after DESEQ2 analysis. Human Nuclei in red, Myofibers in green, PAX7 Cells in blue.

Chapter 2: The Regenerative Niche Supports Engrafted Human Muscle Stem Cells

2.1: Abstract

Skeletal muscle stem and progenitor cells including those derived from human pluripotent stem cells (hPSCs) offer an avenue towards personalized therapies and readily fuse to form human–mouse myofibers in vivo. However, skeletal muscle progenitor cells (SMPCs) inefficiently colonize chimeric stem cell niches and instead associate with human myofibers resembling fetal niches. We hypothesized competition with mouse satellite cells (SCs) prevented SMPC engraftment into the SC niche and thus generated an SC ablation mouse compatible with human engraftment. Single-nucleus RNA sequencing of SC-ablated mice identified the absence of a transient myofiber subtype during regeneration expressing *Actc1*. Similarly, ACTC1+ human myofibers supporting PAX7+ SMPCs increased in SC-ablated mice, and after re-injury we found SMPCs could now repopulate into chimeric niches. To demonstrate ACTC1+ myofibers are essential to supporting PAX7 SMPCs, we generated caspase-inducible ACTC1 depletion human pluripotent stem cells, and upon SMPC engraftment we found a 90% reduction in ACTC1+ myofibers and a 100-fold decrease in PAX7 cell numbers compared with non-induced controls. We used spatial RNA sequencing to identify key factors driving emerging human niche formation between ACTC1+ myofibers and PAX7+ SMPCs in vivo. This revealed that transient regenerating human myofibers are essential for emerging niche formation in vivo to support PAX7 SMPCs.

2.2: Introduction

Skeletal muscle is one of the most regenerative organs due to the presence of an endogenous muscle stem cell called the satellite cell (SC) [41, 225]. SCs are supported within anatomically defined specialized compartments, termed niches, that regulate the balance of self-

renewal and differentiation, similar to other highly regenerative tissues [226-228]. The SC niche is composed of dynamic ligand and receptor signaling between the myofiber sarcolemma, SC and the laminin-rich basal lamina that enables SC quiescence [163, 229]. Muscle injury disrupts the SC niche inducing a regeneration cascade beginning with rapid activation of SCs to proliferative myoblasts, which turn on fusion-capable genes to form new skeletal myofibers, that will contain hundreds of myonuclei [230, 231]. As new skeletal myofibers form, a subset of SCs re-enter the niche and revert from a proliferative to quiescent state, ensuring an SC pool for continual future regenerative events [232].

The ability to establish new SC niches is essential for long-term cell therapies, in which transplanted muscle stem cells must balance the formation of new muscle fibers and maintain the stem cell pool [39, 233]. More recently, small-molecule-mediated directed differentiation of human pluripotent stem cells (hPSCs) to skeletal muscle progenitor cells (SMPCs) has opened up the potential for personalized cell therapies and disease modelling for devastating muscle diseases from an *in vitro* cell source [27, 29, 32] including in concert with gene editing [187, 234]. We have demonstrated that hPSC SMPCs fuse to form hundreds of new myofibers *in vivo* after gene editing [184]; however, so far retention of human PAX7 cells after transplantation of hPSC SMPCs has proven challenging. Many reports show a high-magnification image of one or a few PAX7 cells following transplantation [184, 235, 236], but to fully restore muscle function will probably require at least 8–15 engrafted PAX7 cells per 100 myofibers, similar to endogenous SC levels in adult [237, 238]. Determining how transplanted SMPCs take up position in SC niches may enable support of PAX7 cells to improve long-term cell therapies for skeletal muscle diseases. The emerging SC niche (that is, the first myofibers that form upon engraftment that support PAX7

SMPCs) may also serve as an *in vivo* model to improve our basic understanding of human regeneration, similar to elegant studies of early development in mouse [239, 240].

Our laboratory generated a single-cell atlas of the human limb transcriptome from embryogenesis through adulthood, which determined the developmental identity of hPSC SMPCs *in silico* [33]. We found hPSC SMPCs most closely align with human SMPCs between weeks 7 and 12, a developmental time period that coincides with the transition from embryonic to fetal myogenesis, a period when fetal skeletal muscle is undergoing rapid growth to establish myofibers and fetal myoblasts are genetically maturing [241, 242]. SMPC behavior markedly differs from SCs including differences in mitotic activity [113], receptor expression [152] and extracellular matrix (ECM) composition [125]. In mouse, SMPCs migrate across and take up position under the myofiber basal lamina to establish the first niches late in fetal development [124, 243], but the mechanisms by which human SMPCs form niches and transition from progenitor to SCs to enable SC maintenance during human development or after transplantation is unknown.

We focused on the ability to retain PAX7 cells and reside in SC niches after transplantation *in vivo*. We used human fetal SMPCs and adult SCs to benchmark *in vivo* niche occupancy and repopulate muscle after re-injury, the hallmark assay for measuring SC self-renewing potential *in vivo* [39]. We found that transplanted cells across all developmental states and from hPSCs had the ability to form multinucleated chimeric (mouse–human) myofibers; however, unlike adult SCs, SMPCs did not enter the chimeric mouse niche efficiently. Instead, transplanted SMPCs formed large regions of human-only myofibers and PAX7 cells formed niches with these newly formed human-only myofibers.

In the mouse embryo, it has been shown that hPSC-derived lineages are inefficient at forming niches within interspecies chimeras, but deletion of key niche genes can create a

competitive niche to substantially extend hPSC donor chimerism [244]. Here we applied a similar concept by generating an immunocompromised inducible Pax7 SC ablation mouse model to study human SMPC niche formation in the absence of mouse SCs. In this model, we found an increase in human donor derived PAX7 cells from both SCs and SMPCs but predominantly with human niches and not chimeric niches. However, upon re-injury in the Pax7-ablation model we now begin to see an increase in both human and chimeric niche occupancy demonstrating the emerging human myofibre niche supports PAX7 cells required for repopulation ability. Using Pax7-ablated and control mice as a model system to study new niche formation, we identified a transient regenerating ACTC1+ myofibre that is required for new niche formation during regeneration that is absent in Pax7-ablated mice. Spatial RNA profiling of engrafted stem cells and fibres identified key human skeletal muscle niche genes enabling ACTC1+ myofibre support of human PAX7 cells *in vivo*. We generated stable hPSC lines with a caspase 9-inducible kill switch to deplete ACTC1+ myofibres during engraftment, which we found abolished human PAX7 cells *in vivo*, demonstrating these ACTC1+ myofibres are a key component of the emerging niche needed to support human PAX7 hPSC SMPCs. In summary, this work identifies how emerging niches occur on newly regenerated myofibres *in vivo* and provides a new model for understanding early human stem cell niche formation using SMPCs.

2.3: Results

SMPCs associate with human-only myofibers in vivo

We previously showed hPSC SMPCs can engraft and regenerate hundreds of dystrophin+ myofibers in immunocompromised mouse models of Duchenne muscular dystrophy (mdx-NSG) following cardiotoxin (CTX) injury [184]. We have continued to improve myogenic potential by supplementing survival/maturation factors during directed differentiation of hPSCs *in vitro*, as

well as reducing culture time of ERBB3+NGFR+PAX7+ hPSC SMPCs before engraftment (**Figure 2.1 A**). *In vivo*, we show that, while hPSC SMPCs fused with mouse skeletal muscle to form hundreds of multinucleated chimeric myofibres (maximum number 75–230 per cross sectional area (CSA), N = 7 mice), hPSC SMPCs also generated about twice as many human-only myofibres (maximum number 140–450 per CSA), which occupied large regions of engrafted skeletal muscle. Myofibre size as measured by Imaris software and exclusivity of human nuclei marked by human lamin AC were used to distinguish and quantify chimeric and human-only myofibres in transverse and longitudinal sections (**Figure 2.1 A**). An effective cell therapy must maintain the muscle stem cell pool. Yet so far retention of human PAX7 cells after transplantation of hPSC or foetal SMPCs has proven challenging. Thus, we were encouraged to find 20 or more human PAX7+ hPSC SMPCs within 0.15 mm² (20×). A total of 127 human PAX7+ cells were counted and categorized on the basis of proximity to human-only myofibres, chimeric myofibres, interstitial space or regions with high numbers of human lamin AC cells but few myofibres (**Figure 2.1 B**). We found regions containing the greatest numbers of human-only myofibres also contained 98% of all engrafted PAX7 hPSC SMPCs. In contrast, regions containing only lamin AC, but few or no human-only myofibres, did not contain PAX7 cells. We also evaluated chimeric myofibre regions, and found these regions contained the other 2% of PAX7 cells ($P < 0.05$, **Figure 2.1 B**). We also evaluated hPSC SMPC engraftment in another more severe model of muscular dystrophy, the mdx-DBA/2-NSG mice. We similarly found that hPSC SMPCs form both human-only and chimeric myofibres, but PAX7+ cells predominately associated with the human-only myofibres (**Figure 2.1 B**). These data suggested that human-only myofibres were better at supporting PAX7 SMPC retention than chimeric myofibres or other regions within the skeletal muscle microenvironment.

ACTC1+ myofibers enable repopulation in SC-ablated mice

As the *Actc1*⁺ gene was the highest-expressed candidate found in snRNA-seq data in regenerating mouse satellite cell niches, we evaluated if ACTC1 shares common functions with the human-only myofibres supporting PAX7⁺ SMPC engraftment. ACTC1 is also known to be expressed during foetal skeletal muscle development⁴⁸, which further supported the likelihood that ACTC1 would be important in the emerging niches formed by hPSC SMPCs. Indeed, we found that human-only myofibres from hPSC and foetal SMPCs strongly expressed ACTC1 following engraftment, but in myofibres that we identified as chimeric, ACTC1 was not expressed at 30 days (**Figure 2.2 A**). To further demonstrate that ACTC1 was expressed by human-only myofibres, but not chimeric myofibres, we took advantage of mouse-specific myosin expression *Myh4* in tibialis anterior (TA), which human skeletal muscle does not express. Staining confirmed the presence of chimeric myofibres (lamin AC+spectrin+Myh4⁺) and human-only myofibres (ACTC1+Myh4⁻), in which the smallest human myofibres had the greatest ACTC1 expression (**Figure 2.2 B**). ACTC1⁺ human myofibres were more prominent when engrafted in mdx-NSG Pax7-DTX mice, and moreover adult SCs now predominately formed ACTC1⁺ human-only myofibres in Pax7-ablated mice, suggesting that, when mouse regeneration is impeded, human SC niche formation initially occurs with regenerating human-only myofibres.

Spatial analysis identifies key emerging niche pathways

To better define the ACTC1⁺ niche supporting human PAX7 cells, we optimized spatial RNA sequencing (RNA-seq) and evaluated the niche in both long-term engraftment and after re-injury (**Figure 2.3 A**). To do this, we used the Nanostring GeoMx platform, which enables cell type and location specific analysis of gene expression at single antibody resolution. GeoMx allows for region of interest (ROI) selection and segmentation on PAX7 cells and ACTC1⁺ and ACTC1⁻

myofibres. In brief, we engrafted foetal SMPCs for up to 4 months and induced an injury in a subset of engrafted mice midway at 2 months. We then mounted flash-frozen muscle sections on microscope slides, hybridized a whole transcriptome human RNA oligo library onto the muscle, and stained with morphology markers ACTC1, PAX7, lamin AC and spectrin to identify human muscle engrafted in mouse. After ROI selection and segmentation, the RNA oligos are photocleaved and collected for sequencing (N=44 ROIs, deposited as GSE243875). Principal component analysis (PCA) found distinct clustering of cell types including ACTC1+ and ACTC1– myofibres (**Figure 2.3 B**), and our data confirmed a robust repopulation ability by foetal SMPCs in mdx-NSG Pax7-DTX mice that was sustained through 4 months (**Figure 2.3 C**). At the time of re-injury, we identified unique gene signatures in the ACTC1+ myofibres, which included increased embryonic and foetal myosins, ECM development and cell adhesion. In contrast the ACTC1– myofibres had higher expression of metabolism genes and maturation genes. We additionally performed DEG analysis of PAX7 foetal SMPCs after repopulation versus PAX7 foetal SMPCs that remained over a 4-month time period. Foetal PAX7 SMPCs that had undergone repopulation expressed many genes that could be unique candidates associated with new niche formation including NOTCH4, ITGB1, FGFR1 and MEGF10 as well as stem cell maintenance, chromatin organization and negative regulation of cell proliferation (**Figure 2.3 C,D**). We then used CellChat analysis to predict receptor–ligand interactions between the ACTC1+ myofibres and the PAX7 foetal SMPCs identified potential candidates associated with emerging niches including robust association of DAG1 on PAX7 cells and LAMA5 as well as LAMC1 on ACTC1-positive myofibres. Interestingly, THBS4 was a top candidate on ACTC1+ myofibres interacting strongly with receptors on PAX7 cells including integrins and with other myofibres through CD36, a regulator of lipid metabolism and fatty acid uptake (**Figure 2.3 D**). Thus, spatial analysis revealed

several potential interacting pathways supporting emerging niche formation in vivo in addition to ACTC1.

ACTC1+ myofibres are essential for PAX7 in emerging niches

To definitively prove that ACTC1 myofibres are critical for supporting PAX7 cells, we followed recent approaches that have introduced an inducible suicide gene in hPSCs [245, 246]. HPSCs are uniquely able to be genetically manipulated while retaining pluripotency and ability to differentiate into downstream lineages. We engineered an inducible apoptosis system that included a FKBP12 linked to a caspase 9 (iCasp9) and a self-cleaving T2A peptide immediately following a homology arm of the last 3' base pair in the ACTC1 gene. Treatment with the bio-inert small molecule AP1903 induces cross-linking of the drug-binding domains of this chimeric protein, which in turn dimerizes caspase 9 and activates the downstream executioner caspase 3 molecule, resulting in gene-specific cellular apoptosis [247] (**Figure 2.4 A**). To determine the optimal conditions for inserting iCasp9 by homology-directed repair, we tested 17 conditions in H9 and H1 hPSC lines including 3 guide RNA (gRNA) targets, lipo-STEM and nucleofection, and compared homology-directed repair protocols described by Skarnes et al., to the protocol provided by Lonza [248]. We confirmed the presence of inserted iCasp9 in both H9 and H1 lines following nucleofection (**Figure 2.4 A**) and proceeded to single cell cloning in 384 wells. Of these, 108 clones survived, and we found 2 clones that contained a heterozygous insertion for iCasp9. We performed sequencing to confirm an exact match for our engineered iCasp9 vector within the ACTC1 locus.

We next performed directed differentiation of ACTC1-iCasp9 hPSCs to generate SMPCs for engraftment. We used Pax7-ablated mdx-D2 NSG Pax7-DTX mice as these mice readily enable human-only myofiber formation. Two weeks following engraftment, mice were treated

with AP1903 (10 μ M in 100 μ l saline) or control (0.1% dimethyl sulfoxide in saline) every two weeks for 90 days. Similar to previous results, in non-treated mice we found hundreds of hPSC myofibres that were able to engraft over approximately 6 mm³ of mouse TA, and we counted up to 90 PAX7⁺ hPSC SMPCs associated with hPSC myofibres within a 1.5-mm² region of tissue (**Figure 2.4 B**). AP1903 treatment induced a ~90% reduction in hPSC myofibres, as we found fewer than 20 myofibres per cross section in treated mice. Remarkably the apoptosis of ACTC1⁺ myofibres resulted in an almost complete elimination of PAX7 cells in the AP1903-treated mice. We counted a total of 269 human PAX7 cells in five regions of control mice and only 5 PAX7 cells within the same engrafted regions of AP1903-treated mice (**Figure 2.4 B,C**). However, in the AP1903-treated groups there were hundreds of remaining lamin AC⁺ cells that were neither myofibres nor PAX7⁺.

We further validated the iCasp9 system using GeoMx spatial sequencing on engrafted hPSC muscle cells with and without AP1903 treatment (ROI, N = 59). Spatial analysis showed an abundance of cell death-related pathways including several upregulated caspase genes in the AP1903-treated muscles but not in control muscles (**Figure 2.4 C,D**). We compared the remaining hPSC lamin AC⁺ cells (called hPSC PAX7⁻) with hPSC PAX7⁺ cells, and these expressed genes enriched in non-stem cell or non-myogenic related cell fate change including POSTN, CDH11 and PDGFRA, suggesting that in the absence of human myofibres these cells change fate to other mesoderm lineages, whereas the PAX7⁺ cells expressed key stem cell genes such as ITGA7, NOTCH3 and CDH15. To better define how the hPSC SMPCs are interacting with the emerging fibres we compared the PAX7 cells with the hPSC myofibres in vivo again using spatial RNA-seq. We also discovered that key stem cell signalling and adhesion pathways are turned on demonstrating maintenance of stem cell fate in this model when ACTC1-positive fibres are

present, indicating support of human PAX7 cells by these emerging niches (Fig. 7d). To verify this, we performed CellChat analysis and found that hPSC myofibres send several signals through embryonic laminins and delta ligands (LAMA1 and DLK1) that are predicted to interact with receptors (NOTCH3, FGFR4, DAG1, ITGA7 and CD44) on the hPSC PAX7+ SMPCs (**Figure 2.4 E**). These identified key stemness pathways are turned on, which could be key to supporting hPSC PAX7 cells in emerging niches.

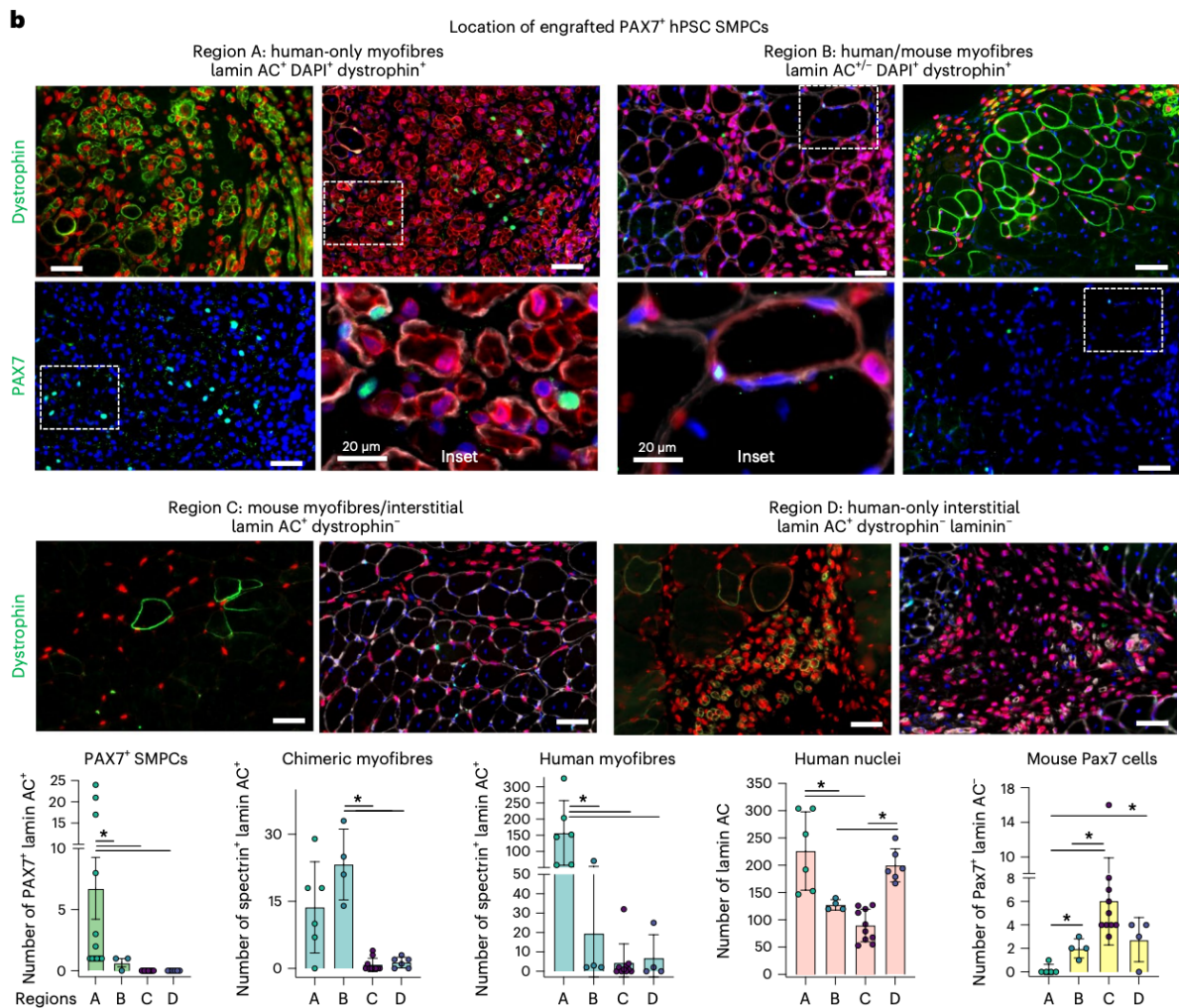
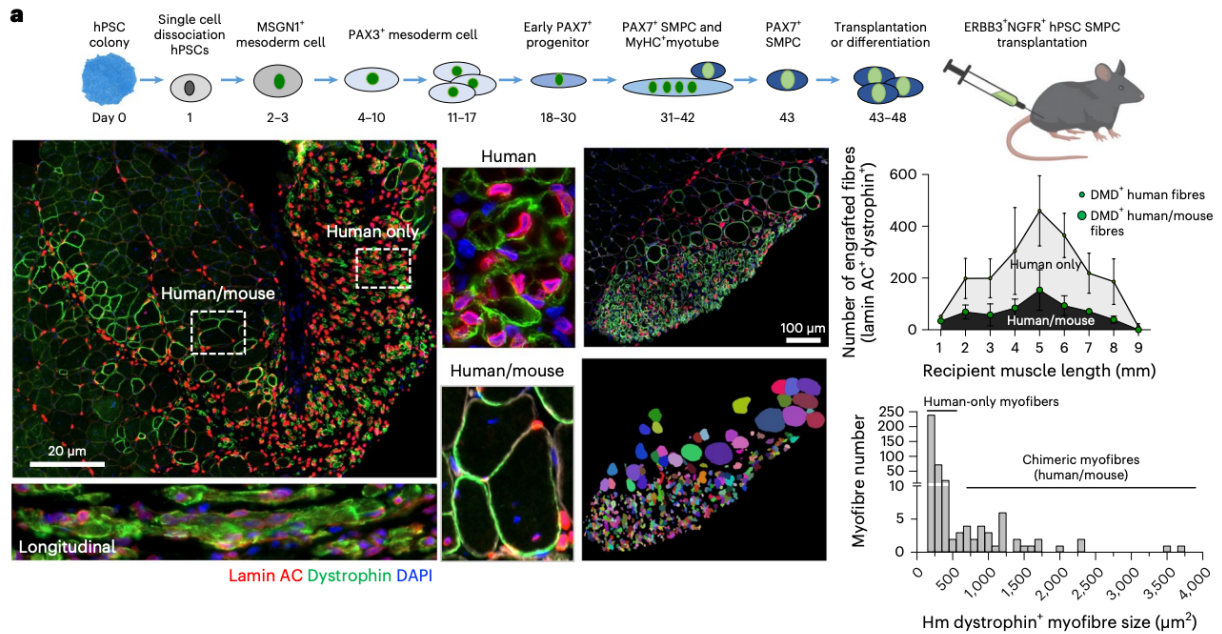


Figure 2.1: HPSC SMPC engraftment produces chimeric and human-only myofibres, and PAX7+ cells associate predominately near human-only myofibres.

a, Top: schematic of hPSC directed differentiation to skeletal muscle in vitro and engraftment in vivo. Left: region of hPSC SMPCs 30 days post engraftment (1.44 mm²) showing insets (rotated 90°) of chimeric and human-only myofibre phenotypes. Human-specific antibodies lamin AC (red) mark human nuclei, and dystrophin (green) mark human myofibres, 4',6-diamidino-2-phenylindole (DAPI) (nuclei, blue). Scale bar, 200 µm. Right: Imaris was used to quantify myofibre cross-sectional area as one parameter of chimeric and human-only myofibres. Scale bar, 100 µm. Graph shows mean ± standard deviation of engrafted human and human–mouse myofibres over the length of mouse TA muscle (N = 7). Histogram shows cross-sectional area of Imaris-identified myofibres using human dystrophin. b, 20× images were quantified for number of human PAX7 cells, chimeric myofibres, human-only myofibres, total human cells and mouse SCs (PAX7 green or dystrophin green depending on image; lamin AC and spectrin, red; DAPI, blue). Scale bar, 50 µm. To quantify location of PAX7 cells, numbers were then quantified using 20× images (0.15 mm²) were separated into four regions: (A) fields of view containing >50 human-only myofibres, (B) fields of view containing >10 chimeric muscle myofibre, (C) mouse myofibres, interstitial space and epimysium, and (D) dense numbers of human cells but few fused myofibres. Graph shows mean ± standard deviation of six to ten images from each region, N = 3 mice were quantified; statistics were performed using one-way ANOVA with post-hoc Tukey test, *P < 0.05.

