Animal regeneration and its loss: the mouse as a model of limited regeneration

Thesis by

Fayth Hui Tan

In Partial Fulfillment of the Requirements for
the degree of
Biology

CALIFORNIA INSTITUTE OF TECHNOLOGY
Pasadena, California

2023

(Defended April 21, 2023)

Fayth Hui Tan

ORCID:0000-0002-2160-5311

"You are drawn to a subject and you become an observer. As such you receive intense esthetic pleasure from what you see, a feeling that is pure enchantment for all of your life. And if you are patient and quiet, keeping that chatterbox part of your mind from rattling on about what it doesn't understand, letting the other part listen in a silent, visual way, an organism begins to talk to you. It takes a piece of your life to find this out. It is of no value to anyone else, certainly not to society, but it is wonderful. Organism to organism."

— N.J. Berrill, The pleasure and practice of biology

ACKNOWLEDGEMENTS

Writing this has felt equal parts inadequate and clumsy. I thought that perhaps leaving this till last might make it less so, but I only find myself overwhelmed and stumbling again. All I know for certain is that I've spent the day mired in an upwelling of mortifyingly sincere emotion about my time in graduate school, and I've just got to articulate my thanks as best I can.

Marianne Bronner— I would not, could not, have seen this through without your support and unwavering belief that I really, really ought not to give up on getting this degree.

My committee, Joe Parker, David Chan, and Viviana Gradinaru— Thank you for treating not only my research, but my turbulent grad school experience, with respect and gravitas.

The Bronner Lab, current and past—particularly Megan Martik, Hugo Urrutia, Erica Hutchins, Can Li, and Sierra Marable— thank you for going out of your way to welcome me to the lab. Every offer of guidance, candid advice, and gesture of kindness was deeply appreciated, even if I was too awkward to adequately express my gratitude in person.

My family— I've gone from an obsessive child with reams of obscure esoterica to an obsessive adult with reams of obscure esoterica…and a degree. With all sincerity, I hope you're proud of me.

Kim Cooper and Dede Lyons— Thank you for taking me seriously, even when all I had was hubris and a pipette. A part of me will always be the undergrad at a lab bench, looking at embryos under a microscope for the first time.

Members of the Cooper and Lyons Lab, especially Hannah Grunwald and Joel Erberich— You made science feel both boundless and like somewhere I could call home.

Yutian Li, Anish Sarma and Changhua Yu— Maybe the real reward of research was the

friends I made along the way? The levity and vulnerability of our discussions about life and research are some of the dearest memories I have of grad school. I'll carry them with me, always.

All my peers that I've organized with to make Caltech a better place for all, present and past— particularly Lev, Krystal, Ollie, Ashay, Jane, Dawna, Kriti, Ryan, Sam, Abdullah, Xun, Alex, Simona, Varun, Quinn, Jasmine, Jessica, and the many others whose efforts I've been inspired by from afar— I've learned so much from you all. Sticking to your principles is hard, but it would've been harder to do alone. I can't wait to see what you all build, hand on my stupid, hopeful heart. Solidarity forever.

Viraj Desai, Amelia Woo and Esther Mak— the great thing about having old friends in different time zones is that you always have someone to have an existential crisis with. Thank you for never turning me away, no matter what time it is.

Matthew Langley and Alice Zhang— Your kindness, creativity, humor, and numerous homemade meals have figuratively and literally sustained me through this endeavor. It is evidence of goodness in the world that you've seen me at some ugly times and invited me into your home, not despite how wretched I felt, but because.

Martin— Of every living creature on this strange, beautiful planet, you are my favorite. I love you. I love you. I love you.

And finally, Miku, Augie and Roamy, the cats in my life: thanks for reminding me that sometimes, when it feels like the world is against you, you really should just take a nap.

ABSTRACT

In this dissertation, we explore animal regeneration through a comparative evolutionary-developmental framework. In Chapter 1, we review animal regeneration and its loss through examining broad developmental and physiological factors that correlate with regenerative ability. We also highlight the mouse as a model of regeneration loss, examining the limited regeneration of the digit tip and the heart. For each context, we discuss how these regenerative processes occur, the physiological and molecular factors involved, and previous attempts to induce or improve the regenerative response.

In Chapter 2, we explore the possibility of inducing regeneration in non-regenerating systems. Along with experiments in the jellyfish *Aurelia coerulea,* (formerly *A. aurita* sp. 1 strain) and the fruit fly *Drosophila melanogaster*, we find that supplementation with the amino acid L-leucine and sucrose induce appendage regeneration across these highly evolutionarily-diverged organisms. We discuss how this intervention highlights the conserved role of energetic parameters in regeneration, and how surpassing nutrient-based limitations may unlock regenerative responses in diverse contexts.

In Chapter 3, we characterize the derivatives of cardiac neural crest cells (CNCCs) in the hearts of neonatal mice at P1 and >P7. We confirm previous work on the diverse cardiac derivatives resulting from CNCCs, and provide additional evidence for CNCC-derived cardiomyocytes, a contribution still contested in current literature. We also demonstrate how CNCC derivatives form a distinct age-related developmental trajectory in the heart, and discuss how these changes may affect cardiac physiology and relate to the loss of neonatal heart regeneration.

Finally, in Chapter 4, we propose future directions based on the work carried out in Chapter 3. While CNCCs have explicitly been studied in the context of heart regeneration in zebrafish, their role in neonatal mouse heart regeneration has not been explored. First, we suggest further investigation into the differences between CNCC-derived and non-CNCC derived CMs, to see if their proliferative ability and molecular profile show different temporal dynamics over the course of embryonic and early postnatal stages. Then, to

examine all CNCC-derived cell types in regenerating P1 and non-regenerating P8 hearts, we propose a single-nuclei RNA-sequencing experiment to better resolve questions about how the myocardial lineage interacts with nonmyocytes, and to capture the full extent of potential cardiomyocyte decline postnatally. Lastly, to understand how CNCC derivatives influence endogenous heart regeneration, we design a dual *Cre* and *(r)tTA* driver system in transgenic mice to perform a conditional ablation experiment of CNCCs in a cryoinjury model of neonatal heart regeneration.

PUBLISHED CONTENT AND CONTRIBUTIONS

Abrams, M.J., **Tan, F.H.**, Li, Y., Basinger, T., Heithe, M.L., Sarma, A.A, Lee, I.T., Condiotte, Z.J., Raffiee, M., Dabiri J.O., Gold D.A., Goentoro, L.. (2021). "A conserved strategy for inducing appendage regeneration in moon jellyfish, *Drosophila*, and mice". eLife. **10**:e65092. https://doi.org/10.7554/eLife.65092.

F.H.T. participated in the conception of the project, planned and performed all mouse digit experiments and data analysis, contributed to Drosophila imaging, and participated in the writing of the manuscript.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv
ABSTRACT vi
PUBLISHED CONTENT AND CONTRIBUTIONS viii
TABLE OF CONTENTS ix
REGENERATION AND REGENERATIVE LOSS IN THE ANIMAL KINGDOM: A COMPARATIVE APPROACH 1
ABSTRACT 1
INTRODUCTION 1
Regeneration in an evolutionary-developmental framework 2
Metabolism and energy expenditure 5
Cellular plasticity 6
Age 7
Immune system 8
The mouse as a model of regeneration loss 9
Mouse digit tip 10
Mouse digit anatomy 10
Digit regeneration as blastema-mediated 10
The process of mouse digit regeneration 11
Positionally-determined regeneration ability 16
Positionally-specific cellular properties 17
Induced digit regeneration 17
Proximal P3 system 18
P2 system 18
Mouse heart regeneration 20
Physiological and molecular factors involved in heart regeneration 21
Hippo/YAP signaling 22
Cardiac metabolism and oxidative stress 24
Neural signaling 26
Extracellular matrix components and properties 26
Immune response 28
Strategies to induce heart regeneration 30
ESCs and iPSCs 31

Cardiac stem cells 32
Direct transdifferentiation of non-cardiomyocyte cell types 34
The CNCC as a potential internal source of regenerating cells 35
CONCLUSION 37
BIBLIOGRAPHY 38
A CONSERVED STRATEGY FOR INDUCING APPENDAGE REGENERATION IN MOON JELLYFISH, *DROSOPHILA*, AND MICE 61
ABSTRACT 61
INTRODUCTION 61
RESULTS 64
Leucine and sucrose induce regeneration in mouse digit 64
DISCUSSION 66
MATERIALS AND METHODS 69
Mus musculus 69
Strain 69
Regeneration experiments 69
Mouse digit dissection and skeletal staining 70
BIBLIOGRAPHY 75
CORRELATING CHANGES IN THE MOUSE CARDIAC NEURAL CREST WITH NEONATAL HEART REGENERATION 78
ABSTRACT 78
INTRODUCTION 79
RESULTS 80
Transcriptomic changes in the neonatal mouse cardiac neural crest 80
Computational analysis of CNCC-derived cardiomyocytes in the postnatal heart 84
Maturing CNCC derivatives reflect a distinct shift in the molecular profile of the postnatal heart 86
Potential impacts of maturing CNCC derivatives on cardiac physiology 88
DISCUSSION 90
MATERIALS AND METHODS 94
Mice 94
Bulk RNA sequencing of *Wnt1*+ and *Wnt1*- cells from postnatal hearts 94
Computational analysis of single-cell data from *Wnt1*+ postnatal cardiac cells 95
BIBLIOGRAPHY 104
FUTURE DIRECTIONS 111

Proposal: Investigating the role of CNCC derivatives in mouse heart regeneration .. 111
Specific Aim 1: Comparative transcriptional profiling of CNCC-derived *Wnt1+* CMs with non-CNCC derived CMs in embryonic and postnatal hearts..... 112
Specific Aim 2: Single nuclei transcriptional profiling of cardiac neural crest-derived cells in regenerating postnatal mouse hearts..... 114
Specific Aim 3: Effects of genetic ablation of neural crest derived cells on heart regeneration in P1 mice 115
BIBLIOGRAPHY 119

List of Illustrations and/or Tables

Number *Page*

1. Mouse digit regeneration from proximal P3 and P2 amputation.....80
2. Classification and quantification of observed digit phenotypes 82
3. Examples of observed regenerative phenotypes……..82
4. Bulk RNA-seq comparisons of P1 and P8 *Wnt1*+ and *Wnt1*- cells ..109
5. Clusters and cell types identified in P1 and P7 CNCC derivatives ..110
6. CNCC-derived CMs in embryonic and postnatal stages 111
7. Developmental trajectory of CNCC derivatives in P1 and P7.....112
8. Ligand-receptor analysis between non-CM and CMs.....113
9. Dual recombinase strategy to identify CNCC-CMs 130

Chapter 1

REGENERATION AND REGENERATIVE LOSS IN THE ANIMAL KINGDOM: A COMPARATIVE APPROACH

ABSTRACT

The myriad regenerative abilities across the animal kingdom have fascinated us for centuries. Recent advances in developmental, molecular, and cellular biology have allowed us to unearth a surprising diversity of mechanisms through which these processes occur. Developing an all-encompassing theory of animal regeneration has thus proved a complex endeavor. In this chapter, we frame the evolution and loss of animal regeneration within the broad developmental constraints that may physiologically inhibit regenerative ability across animal phylogeny. We then examine the mouse as a model of regeneration loss, specifically the experimental systems of the digit tip and heart. We discuss the digit tip and heart as a positionally-limited system of regeneration and a temporally-limited system of regeneration, respectively. We delve into the physiological processes involved in both forms of regeneration, and how each phase of the healing and regenerative process may be affected by various molecular signals, systemic changes, or microenvironmental cues. Lastly, we also discuss the various approaches and interventions used to induce or improve the regenerative response in both contexts, and the implications they have for our understanding regenerative ability more broadly.

INTRODUCTION

The historical fascination with animal regeneration is centuries-old. The first formal experimental studies date back to the 18th century, including René-Antoine Réaumur's studies of the spontaneous regeneration of crayfish claws (Réaumur, 1712) and Abraham Tremblay's discovery of hydra whole-body regeneration (Tremblay, 1744). From these

studies arose a growing awareness of the extensive capacity of animal bodies to regrow and remake themselves, and the myriad diverse animal and organ systems it takes place in. This fascination persists into modern day, with recent advances in developmental, molecular, and cellular biology allowing us to uncover the mechanisms through which these processes occur. The closer we examine these phenomena, however, the more different they appear. The regeneration of the same structure, even between two relatively closely related animals, can be a heterogeneous process. For example, salamander limb regeneration involves the dedifferentiation of postmitotic, multinucleated muscle cells, which re-enter the cell cycle and contribute to the new limb (Wang and Simon, 2016). In contrast, no muscle dedifferentiation is observed in axolotls, as muscle in the regenerated limb is derived from a resident population of satellite stem cells (Fei et al., 2017).

For this reason, forming a unifying theory for animal regeneration is complex—why does it occur in some animals but not others? To what extent does regeneration share or co-opt mechanisms from physiological and developmental processes? Might there be a way to enable non-regenerative species to regenerate? These questions remain relevant even as we gain more fine-grained insights into regeneration across animal phylogeny.

Regeneration in an evolutionary-developmental framework

In 1893, August Weismann put forth the earliest attempt to create a conceptual framework for the emergence of animal regeneration. According to Weismann, regeneration could be conceived of as an adaptive trait—that there was a causal relationship between the frequency or likelihood of damage and the ability to regenerate (Weissman, 1893).

One contemporary critic of Weismann's theory was Thomas Hunt Morgan, who spent much of his early career studying a diverse array of regenerating animals. In one of his classical experiments, Morgan observed that hermit crab hind limbs, often hidden away and protected under their shell, regenerated just as frequently and readily as their less well-protected forelimbs (Morgan, 1902). He would synthesize his own findings and theories in a 1901 book, Regeneration, in which he proposed that regeneration was an intrinsic

property of an organism, and therefore should be studied like a developmental process (Morgan, 1901).

Much of Morgan's theoretical and experimental framework is still relevant to the study of regeneration today. Similar to a developmental process, Morgan considered regeneration to refer broadly to a heterogenous set of organized spatiotemporal processes, including the renewal of an organ, the replacement of entire body parts or even asexual reproduction (Sinigaglia et al., 2022). The broadness of this definition may appear to confuse the study of regeneration, but casting a relatively wide net may help draw parallels between different levels of biological similarity—specific processes, molecular pathways or morphological structure.

Elaborating on Morgan's concept of regeneration as a developmental process, contemporary work in evolutionary-developmental biology has created frameworks through which to draw these parallels in a more consistent and rigorous manner. First, the concept of modularity, which divides complex phenomena such as regeneration into distinct processes, capturing interactions within and between multiple levels of organismal organization (Wagner et al., 2007). This includes regulatory phenomena at the molecular level, or structural entities such as cells or tissues that interact in order to produce a complex structure (Atchley and Hall, 1991). In a regeneration context, limb regeneration can be separated into distinct but recognizably similar stages across taxa. In both the arthropod *Parhyale* and the vertebrate axolotl, amputation of the limb leads to wound healing and closure, followed by the formation of a blastema with the activation of *Pax7*-expressing satellite progenitor cells, which then undergo proliferation, growth, and morphogenesis to form a new limb (Konstantinides and Averof, 2014; Sandoval-Guzmán et al., 2014).

While the arthropod and vertebrate limb are not homologous in the typical sense—i.e., sharing a common evolutionary origin— they are analogous structures in that they perform similar structure. Evolutionary-developmental biology proposes an extended definition of homology that also includes the regulatory networks underlying modular processes, even if their ultimate anatomical or physiological outcomes differ (Gilbert and Bolker, 2001). In the case of *Parhyale*, the discovery of *Pax7* satellite cells was interpreted as a case of deep

homology with vertebrates, suggesting that this pool of progenitor cells share an evolutionary origin (Konstantinides and Averof, 2014). A similar population of satellite cells was also discovered recently in adult *Drosophila* flight muscles, further suggesting that these progenitors have conserved regulatory mechanisms across all arthropods (Chaturvedi et al., 2017).

Given that *Pax7* often labels mesodermal precursors during development across animal phylogeny, it is possible that these arthropod and vertebrate satellite cell populations arose independently. Thus, there may have been multiple independent co-option events of the same ancient gene regulatory kernel into muscle stem cell lineages. However, interpreting these cellular, molecular, and regulatory similarities as convergent occurrences may also highlight generalizable properties that facilitate or hinder regeneration. From a developmental perspective, we could view the presence of *Pax7*+ cells as an embryonic cellular state persisting through adulthood—another potentially generalizable framework through which to view regenerative capacity (Lai and Aboobaker, 2018). Whether homologous or convergent, these comparisons open salient avenues of exploration within each module of the regenerative process. Are there, for example, shared physiological or regulatory changes that allow them to remain quiescent or re-access developmental pathways through adulthood? Are they re-activated with the same signals? Do they have a similar developmental origin? The heterogeneity of regeneration across animals, combined with the difficulty in ascertaining shared evolutionary origin, highlights the need for comparisons that examine all modules of the regenerative process, and investigate how they interact with each other across multiple levels of organization.

Developmental constraints and decline in regenerative ability

The evolutionary-developmental framework of regeneration allows us to return to our initial question—why some animals are able to undergo extensive regeneration while others cannot—with greater clarity about the comparisons we are making. It also highlights how we might be able to investigate an understudied aspect of the field: why regeneration does not occur. The failure of a process to occur is not usually perceived to be as relevant as success, but if regeneration is an organismal property, understanding the specific

properties of failure is at least half of the biological picture. This understanding proves important to biomedical interests as well: if we want to improve human regenerative ability, better insight into failure might produce more targeted avenues for success.

Following the modular processes within a non-regenerative response might assist us in assessing why, how, and when regeneration is no longer feasible. Like axolotls, frogs undergo a wound healing process during which the wound epithelium forms and seals the injury—but unlike an axolotl, frogs do not regenerate the limb, instead forming a cartilaginous spike that lacks both the morphology and function of the original limb. Why does the wound healing process lead to limb regeneration in one case but not the other? To invoke another concept from evolutionary-developmental biology, we could hypothesize that the wound healing and regeneration modules are developmentally constrained in the frog, preventing the processes governing one module from interacting with the other (Richardson and Chipman, 2003).

But what exactly are these developmental constraints? The exact mechanisms behind regenerative decline have not been studied in detail, but there are broad physiological factors that seem to consistently correlate with and affect regenerative ability.

Metabolism and energy expenditure

Energetic allocation and tradeoff can also arise indirectly. Another trend is the generally negative correlation between endothermy and regeneration particularly in vertebrates (Cutie and Huang, 2021). Thermogenic regulation is energetically costly, and poikilothermic animals such as zebrafish and salamanders tend to possess higher regenerative ability compared to endothermic mammals. Likewise, neonatal mammals are not as capable of efficient thermoregulation compared to their adult counterparts and tend to display increased regenerative potential (Tourneaux et al., 2009). Vertebrates with mostly diploid cardiomyocytes (a proxy for cardiac regeneration) and high cardiac regeneration ability are known to have metabolisms an order of magnitude lower than their endothermic mammalian counterparts (Makarieva et al., 2008). A phylogenetic association between diploid cardiomyocytes and standard metabolic rates (factoring out organism size)

in 41 species of animals showed that diploid cardiomyocytes negatively correlated with metabolic rates, and that in mammals, body temperature also negatively correlates with the percentage of myocardium composed of diploid cardiomyocytes (Hirose et al., 2019).

Cellular plasticity

A key factor in regenerative success is having an abundant cellular source from which regenerated tissue originates from. Thus, cellular plasticity within an organism correlates with regenerative capacity. Methods of yielding new cells can include drawing on a pool of progenitor cells, dedifferentiation of differentiated cells to a progenitor state, or transdifferentiation of one cell type into another (Jopling et al., 2011). Diploblastic animals, or animals with bodies derived entirely from ectoderm and endoderm, tend to possess reservoirs of stem cells from which new tissue can be formed (Elchaninov et al., 2021). For example, the incredibly regenerative Hydra has epithelial stem cells and interstitial stem cells, with epithelial stem cells contributing to epidermally-derived cell types such as neurons and secretory cells, and interstitial stem cells contributing to all other remaining cell types (Wittlieb et al., 2006; Hemmrich et al., 2012). The earliest branching triploblastic animal, with tissues derived from ectoderm, endoderm, and mesoderm, are planaria. While similarly possessing whole-body regenerative ability, planarians have a single pool of pluripotent progenitors, the neoblasts, which proliferate and form blastemas to restore missing body parts (Reddien et al., 2005). In vertebrates, where regeneration is typically limited to specific parts of the body, blastema cells appear relatively fate-restricted. In both axolotl limb regeneration and mouse digit tip regeneration, de-differentiated blastema cells only form tissues deriving from the same lineage as the tissue of their origin (Kragl et al., 2009; Lehoczky et al., 2011). For instance, regenerated bone in the mouse digit tip derives from pre-amputation osteoblasts (Lehoczky et al., 2011), and in the axolotl limb, dermal cells will form cartilage but not muscle as limb dermis and cartilage originate from lateral plate mesoderm (Kragl et al., 2009) It has thus been hypothesized that the evolution of more specialized tissue types within a single organism resulted in more complex regulation of the cell cycle and differentiated cells' ability to re-enter it (Galliot and Ghila, 2010), thereby reducing their plasticity and decreasing regenerative capacity.

Age

One association that relates to cellular plasticity is the consideration of age and regenerative ability. Broadly speaking, regenerative potential generally appears to be higher during early life: embryos, larval stages and juvenile animals tend to have greater ability to regenerate than their adult or aged counterparts (Seifert and Voss, 2013). Fetal mammals can regenerate skin, leading to scar-free healing, while adults typically form fibrotic scars when wound healing (Bullard et al., 2003), and even regenerative animals such as zebrafish show a decline in the ability to regenerate pectoral fins in older animals (Itou et al., 2012). This decline has been associated with cell cycle regulation, as age-related stressors such as DNA damage and oxidative stress may impair cellular ability to proliferate and self-renew (Signer and Morrison, 2013). Dysregulated tissue homeostasis in major signaling pathways due to aging can also lead to increased chronic inflammation and fibrosis, which are generally not conducive for regeneration (Singh et al., 2011; Beggs et al., 2004) though restoring homeostatic signaling seems to ameliorate detrimental effects (Kovacheva et al., 2010; Ito et al., 2007).

Mechanisms of age-related loss of plasticity and proliferation may occur through changing epigenetic state of cells with age, as changes in DNA methylation and histone markers influenced genes related to proliferation, pluripotency, and differentiation state (Sousounis et al., 2014). They may also be affected by systemic factors, as demonstrated in parabiosis experiments—serum from younger mice stimulates muscle and neuronal renewal in older mice, while serum from older mice conversely decreased neurogenesis and myogenesis (Conboy et al., 2005; Villeda et al., 2011). Recreating a “younger” systemic microenvironment also encouraged oligodendrocyte renewal and promoted axonal remyelination in older mice (Ruckh et al., 2012). Thus, while aging correlates with the loss of regenerative ability, not all changes are due to the inherent properties of aging tissue. Changing molecular signals and the surrounding systemic milieu also influence how and if cells respond to regenerative cues.

Immune system

The immune system is obviously instrumental in both wound healing and regeneration. A wide body of work across animals and organs implicates the immune system in regenerative responses, explicitly in creating a permissive environment for regeneration to take place (Godwin et al., 2013; Fukuzawa et al., 2009; Aurora et al., 2014; Thomas and Puleo, 2011).

It is also a factor that seems to broadly correlate with regenerative ability on a phylogenetic level. The emergence of a complex immune system, and specifically a specialized adaptive immune response generally appears to have a negative correlation with regenerative ability. Frogs and mice, for example, possess rapid and diverse adaptive immune responses upon injury (Mescher and Neff, 2005). In comparison, salamanders lack some adaptive immune responses, such as reactivity to soluble antigens (Charlemagne, 1979) and an acute rejection of xenografts (Godwin and Rosenthal, 2014), relying primarily on an innate immune response. Other studies point to the maturity of the immune system as a relevant factor. *Xenopus* tadpoles lose regenerative ability with age, and mice only maintain heart regeneration in early neonatal life (Porello et al., 2011) and scarless skin regeneration in embryonic stages (Ferguson and O'Kane, 2004). It is hypothesized that the mature immune system mounts a strong inflammatory response that is not conducive to regeneration, as regenerative failure in Xenopus tadpoles can be partially restored with anti-inflammatory molecules (King et al., 2012)

Immunomodulatory strategies for cardiac regeneration have thus focused on blunting the adaptive immune response (Sattler et al., 2017). CD4+ T cells, which secrete various cytokines to stimulate immune responses, have proved of particular interest—regulatory T-cells (Tregs) specifically are known to suppress inflammatory responses and promote macrophage polarization toward an M2-like anti-inflammatory phenotype (Weirather et al., 2014). Comparisons of CD4+ T-cells in neonatal and adult mice show that neonatal T-cells default to becoming Tregs, though this tendency diminishes within the first 2 weeks of life (Wang et al., 2010). Tregs have also been shown to be essential in zebrafish and neonatal mouse regeneration, due to their immunomodulatory effect and secretion of the

CM mitogen Neuregulin (*Nrg*) (Hui et al., 2017; Li et al., 2019). Human fetal immune systems also generate more Tregs than adult immune systems, which suggests that Treg function might be a conserved therapeutic target to improve heart regeneration.

However, due to the complexity of immune responses, it is unlikely that a single factor is the determinant of regenerative success. There are important caveats to these broad trends. For instance, the spiny mouse *Acomys*, able to regenerate epidermal tissue, has a longer and more marked adaptive immune response than the standard lab mouse (Gawriluk et al., 2020). An inflammatory response has also proved essential in other contexts such as the mouse digit tip (Simkin et al., 2017a) and amphibian limb (Godwin et al., 2013). The apparent pleiotropy of immune-related factors in different contexts points to the fact that a "pro-regenerative" immune response is a multiphasic process that is sensitive to the nature but also the duration of each phase (Aztekin and Storer, 2022). Thus, studying how immune responses are regulated will be crucial to understanding how the immune response lays the groundwork for regeneration.

The mouse as a model of regeneration loss

These physiological correlates demonstrate how regeneration is not simply an intrinsic property that a given organism or body part does or does not possess. Rather, a regenerative response is the result of cellular interactions and states, the local milieu of microenvironmental and molecular cues, and the influence of organismal-level physiology. Understanding how each of these factors function and intersect to help or hinder a regenerative response is crucial to identify key points in the process that play a significant role in determining the ultimate outcome. These critical junctures could provide us with optimal targets for intervention—how best to stimulate a regenerative response where one would not typically occur. To do so, we use the mouse as a model of regeneration loss. Mice possess limited but reliable regenerative ability, in the very distalmost portion of the digit tip (Neufeld and Zhao, 1995), and in the heart as neonates within the first week of life (Porello et al., 2011). Removing anything beyond the digit tip, or damaging the heart in an older mouse instead results in regenerative failure, which provides us with the opportunity to investigate why and how these regenerative responses are so specifically spatially or

temporally limited. In the rest of this chapter, I will discuss how these regenerative processes occur endogenously, the physiological and molecular factors that play a significant role in their success or failure, and the attempts at inducing or improving further regenerative responses in each context.