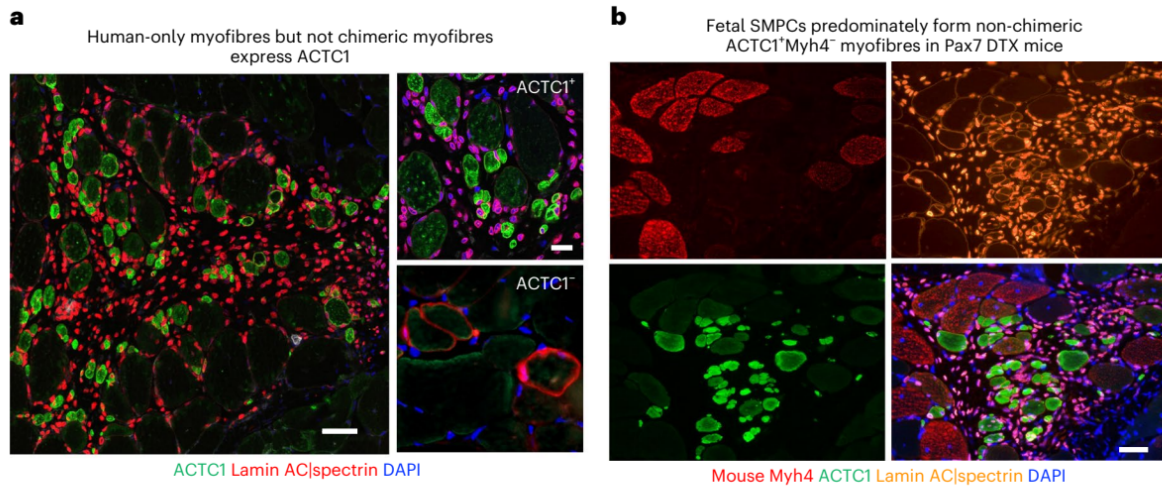
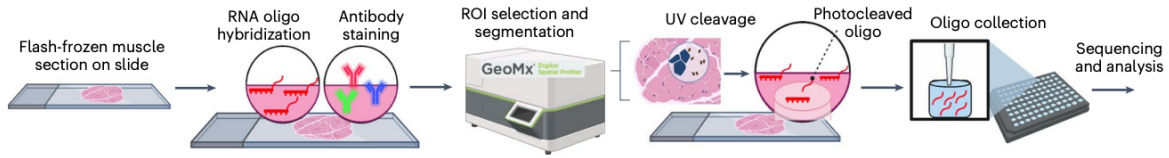


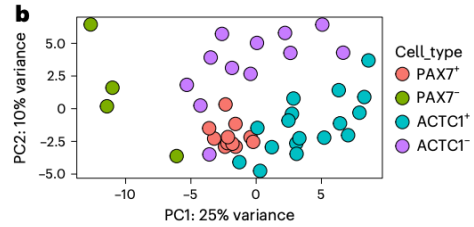
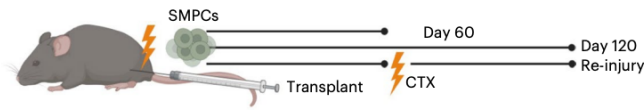
Figure 2.2: ACTC1 is expressed by human-only myofibres and increased in Pax7-ablated mice

a, SMPC engraftment after 30 days show that human-only myofibres, but not chimeric myofibres express ACTC1 (green). N = 4 engrafted tissues. Scale bar, 100 μ m. b, Human-only myofibres, but not chimeric myofibres, express regenerative signature as shown by ACTC1. SC niches form near small regenerating myofibres, which enables PAX7 cells to remain. Scale bar, 100 μ m. Mouse TA express high levels of Myh4, which is not expressed by human, enabling further identification of human-only and chimeric myofibres. ACTC1 expression is reduced in chimeric Myh4+spectrin+lamin AC+ myofibres.

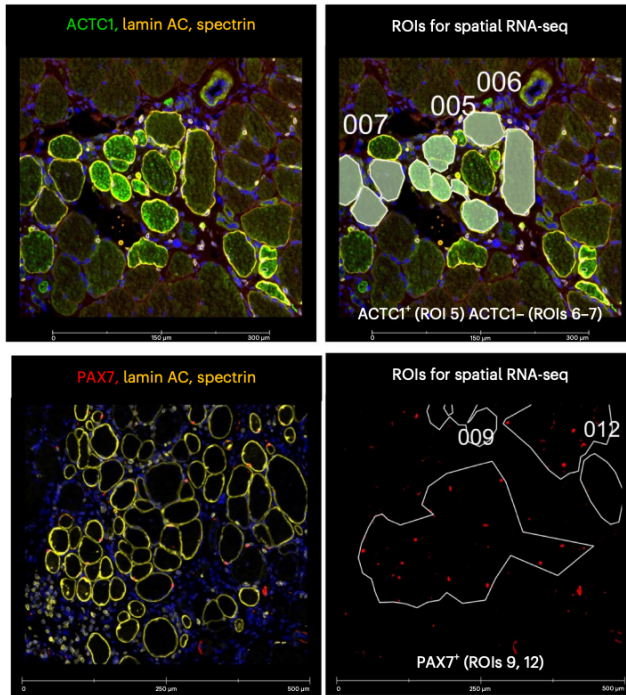
a Spatial transcriptomics of PAX7 and niche $\pm$ re-injury in Pax7-knockout mice



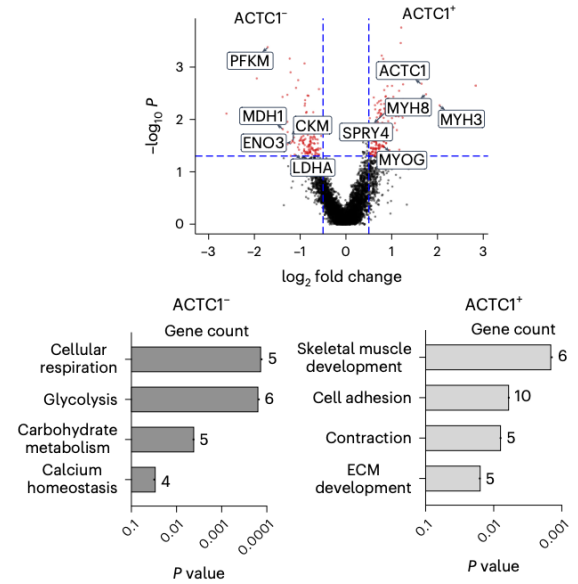
Foetal SMPC repopulation in mdx-NSG PAX7-DTX mice



c



Transcriptomics of engrafted foetal myofibres



d

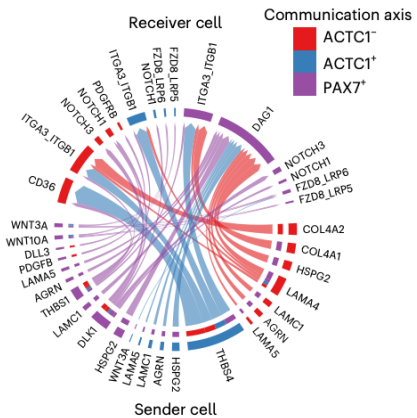
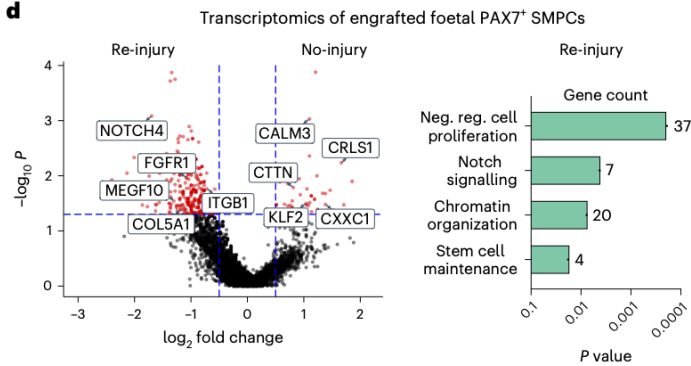
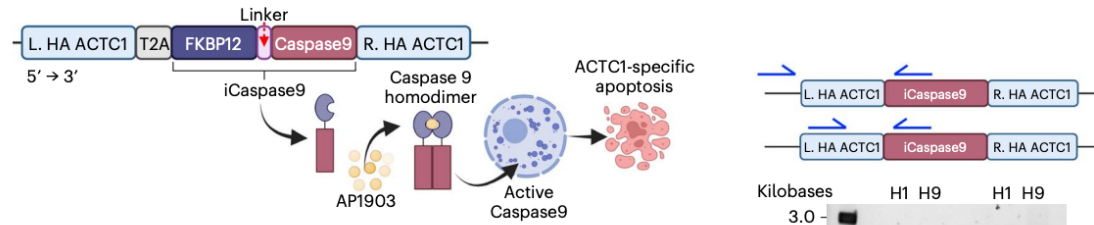


Figure 2.3: Spatial transcriptomics identifies interaction of ACTC1+ myofibres with PAX7 SMPCs in the emerging niche.

a, Cartoon workflow of spatial transcriptomics on engrafted human/mouse tissue. Photocleavable RNA oligo probes are collected on ROIs for sequencing. Tissues used for spatial RNA-seq included foetal SMPCs engrafted in PAX7-ablated (or DTX) mdx-NSG mice for 60 or 120 days, and re-injury with CTX at 60 days. b, PCA plot showing clustering of engrafted human cells (PAX7+, PAX7-) and myofibres (ACTC1+, ACTC1-). Each dot represents a sequenced ROI (N = 43). c, Top left: images taken on GeoMX of day 60 engrafted foetal SMPCs show ACTC1+ and ACTC1- myofibres. GeoMx applied masks to ROIs used for sequencing. Right: volcano plot of ACTC1+ and ACTC1- myofibres. Red dots represent differential expressed genes, and selected key genes are shown. For all volcano plots differential gene expression (DGE) between ROIs was determined using GeoMx linear mixed model (LMM) statistical tests with Benjamini-Hochberg (BH) correction $P < 0.05$. Bar graphs show Gene Ontology of selected pathways upregulated by ACTC1+ and ACTC1- myofibres. Bottom left: PAX7 in the re-injury group and regions used for selecting single PAX7 cells for sequencing. d, Volcano plot of human foetal PAX7+ SMPCs after re-injury or no-injury 120 days after engraftment. Red dots represent differentially expressed genes, and selected key genes are shown. Graphs show Gene Ontology of foetal PAX7+ SMPCs undergoing repopulation after re-injury of selected pathways related to the SC niche comparisons between two groups. For comparing different cell types or treatments to generate volcano plot data, LMM statistical tests with BH correction were performed. Neg. reg., negative regulation. Right: CellChat analysis show predicted sender (bottom) and receiver (top) ligand and receptor interactions between foetal PAX7+ SMPCs and ACTC1+ and ACTC1- myofibres from spatial RNA-seq of engrafted tissues.

a Insertion immediately in 3' of ACTC1 locus of hPSCs



b mdx-D2 NSG Pax7-DTX

CTx, day -1

Transplant hPSC SMPCs

AP1903

ACTC1 apoptosis

Control

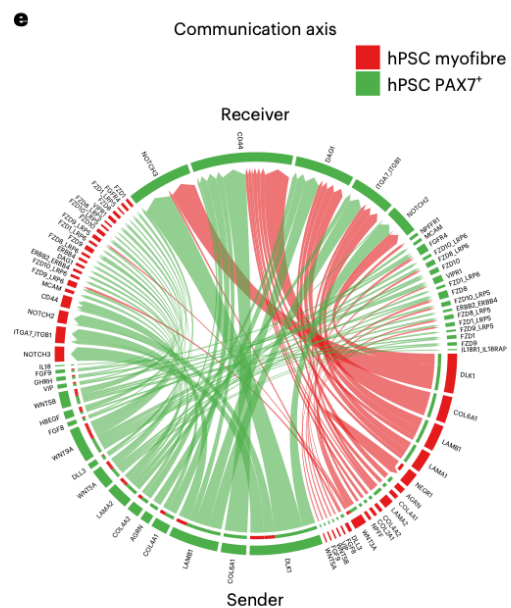
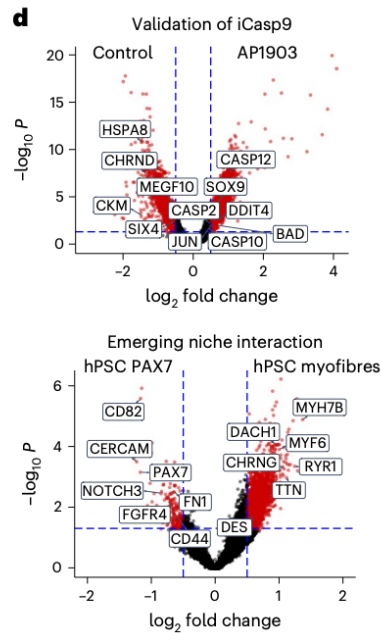
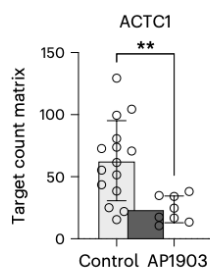
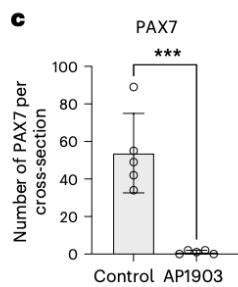
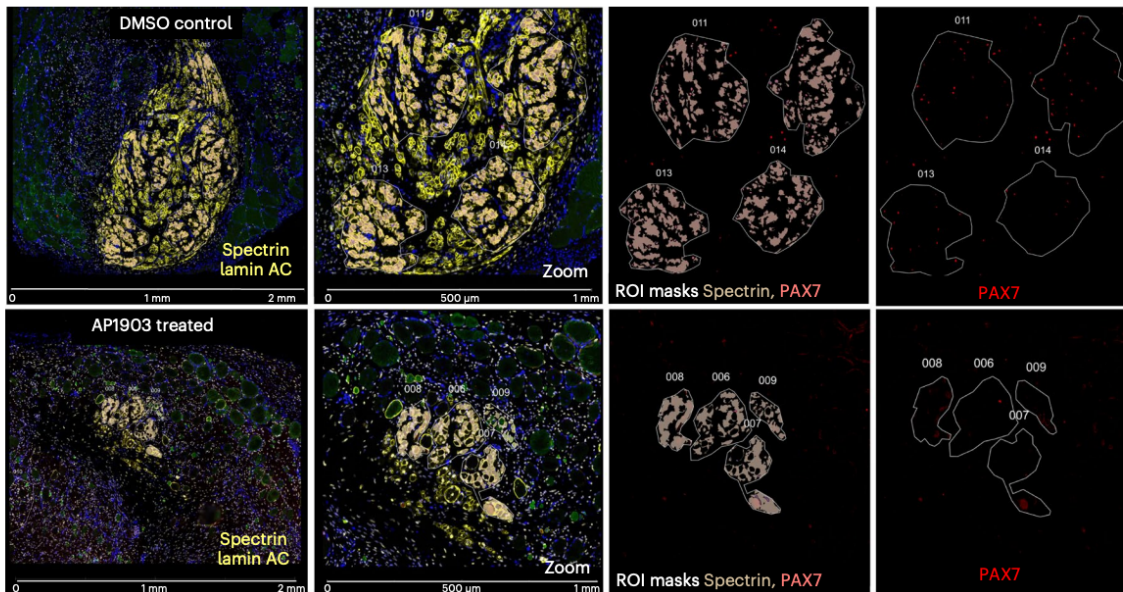


Figure 2.4: ACTC1 is essential for emerging niche formation and support of hPSC PAX7 SMPCs.

a, Cartoon showing iCaspase9 vector (iCasp9) insertion immediately in 3' of ACTC1 locus of hPSCs. AP1903 causes iCasp9 activation and gene-specific cell death. PCR products show insertion of iCasp9 across two hPSC lines (H1 and H9). b, Top: Pax7-DTX (ablated) Mdx-D2-NSG mice were treated with TMX, and hPSC SMPCs transplanted into Pax7-ablated tissues. Mice were treated six times every two weeks with AP1903 over 90 days, N = 5 cross sections. Left: images taken on the GeoMx of 90-day engrafted hPSC SMPCs show regions of transplantation with or without AP1903 treatment. Zoom shows morphology markers and masked ROI overlays. Single PAX7 cells and myofibres were collected for spatial RNA-seq. c, Graph show mean $\pm$ standard deviation of PAX7 counts from cross-sectional area and a decrease in AP1903-treated mice; t-test, ***P = 0.0005, N = 5 cross sections. ACTC1 expression counts were obtained from Q3 normalized outputs from ROIs on GeoMX; **P < 0.003, N = 13 untreated, N = 8 AP1903. d, Top: volcano plot of all human ROIs in control and AP1903-treated group highlight increased caspase activity in AP1903-treated mice. Bottom: volcano plot in control mice shows genes upregulated hPSC PAX7+ SMPCs compared with hPSC myofibres. Red dots represent differentially expressed genes, and selected key genes are shown. For comparing different cell types or treatments to generate volcano plot data, linear mixed model (LMM) statistical tests with Benjamini–Hochberg (BH) correction were performed. e, CellChat analysis show predicted sender (bottom) and receiver (top) ligand and receptor interactions between hPSC PAX7 SMPCs and hPSC myofibres from spatial RNA-seq of engrafted tissues.

2.4: Discussion

The ability to efficiently support human PAX7 cells after engraftment remains a major roadblock to translating cell therapies for skeletal muscle. We demonstrate that the formation of human-only myofibers following transplantation is a key source of niche emergence from transplanted human cells, which has previously been overlooked. Human-only myofibers have a 50-fold better ability to support transplanted PAX7 cells and can be identified by expression of the fetal skeletal muscle actin isoform ACTC1. The ACTC1⁺ hPSC myofibers form niches that phenocopy late-stage fetal myogenesis, which enables the generation of human niches that support PAX7 cells from hPSCs. To demonstrate that ACTC1⁺ myofibers are key to supporting stemness from PAX7⁺ hPSC SMPCs, we developed iCasp9 suicide hPSC lines where we took advantage of the power of hPSCs with genetic engineering to evaluate this question. We demonstrate that ablation of the ACTC1 myofibers results in substantial PAX7 loss. Using multiple functional studies, we conclude that transplanted human SMPC/SCs form niches using skeletal muscle regeneration pathways and do not appear to ‘home’ to empty niches like the haematopoietic stem cell system [249]. We show reciprocation between the myofibre and stem cell; when mouse Pax7⁺ SCs are ablated, the regenerative Actc1⁺ myofibers are lost, and conversely when ACTC1⁺ emerging myofibers are eliminated, the PAX7 hPSC SMPCs are lost. We also have identified unique gene signatures including ligands and receptors found on foetal and hPSC myofibers that may support de novo niche formation and could improve our ability to translate human PAX7 cells for cell and regenerative therapies.

Our new engraftment model specifically eliminated mouse SCs to evaluate SMPC niche formation in the absence of SCs. Interestingly, in Pax7-ablated mice we found increased numbers of PAX7⁺ hPSC and foetal SMPCs, but rather than residing in empty chimeric mouse niches,

SMPCs increased association with ACTC1+Myh4⁻ human-only myofibers at 30 days. We also found that PAX7⁺ foetal SMPCs and adult SCs now predominantly formed niches with ACTC1⁺ human myofibres in Pax7-ablated mice, suggesting that in this environment PAX7 SMPCs/SCs prefer association with regenerative myofibres even though our ablation and injury model still contained hundreds of mouse myofibers. We show that fetal and hPSC SMPCs can maintain PAX7⁺ expression for several months in vivo when associated with ACTC1⁺ myofibres, and when re-injury is applied after 60 days foetal PAX7⁺ SMPCs are now better able to engraft into both human-only and chimeric niches, suggesting association with these niches enables an increase in functional maturation.

Our approach is a more precise ablation model compared with long-standing practices in the field, which has removed competition with the endogenous SCs by irradiation aiming to increase niche occupancy from engrafted donor human or mouse cells. Muscle irradiation studies are confounded by precise dosing and timing regimens required, and high doses of radiation reduce donor mouse-to-mouse engraftment [250-252]. In our hands and others, irradiation did not improve human muscle cell engraftment into mouse [253]. Further, irradiation damages cells in the microenvironment, which orchestrates muscle regeneration and supports SC niche formation [254, 255], and studies have not fully investigated the extent to which radiation regimens affect the myofibre regenerative response. We also cannot rule out that the formation of human-only myofibres will be increased close to the injection site, and therefore would increase the probability of local association with human PAX7 cells. However, this finding is also interesting and could inform on cell numbers needed to support PAX7 SMPCs upon engraftment and the need for multiple injections per muscle.

To understand the mechanisms of failed regeneration and niche formation in Pax7-ablated mice, we performed snRNA-seq in Pax7-ablated and control mice with and without injury. Similar to work from several groups [256-258], we show that loss of Pax7 cells led to severe muscle regeneration defects in acute-injury models. Of the remaining myofibres in the Pax7-ablated mouse muscle, our sequencing data find increased numbers of highly glycolytic Car3+ myofibers, but most striking was the reduction of the regenerative Actc1+ mouse myofibers marked by several genes involved with sarcomere assembly (Tmod1, Myhpb3 and Tnnt3), which suggests that niche formation occurs in parallel with construction of sarcomeres. Notable niche genes identified in the Actc1+ myofibres that interact with laminin include Itgb1pb2, which binds integrins expressed by SCs during niche formation [259], and Tmem38a, which regulates heterochromatin and repositions select genes to the nuclear periphery of the myofiber, which happens to include key regulators of SC migration such as Vcam1. It also brings in Nidogen, a gene involved in repositioning of laminin binding and that we found expressed by human fetal and hPSC myofibers *in vivo* [260].

Although it is well understood how mouse regeneration occurs after injury, this work develops a human model to study regeneration leading to new emerging niche formation *in vivo*. We took advantage of spatial RNA-seq to investigate differentially expressed genes between ACTC1+ and ACTC1- myofibres and ligand-receptor pairs interacting with genes expressed on foetal SMPCs. One of the unique identified pathways expressed on engrafted ACTC1+ foetal myofibres was Thrombospondin 4 (THBS4), which enhances attachment of dystrophin-glycoprotein and integrin complex to stabilize the muscle membranes [261] and is predicted to interact with ITGB1 expressed on foetal SMPCs. We also performed spatial RNA-seq on engrafted hPSC SMPCs and myofibres and identified key *in vivo* niche genes including CD44 expressed by PAX7+ hPSC SMPCs which is activated during regeneration and fetal development [262] and is

predicted to interact with Collagen 6 expressed by hPSC myofibres, which promotes autonomous niche formation through ECM production [263]. Other key identified interactions in our analysis that may also be critical to supporting PAX7⁺ SMPCs include multiple NOTCH signalling pathways that are sending and receiving in both directions from hPSC SMPCs and myofibres. We also found Dystroglycan 1 (DAG1) on hPSC SMPCs interacting with laminin 111 (LAMA1) expressed by hPSC myofibres. One caveat to the use of GeoMx for spatial RNA-seq is the close, but not perfect, precision of morphology marker segmentation, and we do find some muscle genes in the PAX7 SMPC datasets due to their close spatial proximity demonstrating further validations will be required. To avoid this, the datasets we focused on comparing hPSC SMPCs with myofibres to identify enriched genes such as CD82, FGFR4 and CERCAM, and these may represent key targets to modulate for improved support of PAX7 cell engraftment in future studies.

The significance for identification of the ACTC1⁺ myofibers for initiating niche formation could have both basic and therapeutic applications. Many labs have highlighted that both regenerating adult myofibers and developing fetal myofibers support PAX7 cell numbers at orders of magnitude greater per unit area than adult myofibers in homeostasis [124]. It would be interesting to determine whether myofibers in homeostasis could support additional PAX7 cells if stimulated by myofiber genes involved in emerging SC niche formation. Actc1 has previously been shown to be highly expressed in fetal skeletal muscle [264], and overexpression of Actc1 has been used to rescue defects in skeletal actin myopathies that increased the expression of regenerating markers such as embryonic myosin [265]. Similarly, we found mouse SCs during regeneration also clustered near newly formed Actc1⁺ myofibres. Looking to other stem cell niche systems such as dental pulp [266] and hair follicle [267], stem cell density in the niche is far greater than in skeletal muscle, suggesting the upper limit to stem cell density in niches found in biological

systems could be increased. Regulation of the mouse Pax7 cell number set point has been demonstrated via Wnt4 knockdown [170], and increasing the SC number set point could have exciting implications for muscle regeneration and establishing niches from hPSCs.

Our results highlight that SMPCs that do not associate with human myofibers lose PAX7 expression. Loss of stemness also occurs for mouse SCs when displaced from the myofiber niche [151]. Thus, myofibers are important to support the stem cell state. In the absence of myofibers in Myf5/MyoD double-null mice, satellite cells can adopt mesenchymal or alternative somatic lineages after injury, but the double-null satellite cells were retained when myofibres were present and uninjured. This observation suggests that myofibres contribute to maintaining the identity of satellite cells having the ability to adapt non-myogenic cells [268, 269]. Similarly, using iCasp9 in human ACTC1 myofibres, we found that hPSC SMPCs adopted alternative cell fates when not associated with myofibres. PAX7–lamin AC⁺ cells expressed high levels of mesenchymal (PDGFRA and POSTN) and mesodermal (CDH11) genes. Both the lack of niche association and the immaturity of hPSC SMPCs may also result in increased plasticity, similar to work showing that foetal SMPCs have plasticity to form both bone/muscle without niches [33, 270].

The *in vivo* microenvironment has been shown to improve maturation of mouse embryonic stem cells following overexpression of Pax3 or Pax7 (iPAX) [271] and hPSC SMPCs *in vivo* [188], but all are largely fetal and not equivalent to quiescent adult SCs. Recent work has also shown that maturation can be enhanced by epigenetic modulation in other stem cell systems [272, 273]. We evaluated hPSC SMPCs *in vivo* compared with engrafted foetal or adult SCs and tissues, and our work suggests that hPSC SMPCs do mature *in vivo* from an embryonic week 7–12 to a later foetal week 17–20 stage, but do not fully mature to quiescent adult SCs. How human SMPCs become quiescent SCs in the niche is an exciting direction that could require lineage tracing to follow SC

and niche dynamics over time in vivo. Interestingly, we find that the myofibre populations between engrafted foetal and hPSC myofibres and foetal tissues are more dissimilar than are the PAX7+ cells, and thus the myofibres may be one of the most important targets for PAX7+ SMPC maturation, including approaches to enhance innervation.

In summary, we developed a new muscle stem cell ablation mouse model compatible with engraftment and demonstrated that emerging niche formation from hPSCs forms similar to the process of regeneration. This work has global implications for cell therapies and provides a new model system to study how to support human regeneration and niche emergence. Further, this new system could provide a platform to screen for new regulators of niche formation and the key regulators of stem cell–niche interactions supporting PAX7 cells in vivo.

2.5: Methods

Our research complies with all relevant ethical regulations. All hPSC research was approved by the hSCRO committee (2006-009-17), all biosafety was approved by IBC (BUA-2019-128-012-CR), all animal work was approved by the ARC (ARC-2006-119) and all fetal and human tissue research was approved as exempt by the institutional review board (IRB; 20-000197). All foetal and human tissues came de-identified with no patient information. Consents were obtained according to the ABR consent forms (fetal tissue) and NIH NDRI Tissue repository for human cadaver tissue. There was no participant compensation.

hPSC culture, directed differentiation into SMPCs and derivation of BLI hPSC lines

To improve myogenic potential, we revised the directed differentiation from previously described protocols [27, 32]. In brief, hPSC lines were independently optimized using seeding density and CHIR99021 concentration, and all directed differentiation medium changes were based on morphology and metabolic rate rather than set time points described below. Revisions

included addition of transforming growth factor- β inhibitor (TGF β i) in vitro during the final week of myogenic differentiation to improve myotube formation and support SMPCs and addition of insulin-like growth factor-1 (IGF-1) in the final 3 weeks to support SMPC survival and specification. TGF β i was removed from in vivo experiments as previously reported²⁰.

All hPSC experiments were performed with ESCRO approval, and hPSCs were obtained from WiCell and the UCLA stemcellcore. hPSC lines used in this study included H9 (WA09, NIH 0062) (**Figures 2.1, 2.2, 2.3**), H9 with a PAX7-GFP reporter⁸¹ (**Figures 2.1, 2.2**), CDMD 1002 and CDMD 1006-1 [187] (**Figure 2.1**) and WTC-11 (Coriell, AICS-0054-091) (**Figure 2.1**). To generate the luciferase reporter, an EF1 α -LUC-Puro lentivirus was used to infect hPSCs (H9 and H9 PAX7-GFP) in OptiMEM containing protamine sulfate. Three days after infection, hPSCs were selected by progressively increasing the puromycin concentration for 2 weeks. hPSCs were then single cell dissociated in CloneR and allowed to form clonal colonies. Six colonies from H9 and four colonies from H9 PAX7-GFP were generated and karyotyped (Cell Line Genetics). Normal, non-differentiated colonies were selected (two H9 clones and one H9 PAX7-GFP clone) and sequenced by LAM PCR (Creative Biogene) to determine viral integration sites.

hPSC lines were dissociated in TrypLE and plated at 275,000–475,000 cells per well of a six-well plate in mTESR containing 10 μ M ROCK inhibitor for 1 day to allow for recovery (day 1). hPSCs were then treated with 8–10 μ M CHIR99021 (Tocris, 4423) in E6 medium for 2–3 days to induce mesoderm formation. E6 medium was used to generate mesodermal Pax3⁺ cells for 7–10 days. StemPro-34 medium containing 10% StemPro-34 supplement, 5 ng ml⁻¹ basic fibroblast growth factor (bFGF), 2 mM l-glutamine, 0.45 mM monothioglycerol, 10.7 μ g ml⁻¹ transferrin and 0.4% penicillin-streptomycin was used to expand Pax3⁺ cells for 6–8 days. E6 medium was then added to cells for 3 days, and 10 ng ml⁻¹ IGF-1 was added to the E6 medium for 7–10 days

to enable specification of Pax7⁺ cells. N2 medium (1% N2 supplement, 1% insulin-transferrin-selenium (ITS, Gibco), 10 ng ml⁻¹ IGF-1 and 0.4% penicillin-streptomycin) was added to cells for 5–7 days, and N2 medium containing TGFβ_i (SB431542; 5 μM) was added for 5–7 days to differentiate hPSC SMPCs.

At the end of directed differentiation, hPSC SMPCs were prepared for FACS by dissociation in TrypLE and titrated to break apart three-dimensional structures. Cells were filtered through 100-μm meshes, washed in PBS, and resuspended in FACS buffer (2% FBS in PBS). hPSC SMPCs were labelled with antibodies against HNK1 (BioLegend, 359603), ERBB3 (BioLegend, 324705) and NGFR (BD Biosciences, 562123) (1.5 μl per 1 × 10⁶ cells) and far-red viability dye (ThermoFisher, L10120; 0.3 μl per 1 × 10⁶ cells) for 45 min at 4 °C and sorted on a BD FACS Aria II. Fluorescence minus one (FMO) controls were used for all gating controls. Samples were sorted and cultured in skeletal muscle basal medium-2 (SkBM-2, Lonza).

Husbandry and generation of mouse strains

To generate immunocompromised mice for human muscle cell engraftment, NOD-SCID-Gamma (NSG) mice (Jackson Laboratory, 005557) were crossed to C57BL/6J (C57; Jackson Laboratory, 000664), mdx C57BL/10 (mdx; Jackson Laboratory, 001801) or mdx-DBA/2 mice. mdx-DBA/2 mice were a kind gift from the laboratory of M. Spencer generated by crossing mdx to DBA/2J mice (Jackson Laboratory, 000671), which are deficient in *Ltbp4* and *Anxa6*. Pups were continually backcrossed to parental C57 or mdx strains for 5–8 generations to create >90% congenic strains while retaining mutant *Il2* and SCID alleles. Genotyping was performed using Transnetyx. All animal work was conducted under protocols approved by the UCLA or UC Irvine ARC in the Office of Animal Research Oversight. Mice were housed in A⁺-rated barrier facilities in sterile cages with standard 12-h light and 12-h dark cycles.

Congenic homozygous C57-NSG, mdx-NSG and mdx-DBA/2-NSG mice were used for engraftment studies or further crossed to generate Pax7 ablation models. C57-NSG, mdx-NSG and mdx-DBA/2-NSG mice were crossed to Rosa-DTA mice (Jackson Laboratory, 009669) or Pax7-Cre/ERT2 mice (Jackson Laboratory, 017763). Both Rosa-DTA and Pax7-Cre/ERT2 mice were crossed to C57-NSG, mdx-NSG and mdx-DBA/2-NSG strains for 3–5 generations before the start of Pax7 ablation experiments. Congenic homozygous Pax7-Cre/ERT2 males were then crossed to homozygous Rosa-DTA females, and heterozygous F1 Pax7Cre/WT; RosaDTA/WT mice were used for Pax7 ablation studies. Equal numbers of male and female mice were used at random. All mouse experiments were conducted when mice were between 5–10 weeks of age.

Assessment of mouse Pax7 ablation

Ablation of endogenous SCs was achieved using 400 mg ml⁻¹ tamoxifen chow (Envigo) or mice were injected intraperitoneally (i.p.) with 100 µl of 5 mg ml⁻¹ dihydroxytamoxifen (Sigma) diluted in 90% peanut oil and 10% ethanol. Pilot studies tested 3, 7 or 14 days of tamoxifen chow and 1, 3 or 5 days of dihydroxytamoxifen i.p. injections (n = 3 mice per group). Non-transgenic Pax7Cre/WT; RosaWT/WT mice were used as controls. Mice were killed immediately following treatment or 5 days after tamoxifen washout. Tissue was dissected and immunostained for Pax7 (DSHB), laminin and DAPI to count satellite cell numbers. Laminin and H&E staining were also used to measure myofiber and muscle cross-sectional area. Multiple tissue sections and images were taken for each mouse. We found that 7 days of chow, or 3 days of i.p. injections, followed by a 5-day washout period was sufficient to ablate up to 90% of satellite cells compared to non-transgenic controls (n = 5 mice).

Engraftment protocol and preparation of hPSC and fetal SMPCs and adult SCs

After sorting, hPSC SMPCs were cultured and expanded in SkBM-2 plus bFGF for as short a time as possible (2–3 days) before engraftment to prevent loss of PAX7 expression. hPSC SMPCs in culture were dissociated using TrypLE, pelleted at 300g and resuspended in HBSS at 1×10^6 cells per 5 μ l. This allowed us to obtain at least 1 million cells per mouse for engraftment. Mouse models aged 6–8 weeks were subjected to a muscle injury by intramuscular injection of 50 μ l of 10 μ M cardiotoxin (Sigma) or 1.2% barium chloride, 24 h before cell injections. Pelleted cells were kept at 4 °C and transported to the UCLA Humanized Mouse Core. Mice were anaesthetized using 2% isoflurane, and 5–10 μ l of cells in solution were injected into the TA muscle using Hamilton microsyringes.

For Pax7 ablation studies, mice were given tamoxifen chow for 7 days and allowed a washout period of 5 days before engraftment. On day 4 of washout, mice were given a cardiotoxin or barium chloride injection to induce muscle injury. Non-transgenic mdx-NSG mice served as controls. For some Pax7 ablation studies, we also used MACS-enriched (CD45–CD235a–) fetal SMPCs. For Pax7-knockout studies, after notice was received that adult clinical samples had arrived, mice were given tamoxifen for 4 days with washout for 1 day and adult SCs were expanded in SkBM-2 for 5–7 days before engraftment.

Quantification of human PAX7 in engrafted samples

Using a microcryotome, 1,200 μ m of TA muscle was removed; 20 sections were collected on positively charged frosted microscope slides in 50- μ m intervals, and 300 μ m of tissue was collected for RNA or western blots. In this manner, we calculated that each quadrant contained 1,300 μ m of muscle tissue. Quadrants were collected until the entire TA was sectioned. Thus, we could determine the position of each cross-section for downstream analysis of cell engraftment. Microscope slides were kept in aluminum foil-covered boxes at –20 °C.

To quantify cell engraftment, all human lamin AC+spectrin+ and human dystrophin+ myofibers from at least 11 cross-sections were counted. To calculate the maximum number of engrafted fibers, the cross-section containing the greatest number of human lamin AC+spectrin+dystrophin+ myofibers was averaged with other cross-sections containing the greatest number of human myofibers from all mice within a treatment group (n = 3–7). The maximum engrafted human myofiber average $\pm$ s.e.m. was then used to compare to other treatment groups by one-way ANOVA with post hoc Tukey's test for multiple comparisons ($P < 0.05$).

To quantify PAX7+ cells, we first identified regions containing human lamin AC+ nuclei. We then captured images of all human PAX7+ cells in this region at $\times 20$ magnification (0.15 mm²; Zeiss Axio-Observer). Data are represented as total PAX7+ cells per image, rather than total PAX7+ cells per cross-sectional area of a whole tissue, as high-magnification images were required to quantify niche location (that is, within basal lamina) and quantification required multiple fluorescence channels to confirm human PAX7 origin and enabled multiple users to quantify our large image library. At least two independent users quantified data from each high-magnification image to determine the numbers and locations of PAX7+ and lamin AC+ regions, and thus using the percentages of PAX7+ cells was a more robust quantification method.

Slides were co-stained for lamin AC and spectrin to mark engrafted human myofibers and PAX7+ cells were designated as associated with chimeric or human-only myofibers from 18 independent image regions (n = 5 engrafted mice; **Figure 2.1**). We next identified engrafted lamin AC+ locations and categorized these into four regions: (1) interstitial space, within endomysial regions but with few engrafted myofibres; (2) dense lamin AC+ regions, with fields of view containing >100 human cells but few myofibres; (3) chimeric myofibres, with greater than half the field of view occupied by chimeric myofibres; and (4) human-only fibres, where the field of view

contained >50 human-only myofibers. After identification of regions, a total of 15–18 images containing PAX7+ cells from n = 3 mice were quantified; statistical analyses were performed using one-way ANOVA. GraphPad v.6.0 software was used to graph and calculate significance at $P < 0.05$.

Immunofluorescent staining protocol for engrafted muscle tissues

To perform staining, sections on slides were thawed from -20°C freezers and washed in PBS containing 0.2% Tween-20 (PBST). Slides were fixed in 4% paraformaldehyde (PFA) for 10 min and washed with PBST. Sections were then blocked in 10% goat serum in 0.2% gelatin, 3% BSA and 0.1% Tween-20 for 90 min at room temperature. Sections were immunostained with human-specific antibodies to spectrin (1:80; Leica, NCL-SPEC1), lamin AC (1:120; Vector Laboratories, VP-L550) and dystrophin (1:6; DSHB, MANDYS106) to positively identify engrafted human cells and examine whether they were capable of fusing to restore human dystrophin expression. Sections were alternatively stained with antibodies recognizing human PAX7 (10 $\mu\text{g ml}^{-1}$; DSHB), lamin AC, spectrin and laminin (1:150; Sigma, L9393) to identify human SMPCs and SCs residing in the satellite cell position. Antibodies to ACTC1 (1:100; Sigma, A9357), Myh3 (1:100; DSHB, BF-45), Myh4 (1:100; DSHB, BF-F3) and M-cadherin (1:100; BD Biosciences, 61110) were used to identify regenerative myofibers. Slides were washed and tissues were counterstained and mounted with DAPI (Vector Laboratories) and imaged using a Nikon Eclipse 600 or Zeiss Axio-Observer 1. High-magnification images were obtained on a Zeiss LSM 880 confocal microscope.

ACTC1 knock-in method

To generate an inducible ACTC1-expressing suicide cell line, we used both the H1 and H9 lines for CRISPR-mediated homology-directed repair. For nucleofection, hPSCs were single-cell

dissociated and 500,000 hPSCs were nucleofected using an Amaxa 2D Nucleofector (shock program B-016) along with purified spCas9 (IDT), gRNAs and template ACTC1 knock-in plasmid (6–10 µg; VectorBuilder). For the protocol from Skarnes et al. [248], we included HDR enhancer (IDT) and cultured cells at 32 °C for 2 days following nucleofection, while cells on the Lonza protocol was placed immediately at 37 °C without HDR enhancer. hPSCs were recovered with mTESR Plus and CloneR (Stemcell Technologies). We also tested LipoStem (ThermoFisher), which was used to lipofect a plasmid containing two gRNAs (sequences 1 and 2) and spCas9 alongside the template ACTC1 knock-in plasmid into small adherent hPSC colonies. When cells reached confluence, hPSCs from all conditions were dissociated and split for isolation of genomic DNA (Zymogen) and cryostorage. Genomic DNA was used to screen for presence of the correct genomic insertion using primers designed to amplify through both homology arms. We found that nucleofection and gRNA-1 from both protocols worked for both the H1 and H9 lines, but we moved forwards with the parental lines from Skarnes et al. because the bands for these cells appeared to be more pronounced. Following visualization of the correct band on a 1% agarose gel, the band was gel extracted (Zymogen, D4007) and Sanger sequenced to verify the correct insertion. We then thawed H1 and H9 parental cryovials and FACS sorted DAPI-negative single hPSCs into four 96-well tissue culture plates containing mTESR Plus and CloneR. Small to medium-sized colonies were visible in 108 of the 384 wells after 1 week, and clonal colonies were split into 24-well plates, expanded and split for genomic DNA isolation and cryostorage. Extensive PCR genotyping and sequencing using multiple primer pairs were used to identify two hPSC clones (clones 9 and 25 in H9) with the correct knock-in.

We proceeded to directed differentiation of hPSCs into SMPCs with both knock-in clonal lines, and one line was able to produce a high yield of PAX7⁺ SMPCs based on FACS enrichment

of ERBB3+NGFR+ cells, real-time PCR analysis and in vitro myotube fusion. Sorted SMPCs were expanded for 3 days in vitro after sorting and then approximately 1 million SMPCs were injected into 6-week-old mdx-D2-NSG Pax7-ablated mice that were pretreated with barium chloride. Two weeks following engraftment, mice were injected i.p. with AP1903 (10 μ M in 100 μ l saline) or control (0.1% DMSO in saline) every 2 weeks for 90 days (six total injections). Mouse human-transplanted tissues were then dissected and mounted on microscope slides, and we proceeded to same-day GeoMx spatial RNA-seq. A total of 59 ROIs were sequenced, including 18 regions containing PAX7+ cells, 23 regions containing hPSC myofibres and 18 regions containing human lamin AC+PAX7– cells.

GeoMx spatial RNA-seq

Slide preparation

Spatial RNA-seq was performed using human skeletal muscle tissues (limb) or mouse tissues (TA) engrafted with human cells. We determined that freshly sectioned muscle tissues yielded the best RNA quality; thus, tissues were flash-frozen in isopentane-chilled liquid nitrogen and sectioned at 10 μ m for same-day RNA-seq analysis. To identify engrafted regions, TA muscle sections were collected every 500 μ m on Superfrost Plus slides for tissue adherence, which allowed us to avoid the suggested baking steps at 65 °C (Nanostring) to further improve RNA quality. Muscle sections were first washed with DPBS and fixed with 4% PFA for 30 min. To expose RNA targets, we empirically determined that tissues needed to be treated with 0.025 μ g ml⁻¹ proteinase K for 5 min at 37 °C as higher concentrations cleaved antigen binding sites. For in situ hybridization, human whole-transcriptome RNA probes (Nanostring) were applied to the tissues, which were then covered with a Grace Bio-Labs HybriSlip and incubated in a hybridization chamber at 37 °C overnight. The following day, the HybriSlips were removed in 2 $\times$ SSC-T buffer

and tissue slides were washed twice for 25 min with a solution composed of 4× SSC and deionized formamide. Washing and staining of slides were performed using Grace Bio-Labs silicon coverslips to avoid hydrophobic pen usage. To add morphology markers, we blocked with buffer W for 30 min and then added primary antibodies, including antibodies to PAX7 (DSHB; 1:30), spectrin (1:80), lamin AC (ThermoFisher; 1:80) and ACTC1 (Sigma; 1:150), diluted in buffer W, for 90 min. Tissues were washed three times with DPBS, and secondary antibodies conjugated to Alexa Fluor 532, 594 and 647 were added (at a 1:250 dilution) alongside Syto13 nuclear dye (Nanostring; 1:15) in DPBS for 60 min. Tissues were washed in DPBS and taken to the GeoMx machine for scanning and selection of ROIs.

RNA probe collection

Selection of ROIs and segmentation were based on fluorescence intensity for PAX7+ cells (PAX7+lamin AC+), myofibres (spectrin+lamin AC+ACTC1±) and non-myogenic human cells (lamin AC+, negative for other markers). The GeoMx platform photocleaves and collects RNA probes within segmented ROIs for sequencing. A list of all 125 ROIs collected for sequencing is shown in Supplementary Fig. 9b. We sequenced at twice the recommended coverage (200 reads per μm^2) for a total read count of 161 million reads.

Analysis

FASTQ files were aligned to a human reference genome (hg38), and data were uploaded to the GeoMx platform to begin analysis. We first ran a general quality control, and all ROIs passed the quality control analysis. To filter the data, we filtered at 1% of the limit of quantification and higher than the user-defined value of 2 and normalized using the quartile-3 (Q3) method. To compare different cell types or treatments to generate volcano plot data, linear mixed model (LMM) statistical tests with Benjamini–Hochberg correction were performed. The .csv files from

GeoMx analysis served as the input for generating volcano plots in R Studio using the EnhancedVolcano program. PCA and UMAP plots were generated using modified custom scripts on the GeoMx platform.

Statistics and reproducibility

Please see individual Methods sections for sample sizes and how statistical analysis was performed for each experiment. Unless noted otherwise, all statistical t tests used were parametric, unpaired and two tailed. All one-way ANOVA analyses were corrected for multiple comparisons and assumed a Gaussian distribution. GraphPad Prism version 9.3.1 was used to generate graphs and statistics. In general, randomization and blinding were performed where possible. For imaging of PAX7, regions were first blindly selected in the lamin AC fluorescence channel and PAX7 cell images were then captured without predetermining PAX7 locations. All image data were quantified by two or more users, and individual cells such as PAX7+ cells were marked on the original image so that multiple users could verify niche occupancy and PAX7 positivity. For GeoMx spatial RNA-seq, users were blinded to engraftment efficiency until images were scanned through the platform. To increase reproducibility, four hPSC lines were used in this study, including H9, H9 with a PAX7-GFP reporter, H9 with a luciferase reporter, H9 with an iCas9 gene, CDMD 1002 and CDMD 1006-1, and WTC-11. We also used two separate dystrophic mouse models (mdx-NSG and mdx-DBA/2-NSG) to confirm the presence of the human-only myofibres. Litter- and age-matched mice were selected at random for engraftment and re-injury experiments, and analyses between groups such as foetal versus adult engraftment were performed with blinding. No animals or data points were excluded from the study. No statistical method was used to predetermine sample size, but sample sizes for engraftment are similar to those reported in previous publications [29]. In general, data distribution was assumed to be normal, but this was

not formally tested. The one set of experiments where data distribution was not assumed to be normal was in the engraftment distribution of SMPCs into mouse skeletal muscle. For these experiments, we sectioned multiple regions throughout the muscle and report engraftment efficiency across the entire length of the muscle or we report engraftment efficiency as the maximum number of engrafted myofibres from the cross-sectional region with the greatest engraftment, and these regions were used for statistical comparison. For spatial RNA-seq data, all ROIs were validated by the user in addition to the software algorithms provided by the GeoMx DSP segment. If pixel intensities led to inaccurate segmentation, these data were excluded.

Chapter 3: MEGF10 Accelerates a Developing Niche to Improve Engrafted Progenitor Cell Retention in Dystrophic and Regenerating Skeletal Muscle

3.1 Abstract

Skeletal muscle cell therapies are limited by poor long-term engraftment, particularly in diseased muscle, where retention of a PAX7⁺ stem cell pool depends on formation of a supportive niche. Here, we used spatial transcriptomics to map niche development across engraftment time, healthy versus dystrophic microenvironments, and secondary injury. Engrafted myogenic cells underwent rapid early transcriptional remodeling and marked donor-cell loss, whereas regenerating myofibers acquired niche-associated programs that stabilized surviving PAX7⁺ cells. Dystrophic microenvironments impaired niche maturation altered donor-cell state and blunted regenerative adaptation after reinjury. MEGF10 emerged as an early niche-associated regulator that was transiently expressed during healthy regeneration but persisted in dystrophic muscle. Using CRISPR-engineered hPSC models, we found that MEGF10 induction during the first 10 days after transplantation more than doubled PAX7⁺ cell retention and donor-derived myofiber output, whereas EMARDD-associated MEGF10 loss of function failed to maintain engrafted PAX7⁺ cells. Spatial profiling of Tet-On engraftments identified a MEGF10-responsive regenerative myofiber program linked to early niche support. These findings define an early therapeutic window for improving muscle cell transplantation.

3.2: Introduction

Skeletal muscle regeneration is essential for maintaining tissue function and quality of life but can become impaired in aging or neuromuscular diseases. Cell-based therapies offer a promising strategy to restore muscle function by repairing damaged tissue and replacing an exhausted or dysfunctional stem cell pool [274, 275]. However, efficient support of engrafted cells

and retention of a stem cell pool capable of long-term regeneration remain a barrier to therapeutic development. Early myoblast transplantation studies established that, without the ability to replenish a self-renewing stem cell pool, these approaches provide only limited and short-lived benefit [172, 276]. Retention of a PAX7⁺ stem cell pool after engraftment depends on formation of a supportive muscle stem cell niche [37, 123], which is comprised of a basal lamina and extracellular matrix, the adjacent myofiber, and microenvironmental cues including secreted factors and cell-matrix signaling [18, 124]. How muscle stem and progenitor cells establish niches after transplantation, and how the tissue microenvironment influences this process, remain incompletely understood. Defining how transplanted human muscle cells regenerate, establish niches, and adapt to the *in vivo* environment, particularly in diseased muscle, is essential for the development of effective stem cell therapies.

Human pluripotent stem cells (hPSCs) offer a potential solution for studying niche development and improving cell therapies because they can be genetically engineered with niche-associated transgenes or disease-associated modifications and subsequently differentiated into skeletal muscle progenitor cells (SMPCs) for preclinical disease modeling and at scales relevant for human therapy [187]. Directed differentiation of hPSCs to skeletal muscle generates heterogeneous cultures that support PAX7⁺ SMPCs for weeks to months *in vitro* [27-29, 32, 184, 185]. However, hPSC-derived SMPCs are functionally immature and transcriptomically resemble early fetal muscle progenitor cells [33], which are associated with inefficient niche formation after engraftment [37] potentially due to unique cell-niche interactions that differ from adult satellite cells. Following transplantation, some teams found that only a small fraction of PAX7⁺ SMPCs are retained, although those that successfully occupy niches can adopt a more mature gene-expression profile overtime resembling adult-like satellite cells [188]. Improving the ability of

transplanted SMPCs to establish niches *in vivo* and persist as a quiescent stem cell pool is therefore essential for developing long-term cell therapies for patients with myopathies.

Since cell-based therapies will ultimately be deployed in diseased muscle, it is equally important to understand how pathological microenvironments influence niche formation and stem cell persistence after transplantation. Niche disruption is a common feature of many myopathies, in which diseased muscle fails to adequately support endogenous stem cells, leading to impaired self-renewal and loss of tissue homeostasis [277]. Duchenne muscular dystrophy is the most common genetic disease of muscle, results from a loss-of-function mutation in dystrophin, which results in a destabilized sarcolemma and repeated myofiber degeneration, thus forcing resident stem cells to continually rebuild their niches during chronic regeneration and ultimately contribute to stem cell exhaustion [79]. In this setting, destabilization of the extracellular matrix surrounding muscle stem cells, together with inflammatory responses and altered neighboring cell populations, further disrupts the myogenic niche that normally support stem cell function [278]. Similarly, extracellular matrix disorders such as COL6A1-associated Ullrich congenital muscular dystrophy directly impair the myogenic niche leading to poor regeneration and stem cell retention [263]. Early-onset myopathy, areflexia, respiratory distress, and dysphagia (EMARDD), caused by loss-of-function mutations in the niche receptor MEGF10, is among the most severe myopathies, with cases ranging from embryonic lethality to profound fibrosis and impaired regeneration [198, 200]. Thus, muscle diseases highlight the need for therapies that can either correct intrinsic stem cell defects or replace the diseased stem cell pool with a healthy, self-renewing one through transplantation.

Microenvironmental signals that stabilize muscle stem cells within their niche must be appropriately regulated in time and space to support self-renewal and quiescence [279]. The

function of niche receptors is therefore highly dependent on both developmental timing and tissue context, as the niche interactome evolves through regeneration, maturation, and return to quiescence to sustain the stem cell pool [18]. These microenvironmental cues include secreted signals from adjacent cell populations that activate transcriptional programs promoting niche formation, myofiber hypertrophy, and tissue remodeling [160, 280]. Niche forming cues are also provided from stem cell-myofiber or cell-matrix interactions that regulate entry into, and maintenance of, activated or quiescent states. For example, M-cadherin, Tenascin-C, and Calcitonin Receptor play important roles at the satellite cell-myofiber interface and in adhesive interactions between satellite cells and the extracellular matrix, thereby supporting early niche formation, self-renewal, and quiescence [119, 120, 150, 151, 162]. However, we hypothesize that because the niche changes over time, receptor expression profiles that are beneficial during early regeneration may become ineffective, or even maladaptive, if sustained at later stages, and vice versa. Defining the stage-specific functions of niche receptors during engraftment and regeneration, particularly in hPSC-derived SMPCs, therefore represents an important opportunity to advance cell-based therapies.

Recent advances in spatial transcriptomics now make it possible to resolve rare satellite cell populations in their native tissue context with increasing cellular precision [281]. We therefore applied spatial transcriptomic analysis to engrafted human skeletal muscle across time in both healthy and dystrophic mice to define supportive and antagonistic microenvironmental interactions, including those arising from unperturbed and regenerating human myofibers after SMPC transplantation into mice. These studies revealed that dystrophic muscle exerts maladaptive effects on both the transcriptional interactome and functional output of donor cell therapy. Among the factors that changed across time and disease, MEGF10 emerged as a candidate niche receptor

of particular interest because of its association with early myopathic disease and its understudied mechanisms, binding partners, and cellular expression patterns in skeletal muscle. To test the temporal role of MEGF10 during engraftment, we generated tetracycline-inducible and disease-modeling loss-of-function hPSC lines and examined the effects of modulating MEGF10 expression after transplantation. Early MEGF10 induction more than doubled the engraftment efficiency of PAX7⁺ cells and donor-derived myofibers. Our findings identify the first 5-10 days after transplantation as a critical therapeutic window during which manipulation of niche development may improve engraftment and regenerative outcome in muscular dystrophy.