Mouse digit tip

The mouse digit tip is an important model in the study of mammalian regeneration. It possesses some endogenous regeneration ability throughout the entire life of the animal, as mice are able to regenerate the distal tip of the terminal phalanx from neonatal stages to adulthood (P3). This ability mirrors documented cases in clinical literature where young children (often ages 6 or younger) have been able to regenerate severed fingertips (Illingworth, 1974), highlighting its potential relevance to improving human regenerative ability.

Mouse digit anatomy

The mouse digit consists of three phalanges, referred to as P1, P2, and P3 proximally to distally. The regenerative portion of the digit is found in P3, a structurally distinct bone that has a wide, flared base with a large bone marrow cavity that tapers into a pointed distal tip (Seifert and Muneoka, 2018). The distal end of P3 is encased by the nail organ, while the proximal end articulates with P2 to form the P2/P3 joint. Distal amputations, or amputations that removed up to the first third of P3 successfully regenerate, although other components such as the bone marrow, fat pad, or nail matrix should also remain undamaged to ensure regeneration (Dolan et al., 2018). In contrast, amputation that removes over 50% of P3 generally results in wound healing without regeneration (Neufeld and Zhao, 1995).

Digit regeneration as blastema-mediated

Digit regeneration in mice is unusual in two respects: firstly, that it is a mammalian example of blastema-mediated regeneration, and second, that it occurs in a markedly different manner from digit development. Unlike other repair processes such as healing a

fracture or muscle injury, digit regeneration involves the formation of a transient blastema, a mass of proliferative, pluripotent cells that differentiate and undergo morphogenesis to pattern and regenerate the lost structure (McCusker et al., 2015). Other known instances of mammalian blastema mediated regeneration are the regeneration of ear punch wounds in rabbits (Vorontsova and Liosner, 1960) or certain mouse models such as the spiny mouse *Acomys* (Seifert et al., 2012a) or the Murphy's Roth Large (MRL) mouse (Clark et al., 1998), and the annual regeneration of antlers in deer (Kierdorf et al., 2007).

The process of mouse digit regeneration

During the endogenous regeneration process, the digit undergoes sequential phases of wound healing, blastema formation, and differentiation of regenerating tissues (Fernando et al., 2011; Dolan et al., 2018). The initial inflammatory response post digit amputation is crucial to regeneration success—a physiological component that is also true in other systems and organs, such as the zebrafish tail fin, axolotl limb, and ear tissue of the spiny mouse (Petrie et al., 2014; Godwin et al., 2013; Simkin et al., 2017b). Depleting macrophages in the digit tip at an early stage of regeneration leads to the subsequent inhibition of bone histolysis and wound closure, and the failure of the blastema to form (Simkin et al., 2017a). Complete depletion of macrophages in other systems such as axolotl limb and neonatal mouse heart also results in the inhibition of regeneration (Godwin et al., 2013; Aurora et al., 2014). Due to the functional diversity of macrophage subtypes, it is impossible to say if macrophages in general are inhibitory or essential to regeneration (Novak and Koh, 2013). It is likely that a specific subtype is involved in blastema formation, and the exact immunoregulatory dynamics have not been studied at that resolution.

Concurrently with the inflammatory phase of wound healing, the digit bone undergoes histolysis, the degradative loss of organized bone tissue. In regenerative amputations, the digit can lose up to half its original volume in bone (Simkin et al., 2015a). While the exact role of histolysis in mouse digit tip regeneration is not entirely clear, it appears to facilitate the regenerative response (Dawson et al., 2016). This is supported by the observation that blastema size correlates positively with the amount of histolysis observed, and that

degradation of the extracellular matrix may release pro-regenerative factors and cells that encourage new bone deposition (Simkin et al., 2015a; Dawson et al., 2016).

The wound epithelium forms after histolysis occurs, which is a relatively delayed timeframe compared to most rapid wound healing responses. Wound closure occurs in the digit tip when the injured epidermis retracts proximally, sealing the wound over the exposed periosteum of P3. Following histolysis, the wound epithelium forms over the digit stump and acts as a crucial signaling center in the formation of the blastema (Takeo et al., 2013). Secreting chemoattractants such as *Sdf-1*, the wound epithelium regulates the migration of mesenchymal blastema cells to the wound site (Lee et al., 2013). The activity of the wound epithelium is another response that plays a vital role in other systems, as inhibiting its formation negatively affects the regenerative response in salamanders and humans as well (Thornton, 1957; Illingworth, 1974).

The origin of blastema cells is highly heterogeneous, though their exact cellular composition is complicated due to the histolytic events that precede it (Simkin et al., 2015a). Lineage tracing studies show that they are lineage-restricted, though they derive from a wide variety of tissues, including the epidermis, bone, connective tissue, and vasculature (Lehoczky et al., 2011; Rinkevich et al., 2011; Takeo et al., 2013; Wu et al., 2013). No transdifferentiation occurs between ectodermal and mesodermal lineages, as all regenerative epithelium is derived from keratinocytes and bone and periosteum are derived from osteoblast (Lehoczky et al., 2011; Rinkevich et al., 2011). However, a potentially understudied source of progenitor cells may also originate from fibroblastic cells of the connective tissue, as fibroblasts are known to be phenotypically plastic and multipotent in the context of tissue repair (Plikus et al., 2021).

The complete formation of the wound epithelium marks the end of the wound healing phase, and the initiation of blastema formation. The digit tip blastema is a transient aggregation of undifferentiated cells, with their physiology broadly characterized as proliferative, avascular, and hypoxic. Blastema cells are recruited to the wound site through signaling from the wound epithelium, and receive signals that are crucial to maintain high proliferative ability. Broad signals include bone morphogenetic proteins (BMPs), as

inhibiting BMPs with noggin in amputated digits results in their failure to regenerate (Han et al., 2003). Other signals are regulated by digital nerves, which play a crucial role in creating a proliferative environment. Denervation of the digit tip reduces blastema proliferation, suggesting that neurotrophic factors released by paracrine signaling contribute to digit regeneration (Mohammad and Neufeld, 2000; Rinkevich et al., 2014). Surrounding tissues such as the nail bed also regulate the secretion of FGFs from digital nerves through Wnt signaling (Takeo et al., 2013). Recent work also suggests that these neurotrophic factors are not dependent on axonal innervation, but secreted by nerve-associated Schwann cells precursors that dedifferentiate and secrete growth factors such as oncostatin M (OSM) or platelet-derived growth factor AA upon amputation (PDGF-AA) (Johnston et al., 2016). The rapid proliferation of blastema cells may be facilitated by the maintenance of their undifferentiated state. A genetic marker of blastema cells is the transcription factor *Msx1*, which is a transcriptional repressor necessary for digit regeneration (Han et al., 2003; Lehoczky et al., 2011). *Msx1* is also expressed in the clot that forms over the wound site, and removal of the clot attenuates regeneration, suggesting that the clot may play a functional role in regeneration beyond wound healing (Lehoczky et al., 2011).

The avascular nature of the blastema is also important in maintaining its regenerative ability. Regenerating mouse digits were found to have lower numbers of endothelial cells compared to non-regenerative amputations, which suggests the reduced presence of vasculature (Said et al., 2004). Blastema expression of the anti-angiogenic gene *Pedf* correlated with successful regeneration (Yu et al., 2010), while BMP9, which induces the expression of the angiogenic factor *Vegfa*, inhibited regeneration (Yu et al., 2014). *Pedf* expression was also present in regenerative digit amputation wounds, while it was absent in proximal non-regenerative amputations, confirming that vasculature formation is inhibited in regenerative responses (Muneoka et al., 2008; Yu et al., 2010). The delayed formation of vasculature in regenerative responses contrasts with the rapid angiogenic response of non-regenerative wound healing, where the granulation tissue that eventually becomes scar tissue is highly vascularized (Kawasumi et al., 2013). Additionally, granulation tissue expresses high levels of *Vegfa* (Semenza, 2010), supporting the

hypothesis that *Pedf* expression during the early stages of wound healing and blastema formation suppresses *Vegfa* to maintain a regeneration-permissive wound environment.

The blastema's lack of vasculature may be permissive to regeneration by forming a hypoxic microenvironment. Reactive oxygen species have been demonstrated to be essential to regeneration in other systems such as *Xenopus* and zebrafish (Love et al., 2013; Gauron et al., 2013), and the changing vascular profile of regenerating mouse digits are likewise suggestive of a fluctuating oxygen microenvironment. Studying the oxygen profile of regenerating mouse digits showed that the hypoxic areas of the amputation site significantly increase during blastema formation, and that oxygen profiles vary temporally over the course of regeneration (Sammarco et al., 2014). Spatially, hypoxic cells are first restricted to the bone marrow of the amputated digit, before becoming associated with the forming blastema itself, and prematurely ending those hypoxic phases attenuates the regenerative response (Sammarco et al., 2014). It is hypothesized that bone marrow specific hypoxia may activate and encourage the proliferation and delay differentiation of stem cells such as osteoblast progenitors (Tuncay et al., 1994; Zahm et al., 2008), which are known to contribute to the regenerated digit (Lehoczky et al., 2011). Furthermore, hypoxia-inducible factor (HIF-1), which is the primary mediator of cell survival under hypoxia, upregulates the blastema markers *Sdf-1* and *Cxcr4* under hypoxic conditions (Ceradini et al., 2004; Staller et al., 2003). It is therefore likely that the hypoxic microenvironment of the regenerating digit is essential to activate *Sdf-1/Cxcr4* signaling, which in turn increases blastema cell recruitment and retention at the wound site.

Oxygen dynamics may also play a role in shifting regeneration from the blastema formation stage to the re-differentiation stage. The following phase is marked by a shift in oxygen dynamics, as a release from hypoxia is necessary for re-differentiation (Sammarco et al., 2014). Physiologically, certain oxygen thresholds need to be met for osteoblasts to secrete collagen that forms the mineralized bone matrix (Ramaley and Rosenbloom, 1971), which suggests that oxygen might act as a primary cue for other molecular signals needed for re-differentiation.

The differentiation phase of digit regeneration does not occur in the same manner as digit development: instead, it occurs through a different mechanism that allows for more rapid osteogenesis. In development, the digit bone, as with most long bones, forms through a process of endochondral ossification, with a chondrogenic scaffold later replaced by bone (Han et al., 2008). However, unlike most long bones, the P3 bone possesses only a single growth plate at its proximal end, instead of at both proximal and distal end (Dixey, 1881). Unique to the P3 bone, the length of the phalanx is also increased by an additional ossification center located in the distal end of the digit bone, with up to 55% of postnatal elongation achieved through this process of intramembranous ossification (Han et al., 2008).

Similarly, in regenerated digits, bone forms through direct intramembranous ossification as re-differentiating blastema cells do not form chondrogenic cells during regeneration (Sensiate and Marquez-Souza, 2019). New bone is simply built directly onto the digit stump. This process creates a regenerated digit that is histologically distinct from the original bone. In the original digit bone, collagen fibers are arranged in parallel with one another, while regenerated bone fibers are "crosshatched," producing a structure often termed woven bone (Simkin et al., 2015b). Regenerated bone often has numerous trabecular spaces, resulting in a more porous and likely weaker structure, but tends to be up to 50% larger in volume than the original digit (Fernando et al., 2011). While the bone increases in density over time, it has been hypothesized that mouse digit regeneration thus represents an evolutionary outcome that traded anatomical fidelity for a relatively rapid and functional regenerative structure (Simkin et al., 2015a).

However, despite the differing architectures of original and regenerated digit, the distinct shape of the P3 bone allows us to conclude that P3-specific patterning does occur during regeneration. Most obviously, the tapered shape of the digit tip is recapitulated in the regenerate (Fernando et al., 2011), as well as the dorsal curvature of the nail (Rinkevich et al., 2011). Patterning genes involved in embryonic limb patterning are also re-expressed in digit regeneration, such as *engrailed-1*, which patterns the dorsoventral axis of developing limbs (Rinkevich et al., 2011).

Positionally-determined regeneration ability

The proximity of regeneration-competent and regeneration-incompetent zones in the mouse digit also makes it an ideal model to study regeneration failure. If the digit can form a blastema at one level of amputation, why does it fail to do so at another? It is hypothesized that proximal amputations of P3 remove crucial signaling centers and sources of progenitor cells, namely the nail epithelium and the periosteum (Sensiate and Marquez-Souza, 2019). The nail organ appears to have a determinate role in digit regeneration, as *Wnt* signaling from the nail bed to surrounding mesenchymal cells is both necessary and sufficient to encourage distal growth of the phalanx (Takeo et al., 2013; Lehoczky et al., 2015). In parallel, the periosteum is a mesenchymal tissue that is a source of bone progenitor cells generally (Murao et al., 2013), and is known to drive antler regeneration in deer (Li et al., 2014). As bone progenitor cells are the primary actors in digit regeneration (Lehoczky et al., 2011), it has been hypothesized that the periosteum acts as a source of progenitor cells while the nail bed acts as a source of osteogenic signaling (Sensiate and Marquez-Souza, 2019). Proximal amputations that remove over 50% of P3 eliminate both the nail bed and the periosteum, thus removing key regeneration-responsive elements.

Another potential contributing factor to the positionally-dependent regenerative response is the role of connective tissue fibroblasts. Fibroblasts regulate wound healing and regeneration in diverse contexts, such as amphibian limb regeneration (Gerber et al., 2018), mammalian wound healing (Bainbridge, 2013) and zebrafish and neonatal mouse heart regeneration (Hu et al., 2022; Wang et al., 2020). They also maintain positional memory in the human body (Chang et al., 2002). Manipulating signaling pathways that affect positional information in regenerative systems have led to ectopic expansions of proximal domains, increased blastema size and lengthened or duplicated anatomical structures (Kujawski et al., 2014; Thoms and Stocum, 1984, Blums and Begemann, 2015; Wang et al., 2019). Thus, positionally dependent differences within certain cell types may contribute to their differing ability to mount a regenerative response.

Positionally-specific cellular properties

At a cellular level, fibroblasts derived from the connective tissue of P3 and P2 phalanges both retained the ability to form the blastema when injected into regenerating digits, but P3 cells displayed much higher proliferation and greater responsiveness and ability to integrate into the regenerate (Wu et al., 2013). In *in vitro* cultures, stromal cells isolated from P3 and P2 displayed differences in their proliferative rates, as P3 cells were much more highly proliferative and P2 cells expressed cell cycle inhibitors at higher levels (Lynch and Ahsan, 2013). The cytoskeletal properties of P2 cells differ from those in P3, as microfilament and microtubule-related genes were expressed at higher levels, highlighting the potential role of modifying cell migration and cytoskeletal properties in regeneration (Lynch and Ahsan, 2013). P2 and P3 cells also interact differently with other cell types in the wound microenvironment—co-culture with adult and neonatal fibroblasts increased the proliferation and migration of P2 cells, but only neonatal fibroblasts increased the migratory behavior of P3 cells (Lynch and Ahsan, 2014). *In vivo* studies showed that transplanted P3 cells respond and migrate toward the wound site while this honing behavior is not observed in transplanted P2 or cancer cell line cells (Wu et al., 2013). The differential responsiveness of P2 and P3 cells was further supported *in vitro* by showing that the differential migratory behavior of P2 and P3 cells could be recapitulated by exposing the cells to BMP2—a signaling molecule expressed in the marrow of the amputated stump (Lynch and Ahsan, 2014). Thus, positionally separated cells show intrinsic differences in their varying responses to other cells and secreted factors encountered in the wound microenvironment.

Induced digit regeneration

The mouse digit has also been used to characterize induced regeneration, in which key morphogenetic agents were used to induce regeneration from a typically non-regenerative amputation. Both proximal amputations through P3 (removing 60% or more of the phalanx) and proximal amputations through P2 are used to investigate the possibility of inducing regenerative responses in contexts where they would typically fail.

Similar approaches have also been carried out in other non-regenerative vertebrate contexts, demonstrating the potential viability of modulating an injury response to generate regenerative outcomes. In experiments with adult mice, rats, and pigs, modulating the wound response to full thickness skin wounds to mimic that of fetal skin led to scar-free skin regrowth in adults, an ability that is usually only observed in fetal skin (Ferguson and O'Kane, 2004). In embryonic chicks, amputated limb buds, which do not typically regenerate, could be coaxed to do so by locally stimulating mesodermal cells using FGFs (Taylor et al., 1996; Kostakopoulou et al., 1996). These successful attempts to induce regeneration in multiple organs across different species and life stages have thus motivated the idea that tissues or organs could possess dormant regenerative potential—and identifying the agents that unlock it could be key to understanding how to systematically expand regenerative ability.

Proximal P3 system

Proximal amputation through P3 (removing 60% or more of the terminal phalanx) is an obvious means to study positionally dependent regenerative responses. Recent work differentiates the proximal P3 amputation from P2 amputation due to the observation of blastema-like mesenchymal tissue located in between the amputation site and regenerating dermis (Sensiate and Marquez-Souza, 2019). This is further supported by recent work showing that proximal P3 amputation results in a highly attenuated regenerative response, including blastema formation and partial bone regrowth, rather than complete failure as previously characterized (Dawson et al., 2020). Most models of scar-free healing display a limited fibrotic response (Seifert et al., 2012a; Seifert et al., 2012b), and no fibrosis is observed in distal P3 regeneration. P3 proximal amputation results in the formation of transient fibrosis, followed by a regenerative response limited to the marrow cavity, while the little remaining periosteum remains inert (Dawson et al., 2020).

P2 system

Non-regenerative, proximal amputation through the P2 digit is also a common system to investigate regenerative failure. The standard outcome of a non-regenerative amputation is

a truncated skeletal element, and the formation of fibrotic scar tissue over the wound site (Dawson et al., 2016). The P2 bone stump appears inert at first glance, as it is visually similar before and after the injury response, but this seeming similarity obfuscates a dynamic and elaborate tissue repair process that is reminiscent of an initiated, but failed, attempt at regeneration (Dawson et al., 2016). The P2 amputation response resembles skeletal repair after fracture, as an initial inflammatory response is followed by periosteum-derived chondrocyte formation of a cartilage callus, which is then vascularized and organized first into a woven bony callus and then reorganized into lamellar bone (Dolan et al., 2018). The bone also undergoes histolysis and is truncated, and fibrotic scarring also occurs on the soft tissue around the wound site.

Even though cells possess position specific qualities, this limited regenerative response shows that there are indeed regeneration-competent cells located in digit amputations that fail to completely regenerate. Thus, much work in inducing and expanding regenerative capacity focuses on modulating the wound site to create a more supportive environment for regeneration. In neonatal mice, treating proximal P3 amputations with exogenous BMP7 induced regeneration by potentially reactivating the embryonic program active during development (Yu et al., 2010). Instead of intramembranous ossification, blastema formation was followed by endochondral ossification, with endochondral marker genes expressed in a similar pattern as in development along the proximal-distal axis (Yu et al., 2010).

A similar response to exogenous BMP2 was also observed when neonatal digits were amputated at the P2 level, as well as in the long bones of amputated adult hindlimbs (Yu et al., 2012). By forming a new endochondral ossification at the distal end of the skeletal element, amputated elements could regenerate distally patterned structures, but were restricted to regrowing specific segments—i.e., no joints or further distal structures were regenerated (Yu et al., 2012). However, the induced regeneration of bone and joint could be achieved through the sequential treatment of P2 amputations with exogenous BMP2 and then BMP9 (Yu et al., 2019). It was hypothesized that BMP9 treatment reactivated the developmental program for joint formation, allowing amputated digits to form a synovial cavity and an articular cartilage lined structure that articulates with the bone stump (Yu et

al., 2019). The ability of transient treatments with growth factors being able to stimulate regenerative responses highlights the potential of extrinsically manipulating typically non-regenerative injuries to engineer a regenerative response.

Mouse heart regeneration

Another organ with limited regenerative ability in the mouse is the heart. Instead of being spatially limited as in the case of the digit tip, cardiac regeneration in the mouse is temporally limited. Neonatal mice possess cardiac regenerative ability up till the first week of postnatal life, after which this transient ability rapidly diminishes (Porello et al., 2011). In contrast, the adult mammalian heart was long considered a post-mitotic organ, as cardiomyocytes were thought to be terminally differentiated upon maturity (Poolman and Brooks, 1998). However, radiocarbon tracing showed that cardiomyocytes do undergo renewal, albeit at a low rate of about 1%, decreasing throughout adult life in humans (Bergmann et al., 2009). However, this rate of renewal will not successfully replace the significant loss of cardiomyocytes lost to cardiac injury such as myocardial infarction, which can compromise around 25% of cells in the heart (Murry et al., 2006). When myocardial infarction occurs in non-regenerative hearts, the site of injury is typically replaced with a fibrotic scar that affects tensile properties of the heart and decreases cardiac function, potentially leading to heart failure (Leask, 2010).

Upon cardiac injury affecting approximately 15% of the heart, neonatal mice can restore myocardial damage in around the next month with little evidence of fibrosis and the restoration of cardiomyocytes and cardiac contractile function (Porello et al., 2011; Porello et al., 2013). Lineage tracing of cells in the mammalian heart have shown that regeneration occurs through the proliferation of existing cardiomyocytes (Porrello et al., 2011; Zhu et al., 2018). The regenerative response is marked by cardiomyocyte cytokinesis and sarcomere disassembly, as well as a rapid revascularization response that perfuses the injured tissue (Porello et al., 2012). It is thus commonly thought that the failure to regenerate in older life stages is due to the limited potential of mature cardiomyocytes to proliferate and renew themselves (Cardoso et al., 2020).

Physiological and molecular factors involved in heart regeneration

This limitation is sometimes attributed to an intrinsic property of the cardiomyocytes themselves, as regeneration loss coincides with the time point when cardiomyocytes undergo cell cycle arrest and binucleation at postnatal day 7 (Soonpaa et al., 1996). Regenerative species, such as zebrafish, also possess mononucleated, diploid cardiomyocytes (Kikuchi, 2015). Ploidy appears to correlate with cardiomyocyte proliferation and functional recovery after injury (Hirose et al., 2019) and experimental polyploidization of zebrafish cardiomyocytes was sufficient to inhibit regeneration (González-Rosa et al., 2018). Comparative studies between closely related frog species *Xenopus tropicalis* and *Xenopus laevis* also reveal that while adult *X. tropicalis* can completely regenerate myocardium after 10% cardiac apical resection (Liao et al., 2017), *X. laevis* adults only mount a partial regenerative response to equivalent amounts of damage (Marshall et al., 2017). Notably, *X. tropicalis* has a diploid genome with mononucleated cardiomyocytes, while *X. laevis* has a psuedotetraploid genome with majority tetraploid cardiomyocytes (Marshall et al., 2018).

However, studies in neonatal pig regeneration seem to suggest that other factors may be involved. Similarly to mice, pigs also have a short postnatal window during which they are able to regenerate cardiac tissue from pre-existing cardiomyocytes (Zhu et al., 2018). While pig cardiomyocytes also eventually become multinucleated and withdraw from the cell cycle, the majority of them do so after the neonatal period (Adler et al., 1996). Hence it is clear that although retaining cardiomyocyte proliferative ability is a permissive condition for regeneration, it is not the only factor determining regenerative outcome.

Beyond cardiomyocyte-intrinsic factors, other physiological responses are known to influence cardiac regenerative ability. The overall physiology of the heart undergoes a number of changes shortly after birth: a metabolic shift likely in response to changing concentrations of oxygen, and a mechanical shift, as the composition of cardiac extracellular matrix (ECM) changes rapidly in postnatal life. Different molecular regulators, such as the YAP and Hippo signaling pathways, specific transcription factors such as *Gata4* or *Meis*1, or microRNAs, have also been implicated as key regulators of

cardiomyocyte physiology. Furthermore, while 90% of myocardium is composed of cardiomyocytes, cardiomyocytes only represent about 30% of the total cells in the heart (Pinto et al., 2016). Other types of cells, aside from cardiomyocytes, immune cells, fibroblasts, and neural cells—may also contribute to the changing physiology of the postnatal heart.

Hippo/YAP signaling

A pertinent physiological factor in cardiac maturation is the switch between primary modes of growth in the fetal and adult mammalian heart. While the main mode of growth during fetal development is cardiomyocyte proliferation, growth after birth is mostly achieved through increase in cardiomyocyte size, which is known as cardiac hypertrophy (Maillet et al., 2013). One pathway of interest for this transition is the Hippo/YAP signaling pathway, which plays a conserved role in organ size control, stem cell fate and differentiation, as well as the regulation of cellular proliferation and apoptosis (Pan, 2010). YAP (Yes-associated protein) mediates Hippo signaling as a transcriptional co-factor—when Hippo signaling is active, YAP is phosphorylated and inactivated, leading to cell apoptosis, while Hippo inactivity leads to YAP activation and translocation into the nucleus where it binds to transcription factors that promote cell survival and proliferation (Ikeda and Sadoshima, 2016). In cardiac development, Hippo limits cardiomyocyte proliferation through inhibiting YAP, and *Yap* overexpression during development increases cardiomyocyte proliferation and results in hyperplasia of ventricular walls (Heallen et al., 2011). Likewise, conditional ablation of *Yap* in cardiac cells leads to hypoplasia through reduced cardiomyocyte proliferation (von Gise et al., 2012). In both cases, cardiomyocyte size was unchanged, meaning that Hippo/YAP specifically affects cardiac growth in development through cardiomyocyte proliferation.

As the loss of cardiomyocyte proliferative ability is thought to be the primary reason behind regenerative failure, investigating Hippo/YAP activity in heart regeneration is of much interest. Hippo is generally thought to suppress mitosis in the adult mammalian heart, as the amount of YAP protein detected in the heart declines with age (von Gise et al., 2012). *Yap* has been shown to be necessary for neonatal mouse regeneration. Cardiomyocyte-

specific knockout of *Yap* led to extensive fibrosis and a lack of healthy myocardium in P2 mice post-infarction (Xin et al., 2013). Endogenous activation of *Yap* through cardiomyocyte-specific knockouts of Hippo effectors *Lats1/2* and *Sav1* conversely was cardioprotective in adult mouse models of infarction and was even able to induce mitosis in adult myocardium (Heallen et al., 2013). As cardiomyocytes expressing *Yap* were observed at the border of infarcted cardiac tissue, it is hypothesized that *Yap* prevents cardiomyocyte apoptosis and promotes proliferation in the border zone, which encourages myocardial survival and prevents further infarction (Xin et al., 2013; Del Re et al., 2013).

The Hippo/YAP pathway is notable for its crosstalk with numerous other physiological factors known to play a role in heart regeneration. One of the closest associations is its interactions with cardiac ECM and regulation through mechanical stress. Changes in tissue stiffness and cytoskeletal rearrangement are known to mediate YAP activation (Halder et al., 2012; Aragona et al., 2013; Vite et al., 2018). Direct interactions between the ECM and the Hippo/YAP pathway have also been established through the dystrophin-glycoprotein complex (DGC), a transmembrane complex that links the actin cytoskeleton to the ECM. Agrin (an ECM protein known to promote cardiomyocyte proliferation) and YAP both interact directly with DGC, with agrin binding resulting in YAP dissociating from the complex and being released into the nucleus, where its transcriptional activity promotes cardiomyocyte proliferation (Bassat et al., 2017). Other work indicates that Hippo signaling is required for YAP binding to the DGC, as phosphorylated, inactive YAP interacts directly with components of the DGC (Morikawa et al., 2017).