3.3: Results

Regenerative trajectory mapping after engraftment of hPSC SMPCs

Although directed differentiation from hPSCs generates cultures that support PAX7⁺ SMPCs *in vitro*, retention of these cells after transplantation *in vivo* remains inefficient [185]. Following directed differentiation, we FACS-enriched SMPCs, briefly expanded them, and cryopreserved them for *in vivo* transplantation studies. For engraftments, we used ROSA-DTA[±]-PAX7-Cre[±]-NSG mice to inducibly ablate endogenous mouse Pax7 cells and thereby reduce competition from host satellite cells [37]. Tamoxifen treatment significantly depleted host mouse Pax7⁺ stem cells (**Figure 3.2A**). We then performed spatial transcriptomic analysis on fresh-frozen xenografted muscle using the GeoMX Digital Spatial Profiler [282]. An advantage of the GeoMX platform is its ability to segment defined cell populations *in situ* using antibody-based staining, enabling selective profiling of specific myogenic populations within engrafted regions. We chose spatial transcriptomics because it retains spatial relationships between engrafted cells and surrounding muscle, permits *in situ* selection of engrafted regions, and enables capture of multinucleated myofibers without the need for FACS.

To define the *in vivo* trajectory of transplanted SMPCs, we performed spatial transcriptomics at days 1, 5, 10, and 20 after engraftment (**Figure 3.1A**). At each time point, we profiled myogenic regenerative progression using markers for human nuclei (Lamin AC), SMPCs (PAX7), intermediate myoblasts (MYOD1), myocytes (MYOG), and human myofibers (Spectrin) [281]. We collected 38-47 regions of interest (ROIs) per time point using segmented single- and double-positive marker morphologies and used ROIs for statistical analysis (**Figure 3.2B-C**).

Before comparing transcriptional changes across time, we first confirmed dataset quality and cell-type annotation through spatial transcriptomic profiles. Spatial transcriptomic profiling detected 18,678 genes across samples, of which 11,196 remained after quality control and background filtering. Each mononuclear population ROI contained approximately 5-15 individual SMPCs or myocytes, which were pooled to generate a gene expression profile, resembling bulk RNAseq. Filtered count matrices were normalized and analyzed in DESeq2 to compare engrafted myogenic populations across time [283]. To validate cell-type identities, we grouped PAX7+ SMPCs, MYOG+ myocytes, and myofibers across all time points and examined expression of canonical markers including *PAX7*, *MYOD1*, *MYOG*, *MYH3*, and *MYH1* (**Figure 3.2D**). As expected, SMPCs expressed higher levels of *PAX7*, myocytes expressed higher *MYOD1* and *MYOG*, and myofibers expressed higher *MYH3* and *MYH1* ($p < 0.05$). Despite qualitative differences of *PAX7* expression levels within SMPC ROIs, these differences did not separate into statistically distinct high- and low-expressing subgroups.

Engraftment induces rapid transcriptional remodeling across myogenic populations

To complement our spatial transcriptomic analysis, we conducted histological analysis quantifying myogenic populations across time and found a sharp decline in both PAX7+ SMPCs and MYOG+ myocytes after engraftment, paralleling a global loss of human nuclei and a

progressive increase in myofibers (**Figure 3.1B**). This early loss plateaued after day 5, suggesting that the cells that survive the *in vitro*-to-*in vivo* transition become stabilized and are subsequently maintained through supportive interactions with regenerating myofibers. We then performed principal component analysis (PCA) on all spatial transcriptomic myogenic populations across time to gain insight on the mechanisms of transcriptional remodeling and stem cell support (**Figure 3.1C**). Genes uniquely expressed in each population and conserved across time further confirmed their distinct transcriptomic identities (**Figure 3.1D**). In particular, engrafted SMPCs and myocytes at day 1 were clearly separated from later time points, indicating that exposure to the tissue microenvironment rapidly reshapes donor cell state. To define this global *in vivo* response, we identified genes shared across time in SMPCs and myocytes. Surprisingly, more genes were downregulated after engraftment than were induced. In total, 443 DEGs captured the global transition from *in vitro* culture to the *in vivo* environment, and gene ontology analysis showed enrichment for translation, cell adhesion, cell-matrix interactions, and migration (**Figure 3.1E**). Among these were *TNC* and *NOTCH3*, extracellular and transmembrane niche-associated factors implicated in establishment and stability of the muscle stem cell niche [162, 284]. These data indicate rapid transcriptional reprogramming of engrafted populations that stabilizes upon regeneration of myofiber populations, suggesting potential supportive interactions at play.

We next examined temporal transcriptional changes after engraftment to define how transplanted myogenic populations remodel muscle tissue across time and how this remodeling relates to niche formation. In SMPCs, spatial transcriptomic analysis identified more than 700 DEGs from days 1 to 5 and more than 50 DEGs from days 5 to 10. Among these, musculoskeletal growth regulator *ACVR2* declined after engraftment, whereas *LRRN1*, a transmembrane receptor involved in matrix remodeling, increased (**Figure 3.3A**) [124, 285]. Human myocytes underwent

even broader remodeling, with more than 1,800 DEGs from days 1 to 5, more than 200 from days 5 to 10, and more than 40 from days 10 to 20. Key myogenic receptors exhibited chronological trends, with *MEGF10* and *DLL1* rapidly lost on myocytes by day 5, while MCAM increased following engraftment [124, 152, 219]. Meanwhile, factors linked to fusion and myofiber assembly like *MYOM3* increased after engraftment (**Figure 3.3B**) [286]. These data suggest rapid early transcriptomic shifts followed by a more stabilized transcriptomic program over time.

Distinct myofiber maturation states reveal candidate mechanisms of niche support

We identified morphological and transcriptomic heterogeneity within myofiber populations after engraftment. Smaller myofibers were closely localized to neighboring engrafted human myocytes and appeared to represent donor-derived, actively regenerating fibers composed exclusively of human cells (human only), whereas larger chimeric myofibers had fused with host mouse myofibers, as indicated by the presence of mouse Lamin A/C-negative myonuclei (chimeric; **Figure 3.1F**) [37]. Our previous work showed that human-only myofibers support engrafted PAX7⁺ cells more effectively than chimeric myofibers, although the mechanisms underlying this effect were unclear [37]. We therefore used spatial transcriptomics to examine adhesive ligands and receptors that might mediate early niche formation between regenerative myofibers and PAX7⁺ SMPCs. This analysis revealed a distinct niche-supportive receptor profile in regenerative human-only myofibers, including significantly elevated expression of *MEGF10* and *DLK1* (**Figure 3.1F**), implicating candidate mechanisms and signals that promote SMPC support and niche establishment.

PCA further confirmed that human-only and chimeric myofibers represent distinct populations with separate temporal trajectories (**Figure 3.3C**). Human-only myofibers displayed a persistent regenerative transcriptome marked by embryonic myofiber genes *MYH3* and *ACTC1*

[11, 37], along with additional regenerative markers including *PEG10*, *MYL4*, and neuromuscular junction gene *CHRND* [287-289]. In contrast, chimeric myofibers expressed higher levels of mature, non-regenerative myofiber markers including *MYH1*, *MYH2*, *ALDOA*, and *CKM* [290]. Consistent with these distinct states, *ACTC1* expression remained relatively stable over time in human-only myofibers, whereas it progressively declined in chimeric myofibers, indicating continued maturation (**Figure 3.3D-E**). This distinction was further supported by increased *VEGFA* expression over time in chimeric myofibers, a secreted factor linked to muscle regeneration and capillary remodeling [291]. Chimeric myofibers also expressed *MYH4* which may reflect some cross-hybridization of human GeoMx probes with mouse myofiber transcripts within multinucleated myofibers [292]. These findings indicate that human-only myofibers remain in a more immature, niche-supportive state, whereas fusion into host fibers is associated with progression toward a more mature phenotype and dampening of supportive niche receptors.

Investigating the heterogeneity of engrafted myogenic cells using single nucleus RNAseq

It remains unclear how to retain and support PAX7 expression in vitro or in vivo and prevent precocious differentiation to committed myoblasts. Directed differentiation from hPSC to SMPCs yields a heterogeneous mixture of cells that supports increased PAX7 levels on SMPCs in vitro but are less well understood in vivo [186]. Our engraftment strategy relies on enrichment for PAX7⁺ cells after directed differentiation with short recovery times prior to cell transplantation but enough time to expand sufficient numbers for engraftment. This approach yields myogenic populations of mixed states of activation and gene expression changes, and the expansion of enriched SMPCs reduces PAX7, NOTCH3, and MEGF10 expression levels significantly (**Figure 3.4B**). Thus, we performed single nucleus RNA-sequencing (snRNAseq) on expanded SMPCs seven days after sorting to better define the immediate SMPCs at the time of transplantation

(**Figure 3.4A**). This analysis revealed five transcriptomically distinct subpopulations that have uniquely expressed genes including two PAX7⁺ populations comprising about one third of the in vitro culture, with one marked by highly proliferative markers MKI67 and TOP2A (**Figure 3.4C-E**). An SMPC cluster undergoing precocious differentiation identified MYOD1, MYOG, and CKM and makes up about a quarter of the in vitro culture. A PAX3⁺ OSMR⁺ population of myogenic cells that may represent an earlier precursor or developmentally immature muscle stem cell make up a third of the culture. The final ~10% is a proliferative cell type negative for PAX7 and other muscle markers, suggesting potential impurity in the enrichment strategy for SMPCs. Muscle stem cell niche receptors such as MEGF10, CDH15, and ITGA7 followed a pattern of expression on both the PAX7⁺ and differentiated populations.

Dystrophic muscle reshapes the interactome of engrafted skeletal muscle populations and impairs regeneration after secondary injury

Understanding how engraftment evolves within a dystrophic environment is critical for developing effective cell therapies for muscular dystrophies. To define how a diseased host environment alters donor cell behavior, myofiber regeneration, and niche development, we engrafted human muscle stem cells into both wildtype C57-NSG and dystrophic mdx-D2-NSG mice, a severe model of Duchenne muscular dystrophy [293]. To eliminate competition from host satellite cells during niche formation, we further crossed both strains onto inducible Pax7 ablation transgenes [37]. We then performed spatial transcriptomic profiling 30 days after engraftment, corresponding to the completion of regeneration, to compare engrafted human SMPCs and myofibers in healthy and dystrophic muscle (**Figure 3.5A**). Across conditions, we captured and analyzed 26–28 ROIs containing both PAX7⁺ SMPCs and myofiber populations (**Figure 3.7A**).

Engraftment into both healthy and dystrophic muscle produced PAX7+ SMPCs and human spectrin+ regenerative myofibers. However, the dystrophic environment markedly impaired niche formation by engrafted PAX7+ SMPCs, reducing niche establishment from 77% in healthy muscle to 45% in dystrophic muscle (**Figure 3.5B**). PCA stratified by microenvironment and cell type revealed clear separation between healthy and dystrophic engraftments, indicating that the host tissue environment strongly influences the transcriptional state of transplanted cells (**Figure 3.5C-D**). PC1 primarily captured the distinction between SMPCs and myofibers, whereas PC2 reflected transcriptional differences associated with the host microenvironment. To define this global microenvironmental response, we used DESeq2 to compare differential gene expression across tissue contexts and retained genes shared by both SMPCs and myofibers. This analysis identified 97 SMPC-associated DEGs in dystrophic engraftments compared with 44 in healthy engraftments, while comparison of healthy and dystrophic myogenic populations overall revealed more than 2,000 DEGs, underscoring broad divergence in both donor cell state and regenerative maturation across tissue environments.

Gene ontology analysis of genes associated with a healthy microenvironment highlighted programs related to myofiber contraction, muscle maturation, and integrin-mediated cell interactions, whereas dystrophic microenvironment-associated genes were enriched for extracellular matrix organization and cytokine-responsive pathways (**Figure 3.5E**). The mdx-D2 environment also induced donor-cell expression of genes linked to osteogenic and chondrogenic states, suggesting that the *in vivo* plasticity of engrafted SMPCs is altered in dystrophic muscle.

We next projected selected genes representing both shared microenvironment-responsive programs and uniquely expressed genes across cell type and tissue context (**Figure 3.5F-G**). Among genes enriched in dystrophic engraftments, *PAMI6*, a regulator of mitochondrial protein

import with reported links to immune-associated signaling was the top unbiased DEG, while *COL1A1* was the most prominent matrix-associated DEG, paralleling collagen trends in aged fibrotic settings [194, 294]. In contrast, healthy engraftments showed increased expression of *LYRM2*, which is associated with mitochondrial function, and *NCOA1*, a regulator of skeletal muscle maturation [33]. Within SMPCs, healthy tissue favored expression of the ECM-associated factor *LAMBI* and the cadherin-linked niche regulator *CTNNB1*, whereas dystrophic tissue increased expression of *NOTCH3*, *DLL3*, and the differentiation-associated marker *SP7* [119, 295]. Regenerative myofibers also showed marked divergence between healthy and dystrophic environments, with differences in both adhesive signaling and maturational state contributing to the more than 2,000 DEGs identified across tissue contexts. These findings demonstrate that the host microenvironment is a dominant determinant of engrafted cell state, rewiring adhesive and transcriptional programs to drive fundamentally distinct regenerative outcomes in healthy and dystrophic muscle.

Dystrophic microenvironments limit regenerative recovery after secondary injury

Given the marked differences in transcriptomic state and niche colonization by engrafted PAX7+ SMPCs in dystrophic muscle, we next tested their functional regenerative potential. Since secondary injury after initial regeneration provides a stringent measure of stem cell potency *in vivo* [296], we induced reinjury with BaCl₂ 30 days after SMPC engraftment in healthy and dystrophic muscle (**Figure 3.6A**). In both settings, secondary injury increased the frequency of PAX7+ SMPC niche formation; in mdx-D2-NSG muscle, niche establishment rose from 44% to 74% ($p < 0.05$). However, healthy engraftments still supported superior niche formation compared with dystrophic engraftments (**Figure 3.6B**). Consistent with this, dystrophic muscle produced significantly fewer

engrafted myofibers and SMPCs after secondary injury, indicating a stunted regenerative response (**Figure 3.6C-D**).

Spatial transcriptomic analysis further showed that the host microenvironment determined how donor cells responded to reinjury. In healthy engraftments, both SMPCs and myofibers underwent a clear transcriptomic shift after secondary injury, whereas dystrophic engraftments showed only limited change through PC analysis (**Figure 3.6E**). SMPCs in healthy muscle exhibited extensive transcriptional remodeling, with more than 300 genes changing with re-injury. By contrast, SMPCs in dystrophic muscle showed only ~20 total DEGs across this transition, consistent with a muted regenerative response imposed by the host environment (**Figure 3.6F**). Among the genes that resolved after secondary injury in healthy engraftments were the developmental niche-associated factors *MEGF10*, *TNC*, and *MCAM* [124], whereas their expression remained sustained in dystrophic engraftments (**Figure 3.6G**). These data indicate that dystrophic muscle not only reduces regenerative output after reinjury, but also prevents engrafted SMPCs from appropriately transitioning and maturing their niche transcriptional state.

Regenerative myofiber populations also underwent divergent responses to secondary injury in healthy and dystrophic environments. Dystrophic engraftments had reduced myofiber maturation after secondary injury, as indicated by reduced expression of *MYH1* and increased fetal *MYH8* expression (**Figure 3.7B**). Notably, the adhesive ligand-receptor programs associated with niche support also differed substantially after reinjury. *MEGF10*, *LRRN1*, and *MCAM* were expressed at significantly higher levels in healthy engrafted myofibers before reinjury, whereas the early fusion regulator *NID1* is upregulated after reinjury in dystrophic engraftments [297]. These findings suggest that healthy and dystrophic microenvironments differentially regulate the

adhesive and maturational programs of regenerative myofibers, with likely consequences for niche re-establishment after injury.

These transcriptomic analyses across time and disease identify a temporally regulated niche-associated program during engraftment and regeneration. Notably, MEGF10, TNC, and MCAM were most highly expressed in regenerating human-only myofibers rather than in PAX7+ SMPCs, suggesting that niche-promoting signals may arise from the myofibers themselves. In dystrophic muscle, persistence of this early regenerative program together with reduced PAX7+ cell number and impaired myofiber maturation is consistent with defective niche progression.

CRISPR-engineered hPSCs model inducible MEGF10 gain and EMARDD-associated loss of function

We hypothesized that prolonging the early niche-like state would extend the window during which newly engrafted SMPCs can establish stable niches. Since MEGF10 was consistently associated with early niche formation, and because loss of MEGF10 causes the severe and often fatal congenital myopathy EMARDD (early-onset myopathy, areflexia, respiratory distress, and dysphagia), we asked whether MEGF10 actively regulates early stages of engraftment. To test this, we generated CRISPR-engineered hPSC models for both inducible MEGF10 overexpression and disease-relevant MEGF10 loss of function.

We first generated an inducible Tet-On MEGF10 overexpression system in hPSCs. This system uses a reverse tetracycline transactivator (rtTA) that responds to doxycycline to drive transgene expression [298]. Using CRISPR-mediated homology-directed repair, we inserted EF1A-rtTA-mTagBFP2 into the AAVS1 safe-harbor locus and TET-MEGF10-tdTomato into the hROSA26 locus [299]. Separating the two core components of the Tet-On system reduced leaky MEGF10 expression and improved HDR efficiency. Inclusion of the BFP and tdTomato reporters,

each linked by self-cleaving sequences, enabled selection and FACS purification of rtTA⁺ and Tet-On MEGF10⁺ cells (**Figure 3.8A**).

We first validated insertion of rtTA-BFP at the AAVS1 locus by RT-PCR, which confirmed rtTA expression in the clonal knock-in hPSC line relative to parental H1 hPSCs. We then inserted the TET-MEGF10-tdTomato cassette into the hROSA26 locus and verified doxycycline-inducible tdTomato expression. Although inducible tdTomato levels declined during clonal expansion, sequential sorting of tdTomato⁺ cells restored a highly pure inducible population (**Figure 3.9A**). We next differentiated Tet-On hPSCs toward skeletal muscle and enriched SMPCs by sorting for HNK1-ERBB3+NGFR⁺ (H-E+N⁺) cells. To ensure a uniformly responsive population, we pulsed cultures overnight with doxycycline before isolating a fully inducible SMPC pool (**Figure 3.9B**). Controls included Tet-On H-E+N⁺ SMPCs without doxycycline and an rtTA control line carrying only the AAVS1 knock-in without the MEGF10 transgene. Directed differentiation yielded comparable numbers of SMPCs across all lines.

To define conditions for reliable MEGF10 induction, we performed doxycycline titration and found that 2 μ M efficiently induced tdTomato expression within 24 hours (**Figure 3.9C**). We then confirmed myogenic potential by differentiating enriched SMPCs in N2 medium (**Figure 3.8B**). Tet-On and control cultures both formed abundant myosin⁺ myotubes and PAX7⁺ stem cells, with no significant difference in fusion efficiency. Upon doxycycline treatment, Tet-On MEGF10⁺ SMPCs and myotubes overexpressed MEGF10 at both the RNA and protein levels *in vitro*. Within 6 hours of doxycycline exposure, the Tet-On line showed a roughly 10-fold increase in *MEGF10* transcript and a 350-fold increase in *tdTomato* transcript (**Figure 3.8C**), whereas control lines expressed low endogenous MEGF10 and no tdTomato. At the protein level, doxycycline induced a 55% increase in MEGF10 expression in the Tet-On line. Notably, MEGF10

staining localized primarily to myotubes in differentiation cultures. these data establish the CRISPR-engineered Tet-On MEGF10 hPSC line as a robust platform to test MEGF10 function during human myogenesis and niche formation *in vitro* and *in vivo*.

To complement this gain-of-function approach with a disease-relevant loss-of-function model, we generated and analyzed EMARDD-modeling hPSC lines alongside control and Tet-On lines. Specifically, we engineered two hPSC lines carrying pathogenic MEGF10 mutations reported in the literature in exon 2 (*Takayama* et al.,) and exon 14 (*Logan* et al.,) [198, 200] (**Figure 3.8D**). The *Takayama* mutation models a complete loss of MEGF10, whereas the *Logan* mutation, located within a mutational hotspot, produces a truncated protein predicted to retain reduced function. After CRISPR editing, we differentiated both lines toward skeletal muscle and enriched the SMPC population. Neither mutation altered overall SMPC yield in directed differentiation cultures (**Figure 3.9D**). However, when differentiated into myotubes *in vitro*, the two mutant lines showed distinct phenotypes. *Logan* SMPCs fused less efficiently than controls, whereas *Takayama* SMPCs showed increased fusion efficiency but also contained fewer PAX7⁺ cells, and those remaining were less proliferative (**Figure 3.8F**). In contrast, *Logan* cultures showed no change in PAX7⁺ cell density. These distinct behaviors indicate that different MEGF10 mutations exert nonuniform effects on human muscle stem cell function, consistent with the phenotypic heterogeneity observed in EMARDD.

MEGF10 overexpression shifts SMPCs toward a proliferative transcriptional state *in vitro*

To gain initial mechanistic insight into MEGF10 function *in vitro*, we performed directed differentiation followed by doxycycline induction during the final 7 days in Tet-On MEGF10 and rtTA control cultures. SMPCs were FACS-enriched using H-E+N⁺ tdTomato⁺ and immediately profiled by bulk RNA-seq (n=4-5, **Figure 3.10A**). PCA showed clear separation between control

and Tet-On MEGF10 SMPCs (**Figure 3.10B**). Differential expression analysis identified 946 genes enriched in control SMPCs and 243 enriched in Tet-On MEGF10 SMPCs, including *MEGF10* itself among the induced transcripts in Tet-On SMPCs (**Figure 3.10C**). Gene ontology analysis indicated that control SMPCs were enriched for cell adhesion, extracellular matrix assembly, and integrin-signaling programs, whereas Tet-On MEGF10 SMPCs were enriched for proliferation-, Notch-, and migration-associated pathways (**Figure 3.10D**). Consistent with this, Tet-On MEGF10 SMPCs upregulated several Notch pathway genes, including *HES1*, *RBPJ*, and *HES7*, as well as early myogenic regulators such as *PAX3* and *EYAI* [239] (**Figure 3.10E**). They also expressed higher levels of proliferation-associated genes, including *HMMR*, *CCNB1*, and *AURKA* [300, 301]. Interestingly, *TNC*, which closely paralleled MEGF10 expression in engrafted SMPCs, was also upregulated on Tet-On SMPCs, further suggesting an interplay between these early niche-forming factors. In contrast, control SMPCs showed higher expression of adhesion- and matrix- and integrin-signaling genes such as *LAMA5* and *VTN* [302]. These data indicate that MEGF10 overexpression shifts SMPCs toward a more proliferative and activated transcriptional state, whereas control SMPCs adopt an altered adhesive interactome and gene-expression profile.

MEGF10 acts during the first 10 days after engraftment to support PAX7+ cell retention and niche incorporation *in vivo*

Since MEGF10 was enriched in early regenerative, niche-supportive myofibers during engraftment, we next asked whether prolonging this transient window could improve donor-cell retention *in vivo*. The inducible Tet-On system provided a unique opportunity to control MEGF10 expression across defined phases of regeneration and thereby determine when MEGF10 activity is most critical for engraftment. We therefore pulsed MEGF10 overexpression during early, intermediate, or late phases of engraftment in our human muscle stem cell niche model and

compared these groups with control and EMARDD loss-of-function engraftments (**Figure 3.11A**). Robust MEGF10 expression was confirmed *in vivo* by immunostaining, validating the feasibility of this inducible approach. Strikingly, MEGF10 was strongly expressed on regenerative human-only myofibers, but not detected in human-mouse chimeric myofibers (**Figure 3.11B**).

We then assessed engraftment efficacy across groups. Control engraftments yielded ~70 PAX7+ SMPCs and 200 myofibers per cross sectional area (CSA; **Figure 3.11C-D**). Early MEGF10 induction, from day 0 to day 10 after engraftment, significantly improved both donor cell retention and regenerative output, increasing engraftments to ~200 PAX7+ SMPCs and 400 myofibers per CSA. In contrast, intermediate or late MEGF10 induction did not improve engraftment over control, and induction of MEGF10 for the full engraftment period decreased engraftment efficiency with several mice showing no detectable donor cells. Complementing our gain-of-function approach, EMARDD engraftments generated myofiber numbers comparable to controls but failed to retain any PAX7+ SMPCs, indicating that MEGF10 is required for stem cell maintenance.

We then quantified niche colonization across all groups. Compared with controls, early and intermediate MEGF10 overexpression did not alter niche colonization after regeneration. However, late overexpression of MEGF10 yielded significantly more PAX7 cells outside of their niches (**Figure 3.11E**). These data indicate that the timing of MEGF10 expression is critical, with induction during the first 10 days after engraftment providing the strongest support for PAX7+ cell retention and productive niche incorporation.

MEGF10 signaling supports engrafted PAX7+ cells by extending early niche-forming myocyte states

To distinguish direct early effects of MEGF10 signaling from secondary consequences that emerge later during regeneration, we performed spatial transcriptomic analysis of control and Tet-On engraftments 10 days after transplantation, when regeneration is ongoing and interactions between regenerating myofibers and PAX7+ SMPCs are likely central to niche establishment (**Figure 3.12A**). We first quantified engraftment potential at this early time point by measuring PAX7+ SMPCs and myofibers. Tet-On engraftments contained significantly more PAX7+ SMPCs and myofibers than control and EMARDD samples, indicating that MEGF10 promotes donor cell support during the same window in which it is overexpressed ($p < 0.05$; **Figure 3.12B**).

Since our *in vitro* data suggested altered proliferative behavior, we next assessed PAX7+ cell proliferation *in vivo* by administering EdU during the final 24 hours before tissue harvest. At day 10, Tet-On SMPCs were less proliferative than controls, despite their greater abundance, indicating that MEGF10 alters the temporal dynamics of donor-cell proliferation during regeneration (**Figure 3.12C**). This result contrasted with our *in vitro* bulk RNA-seq data, which suggested a more proliferative SMPC state, and instead points to a dominant influence of the regenerative myofiber compartment and the *in vivo* environment on donor-cell proliferation. When we examined niche localization in these actively regenerating grafts, Tet-On engraftments showed a lower percentage of PAX7+ cells positioned within niches than controls at this stage. In parallel, EMARDD engraftments at day 10 generated myofiber numbers comparable to controls but failed to support detectable PAX7+ SMPCs, further supporting a specific requirement for MEGF10 in early stem cell retention.