Inflammatory and oxidative stress related signaling have also been associated with mediating Hippo/YAP activity. YAP appears to play an immunosuppressive, anti-inflammatory role after infarction, as deletion of YAP from cardiac epicardial cells led to lower levels of anti-inflammatory cytokines, prolonged inflammation, and drastically increased fibrosis—all of which contributed to high incidence of cardiomyopathy and poorer outcomes post-infarction (Ramjee et al., 2017). The Hippo effector *Mst1* is also known to be activated by oxidative stress (Odashima et al., 2007), which is greatly increased in post-infarction hearts (Murphy and Steenbergen, 2008). Inhibiting *Mst1* in mice reduced fibrosis and apoptosis post-injury (Odashima et al., 2007), but did not

stimulate increased cardiomyocyte proliferation, unlike the inhibition of other Hippo effectors (Yamamoto et al., 2003). This may be due to compensatory effects from *Mst2*, or *Mst1* acting to promote cell survival through alternate means, such as the prevention of autophagy (Wang et al., 2018). *Mst1* inhibition is thought to mediate cell survival through the YAP-FOXO1 complex, which is inhibited by MST1 and induces the expression of antioxidant genes (Lehtinen et al., 2006; Shao et al., 2014). The Hippo/YAP pathway has an abundance of complex interactions with the physiology and signaling that varies across hearts of different ages and conditions and is a promising avenue for further investigation.

Cardiac metabolism and oxidative stress

A noticeable physiological transition within the first week of postnatal life is metabolic: embryonic hearts typically use glycolysis to generate energy (Lopaschuk et al., 1992), while adult cardiomyocytes use mitochondrially-dependent oxidative phosphorylation (Wisnecki et al., 1985). The postnatal cell-cycle arrest of mouse cardiomyocytes was found to be partly mediated by this metabolic switch, with the shift to oxidative phosphorylation resulting in increased reactive oxygen species (ROS) production (Puente et al., 2014). ROS signaling is thought to be the cue to promote immune cell recruitment into injured tissue (Niethammer et al., 2009), as necrotic cells and neutrophils secrete ROS and activate a local inflammatory response by cardiac fibroblasts and mast cells, leading to the secretion of pro-inflammatory cytokines (Kawaguchi et al., 2011). Excessive ROS production results in chronic inflammation and increased myocardial injury (Muntean et al., 2016). Increased ROS generation may account for the loss of mouse heart regeneration, as ROS in postnatal day 7 mouse hearts was shown to cause DNA damage and cell cycle arrest in cardiomyocytes (Puente et al., 2014). Conversely, exposure to chronic hypoxia post-infarction induced heart regeneration in adult mice (Kimura et al., 1985; Nakada et al., 2017), supporting the hypothesis that reducing oxidative damage from ROS could facilitate cardiomyocyte renewal., However, while their presence in excessively high levels impairs regeneration, ROS signaling appears to have pleiotropic effects in cardiac injury—low levels of ROS are needed for cardioprotective redox signaling (Muntean et al., 2016), while hydrogen peroxide (a potent ROS) in zebrafish has shown to promote leukocyte recruitment and cardiomyocyte proliferation (Yan et al., 2014; Niethammer et al., 2009).

Understanding how redox signaling is differentially modulated in regenerative and non-regenerative contexts might therefore give us better insight into the variable physiological effects of ROS.

Additionally, the specific metabolites used in cardiac energetic metabolism also appear to affect cardiomyocyte proliferation. The shift to oxidative phosphorylation in the neonatal-adult also results in a shift in primary energetic substrate from pyruvate in neonates to fatty acids in adults (Lopaschuk et al., 1992). Inhibiting fatty acid metabolism has been shown to decrease DNA damage and promote cardiomyocyte proliferation, demonstrating that the changing metabolic profile of the heart impacts its proliferative potential (Cardoso et al., 2020b). Conversely, inhibiting glycolytic enzymes resulted in increased cell death and fibrosis after cardiac injury in mice and decreased heart regeneration due to low cardiomyocyte proliferation in zebrafish (Wu et al., 2011; Fukuda et al., 2020).

Though the adult mammalian heart typically uses fatty acids as an energy source, it shifts to anaerobic glycolysis during pathological conditions such as ischemia or hypertrophy (Tuomainen and Tavi, 2017). This shift is thought to be cardioprotective, which is supported by the naturally-occurring metabolic reprogramming observed in zebrafish—cardiomyocytes at the border of the injury site upregulated glycolytic genes, while down-regulating genes involved in mitochondrial oxidative phosphorylation (Honkoop et al., 2019). It follows that stimulating glycolysis and glucose metabolism may promote heart regeneration as it returns the heart to a neonatal-like state. The expression of glycolytic enzymes changes over development—pyruvate dehydrogenase kinases (PDK), for example, increase during mammalian heart development, and PDK4 is the most upregulated enzyme in P7 mice hearts, coinciding with the loss of regenerative ability (Sugden et al., 2000). PDKs inhibit pyruvate dehydrogenase (PDH), which is a limiting step of glycolysis, and inhibiting PDKs in turn results in PDH activation (Bae et al., 2021). PDK inhibition or cardiomyocyte-specific deletion has shown to promote cardiomyocyte proliferation and heart regeneration in adult mice (Piao et al., 2017; Cardoso et al., 2020b). Similarly, cardiomyocyte-specific overexpression of rate-limiting glycolytic enzyme pyruvate kinases muscle isoenzyme 2 (PKM2 encouraged cardiomyocyte proliferation and heart regeneration in adult mice (Mangadum et al., 2020).

Neural signaling

The mammalian heart is physiologically regulated by sympathetic and parasympathetic nerves, with cardiac innervation playing a role in heart rate and contraction. Nerves play a role in many regenerative processes, and often secrete pro-regenerative growth factors and mitogens (Kumar and Brockes, 2012). Ablation of either sympathetic or parasympathetic nerves impairs cardiac regeneration in neonatal— chemical ablation of sub-epicardial sympathetic nerves resulted in fibrotic scarring and regenerative failure (White et al., 2015), while vagotomy of the parasympathetic nerve impaired cardiomyocyte proliferation. Vagatomy decreased the level of cell cycle regulators such as *Cdk4* and *Ccnd2*, as well as growth factors *Nrg1* and *Ngf,* with cardiac regeneration partially rescued by exogenously administering NGF and neuregulin1 proteins (Mahmoud et al., 2015).

Extracellular matrix components and properties

The cardiac extracellular matrix (ECM) changes significantly over the course of postnatal maturation and is known to interact with cells directly or contain signaling molecules that regulate migration or pro-regenerative growth factors (Hynes, 2009). The ability of the ECM to mediate the transition from fibrosis to scar resolution may play a significant role in determining regenerative outcome. In adult mammalian hearts, the deposition of fibrous collagenous matrix is permanent, and the myocardium remains fibrotic after injury. However, in regenerative models such as zebrafish and neonatal mice, the scar formation is transient, and is eventually replaced by cardiomyocytes. Scar formation is not itself detrimental to regeneration, as inhibiting scar formation attenuated the regenerative response in zebrafish, decreasing cardiomyocyte proliferation and resulting in an abnormally remodeled ventricular chamber (Chablais and Jazwinska, 2012). This highlights that transient scarring is not inherently inhibitory to regeneration, and that it is specifically the capability to resolve scarring that is crucial to a successful outcome.

The ability to mediate this transition may be due to differing ECM components. Unlike adult ECM, neonatal ECM components have been shown to modulate cardiomyocyte proliferation by stimulating cell cycle reentry in differentiated cardiomyocytes. Levels of

agrin, an extracellular proteoglycan, decrease postnatally in mice coincident with the loss of regeneration. Agrin was shown to be necessary for neonatal mouse heart regeneration and administering exogenous agrin in adult mice resulted in cardiomyocyte cell cycle re-entry (Bassat et al., 2017). In pigs, local delivery of exogenous recombinant agrin into the infarcted heart also encouraged cell cycle reentry and resulted in improved heart function and decreased infarct size (Baehr et al., 2020). Agrin is thought to activate cell cycle re-entry by promoting sarcomere disassembly and activation of pro-regenerative signaling molecules such as YAP and ERK (Bassat et al., 2017).

In typically regenerative organisms such as zebrafish, specific ECM components mediate different aspects of the regenerative process. Many of these processes are regulated by TGF-β/Activin signaling, as it affects the synthesis of different ECM components with properties crucial to various stages of healing and regeneration. Inhibiting TGF-β/Activin resulted in decreased collagenous scar deposition, as well as decreased fibronectin, an adhesive matrix protein that assists in recruiting and integrating cardiomyocytes in damaged tissue (Chablais and Jazwinska, 2012; Wang et al., 2013). The expression of Tenascin C, an anti-adhesive protein typically expressed at the border of the infarct zone and myocardium (Imanaka-Yoshida et al., 2004) is also completely abolished, which impaired cardiomyocyte ability to migrate into the infarcted area and subsequent tissue remodeling (Chablais and Jazwinska, 2012). The active regulation ECM composition during specific stages of the injury response might therefore play a significant role in ensuring that a regenerative response supersedes a fibrotic one.

The mechanical properties of ECM, such as its stiffness or flexibility also exert influence over cellular behavior such as migration, adhesion, and proliferation during injury, thereby influencing the success of a regenerative response. From embryonic to postnatal life, cardiac ECM displays a marked and rapid change in both composition and mechanical properties. In neonatal rats, the elastic modulus of ventricular tissue immediately increased upon birth, demonstrating that the transition from a gestational environment has a distinct effect on heart maturation (Jacot et al., 2010). Cardiac stiffness, from the decreased elasticity of the myocardium and cardiac vasculature is associated with aging and the onset of cardiac disease, such as dilated cardiomyopathy and diastolic heart failure (Villalobos

Lizardi et al., 2022; Singam et al., 2019). *In vitro*, substrate stiffness appears to influence mouse cardiomyocyte maturation. Cardiomyocytes grown on stiffer substrates were post-mitotic and binucleated, while those grown on softer substrate began to lose mature contractile gene expression and de-differentiate and continued replicating (Yahalom-Ronen et al., 2015). In neonatal mice, ECM stiffness noticeably increases even within the first two days of postnatal life, and decreasing the stiffness by inhibiting the cross-linking enzyme LOX resulted in an improved regenerative response by postnatal day 3 (P3) mice as far less fibrotic deposition was observed three weeks after apical resection (Notari et al., 2018).

Immune response

The immune response to cardiac injury is highly spatially and temporally regulated. How and when the immune response is activated can result in beneficial or detrimental impacts on heart regeneration. Broadly, the immune response post myocardial infarction can be divided into an inflammatory state triggered by necrotic cells, in which immune cells are recruited to the injury site, followed by an inflammation resolution phase in which reparative cellular responses suppress inflammatory signals and begin to repair tissue (Lai et al., 2019). It is well established in regenerative models that inflammation is essential to cardiac regeneration— suppressing the early immune response by depleting resident macrophages in both zebrafish and neonatal mice resulted in impaired regeneration (Huang et al., 2013; Aurora et al., 2014). Triggering an acute inflammatory response also seems to increase regenerative efficacy, acting as a stimulus for regenerative responses in neonatal mice and zebrafish (Han et al., 2015; de Preux Charles et al., 2016).

However, overactive or chronic inflammation in injury typically leads to increased tissue damage and dysfunction. Prolonged inflammation has been linked to increased apoptosis of cardiomyocytes, fibrogenic signaling in non-infarcted myocardial tissue, and the activation of proteases that further degrade cardiac ECM (Chen and Frangiogiannis, 2012; Frangogiannis and Entman, 2005; Huang and Frangogiannis, 2018). Failure to resolve or contain inflammation has been shown to directly result in adverse cardiac remodeling in mice (Dobaczewski et al., 2010; Cochain et al., 2012), and persistently elevated

inflammatory markers in human patients after an acute coronary syndrome was associated with higher mortality (de Lemos et al., 2007). Thus, the poorer prognosis of these patients might be due to increased injury and/or the inability to activate anti-inflammatory pathways to resolve the deleterious effects of prolonged inflammation (Frangogiannis, 2007).

The heterogeneous effects of the immune response on regeneration are likely due to spatiotemporal and phenotypic differences which may be illustrated more clearly at a cellular level. Neutrophils, for example, are rapidly recruited to injured tissue and make up the majority of the immune cells present in the first few days post-injury in both mice and zebrafish (Yan et al., 2013; Lai et al., 2017). Neutrophil activity is associated with inflammation, as they encourage monocyte recruitment and secrete inflammatory cytokines (Soehnlein and Lindbom, 2010). Prolonged neutrophil retention is generally associated with poorer outcomes after cardiac injury (Mocatta et al., 2007) and impairs regeneration, as it led to excessive fibrosis and unresolved scarring in zebrafish (Lai et al., 2017). However, it would be inaccurate to think of them as purely pro-inflammatory and detrimental. Neutrophils secrete myeloperoxidase, which neutralizes the ROS hydrogen peroxide released after injury (Pase et al., 2012), and facilitate the entry into the reparative phase by shifting macrophages to an M2-like anti-inflammatory phenotype (Horckmans et al., 2017). Blunting the acute inflammatory response might thus have unintended negative downstream consequences, with early attempts at broad inhibition of inflammation post-injury leading to adverse effects (Hartman et al., 2018; Huang and Frangiogiannis, 2018; D'Amario et al., 2021).