We then performed spatial transcriptomic analysis on day 10 control and Tet-On engraftments, capturing 42-43 ROIs from PAX7 and myofiber populations from each treatment (**Figure 3.13A**). This early analysis revealed additional heterogeneity within the regenerative

myofiber compartment, including a distinct early regenerative population that we classified as myocytes (**Figure 3.13B**). These spectin⁺ myocytes likely contain MYOG⁺ myogenic nuclei and small actively fusing bundles that are morphologically less mature than larger myofibers. Although Tet-On myocytes expressed MEGF10, expression was even higher in myofiber regions. Comparison of control myocytes and myofibers confirmed that these populations were transcriptionally distinct, with over 700 DEGs distinguishing the myogenic groups.

PCA of the spatial transcriptomic data showed segregation by myogenic populations and genotype, with Tet-On and control populations clustering separately on PC2 (**Figure 3.12D**). In control engraftments, myocytes were transcriptionally more similar to PAX7⁺ cells than to myofibers, whereas in Tet-On engraftments myocytes shifted toward a more myofiber-like state. Spatial transcriptomic analysis also validated the Tet-On system, showing significantly increased MEGF10 transcript levels across all myogenic populations *in vivo* (**Figure 3.12E**).

To define the broader MEGF10 response, we identified DEGs between control and Tet-On engraftments across each myogenic population. PAX7⁺ SMPCs differed by more than 40 genes, whereas myocytes and myofibers differed by more than 2,000 and 900 genes, respectively, indicating that the strongest transcriptional effects of MEGF10 occurred in the regenerative myocyte and myofiber compartments. Across Tet-On myocytes and myofibers, 299 DEGs were shared, and gene ontology analysis showed enrichment for translation, muscle contraction, and TOR signaling (**Figure 3.12F**). Several genes linked to niche development, early fusion, and regenerative myogenesis, including niche receptors *CDH15*, *JAM2*, *DLK1*, and *LRRN1*, were significantly upregulated in Tet-On regenerative engraftments, shedding light on the mechanism of action of the MEGF10 signaling cascade (**Figure 3.12G**).

Finally, we examined how MEGF10 signaling affected myofiber development within regeneration. Tet-On MEGF10⁺ myocytes displayed a more regenerative profile, with higher expression of fusion-associated *MYOM2*, the developmental myosin *MYH8* (**Figure 3.13C-D**). In contrast, control myofibers appeared more developmentally advanced and less regenerative, with higher expression of the mature myofiber marker *MYH1* and *FHL1*, whereas Tet-On myofibers retained higher expression of immature markers such as *ACTC1*. Together, these data indicate that MEGF10 prolongs an early regenerative myocyte state and delays full myofiber maturation, thereby extending a developmental window that is associated with improved PAX7⁺ cell support during engraftment.

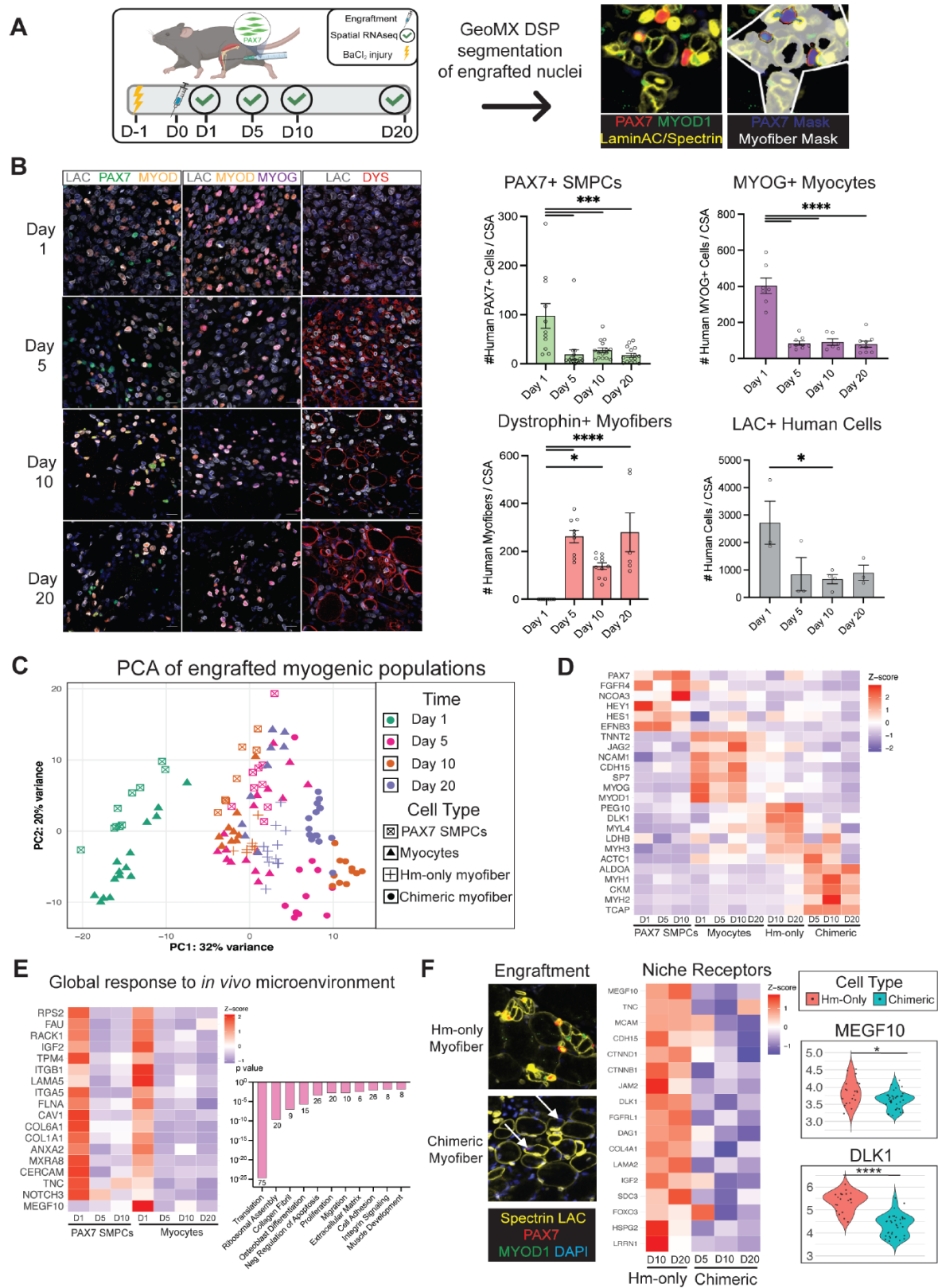


Figure 3.1: Engrafted Human Muscle Progenitor Cells Stabilize with the Regeneration of Myofibers.

A. Visual schematic of the experimental workflow for Figure 1. Engraftment of SMPCs into tibialis anterior and tissue harvesting at days 1, 5, 10, and 20. GeoMX DSP segmentation strategy shown (right) based on antibody staining for myogenic markers of interest. **B.** Immunostaining imaging of myogenic populations across time, human lamin AC (grey), PAX7 (green), MYOD1 (yellow), MYOG (purple), and dystrophin (red; right). Quantifications of myogenic populations across time; PAX7+ SMPCs, MYOG+ myocytes, Dystrophin+ myofibers, and Lamin AC+ human nuclei per cross-sectional area. One-way ANOVA statistical analysis were applied. **C.** PCA projection of spatial transcriptomic data collected from engraftment trajectory analysis. **D.** Heatmap projection of the gene expression profile of population unique myogenic identifiers. **E.** Heatmap projection of the global response to *in vivo* microenvironment across SMPCs and myocytes after engraftment (left). Gene ontology analysis of global response genes analyzed through DAVID Gene Ontology (Statistical analysis; fisher's exact test and Benjamini-Hochberg multiple testing correction). Gene number shown next to each bar. **F.** Images from the GeoMX DSP of Human-only and Chimeric myofiber regions (left). Arrows point to LaminAC- myonuclei in chimeric myofibers. Heatmap projection of significantly upregulated muscle stem cell niche receptors on Human-only myofibers (center). Violin plots of normalized gene expression values of MEGF10 and DLK1 (right). P values included in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

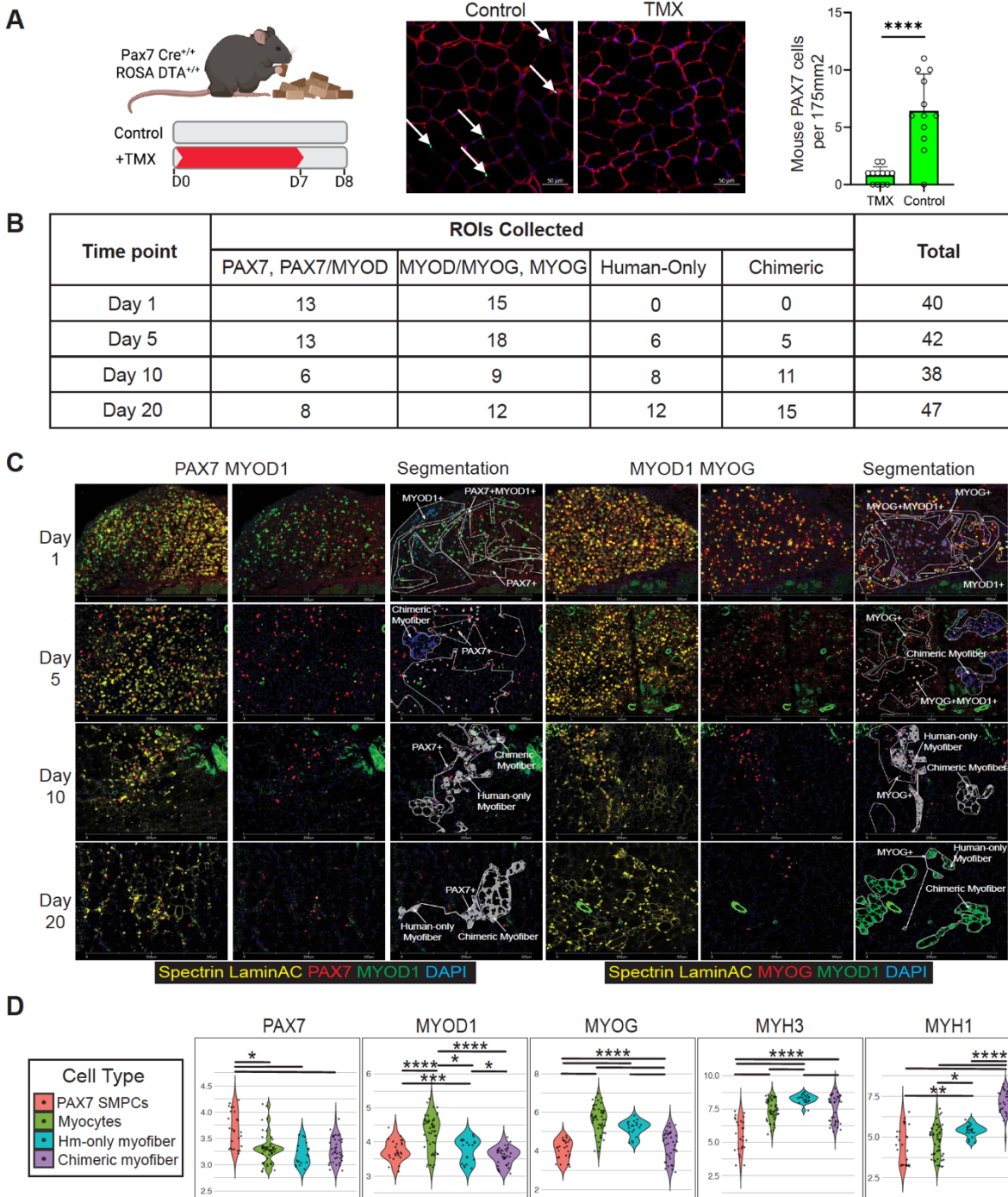


Figure 3.2: Assessment and collection of engrafted myogenic populations through spatial transcriptomic approaches.

A. Number of PAX7 cells per 175mm^2 in immunostained images of PAX7-CRE ROSA-DTA NSG mice after treatment with tamoxifen for 7 days compared to a non-treated control. Arrows point to Pax7⁺ nuclei. T-test statistical analysis applied. **B.** Table of all engrafted regions of interest of myogenic populations across time collected through spatial transcriptomics analysis for Figure 1, S1, and S2. **C.** GeoMX DSP platform images of immunofluorescent spatial transcriptomic samples used for ROI collection. ROIs are outlined in white boundaries in the segmentation column and labeled according to their collected myogenic population. Scale bars are $500\mu\text{M}$. **D.** Normalized gene expression values of myogenic cell identity genes: PAX7, MYOD1, MYOG, MYH3, and MYH1. All samples across time of the same myogenic identify are grouped together. **E.** Infiltration of myofiber transcripts in PAX7 cells: Violin plot of normalized gene expression values after variance stabilization transformation across time in PAX7 SMPC populations; PAX7 transcripts (left) and MYH1 transcripts (right). Statistical significance determined through DESeq2's metric of Negative Wald Binominal and Multiple Testing Corrections (Benjamini Hochberg) – no significance determined in PAX7, but in MYH1 transcripts Day 1 is significantly lower than other Day 5 and Day 10. P values included in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

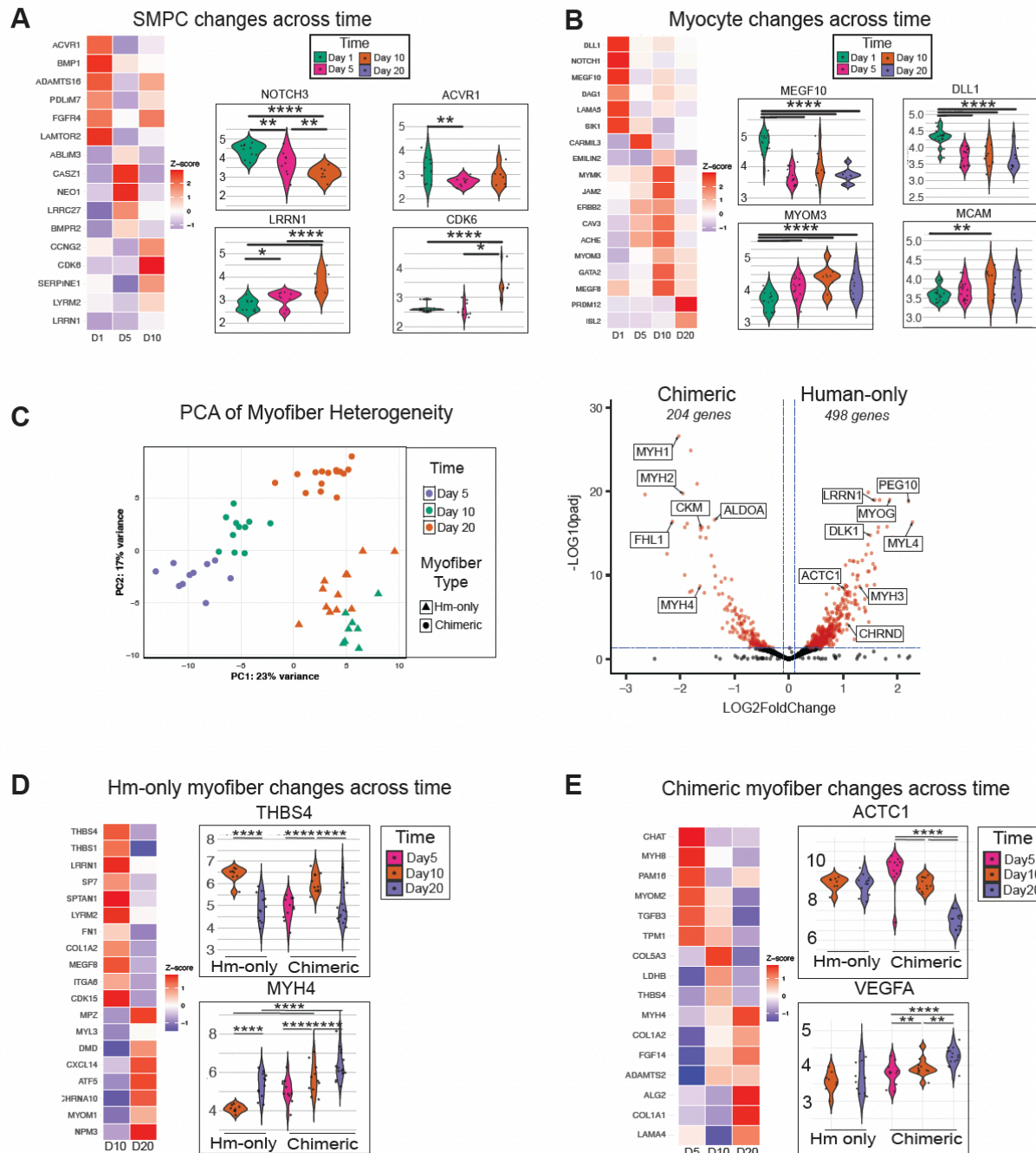


Figure 3.3: Mapping the Regenerative Trajectory of Engrafted Human hPSC SMPCs using Spatial Transcriptomic Analysis.

A. Heatmap projection significant hits across time in SMPCs (left). Violin plots of normalized gene expression values of NOTCH3, LRRN1, ACVR1, and CDK6 (right). **B.** Heatmap projection significant hits across time in myocytes (left). Violin plots of normalized gene expression values of MEGF10, MYOM3, DLL1, and MCAM (right). **C.** PCA projection of Human-only and Chimeric myofibers across time (left). Volcano plot projection of upregulated hits in human-only vs chimeric myofibers (right). Y-axis threshold is based on $\text{padj} = 0.05$. **D.** Heatmap projection significant hits across time in human-only myofibers (left). Violin plots of normalized gene expression values of THBS4 and MYH4 (right). **E.** Heatmap projection significant hits across time in chimeric myofibers (left). Violin plots of normalized gene expression values of ACTC1 and VEGFA (right). P values included in this figure: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Figure 3.4: Single Nucleus Transcriptomics Identifies Heterogeneity of Human Muscle Progenitor Cells.

A. Schematic of experimental overview. Directed differentiation of pluripotent human stem cells to skeletal muscle *in vitro* using our labs directed differentiation protocol. Single nucleus RNAseq was collected from enriched muscle stem cells ~7 days after expansion in growth media. **B.** RtPCR data of PAX7, MYOG, MEGF10, and NOTCH3 on enriched muscle stem cells the day of FACS enrichment (imm.sort) vs 7 days of subsequent expansion (expanded). N=3 collected, * = p value < 0.05, student's T-test. **C.** Single nucleus RNA sequencing of enriched and expanded myogenic progenitors. Analysis conducted using Seurat analysis in R. **D.** Unbiased heatmap of top ten genes per cluster in snRNAseq analysis. **E.** FeaturePlot analysis of select genes across clusters.

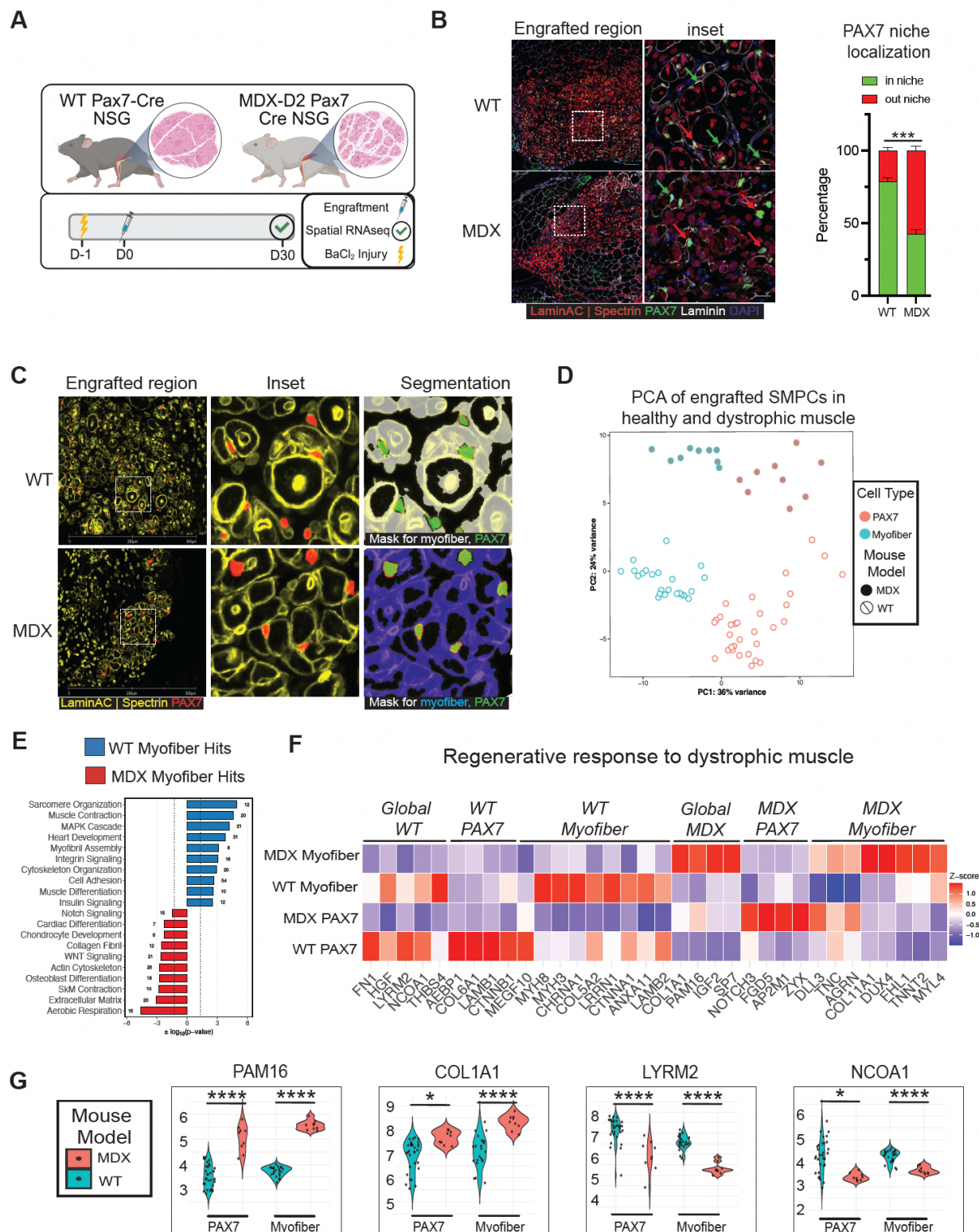


Figure 3.5: Engrafted Human SMPC Regeneration is Determined by the Microenvironment

A. Visual schematic of experimental layout for Figure 2: engraftment into healthy and dystrophic microenvironments. **B.** Immunostaining of engrafted human skeletal muscle in healthy and dystrophic microenvironments (left). Green arrows point to PAX7 cells inside niches, while red arrows point to those outside niches. Niche colonization quantified across samples, applying a t-test for statistical analysis (right). **C.** Engrafted images from the GeoMX DSP showing the process of ROI segmentation of PAX7 cells and myofibers. Scale bars are 500 μ M. **D.** PCA projection of spatial transcriptomic data collected from healthy and dystrophic engraftments. **E.** DAVID Gene ontology analysis of myofiber DEGs in healthy vs dystrophic engraftments. The dotted line indicates p-value cutoff of 0.05. **F.** Heatmap projection significant hits across cell type and microenvironment. **G.** Select DEGs projected via Violin plot; PAM16, COL1A1, LYRM2, and NCOA1. P values included in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

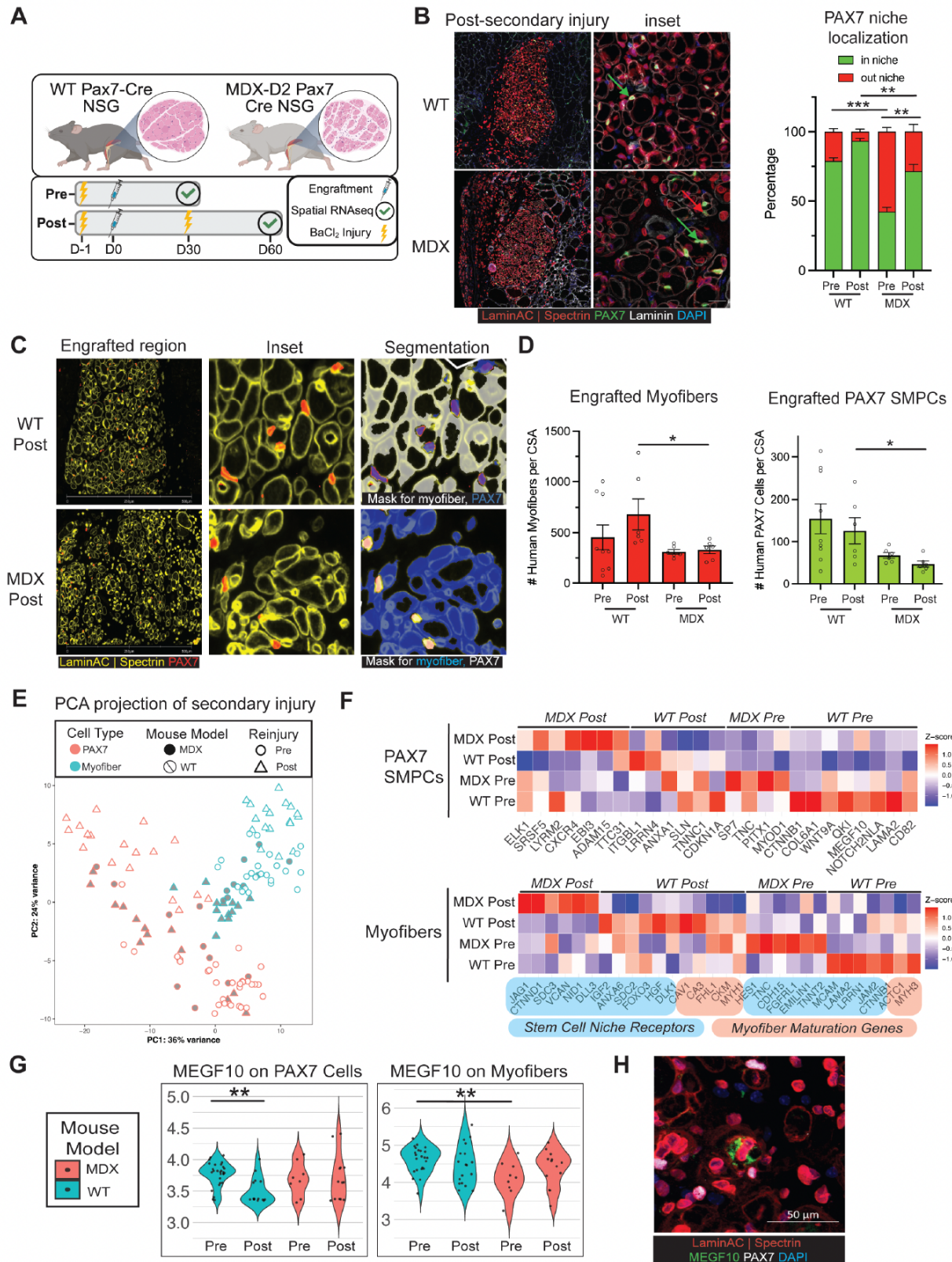


Figure 3.6: Dystrophic *in vivo* environment stunts secondary injury in engrafted human muscle stem cell models

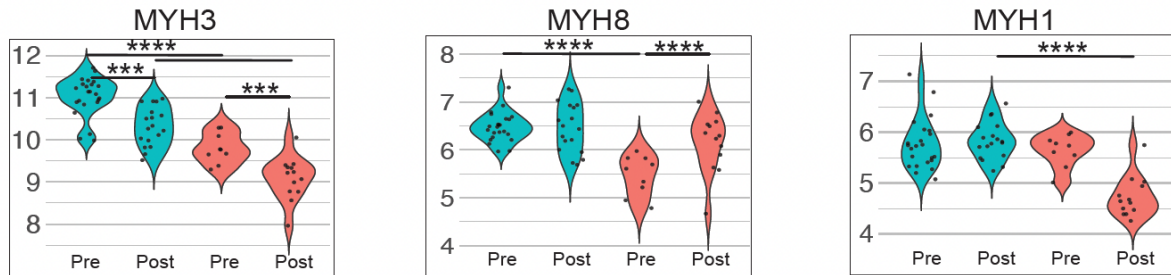
A. Visual schematic of experimental layout for Figure 3. Pre-secondary injury (Pre) samples are collected 30 days after engraftment. Post-secondary injury (Post) samples are administered a secondary injury event 30 days after engraftment and collected after another 30 days. **B.** Immunostaining of engrafted human skeletal muscle in healthy and dystrophic microenvironments (left). Green arrows point to PAX7 cells inside niches, while red arrows point to those outside niches. Niche colonization quantified across samples, applying a one-way ANOVA for statistical analysis (right). **C.** Engrafted images from the GeoMX DSP showing the process of ROI segmentation of PAX7 cells and myofibers. Scale bars are 500 μ M. **D.** Quantifications of engrafted human PAX7+ SMPCs (left) and spectrin+ myofibers (right) per cross sectional area. T-test applied for statistics. **E.** PCA projection of spatial transcriptomic data from healthy and dystrophic SMPC engraftments before and after secondary injury. **F.** Heatmap projection of significant DEGs in PAX7+ SMPCs across microenvironment and secondary injury (top). Heatmap projection of significant DEGs in myofibers across microenvironment and secondary injury (bottom). Genes associated with niche formation and myofiber maturation are color labeled. **G.** MEGF10 normalized gene expression projection via Violin plot in SMPCs (left) and myofibers (right) across microenvironment and secondary injury. **H.** Immunofluorescence image (MEGF10, PAX7, LAC/Spectrin) of healthy engraftment pre-secondary injury. Scale bar = 50 μ M. P values included in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

A

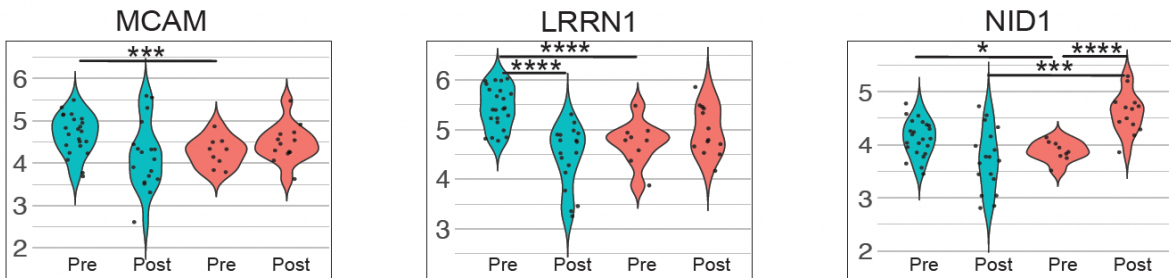
Mouse Model	ROIs Collected			Total
	PAX7+ SMPC	Human-Only Myofiber	Chimeric Myofiber	
WildType Pre	13	15	0	70
WildType Post	13	18	5	41
MDX Pre	6	9	11	20
MDX Post	8	12	15	32

B

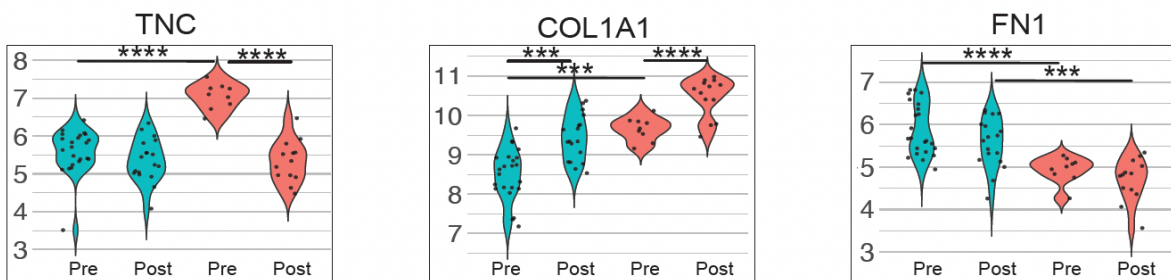
Myofiber Development and Maturation



Stem Cell Niche Formation and Adhesion



Extracellular Matrix



Secreted Signaling

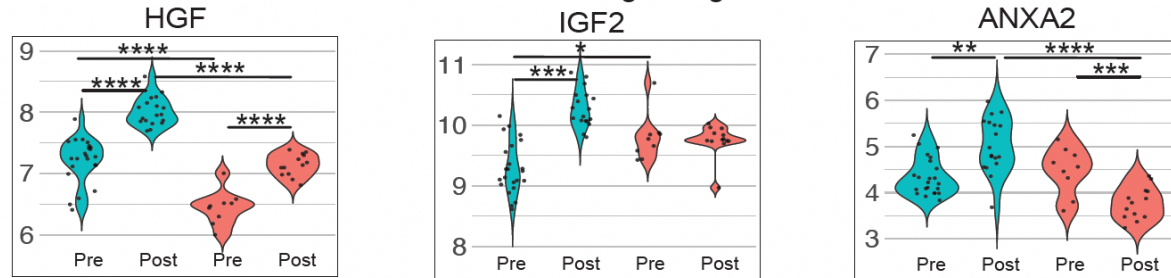


Figure 3.7: Engrafted Human SMPC Regeneration and Support is Determined by the Microenvironment

A. Table of all engrafted regions of interest collected through spatial transcriptomics analysis for Figure 2,3, and S3. **B.** Violin plot projection of normalized gene expression values of genes associated with myofiber development and maturation (MYH3, MYH8, MYH1), stem cell niche formation and adhesion (MCAM, LRRN1, NID1), extracellular matrix (TNC, COL1A1, FN1), and secreted signaling (HGF, IGF2, ANXA2). P values included in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

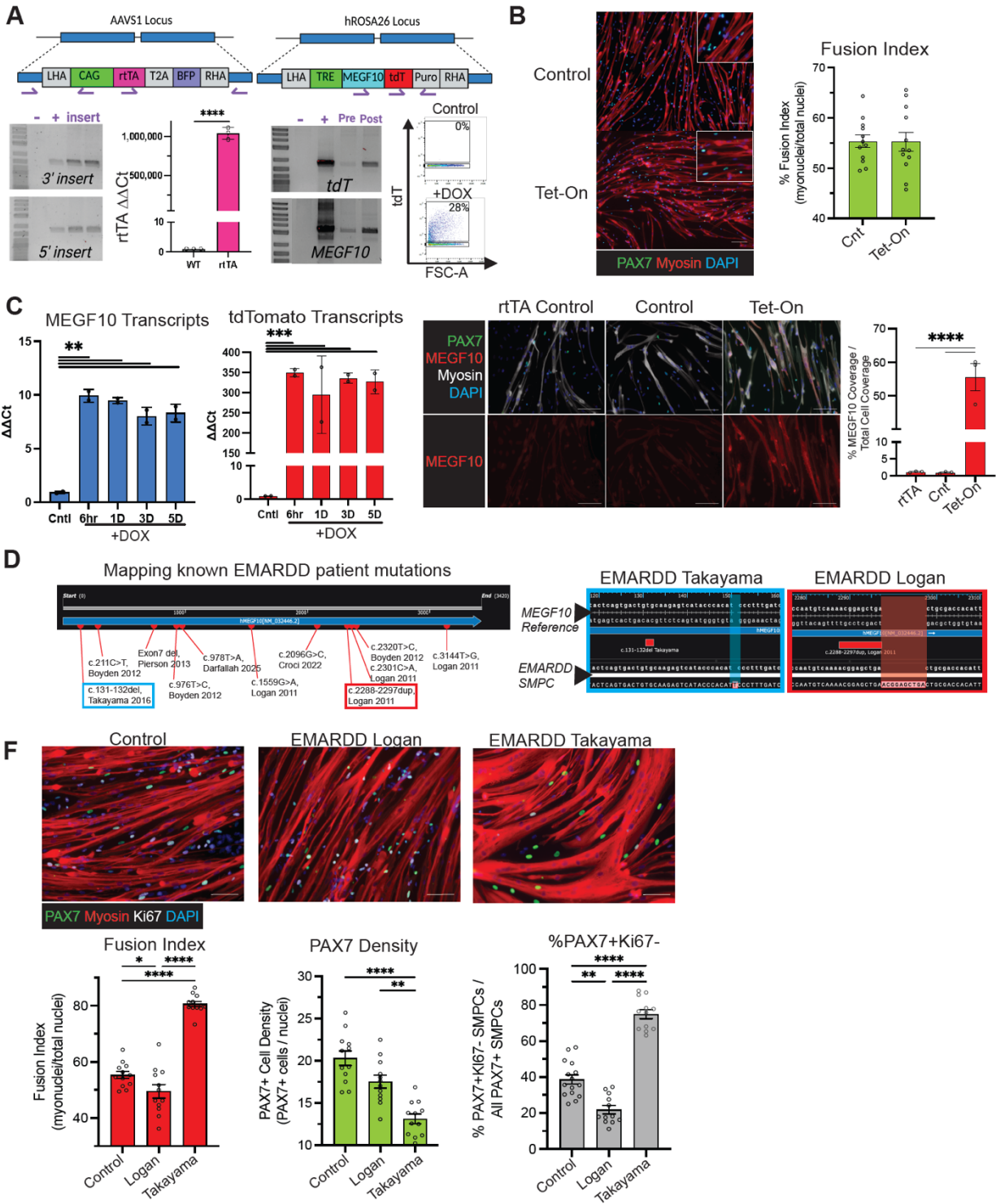


Figure 3.8: CRISPR Generation of a MEGF10 Inducible Overexpression Muscle

Progenitor

A. Schematic of the first homology directed repair guided insertion at the AAVS1 locus (left). PCR verification of correct insertion with primer location annotated by purple arrows, including a wildtype negative control, parental positive control, and clonal knock-in line (left to right). Real time PCR of rtTA transcripts comparing wildtype hPSC to insertion line. Schematic of the second homology directed repair guided insertion at the hROSA26 locus (right). PCR verification of the correct insertion with primer location annotated by purple arrows, including a wildtype negative control, plasmid positive control, and clonal knock-in line before and after puromycin selection. FACS plots of this TET-on knock-in hPSC line with and without treatment of doxycycline, y-Axis = tdTomato. **B.** Schematic detailing all described EMARDD pathological variants. The MEGF10 coding region taken from UCSC Genome Browser mapped with all known disease variants and their respective mutations (top). Selected Takayama and Logan EMARDD mutations validated through sanger sequencing of SMPCs following directed differentiation (bottom). **C.** Differentiation of FACS enriched TET-on SMPCs to myotubes using serum free N2 media *in vitro*. Immunostaining for Myosin (red), PAX7 (green), DAPI (blue). 10X image scale bar = 200 μ M (left). Inset is 20X image scale bar = 50 μ M. Fusion assay (myonuclei / total nuclei) quantified (right), statistics applied with T-test. **D.** Real Time PCR of five-day myotube differentiation cultures comparing treatment of doxycycline for the final 6hours, 1 day, 3 days and 5 days of culture. MEGF10 transcripts (left) and tdTomato transcripts (right) measured across timepoints. **E.** Immunostaining of myotube differentiation for MEGF10 (red), PAX7 (green), Myosin (grey), DAPI (blue). Images taken at 20X, scale bar = X. Quantification of MEGF10 staining (right). **F.** Differentiation of FACS enriched EMARDD SMPCs to myotubes using serum

free N2 media *in vitro* (top). Quantifications for fusion assay (myonuclei / total nuclei), PAX7 density (PAX7+ nuclei / total nuclei), and PAX7 proliferation (PAX7+Ki67- nuclei / PAX7+ nuclei) shown (bottom). T-test applied for statistics. P values included in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

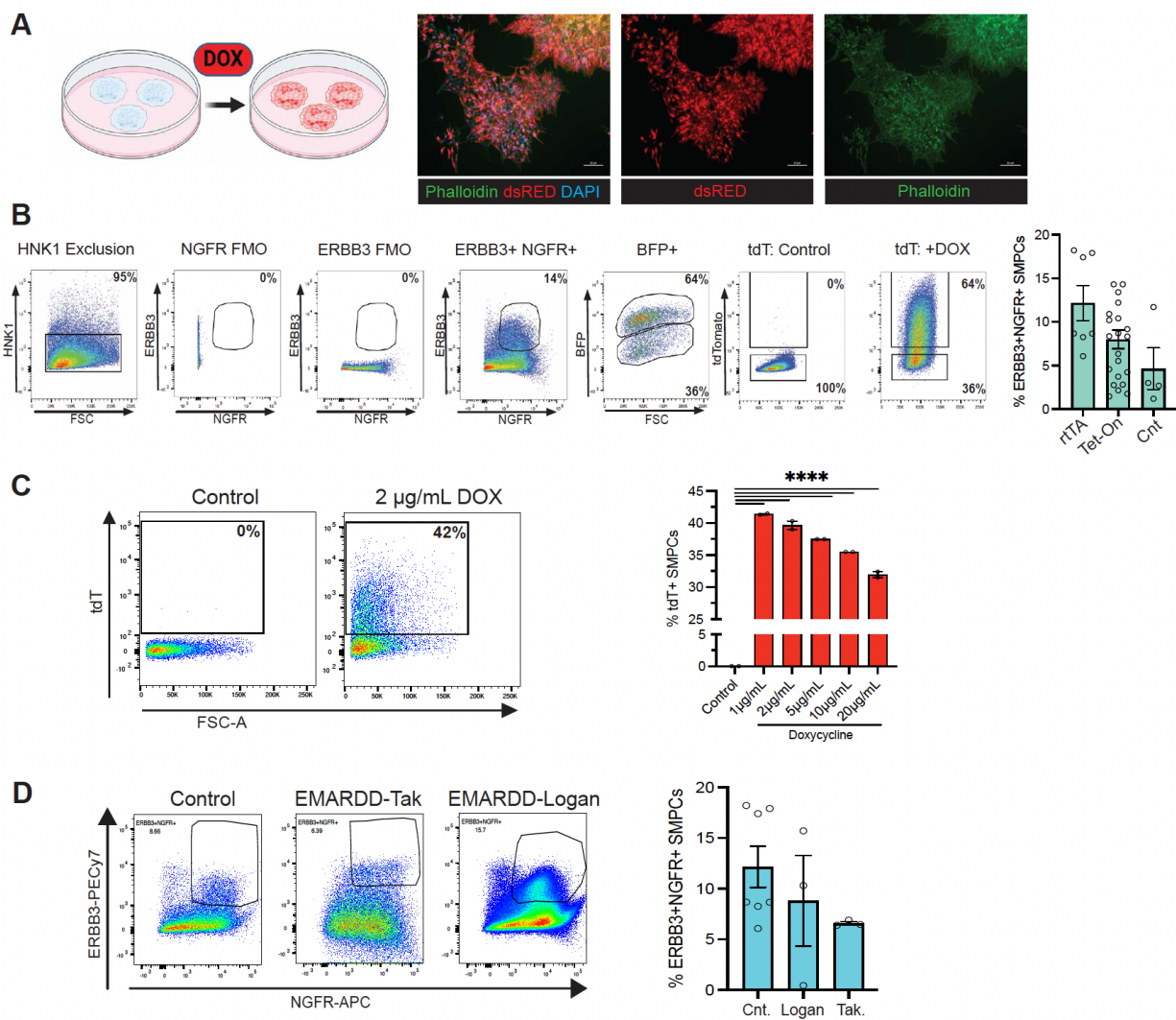


Figure 3.9: CRISPR engineering Tet-On MEGF10 overexpression and EMARDD disease modeling lines

A. Schematic of treating TET-on hPSCs with doxycycline to activate the tdTomato reporter (top). Immunostaining of doxycycline-treated TET-on hPSCs with dsRED (red), phalloidin (green), DAPI (blue). 10X images, scale bar = 150 μ M. **B.** Flow cytometry analysis of doxycycline treated Tet-On SMPCs. Representative plots for non-treated control and 2 μ g/mL (left) and quantifications for the percentage of tdT positivity (left). One-way ANOVA statistical test applied. **C.** FACS enrichment of TET-on SMPCs following directed differentiation. HNK1 exclusion, NGFR FMO, ERBB3 FMO, ERBB3+NGFR+ double positive gating, BFP+ selection, and tdTomato gating for TET-on SMPCs +/- doxycycline treatment (left to right). Quantifications of percentage E+N+H-SMPCs collected through flow sorting across samples (right). **D.** FACS enrichment of EMARDD disease modeling SMPCs following directed differentiation, showing the ERBB3+NGFR+ gating strategy (left) and the quantification of the SMPC percentage of total viable cells (right). P values included in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

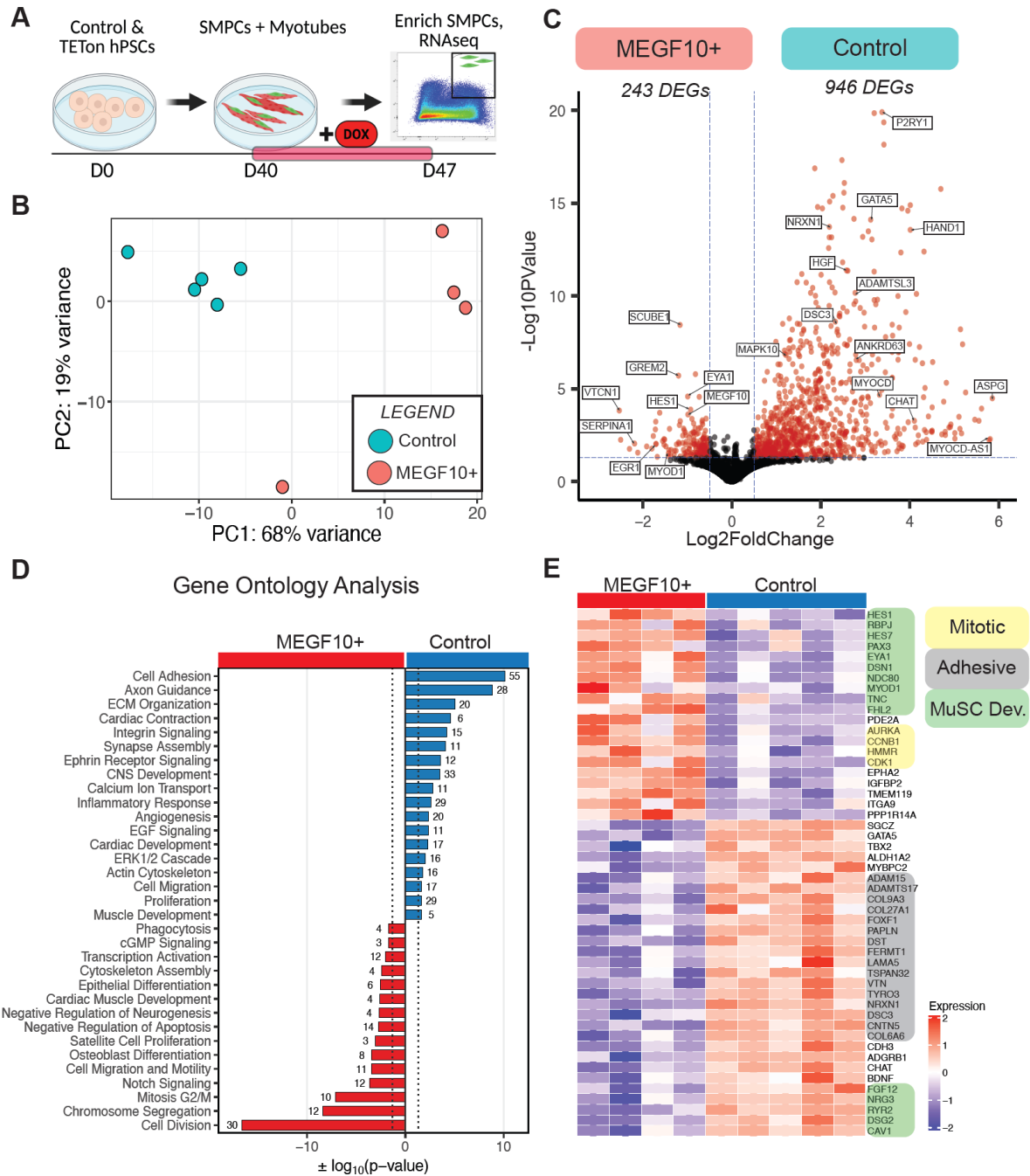


Figure 3.10: MEGF10 Regulates SMPC Interactions via the Notch Signaling Pathway

A. Visual schematic of the experimental layout for Figure 5. Tet-On cultures were administered 2 μ M doxycycline for the final 7 days of differentiation. **B.** PCA projection of RNAseq datasets using DESeq2 analysis. **C.** Volcano plot projection of Control and Tet-On DEGs – adjusted p value cutoff < 0.05. Selected significant DEGs are highlighted and labeled. **D.** DAVID Gene Ontology Analysis of Control and Tet-On DEGs. P value cutoff of 0.05 is indicated by the vertical dotted lines. **E.** Heatmap projection of normalized gene expression for select DEGs of interest across Control and Tet-On samples. Genes associated with Adhesion, Mitosis, and Muscle Stem cell development are color labeled. P values included in this figure: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

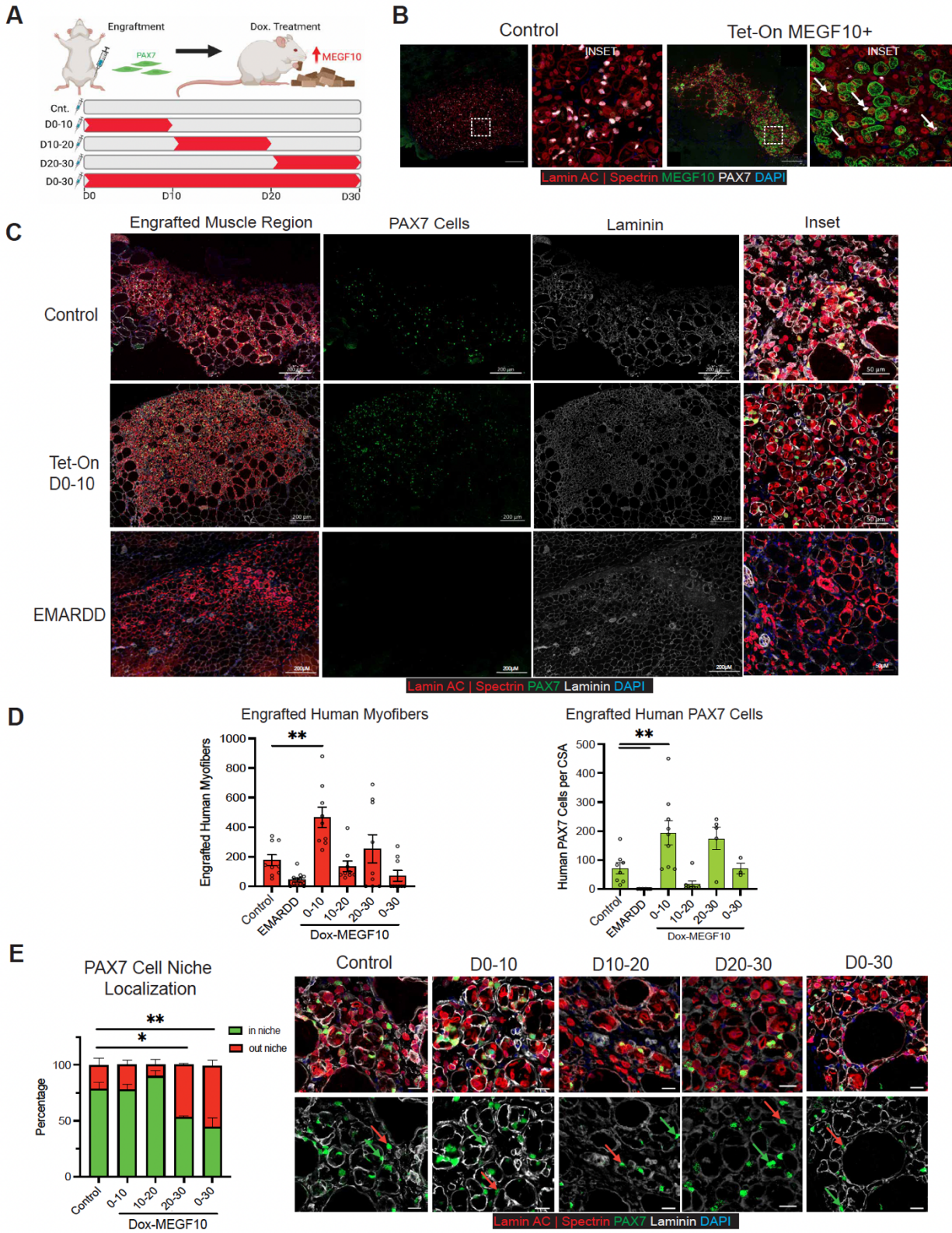


Figure 3.11: Early MEGF10 Overexpression on Engrafted Human SMPCs Supports PAX7 Support and Improved Regeneration