The multifaceted and highly interconnected nature of the immune response is perhaps best illustrated by the role of macrophage polarization in heart regeneration. It was observed that distinct subpopulations of macrophages populate the infarct zone of adult mouse hearts over time, with pro-inflammatory M1 macrophages gradually giving way to anti-inflammatory M2 macrophages (Nahrendorf et al., 2007; Yan et al., 2013). Further depletion of M2-like macrophages also led to decreased repair and poorer outcomes post-injury (Shiraishi et al., 2016). However, these dynamics differ between adult and neonatal mice. After injury, the neonatal heart is dominated by M2-like resident macrophages, with the early response coordinated by increasing the numbers of these resident macrophages,

instead of recruiting pro-inflammatory monocyte-derived macrophages to infiltrate the wound site like in the adult heart (Lavine et al., 2014). It is unlikely, however, that the M1/M2 paradigm captures the full complexity of macrophage function during heart regeneration as the heterogeneity of immune cell phenotypes and kinetics has only become apparent relatively recently (Martinez and Gordon, 2014). Recent transcriptomic studies of macrophage subpopulations in the adult and neonatal heart show that macrophages possess distinct transcriptional signatures depending on age, and that differences in transcriptionally-regulated macrophage functions might contribute to their differing ability to facilitate regeneration (Simóes et al., 2020).

Elucidating the full dynamics of the immune response during regeneration—including both the kinetics and subpopulations of cells involved will likely be crucial in understanding how it facilitates regeneration. In a comparative study between zebrafish and the non-regenerative medaka, the medaka heart displayed both delayed recruitment of macrophages and neutrophils to the injury site, as well as delayed clearance of neutrophils compared to zebrafish (Lai et al., 2017). Changing the dynamics of this response by administering the inflammatory agonist poly I:C by accelerating immune cell recruitment and subsequent clearance led to an improved regenerative response in medaka hearts, including more rapid revascularization, scar resolution and improved cardiomyocyte proliferation (Lai et al., 2017). This study demonstrates how interventions that affect the timing and duration of specific phases of the immune response might be key to modulating the immune response to produce a pro-regenerative outcome.

Strategies to induce heart regeneration

In addition to understanding how different physiological factors affect regenerative outcomes in the heart, there has also been much research into targeted strategies and therapeutics to improve mammalian heart regeneration. In clinical settings, heart failure after myocardial infarction is often due to a combination of post-injury complications: myocardium is replaced by mechanically inferior fibrotic scarring, with exacerbated hypertension and valvular diseases often following the infarction (Velagaleti et al., 2020). Currently, in the case of severe infarction, whole heart transplants remain the only viable

strategy to regain heart function. However, the long waitlist of patients due to insufficient donors and numerous post-transplant complications is obviously limiting (Benjamin et al., 2019). Therefore, using our understanding of basic cardiac physiology to target and promote cardioprotective and regenerative processes, while attenuating damaging ones remains both biologically and clinically relevant to current research.

Strategies to induce heart regeneration have typically involved cellular transplants to generate new myocardium, using exogenous bioactive factors to encourage existing cardiac tissue to become more regenerative, or modifying the structural microenvironment of the heart to promote regeneration. The myriad interactions between cardiac cell types and the increasing appreciation for the role of their microenvironment likely means that successful interventions will make use of a combination of these strategies, or require the coordination of different interventions at different timepoints in the recovery process.

ESCs and iPSCs

Cell-replacement therapies often exogenously introduce new cardiomyocytes generated from either embryonic stem cells (ESCs) or somatic cells via induced pluripotent stem cells (iPSCs) (Shiba et al., 2016). While iPSCs and ESCs were injected directly into the myocardium in earlier attempts, they are now often re-differentiated into CMs before being introduced into the heart, as the pluripotency of iPSCs led to the risk of teratoma formation (Nussbaum et al., 2007). iPSC and ESC-derived CMs have shown beneficial effects in improving cardiac function in mammalian models (Kawamura et al., 2012; Chong et al., 2014). However, cell transplantation with ESCs or iPSCs often have low rates of survival, with only around 10% of cells at best surviving 24 hours after transplantation (Guo et al., 2017).

To improve survival and retention, much research has focused on integrating and recreating cardiac cell-cell and cell-ECM interactions. For example, mouse ESC-CMs cultured in fibroblast medium obtained from neonatal hearts displayed improved contractile ability compared to those cultured in adult fibroblast media (Liau et al., 2017). Furthermore, given the role of cardiac ECM in affecting cellular physiology, migration and organization, much

research has investigated scaffold-based approaches to recreate a supportive microenvironment. Hydrogel scaffolds have been shown to increase cell retention as well as induce maturation and integration with host cardiac tissue (Ban et al., 2014), while cellular transplants that utilized three-dimensional cardiac fibroblast ECM as a transfer media were better retained in ischemic cardiac tissue (Schmuck et al., 2014). The question of the optimal combinations of biomechanical and biochemical components to recapitulate a regenerative cardiac microenvironment is an active area of research—the most ambitious goals include cardiac patch designs that aim to develop fully-functioning heart tissue ex vivo, creating a patch of tissue that would be ready to transplant and used to replace large sections of infarcted tissue (Liau et al., 2011; Zhang et al., 2013; Hayoun-Neeman et al., 2019).

Cardiac stem cells

Besides ESCs and iPSCs, the potential use of resident cardiac stem cells (CSCs) in heart regeneration has also been the subject of much research over the last two decades. Though their existence remains deeply contested, the general hypothesis follows that resident progenitor stem cells in the heart could be cardiomyogenic, with different subpopulations of putative stem cells being investigated for their ability to generate new cardiomyocytes after injury (He et al., 2020).

The first putative cardiac stem cell population proposed were *cKit*+ cells. Initial studies reported that *cKit*+ CSCs were multipotent *in vitro*, differentiating into smooth muscle, cardiomyocytes, and endothelial cells (Beltrami et al., 2003). They were also reported to contribute to recovery after myocardial infarction by differentiating into new myocardium (Beltrami et al., 2003; Dawn et al., 2005). However, more recent studies reported that *cKit*+ CSCs did not differentiate into cardiomyocytes in the infarcted adult mouse heart (Zaruba et al., 2010), and that putative new *cKit*+ cardiomyocytes in earlier human heart studies were likely mast cells (Pouly et al., 2008). Lineage tracing of *cKit*+ cells demonstrated that *cKit*+ cardiomyocytes were likely the result of cell fusion rather than differentiation, and that all *cKit*+ cells in the adult heart did not have a cardiomyocyte identity (van Berlo et al., 2014; He et al., 2017).

Another such population were *Sca1* (stem cell antigen)/*Ly6a*+ cells, which were shown to be multipotent and possess cardiomyogenic potential *in vitro* (Oh et al., 2003). While a study reported that endogenous *Sca1*+ cells in the heart contributed to myocardium after injury (Uchida et al., 2013), this finding was not supported by subsequent work, as transplants of *Sca1*+ cells did not differentiate into new cardiomyocytes in injured myocardium (Soonpaa et al., 2018), and lineage tracing of *Sca1*+ cells revealed that they mostly adopted endothelial and not cardiomyocyte fates (Vagnozzi et al., 2018; Zhang et al., 2018). Thus, both *Sca1*+ and *cKit*+ cells are generally no longer thought of as endogenous myocardial progenitors.

However, certain subpopulations of these cells have proved beneficial in ways other than their cardiomyogenic potential. A subset of *Ly6a*+ cells of mesenchymal origin are known as mesenchymal stromal cells (MSCs). MSCs, similarly to other *Ly6a*+ cells, had some, relatively limited, ability to differentiate into cardiomyocytes *in vivo* (da Silva Meirelles et al., 2006), but had a beneficial effect when transplanted in injured myocardium. It was later found that MSCs benefit host tissue through paracrine signaling rather than direct differentiation into myocardial tissue (Guo et al., 2020). MSCs secrete a wide variety of signaling molecules, which have been shown to promote cell survival, possess immunomodulatory properties and encourage neovascularization (Pittenger and Martin, 2004; Thakker and Yang, 2014; Wehman et al., 2016).

MSCs attenuate pathological outcomes after infarction directly by interacting with various immune cells involved in the innate and adaptive immune response. Their presence can decrease the number of pro-inflammatory monocytes (Miteva et al., 2017), promote M2 macrophage polarization and inhibit T cell proliferation (Chiossone et al., 2016). The introduction of MSCs to injured tissue also has a marked antifibrotic effect, reducing scarring after injury directly by lessening ECM deposition and inhibiting fibroblast activation through matrix metalloproteinase (MMP) regulation (Guo et al., 2020). They may also regulate cardioprotective signaling in a paracrine manner, as MSC transplants to infarcted cardiac tissue displayed lower expression of the inflammatory markers TNF-α, IL-1 and IL-6, and decreased apoptosis in the myocardium (Guo et al., 2007).

Thus, while certain identified subpopulations improve cardiac function after injury, the current research consensus suggests that the current research consensus suggests that the adult mammalian heart does not possess a dedicated progenitor population capable of readily differentiating into cardiomyocytes (Maliken and Molkentin, 2018; He et al., 2020). Genetic lineage tracing of cardiomyocyte and non-cardiomyocyte cells in the developing mouse heart shows that lineage segregation occurs by E11.5, as non-myocyte conversions to cardiomyocyte conversions peak at E8.5 and gradually declines (Li et al., 2018). It was also found that non-myocytes do not contribute to the regenerating neonatal mouse heart, in line with results from studies in zebrafish that the primary source of new cardiomyocytes are existing cardiomyocytes (Li et al., 2018; Jopling et al., 2010).

Direct transdifferentiation of non-cardiomyocyte cell types

Another method that bypasses the potential oncogenic or immunological side effects of cell transplants, while avoiding the limitations of targeting either mature cardiomyocytes or low numbers of potential cardiac progenitors is to target a far more abundant and plastic cell type in the heart, the cardiac fibroblast. Cardiac fibroblasts are one of the most numerous cell types in the heart (Baudino et al., 2006) and are amenable to transdifferentiation, having been reprogrammed into pluripotent stem cells, smooth muscle cells and neurons by combinations of lineage specific transcription factors (Takahashi and Yamanaka, 2006; Wang et al., 2003; Vierbuchen et al., 2010). Overexpression of the transcription factors *Gata4*, *Mef2c* and *Tbx5* through retroviral injection resulted in cardiac fibroblasts expressing cardiomyocyte markers, spontaneous contraction, and cardiomyocyte-like electrophysiology *in vivo* (Ieda et al., 2010). Subsequent work has improved upon the efficiency of the original protocol through varying proportions and combinations transcription factors used (Song et al., 2012; Wang et al., 2015; Protze et al., 2012). Induced cardiomyocyte conversion yield and function has also been increased with the addition of small molecules. Adding protein kinase B (AKT1) to activate insulin-like growth factor (IGF1) and mammalian target of rapamycin (mTORC1) signaling resulted in more polynucleated, hypertrophic cells with increased spontaneous contraction (Zhou et al., 2015). Inhibiting pro-fibrotic pathways through the TGF-β inhibitor A83-01 also promoted the expression of cardiac contractility genes *Actc1*, *Myh6* and *Ryr2*, and

increased the incidence of spontaneous contraction (Zhao et al., 2015). Converting endogenous cardiac fibroblasts into cardiomyocytes may also alter the biomechanical properties of the heart as fibroblasts typically secrete and maintain cardiac ECM, which is known to affect regenerative outcomes (Notari et al., 2018). The initial study inducing cardiomyocyte conversion was more successful *in vivo* than predicted based on *in vitro* observations (Ieda et al., 2010), which suggests that altering the native cell composition and microenvironment of the heart may in turn promote further endogenous changes conducive to regeneration.

The CNCC as a potential internal source of regenerating cells

Much of the work examining potential sources of new cardiomyocytes from non-cardiomyocyte cell types overlook the lineage from which the cells originate from. This has led to cells expressing certain markers being categorized as a single population when more detailed examination has revealed more heterogeneity than previously appreciated. For example, cardiac fibroblasts arise from different lineages, and single cell RNA-sequencing has demonstrated multiple distinct subpopulations (Sadoshima and Weiss, 2014). *Sca1*+ cells were also able to be separated into 4 populations based on *PDGFRa* (platelet-derived growth factor receptor α) and *CD31* expression, with pro-angiogenic $PDGFRa^{+}CD31^{-}Sca1^{+}$ cells displaying stemness attributes from multiple cardiac cell types, including cardiomyocytes and endothelial cells (Pfister et al., 2005; Noseda et al., 2015). This raises the possibility that differential myogenic potential of these cell populations may be related to their developmental origin. Proliferating cardiomyocytes in the regenerating heart might thus be heterogeneous (Tang et al., 2019; Sánchez-Iranzo et al., 2018; Kikuchi et al., 2010). Certain lineages of cardiomyocytes might be more amenable to re-enter the cell cycle, or in the case of non-myocyte cell types, perhaps more readily transdifferentiate into cardiomyocytes.

One cell population of interest are cardiac neural crest cells (CNCCs), a multipotent, migratory group of stem cells that contribute to the outflow septum, outflow tract and smooth muscle of the developing heart. In zebrafish, CNCCs are known to contribute to myocardium (Sato and Yost, 2003; Cavanaugh et al., 2015), with recent work extending

this finding to amniotic vertebrates, as CNCC-derived cardiomyocytes were found in the chick and mouse (Tang et al., 2019). In the context of heart regeneration, CNCC-derived cells in zebrafish contribute to new proliferative cardiomyocytes both through the proliferation of CNCC-derived cardiomyocytes, but also the de novo cardiomyogenesis of CNCC-derivatives, as they reactivate a molecular signature reminiscent of embryonic neural crest cells (Sande-Melon et al., 2019; Tang et al., 2019). Notably, the most recent claim of a CSC population in the adult mammalian heart is a population of interstitial *Twist2*+ cells, which appear to contribute to cardiomyocytes, endothelial cells, and fibroblasts (Min et al., 2018). While a relatively small number of *Twist2*+ cells differentiated into cardiomyocytes *in vivo*, *Twist2*+ cells were strongly activated after myocardial infarction, largely at the border of the infarcted zone, contributing to cardiac remodeling by differentiating into endothelial cells and fibroblasts (Min et al., 2018). *Twist2* is a member of the Twist-family basic helix-loop-helix (bLHL) transcription factors, which is known to be involved in CNCC migration and cardiac development (VanDusen and Firulli, 2012), raising the possibility that some of these *Twist2*+ cells may be of CNCC origin. Their plasticity and multipotency both *in vitro* and *in vivo* indicate that these cells might be an ideal therapeutic target for direct transdifferentiation.