A. Visual schematic of experimental workflow for Figure 6. Tet-On SMPCs not treated with doxycycline were used as Control. **B.** Engrafted Control and Tet-On samples immunostained with MEGF10, PAX7, lamin AC, and spectrin. Arrows point to PAX7 cells that are interfacing with MEGF10+ myofibers. **C.** Engrafted samples immunostained with PAX7, laminin, lamin AC, and spectrin across Control (top), Tet-On D0-10 (middle), and EMARDD (bottom). Scale bars are 200 μ M. Inset scale bars are 50 μ M. **D.** Quantifications per cross-sectional area of human Spectrin+ myofibers (left) and PAX7 cells (right). One-way ANOVA statistical test applied. **E.** PAX7 niche localization assay across groups. PAX7+ nuclei were quantified as either within basal lamina (in niche) or outside it (out niche; left). One-way ANOVA statistical test applied. Representative immunofluorescent images of each group, scale bars = 20 μ M (right). Green arrows point to PAX7 cells in niches, red arrows point to PAX7 cells outside niches. P values included in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

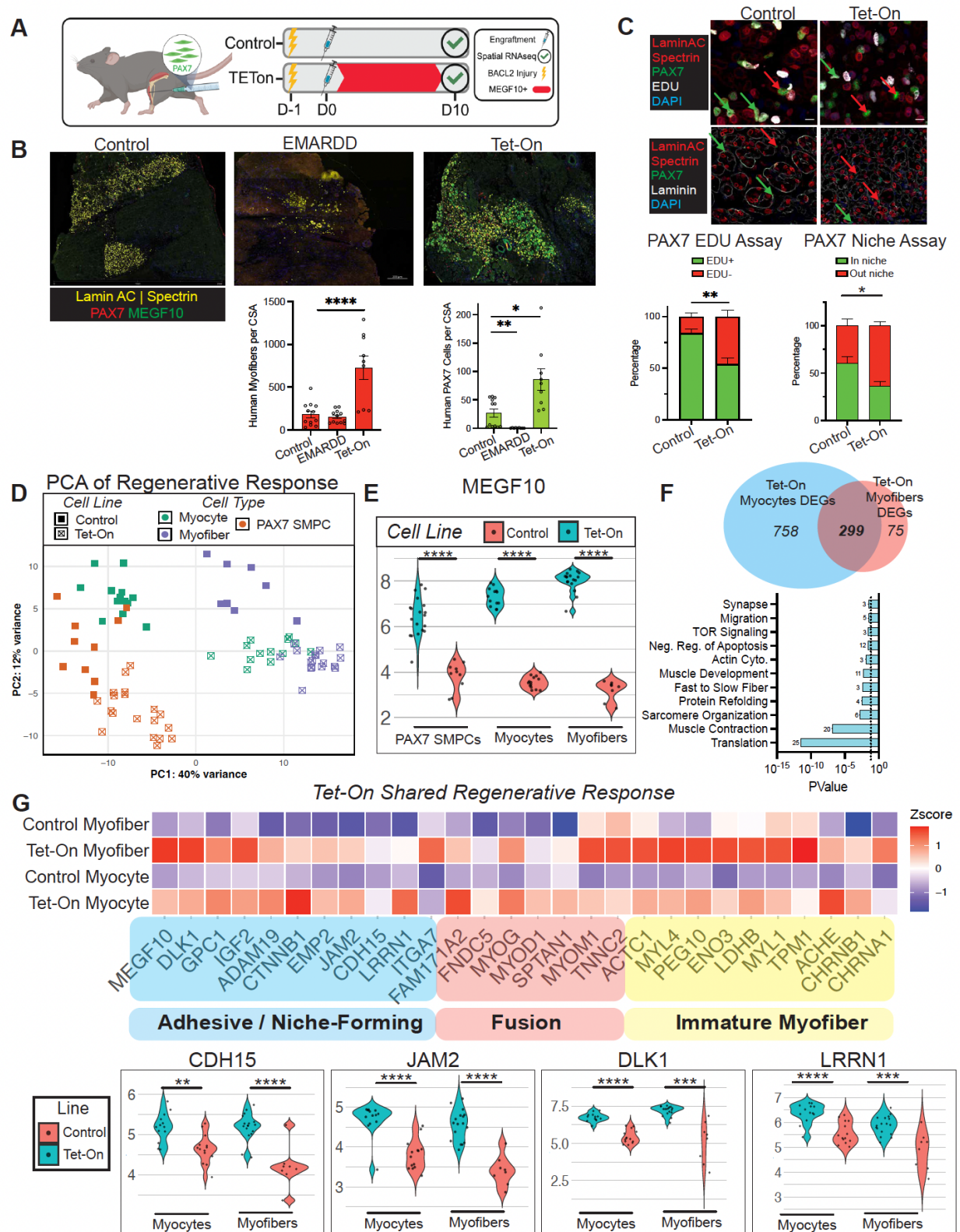


Figure 3.12: MEGF10 Signaling Pathway Triggers Niche Forming Factors and Accelerates Early Myofiber Regeneration

A. Visual schematic of the experimental procedure for Figure 7 (top). Tet-On samples administered doxycycline to pulse MEGF10 overexpression Days 0-10. **B.** GeoMX DSP platform scanned images of spatial transcriptomic samples of Control and Tet-On engraftments at day 10; LaminAC, Spectrin, PAX7, and MEGF10 (left). Quantifications of human myofibers (center) and PAX7 cells (right) per cross sectional area of engraftment in Control, Tet-On, and EMARDD samples, one-way ANOVA statistical test applied. **C.** EDU proliferation assay and Niche localization assay of engrafted human PAX7 cells in Control and Tet-On. Immunofluorescence images of engraftments stained with EDU, PAX7, LaminAc, Spectrin (top), and Laminin, PAX7, LaminAc, Spectrin (middle). Green arrows point to PAX7 cells that are EDU+ (top), or inside niches (bottom). Red arrows point to PAX7 cells that are EDU- (top), or outside niches (bottom). Scale bars are 20 and 50 μ M respectively. Quantifications of PAX7+ EDU+/- nuclei (bottom left) and PAX7+ nuclei within/outside basal lamina (bottom right). T-test statistical test applied. **D.** PCA projection of spatial transcriptomic data. Principal component analysis was performed on normalized gene expression values across all genes, and the top 2 PCs were used to project these datasets. **E.** Violin plot of normalized gene expression counts for MEGF10 transcripts across samples. **F.** Venn diagram showing the DEGs upregulated in Tet-On myofibers and myocytes (top). 299 genes are shared between these Tet-On myogenic populations, which were input into DAVID Gene Ontology analysis for systematic categorization and association of genes (bottom). The dotted line corresponds to a p-value of 0.05, and gene numbers are labeled next to their respective bar on the graph. **G.** Heatmap projection of normalized gene expression values of global Tet-On DEGs shared across myogenic populations (top). DEGs are categorized into adhesive niche

forming, fusion, and immature myofiber development associated hits. Violin plots of normalized gene counts for global Tet-On DEGs. P values included in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3.13: Early MEGF10 Overexpression on Engrafted Human SMPCs Supports PAX7 Support and Improved Regeneration

A. Table of all engrafted regions of interest collected through spatial transcriptomics analysis for Figure 7 and S5. **B.** GeoMX DSP platform scanned images of myocytes and myofibers in Control and Tet-On spatial transcriptomic samples, stained for lamin AC, spectrin, PAX7, and MEGF10 (left). The dotted circles highlight regions of regenerative myofibers. Volcano plot projection of DEGs in control myocyte vs myofiber populations – padj value of 0.05 used to establish the horizontal threshold (right). **C.** Volcano plot projection of Control vs Tet-On myocytes (left) and myofibers (right). P adjusted value cutoff is 0.05, and labeled genes are all significant DEGs. **D.** Heatmap projection of normalized gene expression values across myocyte and myofiber populations in Control and Tet-On samples (top). Population specific hits are labeled; Tet-On regenerative hits, Control myocyte hits, and Control myofiber hits. Violin plot of normalized gene expression for DEGs. P values included in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4: Discussion

Our study found successful human muscle progenitor cell engraftment depends on a transient regenerative niche that forms during the initial phase of myofiber repair. By integrating spatial transcriptomic profiling across time, regenerative states, and healthy versus dystrophic host environments from multiple independent datasets, we resolved how the myogenic niche emerges, progresses, and fails. Our analyses show dystrophic muscle alters engrafted SMPC transcriptional programs, disrupts niche progression after reinjury, and inhibits many regenerating myofibers from maturing. MEGF10 emerged as a functional regulator of this early window, and stage-specific MEGF10 induction in SMPCs improved PAX7 retention and regenerative output, whereas EMARDD-associated MEGF10 loss-of-function failed to retain any engrafted stem cells. Our findings demonstrate that the early developing niche is a critical determinant of engraftment success, establishes the dystrophic microenvironment as a barrier to myogenic niche progression, and identifies MEGF10-dependent signaling as a potential therapeutic target to improve muscle stem cell transplantation.

For cell- and gene-based therapies to be successful, it is essential to understand how diseased host environments alter the behavior, persistence, and regenerative capacity of muscle stem and progenitor cells [303, 304]. Through spatial transcriptomics, we found that healthy donor SMPCs engrafted into dystrophic muscle adopt a transcriptional state marked by persistent failure to resolve gene programs that normally extinguish *in vivo*, accompanied by a shift toward osteogenic and chondrogenic differentiation. These SMPCs remain abnormally activated and showed evidence of altered mitochondrial stress and adaptation within the dystrophic microenvironment. These state changes were accompanied by reduced PAX7⁺ niche colonization and a diminished capacity to repopulate the niche after secondary injury, indicating that dystrophic muscle

compromises both initial niche establishment and long-term regenerative competence. Notably, engrafted SMPCs upregulated extracellular matrix genes, raising the possibility that healthy cells can also contribute to maladaptive remodeling in a disease context. These findings corroborate prior studies which show healthy recipient muscle supports more robust engraftment than dystrophic muscle [253], and wild-type ECM is more permissive for progenitor remodeling and differentiation than dystrophic mdx ECM [195]. More broadly, they underscore the suggested requirement for combinatorial strategies that improve both donor-cell fitness and the host microenvironment in dystrophic muscle.

Upon engraftment, hPSC-derived myogenic progenitors undergo developmental maturation *in vivo*, which in turn gives rise to increased regenerative potency [188]. Although previous bulk RNAseq studies of engrafted muscle provided important insight into *in vivo* maturation, spatial transcriptomics preserves tissue architecture and enables engrafted heterogeneous myofiber populations to be analyzed *in situ* without disrupting the multinucleated fibers [193]. By resolving all populations directly within engrafted muscle, our analysis substantially advances prior work by uncovering previously unrecognized heterogeneity among engrafted hPSC-derived myofibers, enabling us to better define how distinct myofiber populations contribute to SMPC niche development and support. We found that transplanted myocytes begin expressing regenerative myogenic genes within 1 day of engraftment and can form robust myofibers by day 5. This rapid regenerative progression was accompanied by a global transcriptional response significantly enriched in engrafted myofibers rather than in SMPCs, suggesting that niche development is closely linked to myofiber regeneration and that these factors may help stabilize and retain the PAX7⁺ SMPC pool. Human-only myofibers appear to represent an early niche-supportive state that is particularly effective at maintaining engrafted PAX7⁺ SMPCs, likely because they

upregulate key niche receptors and are most closely associated with PAX7⁺ cells histologically [37], yet they transition more slowly toward a mature myofiber state. One possible explanation is that these donor-derived fibers remain incompletely integrated within the larger host muscle, whereas fusion with host mouse myofibers to form chimeric fibers may provide structural or signaling cues that promote further maturation. Secondary injury appeared to be the most effective condition for improving this integration and advancing niche progression. Functionally, reinjury also enhanced the ability of engrafted SMPCs to occupy the stem cell niche, suggesting that regenerative remodeling of the host environment promotes both myofiber integration and productive niche maturation.

MEGF10 appears to act during a defined early phase of regeneration, promoting establishment and extension of the primary engrafted myogenic niche rather than maturing into a later secondary niche. Although early donor-cell loss is widely recognized as a major barrier to skeletal muscle transplantation, relatively few studies have directly analyzed engraftment during the first several days after transplantation [305], with most work focusing on outcomes measured weeks later. In our model, MEGF10 overexpression during the first 10 days accelerated differentiation and early myofiber commitment, thereby reinforcing retention of the PAX7⁺ SMPC pool, coincident with the period when the regenerating host-muscle environment directs stem-cell fate decisions and promotes incorporation into the emerging niche [306]. Consistent with this model, the decline in SMPC number between days 1 and 5 after engraftment paralleled reduced global MEGF10 expression, supporting the existence of a limited window for donor-cell capture. Importantly, when MEGF10 is either missing or expressed at a later regenerative stage, engraftment dynamics and PAX7 SMPC retention are significantly worsened. However, continued induction beyond day 10 also impaired further maturation, indicating that prolonged maintenance of this immature niche

becomes counterproductive. As the niche, and likely the associated stem cell state, matures, it may become less supportive of initial donor-cell adhesion and incorporation and instead more supportive of a functional, quiescent state.

The physiological importance of MEGF10 in skeletal muscle is underscored by EMARDD, a severe early-onset myopathy caused by loss-of-function mutations in MEGF10 [198]. Multiple EMARDD-associated MEGF10 mutations have been described, and the distinct behaviors of the *Takayama* and *Logan* disease-modeling EMARDD variant lines parallel the variability in affected patients ranging from death *in utero* to survival into early adulthood. Our data support the idea that different MEGF10 mutations exert nonuniform effects on human myofiber and muscle stem cell function. Despite this clinical significance, MEGF10 and its precise role in skeletal muscle homeostasis remains relatively understudied. In developing mouse skeletal muscle, MEGF10 is expressed at the interface between PAX3⁺ muscle progenitors and developing myofibers, suggesting a role in embryonic niche colonization, although it remains unclear which cell type, or whether both, may be its principal source [124]. After *in vivo* engraftment of primary human fetal muscle progenitors, MEGF10 is significantly upregulated in repopulating PAX7⁺ SMPCs after secondary injury [37], although its relevant binding partners and interactome in skeletal muscle remain incompletely defined. In the human brain, MEGF10 functions in astrocyte-mediated debris clearance by binding the C1q receptor and triggering phagocytosis, which is consistent with a broader role in cell-cell interactions and adhesion [209].

We therefore sought to identify downstream signaling partners of MEGF10 that may contribute to the formation of a permissive niche by engrafted myogenic cells. *In vitro* RNA-seq of Tet-On MEGF10 SMPCs provided a useful baseline, revealing increased proliferation and Notch signaling. However, spatial transcriptomic analysis *in vivo* uncovered a markedly different gene-

expression profile, suggesting that transcriptional programs associated with MEGF10 induction are highly dependent on the regenerative environment encountered after transplantation. This distinction is consistent with recent comparative studies in other tissues showing that even advanced *in vitro* models remain transcriptionally distinct from their *in vivo* counterparts [307]. Early MEGF10 induction *in vivo* was accompanied by increased expression of *DLK1*, *JAM2*, *CDH15*, and *LRRN1* in regenerating myofibers, identifying these genes as candidate niche associated factors. We did not identify prior evidence for a direct DLK1-LRRN1 signaling relationship, but both genes have been associated with early somite, developmental, and regenerative myogenic states, making them plausible components of a broader immature niche-supportive program [308].

Although current technologies have limitations in the ability to fully resolve the cellular and transcriptomic complexity of engrafted muscle, GeoMX-based high-plex spatial profiling provides one of the most informative approaches currently available for studying donor-cell state and niche development *in situ*. One key strength of this platform is that it preserves spatial integrity and resolution that would be lost with tissue dissociation, enabling unbiased transcriptomic analysis of engrafted cells within their regenerative context. At the same time, it has important limitations, including segmentation overlap and incomplete resolution of true cellular heterogeneity. Since SMPCs and regenerating myofibers lie directly adjacent to one another, low levels of myofiber transcripts were detected within SMPC regions. We addressed imperfect segmentation by comparing significant genes across populations and excluding likely contaminating genes in SMPC-focused analyses. In addition, because each region captures small groups of 5-15 SMPCs or myocytes and generates bulk-like expression profiles, this approach cannot fully resolve heterogeneity within engrafted populations. This limitation is particularly relevant given that

spatial transcriptomic analysis as early as one day after engraftment revealed abundant myoblast-like populations, underscoring the need to improve SMPC expansion and enrichment strategies to better preserve PAX7⁺ cells after sorting. It remains unclear whether engrafted population heterogeneity may itself contribute to early niche formation and donor-cell retention. Finally, because our analyses relied on human-specific probes, and the extent to which the recipient mouse microenvironment, particularly in dystrophic and injured settings, contributes maladaptive signals that shape donor-cell behavior has yet to be defined.

In summary, our findings have important clinical implications and point to new avenues for improving cell-based therapies. Our data support a model in which human muscle stem cell engraftment is determined not only by donor-cell intrinsic properties, but also by a transient niche established within the first days of cell transplantation. The dystrophic microenvironment disrupts the progression of this niche, limiting both stem cell retention and regenerative recovery, whereas stage specific MEGF10 signaling prolongs an early permissive state that improves engraftment efficiency. Ongoing work will determine whether sustained MEGF10 overexpression, or more clinically tractable approaches to modulate MEGF10 signaling, can help overcome the barriers imposed by the dystrophic microenvironment and improve therapeutic regeneration in muscular dystrophy. These findings provide a basis for understanding why donor-cell loss is so pronounced after transplantation and suggest that effective cell therapies for muscular dystrophies may require not only improved donor cells, but also strategies to recreate or extend the early niche environment that supports their incorporation.

3.5: Methods

Cell Culture Techniques and CRISPR Homology Directed Repair

H1 hPSC (WA01, WiCell) were used in this publication to generate the rtTA Control and TET-On hPSC lines. HPSC's were seeded on Matrigel (Corning), cultured using mTESR Plus (Stem Cell Tech), passaged using ReleasR (Stem Cell Tech), and frozen down in mFRESR (Stem Cell Tech). During passaging and thawing, hPSCs were cultured in Rock Inhibitor (Stem Cell Tech).

Generation of a CRISPR genetically engineered TETon MEGF10 overexpression cell line began with the first insertion of the EF1A-rtTA-mTagBFP2 transgene into the AAVS1 genomic safe harbor. We used a single guide RNA (IDT) bound to a purified CAS9 protein (IDT) to cleave the AAVS1 site of insertion, using nucleofection as the method of plasmid and gRNA delivery (Lonza). We then used PCR screening to verify the presence of the correct insertion in the parental bulk cell sample before harnessing the mTagBFP2 fluorescence to single cell plate BFP⁺ cells for culturing and subsequent clonal line PCR screening. We obtained several rtTA clonal insertion lines, and the line we moved forward with for the secondary transgene insertion successfully passed karyotyping assays for genetic abnormalities. Sanger sequencing of PCR products verified that the correct transgene insertion was incorporated at the AAVS1 locus. This line was used as the rtTA Control line through this study.

We then repeated the process to generate a secondary transgene insertion, incorporating the TET-MEGF10-TdTomato into the ROSA26 locus. We again used PCR screening to verify that the transgene was inserted to the right position and to verify that the insertion was homozygous. With another round of single cell plating and screening through surviving colonies of a 96-well plate, we then split the colonies and treated one replicate with 2 μ M doxycycline. We moved forward with the colonies that responded to treatment with a clear fluorescent RFP⁺ signal. It should be noted that after clonal single-cell plating and expansion, ~30-50% of Tet-On hPSCs

responded to treatment – we hypothesize that this may be due to genetic silencing of the transgenes introduced. Thus, we gently pulsed with 1 μ M doxycycline for ~24 hours and FACS enriched for the tdTomato⁺ hPSCs. These enriched hPSCs were ~100% responsive when treated with doxycycline two passages later, and thus we proceeded with directed differentiation with these hPSCs.

Patient-modeling EMARDD mutations were also generated using CRISPR genome engineering. We used the rtTA Control line described above as a template for EMARDD disease modeling lines to minimize genetic line-to-line variability. sgRNA, CAS9 protein (IDT), and template plasmid were nucleofected into hPSCs and single cell clonal insertions were screened from this parental reaction. The EMARDD-Logan line was replicated with an identical, 10-bp duplication event as described in Logan 2011's work diagnosing a novel EMARDD patient variant. This genetic mutation gives rise to an early stop codon, but importantly, it likely still produces a truncated protein since the mutation occurs past halfway in the coding sequence of MEGF10. The EMARDD-Takayama line was not an identical mutation event; rather than the 2-bp deletion, we introduced a 1-bp insertion that introduced an identical frameshift mutation, resulting in a premature stop codon, with only 10 base pairs that differ between our model and the patient genotype. Both lines were applied in our studies, however in engraftment studies, only the EMARDD-Logan line was included.

For directed differentiations, we followed our Lab's publicly available protocol of skeletal muscle derivation from hPSCs. For FACS enrichments of SMPCs after directed differentiation, we used HNK1-FITC (BioLegend), ERBB3-PECy7 (BioLegend), NGFR-APC (BioLegend), and AlexaFlour780 Live Dead Viability stain (Fisher) to accommodate endogenous BFP and tdTomato. We opted to pulse Tet-On differentiations with doxycycline ~24 hours prior to sorting

to enrich a tdT⁺ population that is highly responsive to treatment for engraftment studies. SMPCs were plated at ~150-300K per well of a 6-well plate and cultured in SKGM2 media (Lonza) + bFGF. Generally, they took ~3-4 days to recover and reach 75% confluency before passaging/freezing. In vitro myogenic differentiation and fusion assays were generated using N2 differentiation media; 1.2% N2 supplement (Fisher), 1.2% Immuno transferrin selenium (Fisher), IGF1 (Stem Cell Tech), in 1:1 DMEM-F12 media. Skeletal muscle progenitor cells were grown in SKGM2 media (Lonza), passaged in Tryple (Fisher), and frozen in CryoStor (Stem Cell Tech).

RNA Sequencing of SMPCs

We established directed differentiation cultures of rtTA Control and Tet-On hPSC lines. At ~day 40 of differentiation, the TETon cultures were treated with 2 μ M doxycycline (in DMSO; Stem Cell Tech) to pulse MEGF10 overexpression, and the rtTA Control line was treated with equal amounts of DMSO (1:1000 in N2 media). At day 47, rtTA Control SMPCs were collected (n=5) by enriching live HNK1⁻, ERBB3⁺, NGFR⁺ and TETon SMPCs were collected (n=4) via live HNK1⁻, ERBB3⁺, NGFR⁺, tdTomato⁺ SMPCs. Different wells of a 6-well plate were handled separately to serve as biological replicates. FACS enriched SMPCs were pelleted, and RNA was immediately harvested using the RNA-Easy MicroPrep Kit with gDNA elimination (Qiagen). These RNA samples were used to construct libraries for Illumina sequencing with the assistance of the UCI GHTF Core (SMARTseq library construction kit). We sequenced ~60million reads and FastQ files were groomed user Fastq-groomer and aligned using HiSAT2 (Homo Sapiens hg38 GTF reference file) and processed in Galaxy to generate raw count matrices. Samples were. These matrices were used as input for Deseq2 analysis in R Studio for data visualization and downstream analysis. We closely followed DESeq2's platform as guided by publications and github resources to generate PCA clustering, Volcano plot, and Violin plot generation across our

normalized gene expression data. DESeq2 uses a Fisher Exact Test combined with a Multiple Testing Correction (Benjamini-Hochberg) to generate adjusted p values that correct for over prediction of significantly upregulated DEGs.

Skeletal Muscle Stem Cell Engraftments

~6-10-week-old WT Pax7-Cre ROSA-DTA inducible ablation mice were treated with tamoxifen chow (Envigo) for at least 7 days prior to localized muscle injury and engraftment. For the Tet-On engraftments, doxycycline chow (Envigo) was administered. To label cell proliferation in vivo, EDU (Sigma) was administered to mice in water at 0.5mg/mL. Live HNK1-, ERBB3+, NGFR+ SMPCs were enriched after directed differentiation and plated on Matrigel to recover. After ~3 days, 75% confluence SMPCs were passaged ~1:5 to encourage expansion of the cells to maximize engraftment inputs. After another ~3 days, SMPCs were pelleted and resuspended in HBBS without Ca/Mg (Fisher) at a concentration of 5 μ L/million SMPCs for engraftment. ~1.2 million SMPCs were engrafted into the Tibialis Anterior of mice, and importantly, these muscles had been subjected to 50 μ L of 1.2% Barium Chloride (Sigma) injury ~24hours prior.

After 30 days, mdx-NSG mice were euthanized, and TA muscles dissected. The TA muscles were immediately embedded in optimum cutting temperature (OCT) compound and flash-frozen in isopentane cooled by liquid Nitrogen. Embedded muscles were stored at -80°C until sectioned in 9-10 μ m slices using a Leica microcryotome (LSXII). 1200 μ m of TA muscle were removed and 20 sections collected on positively charged frosted microscope slides in 50 μ m intervals, and 300 μ m of tissue were collected for RNA or western blots. In this manner we calculated that each quadrant contained 1300 μ m of muscle tissue. Quadrants were collected until the entire TA was sectioned. Thus, we could determine the position of each cross section for

downstream analysis of cell engraftments. Microscope slides were kept in aluminum foil covered boxes at -20°C.

Immunofluorescent staining

To perform staining, sections on slides were thawed from -20°C freezers (45 minutes to promote PAX7 antigen binding) and washed in PBS containing 0.2% tween-20 (PBST, 7.4pH). Slides were fixed in 2% PFA for 10 minutes and washed with PBST. Sections were then blocked in 0.2% gelatin, 3% BSA and 0.1% Tween, 10% goat serum for 90 minutes at room temperature. Sections were immunostained with human-specific antibodies to h-Spectrin (1:80; Vector laboratories), h-Lamin AC (1:120; Fisher) and h-Dystrophin (1:6; MANDYS106) to positively identify engrafted human cells. Human/mouse-specific laminin (1:80; Vector laboratories) was used to denote the basal lamina of all myofibers. Sections were alternatively stained with h-PAX7 (10µg/ml; DSHB), h-Lamin AC, h-Spectrin and Laminin to identify human SMPC/SCs residing in the satellite cell position.