This notion is supported by other work that identifies *Isl1*+ CNCCs as a progenitor population in the heart, as *Isl1*+ CNCCs develop into proliferative cardiomyocytes in the embryonic heart (Hatzistergos et al., 2020). *Isl1* is first activated during the specification of precardiac mesoderm, and forms the first and second heart fields, from which most cardiomyocytes develop (Cai et al., 2003; Prall et al., 2007). However, while *Isl1*+ CNCC derivatives persist in the postnatal heart, the high clonal expansion capacity that they possess in fetal stages rapidly declines, and their numbers and proliferative ability are greatly limited after birth (Hatzistergos et al., 2020). Thus, while the early lineage segregation of nonmyocytes and cardiomyocytes might contribute to the loss of neonatal mouse heart regeneration (Li et al., 2018), the loss of cardiomyocyte proliferative capacity through clonal expansion may also play a role. An open avenue of investigation is why this proliferative capacity diminishes, and how it might be reactivated in the adult mammalian heart.

CONCLUSION

The most stunning exemplars of animal regeneration were once mythologized: the Hydra was named for the many-headed hydra of Greek mythology, and the axolotl's namesake is the death-defying Aztec deity Xólotl. However, regenerative ability is far from being mysterious and unattainable. The body of research on animal regeneration shows that it is a process orchestrated and modulated by fundamental physiological conditions and cellular interactions, many of which are shared among animals across phylogeny. Though regenerative mechanisms differ across different animals and organ systems, principles that highlight common points of potential intervention. Regulating proliferative ability, the immune response, the local microenvironment, and organismal metabolism—arise as broadly similar factors that might play a crucial role in determining regenerative outcome. While specific strategies to modulate these factors are likely to need to be tailored to specific dynamics and cellular processes in each context, previous work has created a robust foundation on which these shared themes can be explored at a finer resolution across diverse systems. Having spent his early career researching animal regeneration, Thomas Hunt Morgan once told the marine biologist Norman Berrill, then a young scientist, that he was being "foolish" for studying regeneration in marine invertebrates, and that "we will never understand the phenomena of development and regeneration" (Berrill, 1983). Perhaps if Morgan could see the body of understanding amassed from the humble capabilities of the common lab mouse today—representing a century's worth of research and technological advances from when he spoke to Berrill, he might be convinced otherwise.

BIBLIOGRAPHY

Adler, C.-P., Friedburg, H., Herget, G. W., Neuburger, M., & Schwalb, H. (1996). Variability of cardiomyocyte DNA content, ploidy level and nuclear number in mammalian hearts. *Virchows Archiv*, 429(2), 159–164. https://doi.org/10.1007/BF00192438

Aragona, M., Panciera, T., Manfrin, A., Giulitti, S., Michielin, F., Elvassore, N., Dupont, S., & Piccolo, S. (2013). A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. *Cell,* 154(5), 1047–1059. https://doi.org/10.1016/j.cell.2013.07.042

Atchley, W. R., & Hall, B. K. (1991). A model for development and evolution of complex morphological structures. *Biological Reviews*, 66(2), 101–157. https://doi.org/10.1111/j.1469-185X.1991.tb01138.x

Aurora, A. B., Porrello, E. R., Tan, W., Mahmoud, A. I., Hill, J. A., Bassel-Duby, R., Sadek, H. A., & Olson, E. N. (2014a). Macrophages are required for neonatal heart regeneration. *The Journal of Clinical Investigation*, 124(3), 1382–1392. https://doi.org/10.1172/JCI72181

Aztekin, C., & Storer, M. A. (2022). To regenerate or not to regenerate: Vertebrate model organisms of regeneration-competency and -incompetency. *Wound Repair and Regeneration*, 30(6), 623–635. https://doi.org/10.1111/wrr.13000

Bae, J., Paltzer, W. G., & Mahmoud, A. I. (2021). The role of metabolism in heart failure and regeneration. *Frontiers in Cardiovascular Medicine*, 8, 702920. https://doi.org/10.3389/fcvm.2021.702920

Baehr, A., Umansky, K. B., Bassat, E., Jurisch, V., Klett, K., Bozoglu, T., Hornaschewitz, N., Solyanik, O., Kain, D., Ferraro, B., Cohen-Rabi, R., Krane, M., Cyran, C., Soehnlein, O., Laugwitz, K. L., Hinkel, R., Kupatt, C., & Tzahor, E. (2020). Agrin promotes coordinated therapeutic processes leading to improved cardiac repair in pigs. *Circulation*, 142(9), 868–881. https://doi.org/10.1161/CIRCULATIONAHA.119.045116

Bainbridge, P. (2013). Wound healing and the role of fibroblasts. *Journal of Wound Care*, 22(8), 407–412. https://doi.org/10.12968/jowc.2013.22.8.407

Ban, K., Park, H.-J., Kim, S., Andukuri, A., Cho, K.-W., Hwang, J. W., Cha, H. J., Kim, S. Y., Kim, W.-S., Jun, H.-W., & Yoon, Y.-S. (2014). Cell therapy with embryonic stem cell-derived cardiomyocytes encapsulated in injectable nanomatrix gel enhances cell engraftment and promotes cardiac repair. *ACS Nano*, 8(10), 10815–10825. https://doi.org/10.1021/nn504617g

Bassat, E., Mutlak, Y. E., Genzelinakh, A., Shadrin, I. Y., Umansky, K. B., Yifa, O., Kain, D., Rajchman, D., Leach, J., Bassat, D. R., Udi, Y., Sarig, R., Sagi, I., Martin, J. F., Bursac, N., Cohen, S., & Tzahor, E. (2017). The extracellular matrix protein agrin promotes heart regeneration in mice. *Nature*, 547(7662), 179–184. https://doi.org/10.1038/nature22978

Baudino, T. A., Carver, W., Giles, W., & Borg, T. K. (2006). Cardiac fibroblasts: Friend or foe? *American Journal of Physiology-Heart and Circulatory Physiology*, 291(3), H1015–H1026. https://doi.org/10.1152/ajpheart.00023.2006

Beggs, M. L., Nagarajan, R., Taylor-Jones, J. M., Nolen, G., MacNicol, M., & Peterson, C. A. (2004). Alterations in the TGFβ signaling pathway in myogenic progenitors with age. *Aging Cell*, 3(6), 353–361. https://doi.org/10.1111/j.1474-9728.2004.00135.x

Beltrami, A. P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., Urbanek, K., Leri, A., Kajstura, J., Nadal-Ginard, B., & Anversa, P. (2003). Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell*, 114(6), 763–776. https://doi.org/10.1016/S0092-8674(03)00687-1

Benjamin, E. J., Muntner, P., Alonso, A., Bittencourt, M. S., Callaway, C. W., Carson, A. P., Chamberlain, A. M., Chang, A. R., Cheng, S., Das, S. R., Delling, F. N., Djousse, L., Elkind, M. S. V., Ferguson, J. F., Fornage, M., Jordan, L. C., Khan, S. S., Kissela, B. M., Knutson, K. L., Kwan, T. W., … American Heart Association Council on Epidemiology and Prevention Statistics Committee and Stroke Statistics Subcommittee (2019). Heart disease and stroke statistics-2019 Update: A report from the American Heart Association. *Circulation*, *139*(10), e56–e528. https://doi.org/10.1161/CIR.0000000000000659

Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabé-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B. A., Druid, H., Jovinge, S., & Frisén, J. (2009). Evidence for cardiomyocyte renewal in humans. *Science* (New York, N.Y.), 324(5923), 98–102. https://doi.org/10.1126/science.1164680

Berrill, N. J. (1983). The pleasure and practice of biology. *Canadian Journal of Zoology*, 61(5), 947–951. https://doi.org/10.1139/z83-127

Blum, N., & Begemann, G. (2015). Retinoic acid signaling spatially restricts osteoblasts and controls ray-interray organization during zebrafish fin regeneration. *Development* (Cambridge, England), 142(17), 2888–2893. https://doi.org/10.1242/dev.120212

Brooks, G., Poolman, R. A., & Li, J. M. (1998). Arresting developments in the cardiac myocyte cell cycle: Role of cyclin-dependent kinase inhibitors. *Cardiovascular Research*, 39(2), 301–311. https://doi.org/10.1016/s0008-6363(98)00125-4

Bullard, K. M., Longaker, M. T., & Lorenz, H. P. (2003). Fetal wound healing: Current biology. *World Journal of Surgery*, 27(1), 54–61. https://doi.org/10.1007/s00268-002-6737-2

Cai, C.-L., Liang, X., Shi, Y., Chu, P.-H., Pfaff, S. L., Chen, J., & Evans, S. (2003). Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Developmental Cell*, 5(6), 877–889. https://doi.org/10.1016/s1534-5807(03)00363-0

Cardoso, A. C., Lam, N. T., Savla, J. J., Nakada, Y., Pereira, A. H. M., Elnwasany, A., Menendez-Montes, I., Ensley, E. L., Petric, U. B., Sharma, G., Sherry, A. D., Malloy, C. R., Khemtong, C., Kinter, M. T., Tan, W. L. W., Anene-Nzelu, C. G., Foo, R. S.-Y., Nguyen, N. U. N., Li, S., … Sadek, H. A. (2020). Mitochondrial substrate utilization regulates cardiomyocyte cell cycle progression. *Nature Metabolism*, 2(2), 167–178.

Cardoso, A. C., Pereira, A. H. M., & Sadek, H. A. (2020). Mechanisms of neonatal heart regeneration. *Current Cardiology Reports*, 22(5), 33. https://doi.org/10.1007/s11886-020-01282-5

Ceradini, D. J., Kulkarni, A. R., Callaghan, M. J., Tepper, O. M., Bastidas, N., Kleinman, M. E., Capla, J. M., Galiano, R. D., Levine, J. P., & Gurtner, G. C. (2004). Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nature Medicine*, 10(8), 858–864. https://doi.org/10.1038/nm1075

Chablais, F., & Jazwinska, A. (2012). The regenerative capacity of the zebrafish heart is dependent on TGFβ signaling. *Development* (Cambridge, England), 139(11), 1921–1930. https://doi.org/10.1242/dev.078543

Chang, H. Y., Chi, J.-T., Dudoit, S., Bondre, C., van de Rijn, M., Botstein, D., & Brown, P. O. (2002). Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, 99(20), 12877–12882. https://doi.org/10.1073/pnas.162488599

Charlemagne, J. (1979). Thymus independent anti-horse erythrocyte antibody response and suppressor T cells in the Mexican axolotl (Amphibia, Urodela, ambystoma mexicanum). *Immunology*, 36(4), 643–648.

Chaturvedi, D., Reichert, H., Gunage, R. D., & VijayRaghavan, K. (2017). Identification and functional characterization of muscle satellite cells in Drosophila. *ELife*, 6, e30107. https://doi.org/10.7554/eLife.30107

Chen, W., & Frangogiannis, N. G. (2013). Fibroblasts in post-infarction inflammation and cardiac repair. *Biochimica Et Biophysica Acta*, 1833(4), 945–953. https://doi.org/10.1016/j.bbamcr.2012.08.023

Chiossone, L., Conte, R., Spaggiari, G. M., Serra, M., Romei, C., Bellora, F., Becchetti, F., Andaloro, A., Moretta, L., & Bottino, C. (2016). Mesenchymal stromal cells induce peculiar alternatively activated macrophages capable of dampening both innate and adaptive immune responses. *Stem Cells*, 34(7), 1909–1921. https://doi.org/10.1002/stem.2369

Chong, J. J. H., Yang, X., Don, C. W., Minami, E., Liu, Y.-W., Weyers, J. J., Mahoney, W. M., Van Biber, B., Cook, S. M., Palpant, N. J., Gantz, J. A., Fugate, J. A., Muskheli, V., Gough, G. M., Vogel, K. W., Astley, C. A., Hotchkiss, C. E., Baldessari, A., Pabon, L., … Murry, C. E. (2014). Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. *Nature*, 510(7504), 273–277. https://doi.org/10.1038/nature13233

Clark, L. D., Clark, R. K., & Heber-Katz, E. (1998). A new murine model for mammalian wound repair and regeneration. *Clinical Immunology and Immunopathology*, 88(1), 35–45. https://doi.org/10.1006/clin.1998.4519

Cochain, C., Auvynet, C., Poupel, L., Vilar, J., Dumeau, E., Richart, A., Récalde, A., Zouggari, Y., Yin, K. Y. H. W., Bruneval, P., Renault, G., Marchiol, C., Bonnin, P., Lévy, B., Bonecchi, R., Locati, M., Combadière, C., & Silvestre, J.-S. (2012). The chemokine decoy receptor D6 prevents excessive inflammation and adverse ventricular remodeling after myocardial infarction. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 32(9), 2206–2213. https://doi.org/10.1161/ATVBAHA.112.254409

Cutie, S., & Huang, G. N. (2021). Vertebrate cardiac regeneration: Evolutionary and developmental perspectives. *Cell Regeneration* (London, England), 10(1), 6. https://doi.org/10.1186/s13619-020-00068-y

da Silva Meirelles, L., Chagastelles, P. C., & Nardi, N. B. (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *Journal of Cell Science*, 119(Pt 11), 2204–2213. https://doi.org/10.1242/jcs.02932