SMPC/SCs and myotubes were washed in PBS and fixed in 4% PFA for 15 minutes. Cells were permeabilized with 0.3% Triton-X for 5 minutes. Cells were then blocked in 10% goat serum, 3% BSA for 1 hour. Primary antibodies were added to 2% goat serum in PBS and incubated on cells overnight at 4°C. Primaries were washed three times using 0.1% tween-20 in PBS and secondary antibodies in PBS were added for 65 minutes. Secondary antibodies were washed, and cells were counterstained with 1:1000 Hoechst. Cells were imaged using a Zeiss inverted fluorescent microscope.

Engraftment Quantifications and Statistical Analysis

Engraftment quantifications of PAX7+ SMPCs and myofibers were collected per cross sectional area. This was conducted by determining the ideal engraftment quadrants and manually

counting human PAX7⁺ nuclei and human Spectrin⁺ myofibers across the top 2-3 quadrants with the most engraftment. As these data are already normalized according to the cell amounts engrafted (1.2million per engraftment), we did not normalize these quantifications. Statistics were conducted using one-way ANOVA across comparisons with more than 2 samples, and a student's T-test was applied to comparisons between 2 groups.

For Figure 1, we transplanted n=3/4 mice at each timepoint day 1, 5, 10, 20. For Figure 2/3, we transplanted n=3 of each time point across both healthy and dystrophic mice. In Figure 6, we transplanted n=3 for each group of control, EMARDD, and MEGF10 overexpression. Lastly, for Figure 7, we transplanted n=3/4 mice for control, EMARDD, and Tet-On. In all cases that required spatial transcriptomics, i.e. Figures 1, 2, 3, and 7, we moved forward with spatial transcriptomics on one sample from each time point for cost effective analysis.

GeoMX DSP Spatial Transcriptomic Analysis

Workflow:

This workflow is further detailed in introduction, chapter 1.7. We proceeded with spatial transcriptomic analysis using the Nanostring/Bruker GeoMX Digital Spatial profiler on engraftment samples relevant for Figures 1,2,3, and 7. The kit that was used was the Whole Transcriptome Human Probe Atlas. We opted for the samples that had the best engraftment and proceeded to conduct spatial transcriptomic analysis on them immediately. While flash freezing tissues does not decrease RNA quality, we anticipated degradation after further storage of sectioned slides at -20C. Thus, we stored freshly sectioned slides for ~1-2 days at 4C and began the spatial workflow as soon as possible. We proceeded with one tissue slide consisting of 3-4 tissue slices per sample, for biological replicates of N=1, but technical replicates of N>10 per myogenic population.

This workflow closely followed the detailed protocol in Clock, Hicks Methods in Molecular Biology 2025. To conduct this analysis, engrafted tissue samples were collected by harvesting the engrafted mouse TA and flash freezing in liquid nitrogen. These frozen samples are stable at -80C, with RNA integrity and quality preserved. Frozen engrafted samples were sectioned transversely along the entirety of the tissue to determine where the best region of engraftment resided. We conducted an overnight immunostain to determine where the engraftment was most successful using human lamin AC (Fisher), human spectrin (Fisher), PAX7 (DSHB), and Laminin (Thermo). Critically, during this overnight stain, excess slides were temporarily stored at 4C in a dry box to avoid water build up to preserve RNA integrity and avoid any freeze/thaw degradation of RNA. After determining which region of engraftment was most successful, we moved forward with those specific slides from 4C for spatial transcriptomic analysis. Samples were fixed with 4% formaldehyde then treated with 0.025µg/mL proteinase K to expose RNA binding epitopes. Then, engrafted tissues were incubated with Human Whole Transcriptome Atlas Probes (Bruker) overnight at 37C. The following day the probes were washed off with formalin (Sigma) and 2xSSC Buffer prior to staining the tissues with morphology markers / antibodies of interest – human lamin AC, human spectrin, PAX7, MEGF10, Laminin, MYOG. The GeoMX DSP Platform was then implemented to soundly image the stained samples for precise and controlled region of interest (ROI) selection. We captured between 5-15 nuclei in each ROI to ensure enough transcriptomic content for downstream analysis.

For Figure 1's engraftment trajectory analysis and tracking of myogenic population changes across time, we were unable to capture all myogenic populations desired. We noted a difficulty in capturing enough engrafted cells, that we were unable to stratify populations into the depth and detail drafted in our hypotheses. For example, PAX7 cells are increasingly rare across

time (Figure 1B), and while we could capture separate PAX7+MYOD1- and PAX7+MYOD1+ populations at Day 1, we were unable to do so at all time points due to the low number of PAX7 cells supported after engraftment. Thus, we systematically merged our dataset to focus on cells that were collected across several time points reliably.

Sequencing:

After collection of ROIs on the GeoMX DSP, libraries were prepared according to manufacturer workflows, harnessing a seq-code plate that barcodes ROIs from the same collection plate to allow for sample pooling when sequencing. Illumina sequencing at 2 million reads per micron² (we doubled what the workflow recommended for additional depth) yielded FastQ files, FastQ indices, and a configuration.ini file. The FastQ and .ini file were converted to DCC files using the Linux command line interface, geomxngspipeline. These DCC files were reuploaded to the GeoMX DSP interface platform for analysis.

Sample Flagging and Filtration:

With the uploading of DCC files for sample analysis, we began by downloading unfiltered, unflagged counts matrices for quality control purposes. We assessed filtration cutoffs flagging samples that had >90% of genes that were expressed below negative probe levels and removed them. For PAX7 samples, we flagged samples that had a ratio of PAX7 transcript / negative probe transcript below 0.75. These flagged samples were removed from analysis prior to filtration of the gene expression profiles. Filtration was conducted at 2% LOQ levels on the GeoMX platform, and we subsequently corrected this filtered dataset to remove genes that were, on average, below the average negative probe count. This dataset was used for analysis and uploaded into R for DESeq2 rigor.

Analysis:

DESeq2 required an input of un-normalized data – thus, we relied on the rigor of DESeq2's built-in normalization method which is the Wald Test of Negative Binomial Generalized Linear Model. The Benjamini-Hochberg model is subsequently applied to generate adjusted p-values, and in this study, we considered $\text{padj} < 0.1$ as significant, which is in line with what other publications conducting DESeq2 have applied. These normalized data were then transformed to stabilize variance across samples and datasets using VST, and these values were used to conduct principal component analysis, and project heatmap and violin plots.

In this study, we investigated the global response to the *in vivo* microenvironment by identifying shared hits across time in SMPCs and myocytes, but we first noticed infiltrating myofiber genes in PAX7 SMPCs, as marked by an increase in MYH1. The biological positioning of myofibers directly adjacent to SMPCs and myocytes led to this technical challenge of spatial transcriptomics. The biological positioning of myofibers directly adjacent to SMPCs and myocytes led to this technical challenge of spatial transcriptomics. We resolved this challenge by identifying myofiber infiltrating genes within our SMPC populations through systematic comparison of the two populations across time and flagging all myofiber differentially expressed genes (DEGs) when analyzing SMPCs across time.

Chapter 4: Summary and Future Directions

4.1: Summary

In patients with neuromuscular diseases, endogenous stem cell populations fail to regenerate skeletal muscle tissue. This raises a critical unmet need in the field of translational approaches to develop treatments for these diseases and disorders. The application of iPSC skeletal muscle in these cases is highly appealing, demonstrated by the first ongoing clinical trial for patients with DMD [183]. The drawbacks of iPSC cell therapies in skeletal muscle are diverse and complicate the directions of their translational and clinical applications. Of importance to this study, iPSC cell generations are immature, presumably due to the lack of true *in vivo* developmental signals in a dish. Therefore, extensive work is centered on developing a pipeline that better recapitulates the *in vivo* microenvironment *in vitro* to yield a more mature and functionally potent product for cell therapies. In this study we focus on understanding the biology of these immature muscle stem cells upon engraftment – prior work discovered that muscle stem cells mature upon exposure to the *in vivo* microenvironment in engraftment approaches [56, 188]. However, the retention of muscle stem cells upon initial transplantation remains largely inefficient and serves as a major barrier towards progressing viable cell therapies for patients. Thus, the questions surrounding our findings are clear – how do muscle stem cells respond upon engraftment, what cues support the stem cells that are able to persist, and can we leverage these cues to our advantage?

We began this study by addressing how muscle stem cells regenerate after highlighting an ideal FACS enrichment strategy [184]. Engraftment of ERBB3⁺ NGFR⁺ HNK1⁻ muscle stem cells could consistently and reproducibly generate robust myofibers, and we describe myofiber heterogeneity. The two distinct classes of myofibers included smaller, regenerative myofibers that

clustered predominantly with other human myofibers, and larger, chimeric myofibers that arose from fusion of engrafted human myogenic cells with host mouse myofibers. The regenerative population is marked by ACTC1, a fetal cardiac actin, while the chimeric population is marked by mature myofiber markers such as MYH1, MYH4, and MYH7. These findings illustrate heterogeneity of the regenerative product of human myofibers and shed light on the human regenerative program *in vivo*. Further, we found that PAX7⁺ cells are almost completely retained only in regenerative regions, while chimeric myofibers poorly support them. To showcase the critical importance of regenerative myofibers, we generated an ACTC1-driven cell ablation system and terminated all ACTC1⁺ cells upon engraftment, where we found a significant reduction in PAX7⁺ cells and myofibers. These results demonstrate the significance of ACTC1⁺ regenerative myofibers in forming the early basis of a stem cell niche to support and retain PAX7⁺ muscle stem cells.

We then conducted spatial transcriptomic analysis across several early time points of regeneration to study the trajectory of myofiber regeneration and how PAX7⁺ and MYOG⁺ populations play a role in this process and are stabilized through supportive interactions. This analysis allowed us to further detail the different myogenic populations, including regenerative and chimeric myofibers. Regenerative myofibers are marked not only by ACTC1 and other immature myogenic players (MYH3), but they also displayed more acetylcholine receptors (CHRNA/CHRNA) and a completely different ligand/receptor membrane profile (DLK1, CDH15, MEGF10). Since many of these candidates are involved in niche formation of satellite cells, we deduced that regenerative myofibers better retain PAX7⁺ cells through supportive stem cell – myofiber interactions that represent an early stage of niche colonization upon transplantation.

We then applied engraftment studies to a dystrophic microenvironment (MDX-D2 mouse) to study the effects of a diseased microenvironment on cell transplantations. Initial engraftments did not show significant changes in regeneration, but spatial transcriptomics identified distinctly different gene expression profiles and interactomes on healthy stem cells and myofibers in a dystrophic microenvironment, highlighting the regenerative effect of a diseased host. Some of these changes are central to stem cell – and myofiber – ECM interactions, such as integrin signaling and collagen secretion. Upon implementation of a secondary injury model, we saw a significant stunting of the regenerative program in a dystrophic host, and we expect that this is due to the gene expression changes induced by the diseased microenvironment. Interestingly, we found critical differences in myogenic niche receptors both comparing healthy to dystrophic engraftment, and after secondary injury. In healthy engraftments, regenerative myofibers create a permissive niche that is highly adept at capturing muscle stem cells (marked by MEGF10, CDH15), and after secondary injury this niche is replaced by a different array of candidates that we predict is more supportive for stem cell maturation and quiescence. However, the niche fails to mature and change in a dystrophic environment, and this inability to do so highlights a major limitation of conventional stem cell therapies for patients with neuromuscular disorders. These data indicate that compensatory therapies are required in addition to cell therapies to modulate the impact of the dystrophic microenvironment.

MEGF10 arose several times in our datasets as a critical niche factor expressed through myogenic regeneration. MEGF10 is poorly understood in the context of skeletal muscle biology, although its loss-of-function results in a severe and fatal congenital myopathy called EMARDD. Thus, we sought to evaluate whether MEGF10 could regulate the retention of the PAX7⁺ stem cell population upon engraftment. We generated a CRISPR gene edited Tet-On expression system

to inducibly pulse MEGF10 in engrafted iPSC skeletal muscle progenitors. Transplantation of these cells indicated that overexpression for the first ten days significantly improved PAX7⁺ cell retention and myofiber regeneration. These data excitingly shed light, for the first time, on the chronological stages of niche formation and the importance of tight temporal control of niche factors through regeneration, as improper MEGF10 overexpression worsened regenerative readouts. EMARDD disease modeling muscle stem cell lines failed to support any PAX7⁺ cells, and severely stunted regenerative programs.

We then sought to understand the mechanisms of action of MEGF10 inducible overexpression and how it actively supports PAX7⁺ muscle stem cells. Spatial transcriptomics applied at day ten of engraftment with active MEGF10 overexpression compared to control engraftments revealed the spatiotemporal programs of MEGF10 in muscle regeneration. We found that MEGF10 triggers a massive myofiber response, accelerating myofiber regeneration to reach the regenerative ACTC1⁺ state faster, and therefore better supporting PAX7⁺ cells. Further, these regenerative ACTC1⁺ MEGF10⁺ myofibers are arrested at this state, stunted from further development to prolong the primary niche. Thus, MEGF10 overexpression induces an accelerated and prolonged primary niche, adept at capturing muscle stem cells upon engraftment. We also found novel downstream signaling factors of MEGF10, which seems to play a role in a positive feedback loop with other niche factors such as CDH15, JAM2, and LRRN1. We summarize the findings of this study in **Figure 4.1**.

These data shed critical light on the foundational biological components that comprise autologous muscle stem cell therapies for neuromuscular diseases and disorders. They reveal the importance of regenerative myofibers that support an early permissive niche to enhance self-renewal upon transplantation. Exciting new avenues of research are being conducted, and in

combination with the findings presented in this dissertation, will continue to pave the way for harnessing iPSC skeletal muscle therapies in the clinic.

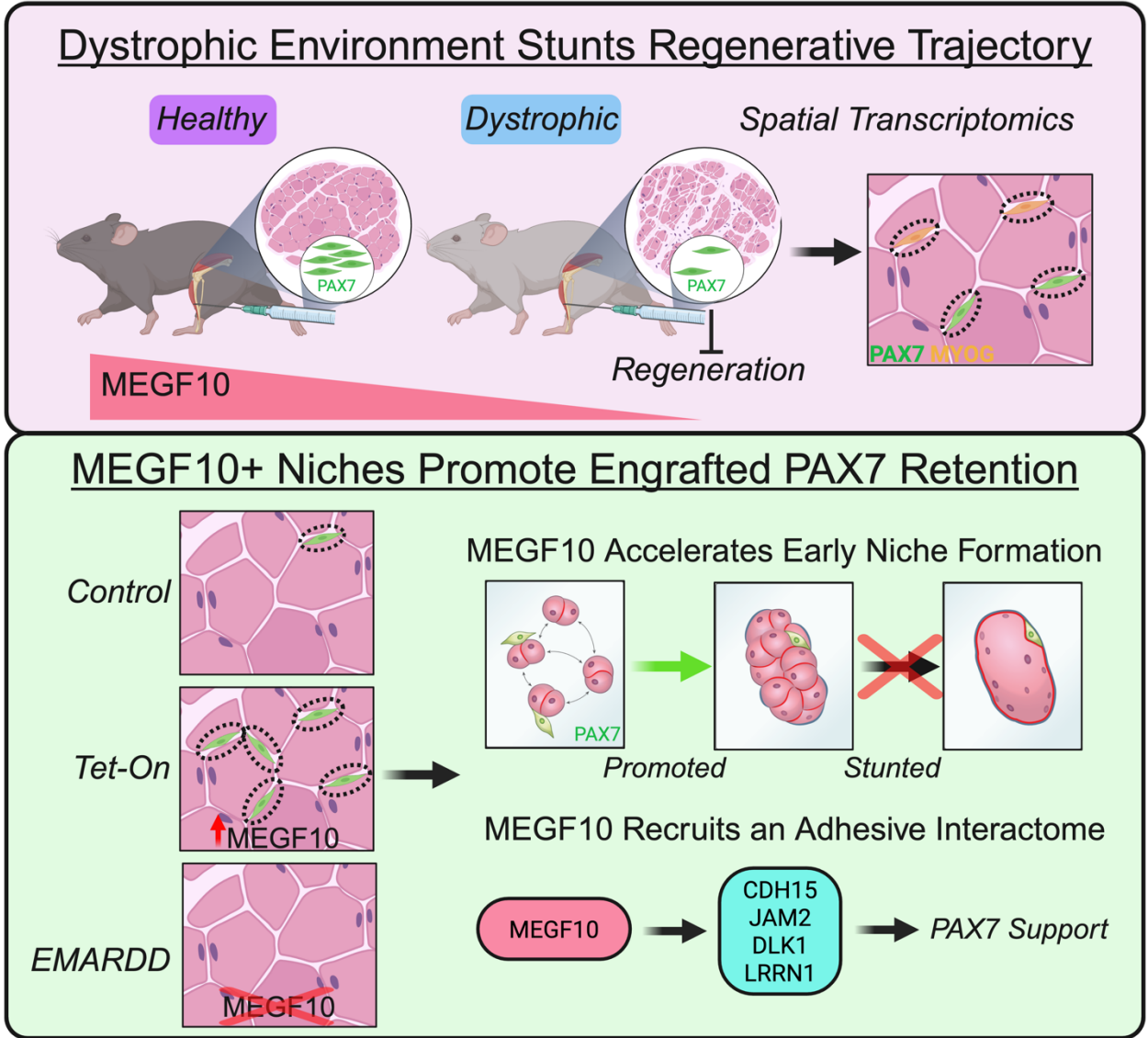


Figure 4.1: Graphical summarization of the findings through this study.

A. Cartoon depicting the significant findings in this dissertation. Top: the Dystrophic environment stunts a regenerative response, as indicated by worsened regenerative potency

in engrafted muscle stem cells. MEGF10 is elevated in healthy environment engraftments. We collected and analyzed data primarily through spatial transcriptomics of in situ transplanted cell populations. Bottom: Our approach to systematically induce overexpression of MEGF10 with Tet-On engrafted muscle stem cells, which drove better retention of PAX7+ cell populations through the acceleration of early myofiber regeneration. We predict a model where MEGF10 overexpression recruits other adhesive niche-forming factors, like CDH15, LRNN1, JAM2, and DLK1.

4.2: Future Directions

This research highlights exciting implementation of MEGF10 as a model system that can better support the retention of PAX7+ cells upon engraftment. Further, we identify that the dystrophic microenvironment can be detrimental for the regenerative program and self-renewal of healthy engraftments. We therefore seek to apply the MEGF10 overexpression system in a dystrophic host to determine whether its effects can salvage detrimental regeneration in a diseased microenvironment, and therefore serve as a potential clinical approach for patients with neuromuscular disorders. If MEGF10 fails to rescue engraftments in a dystrophic host, this further solidifies the dire need for compensatory therapies that may unlock novel solutions and treatments.

Our findings excitingly reveal the chronological stages and temporal components of stem cell niche formation. We leveraged a Tet-On inducible expression system and pulsed MEGF10 across regenerative time points, revealing not only its importance in establishing an early niche, but also that it can be leveraged to accelerate and prolong primary niches for improved PAX7+ cell support. However, this approach is likely not feasible for clinical or translational applications – CRISPR-homology directed repair is highly invasive, and it requires extensive *in vitro* culturing.

Thus, alternative methods of applying a MEGF10 overexpression phenotype are required. As MEGF10 plays a role in the Notch signaling pathway, we propose that Notch overexpression triggering drugs may recapitulate a MEGF10⁺ phenotype and signaling pathway. While Notch inhibition is well understood – DAPT can block gamma secretase cleavage of Notch intracellular domain, shutting down the pathway – Notch overexpression is more complicated. Currently, stimulation with direct agents or epigenetic modulators seem to be the most promising pathways to do so. Valproic Acid (VPA) is a histone deacetylase inhibitor that induces Notch upregulation and is being applied in clinical trials for epilepsy and cancer [309, 310]. Givinostat is a current histone deacetylase inhibitor in clinical trials for DMD, so we anticipate that histone deacetylase inhibitor treatment may be a viable alternative treatment to model a MEGF10 phenotype. Of course, histone deacetylase inhibitor treatment has extensive off-target effects, so sequencing analysis in myogenic cells is a pre-requisite for establishing its clinical viability.

This work describes a novel approach to study the chronological stages of muscle stem cell receptor involvement and regulation of niche colonization. We found that MEGF10 expression is critical for supporting engrafted muscle stem cells and enhancing their potential to hone to available niches, and this effect was temporally specific. We show that while the first ten days of expression is advantageous, expression at later time points is detrimental for the regenerative potency and retention of the transplanted muscle stem cell population. Further, the loss of MEGF10 in our loss-of-function mutation stem cell lines fail to persist at all, highlighting MEGF10's temporal significance in mediating this process. Moreover, this platform provides novel insight into the time-specific roles that stem cell niche receptors play in niche formation, and it could excitingly be applied to other stem cell niche receptors. For example, Calcitonin Receptor (CALCR), which is critical for downstream signaling that supports satellite cells in

quiescence, may be important for supporting engrafted populations through late-stage mediation [150, 151, 190]. We also predict that stem cell – ECM niche receptors, such as OSMR or ITGA7, may be essential for the mid-stage of niche formation [126, 159, 160]. Assessing the chronological stages of iPSC muscle stem cell niche formation using a Tet-On system of carefully and temporally controlled receptor expression would allow for robust investigation into these questions.

While we understand MEGF10's greater role in the regenerative muscle program and stem cell niche support, its direct mechanisms – i.e. its binding partners in the context of skeletal muscle. The most direct approach would be to apply crosslinking Co-IP approaches to capture all proteins bound to MEGF10 in skeletal muscle cultures to decipher its precise binding partners. Additionally, we could turn to bulk RNA-sequencing of Control and Tet-On MEGF10+ myofibers, overlaying these data with both our muscle stem cell and engrafted spatial transcriptomic datasets to uncover the underlying mechanisms of action of MEGF10. With these new data, we would have further clarity and confidence on what signaling pathways are downstream of MEGF10, and which are myofiber or stem cell specific. With this approach we would effectively understand any potential cell autonomous signaling of MEGF10 in skeletal muscle. Additionally, it would be intriguing to apply single cell resolution spatial protein arrays using platforms such as the CosMX to generate analytical datasets. With this approach we could effectively profile the ligand-receptor interactions of iPSC muscle stem cells that can successfully form niches upon transplantation, unlocking novel candidates and binding partners. We could then compare these to Tet-On MEGF10+ engraftments to assess how MEGF10 signaling mediates the niche supportive interactions, shedding further light on these pathways. This would be the most direct and effective profiling of the protein interactions that make up the muscle stem cell niche, and therefore, it would be highly valuable to compare these data across primary fetal and adult tissue samples to determine

how development and maturation changes the stem cell niche. Finally, protein binding assays would be the most direct method to uncover the binding partners of MEGF10 in muscle stem cells and myofibers. If these data remain unclear, investigating the Notch signaling pathway seems to be the most prevalent of suggesting a linkage to MEGF10 in current literature, and thus ChIP-seq analysis of NOTCH1-4 (NICD), or immediate Notch signaling transcription factors (MAML, RBPJ), may be an indirect way to 1) conclusively prove the role of MEGF10 in Notch signaling and 2) determine MEGF10's involvement in the Notch signaling cascade and evaluate potential downstream factors.

The spatial transcriptomics described in this dissertation is particularly exciting because it is the first study that has demonstrated the high-dimensional power to analyze cellular heterogeneity upon engraftment, specifically in myofiber populations. The heterogeneity we were able to compare and characterize was defined across different time points or conditions, rather than from within the same sample. Multinucleated myofibers are particularly difficult to analyze after engraftment, as they cannot be FACS enriched due to their large size. However, contemporary spatial transcriptomic approaches fail to capture the myonuclei heterogeneity in myogenic populations as the technique relies on pooling together enough nuclei/cells to collect transcriptomic data. Single nucleus RNA sequencing of engrafted human nuclei would allow for assaying the genetic profiles of all myogenic populations, including their time point specific heterogeneity. This approach would be challenging because we would have to rely on enriching the human nuclei through either endogenous nuclei-bound fluorescence or a human-specific nuclear membrane marker (to not rupture the nuclei for downstream mRNA library collection). Admittedly, in this case we would lose the spatial resolution of the tissue - the simplest approach to thoroughly address the questions laid out in this dissertation would be to apply single nuclei

resolution spatial technologies to assay the heterogeneity of populations across space and time robustly. This would be extremely strong for profiling the heterogeneity across all myogenic populations, including resident non-myogenic cells, myofiber heterogeneity, myonuclei heterogeneity, and muscle stem cell heterogeneity.

Our work also sheds light on how the microenvironment, and specifically the ECM, regulates the stem cell niche interactome and therefore regenerative potency of engraftments. We therefore postulate that cell therapies may need a compensatory element to alleviate the dystrophic microenvironment. We first need to assess whether the ECM is indeed the key player regulating this diseased response seen in engrafted cells – to address this question, we would need to introduce healthy ECM into a dystrophic host and engraft muscle stem cells into this artificial healthy scaffold. If this can effectively rescue regenerative defects, then the addition of a healthy ECM in dystrophic hosts is a valuable avenue to pursue for cell therapies and highlights a strong candidate for a compensatory therapy. If this fails and no change is seen, then other adjacent cell types (immune signaling cells, FAPS, pericytes, etc) should be further investigated to understand their role in secretive signaling in dystrophic skeletal muscle engraftments.

Our findings outline another exciting direction – can we rescue dystrophic loss of stemness, or slow their diseased progression, through mediated interactions with a healthy ECM? With this approach, we would seek to either harvest primary mouse or human dystrophic satellite cells, or generate iPSC disease modeling muscle stem cells, and culture them atop healthy myoscaffolds [195]. A major readout of this experiment would be assessing the regenerative potency of these stem cells upon engraftment to evaluate whether exposure to a healthy ECM is sufficient to rescue dystrophic loss of stemness. Restoration of the Integrin signaling pathway and its axis of interaction between stem cell and ECM is a critical component of a successful system.

Alternatively, we could engraft dystrophic stem cells into a healthy host to narrow down the importance of the ECM versus the entire *in vivo* system. Our final experiment would be to reisolate dystrophic stem cells after mediated interactome signaling with healthy ECM and transplanting them back into a dystrophic host. Any increases in regenerative or niche forming effects would be attributed to the interactions with a healthy ECM, which could regulate the dystrophic regenerative defects.

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