D'Amario, D., Cappetta, D., Cappannoli, L., Princi, G., Migliaro, S., Diana, G., Chouchane, K., Borovac, J. A., Restivo, A., Arcudi, A., De Angelis, A., Vergallo, R., Montone, R. A., Galli, M., Liuzzo, G., & Crea, F. (2021). Colchicine in ischemic heart disease: The good, the bad and the ugly. *Clinical Research in Cardiology*, 110(10), 1531–1542. https://doi.org/10.1007/s00392-021-01828-9

Dawn, B., Stein, A. B., Urbanek, K., Rota, M., Whang, B., Rastaldo, R., Torella, D., Tang, X.-L., Rezazadeh, A., Kajstura, J., Leri, A., Hunt, G., Varma, J., Prabhu, S. D., Anversa, P., & Bolli, R. (2005). Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function. *Proceedings of the National Academy of Sciences of the United States of America*, 102(10), 3766–3771. https://doi.org/10.1073/pnas.0405957102

Dawson, L. A., Schanes, P. P., Marrero, L., Jordan, K., Brunauer, R., Zimmel, K. N., Qureshi, O., Imholt, F. M., Falck, A. R., Yan, M., Dolan, C. P., Yu, L., & Muneoka, K. (2021). Proximal digit tip amputation initiates simultaneous blastema and transient fibrosis formation and results in partial regeneration. *Wound Repair and Regeneration*, 29(1), 196–205. https://doi.org/10.1111/wrr.12856

Dawson, L. A., Simkin, J., Sauque, M., Pela, M., Palkowski, T., & Muneoka, K. (2016). Analogous cellular contribution and healing mechanisms following digit amputation and phalangeal fracture in mice. *Regeneration*, 3(1), 39–51. https://doi.org/10.1002/reg2.51

de Lemos, J. A., Morrow, D. A., Blazing, M. A., Jarolim, P., Wiviott, S. D., Sabatine, M. S., Califf, R. M., & Braunwald, E. (2007). Serial measurement of monocyte chemoattractant protein-1 after acute coronary syndromes. J*ournal of the American College of Cardiology*, 50(22), 2117–2124. https://doi.org/10.1016/j.jacc.2007.06.057

de Preux Charles, A.-S., Bise, T., Baier, F., Marro, J., & Jaźwińska, A. (2016). Distinct effects of inflammation on preconditioning and regeneration of the adult zebrafish heart. *Open Biology*, 6(7), 160102. https://doi.org/10.1098/rsob.160102

Del Re, D. P., Yang, Y., Nakano, N., Cho, J., Zhai, P., Yamamoto, T., Zhang, N., Yabuta, N., Nojima, H., Pan, D., & Sadoshima, J. (2013). Yes-associated protein isoform 1 (Yap1) promotes cardiomyocyte survival and growth to protect against myocardial ischemic injury. *Journal of Biological Chemistry*, 288(6), 3977–3988. https://doi.org/10.1074/jbc.M112.436311

Dixey, F. A. (1881). II. On the ossification of the terminal phalanges of the digits. *Proceedings of the Royal Society of London*, 31(206–211), 63–71.

Dobaczewski, M., Gonzalez-Quesada, C., & Frangogiannis, N. G. (2010). The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. *Journal of Molecular and Cellular Cardiology*, 48(3), 504–511. https://doi.org/10.1016/j.yjmcc.2009.07.015

Dolan, C. P., Dawson, L. A., & Muneoka, K. (2018). Digit tip regeneration: merging regeneration biology with regenerative medicine. *Stem Cells Translational Medicine*, 7(3), 262–270. https://doi.org/10.1002/sctm.17-0236

Elchaninov, A., Sukhikh, G., & Fatkhudinov, T. (2021). Evolution of regeneration in animals: A tangled story. *Frontiers in Ecology and Evolution*, 9. https://www.frontiersin.org/articles/10.3389/fevo.2021.621686

Fei, J.F., Schuez, M., Knapp, D., Taniguchi, Y., Drechsel, D. N., & Tanaka, E. M. (2017). Efficient gene knockin in axolotl and its use to test the role of satellite cells in limb regeneration. *Proceedings of the National Academy of Sciences*, 114(47), 12501–12506. https://doi.org/10.1073/pnas.1706855114

Ferguson, M. W. J., & O'Kane, S. (2004). Scar-free healing: From embryonic mechanisms to adult therapeutic intervention. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 359(1445), 839–850. https://doi.org/10.1098/rstb.2004.1475

Fernando, W. A., Leininger, E., Simkin, J., Li, N., Malcom, C. A., Sathyamoorthi, S., Han, M., & Muneoka, K. (2011). Wound healing and blastema formation in regenerating digit tips of adult mice. *Developmental Biology*, 350(2), 301–310. https://doi.org/10.1016/j.ydbio.2010.11.035

Frangogiannis, N. G. (2007). Chemokines in ischemia and reperfusion. *Thrombosis and Haemostasis*, 97(5), 738–747. https://doi.org/10.1160/TH07-01-0022

Frangogiannis, N. G., & Entman, M. L. (2005). Chemokines in myocardial ischemia. *Trends in Cardiovascular Medicine*, 15(5), 163–169. https://doi.org/10.1016/j.tcm.2005.06.005

Fukazawa, T., Naora, Y., Kunieda, T., & Kubo, T. (2009). Suppression of the immune response potentiates tadpole tail regeneration during the refractory period. *Development,* 136(14), 2323–2327. https://doi.org/10.1242/dev.033985

Galliot, B., & Ghila, L. (2010). Cell plasticity in homeostasis and regeneration. *Molecular Reproduction and Development*, 77(10), 837–855. https://doi.org/10.1002/mrd.21206

Gauron, C., Rampon, C., Bouzaffour, M., Ipendey, E., Teillon, J., Volovitch, M., & Vriz, S. (2013). Sustained production of ROS triggers compensatory proliferation and is required for regeneration to proceed. *Scientific Reports*, 3(1), Article 1. https://doi.org/10.1038/srep02084

Gawriluk, T. R., Simkin, J., Hacker, C. K., Kimani, J. M., Kiama, S. G., Ezenwa, V. O., & Seifert, A. W. (2020). Complex tissue regeneration in mammals is associated with reduced inflammatory cytokines and an influx of T cells. *Frontiers in Immunology*, 11. https://www.frontiersin.org/articles/10.3389/fimmu.2020.01695

Gerber, T., Murawala, P., Knapp, D., Masselink, W., Schuez, M., Hermann, S., Gac-Santel, M., Nowoshilow, S., Kageyama, J., Khattak, S., Currie, J. D., Camp, J. G., Tanaka, E. M., & Treutlein, B. (2018). Single-cell analysis uncovers convergence of cell identities during axolotl limb regeneration. *Science* (New York, N.Y.), 362(6413), eaaq0681. https://doi.org/10.1126/science.aaq0681

Gilbert, S. F., & Bolker, J. A. (2001). Homologies of process and modular elements of embryonic construction. *The Journal of Experimental Zoology*, 291(1), 1–12. https://doi.org/10.1002/jez.1

Godwin, J. W., Pinto, A. R., & Rosenthal, N. A. (2013). Macrophages are required for adult salamander limb regeneration. *Proceedings of the National Academy of Sciences*, 110(23), 9415–9420. https://doi.org/10.1073/pnas.1300290110

Godwin, J. W., & Rosenthal, N. (2014). Scar-free wound healing and regeneration in amphibians: Immunological influences on regenerative success. *Differentiation; Research in Biological Diversity*, 87(1–2), 66–75. https://doi.org/10.1016/j.diff.2014.02.002

González-Rosa, J. M., Sharpe, M., Field, D., Soonpaa, M. H., Field, L. J., Burns, C. E., & Burns, C. G. (2018). Myocardial polyploidization creates a barrier to heart regeneration in zebrafish. *Developmental Cell*, 44(4), 433-446.e7. https://doi.org/10.1016/j.devcel.2018.01.021

Guo, J., Lin, G., Bao, C., Hu, Z., & Hu, M. (2007). Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction. *Inflammation*, 30(3), 97–104. https://doi.org/10.1007/s10753-007-9025-3

Guo, Y., Wysoczynski, M., Nong, Y., Tomlin, A., Zhu, X., Gumpert, A. M., Nasr, M., Muthusamy, S., Li, H., Book, M., Khan, A., Hong, K. U., Li, Q., & Bolli, R. (2017). Repeated doses of cardiac mesenchymal cells are therapeutically superior to a single dose in mice with old myocardial infarction. *Basic Research in Cardiology*, 112(2), 18. https://doi.org/10.1007/s00395-017-0606-5

Guo, Y., Yu, Y., Hu, S., Chen, Y., & Shen, Z. (2020). The therapeutic potential of mesenchymal stem cells for cardiovascular diseases. *Cell Death & Disease*, 11(5), Article 5. https://doi.org/10.1038/s41419-020-2542-9

Halder, G., Dupont, S., & Piccolo, S. (2012). Transduction of mechanical and cytoskeletal cues by YAP and TAZ. *Nature Reviews Molecular Cell Biology*, 13(9), 591–600. https://doi.org/10.1038/nrm3416

Han, C., Nie, Y., Lian, H., Liu, R., He, F., Huang, H., & Hu, S. (2015). Acute inflammation stimulates a regenerative response in the neonatal mouse heart. *Cell Research*, 25(10), Article 10. https://doi.org/10.1038/cr.2015.110

Han, M., Yang, X., Farrington, J. E., & Muneoka, K. (2003). Digit regeneration is regulated by Msx1 and BMP4 in fetal mice. *Development*, 130(21), 5123–5132. https://doi.org/10.1242/dev.00710

Han, M., Yang, X., Lee, J., Allan, C. H., & Muneoka, K. (2008). Development and regeneration of the neonatal digit tip in mice. *Developmental Biology*, 315(1), 125–135. https://doi.org/10.1016/j.ydbio.2007.12.025

Hartman, M. H. T., Groot, H. E., Leach, I. M., Karper, J. C., & van der Harst, P. (2018). Translational overview of cytokine inhibition in acute myocardial infarction and chronic heart failure. *Trends in Cardiovascular Medicine*, 28(6), 369–379. https://doi.org/10.1016/j.tcm.2018.02.003

Hatzistergos, K. E., Durante, M. A., Valasaki, K., Wanschel, A. C. B. A., Harbour, J. W., & Hare, J. M. (2020). A novel cardiomyogenic role for Isl1+ neural crest cells in the inflow tract. *Science Advances*, 6(49), eaba9950. https://doi.org/10.1126/sciadv.aba9950

Hayoun-Neeman, D., Korover, N., Etzion, S., Ofir, R., Lichtenstein, R. G., & Cohen, S. (2019). Exploring peptide-functionalized alginate scaffolds for engineering cardiac tissue from human embryonic stem cell-derived cardiomyocytes in serum-free medium. *Polymers for Advanced Technologies*, 30(10), 2493–2505. https://doi.org/10.1002/pat.4602

He, L., Li, Y., Li, Y., Pu, W., Huang, X., Tian, X., Wang, Y., Zhang, H., Liu, Q., Zhang, L., Zhao, H., Tang, J., Ji, H., Cai, D., Han, Z., Han, Z., Nie, Y., Hu, S., Wang, Q.-

D., … Zhou, B. (2017). Enhancing the precision of genetic lineage tracing using dual recombinases. *Nature Medicine*, 23(12), 1488–1498. https://doi.org/10.1038/nm.4437

He, L., Nguyen, N. B., Ardehali, R., & Zhou, B. (2020). Heart regeneration by endogenous stem cells and cardiomyocyte proliferation. *Circulation*, 142(3), 275–291. https://doi.org/10.1161/CIRCULATIONAHA.119.045566

Heallen, T., Morikawa, Y., Leach, J., Tao, G., Willerson, J. T., Johnson, R. L., & Martin, J. F. (2013). Hippo signaling impedes adult heart regeneration. *Development* (Cambridge, England), 140(23), 4683–4690. https://doi.org/10.1242/dev.102798

Heallen, T., Zhang, M., Wang, J., Bonilla-Claudio, M., Klysik, E., Johnson, R. L., & Martin, J. F. (2011). Hippo pathway inhibits *Wnt* signaling to restrain cardiomyocyte proliferation and heart size. *Science* (New York, N.Y.), 332(6028), 458–461. https://doi.org/10.1126/science.1199010

Hemmrich, G., Khalturin, K., Boehm, A.-M., Puchert, M., Anton-Erxleben, F., Wittlieb, J., Klostermeier, U. C., Rosenstiel, P., Oberg, H.-H., Domazet-Loso, T., Sugimoto, T., Niwa, H., & Bosch, T. C. G. (2012). Molecular signatures of the three stem cell lineages in hydra and the emergence of stem cell function at the base of multicellularity. *Molecular Biology and Evolution*, 29(11), 3267–3280. https://doi.org/10.1093/molbev/mss134

Hirose, K., Payumo, A. Y., Cutie, S., Hoang, A., Zhang, H., Guyot, R., Lunn, D., Bigley, R. B., Yu, H., Wang, J., Smith, M., Gillett, E., Muroy, S. E., Schmid, T., Wilson, E., Field, K. A., Reeder, D. M., Maden, M., Yartsev, M. M., … Huang, G. N. (2019). Evidence for hormonal control of heart regenerative capacity during endothermy acquisition. *Science* (New York, N.Y.), 364(6436), 184–188. https://doi.org/10.1126/science.aar2038

Honkoop, H., de Bakker, D. E., Aharonov, A., Kruse, F., Shakked, A., Nguyen, P. D., de Heus, C., Garric, L., Muraro, M. J., Shoffner, A., Tessadori, F., Peterson, J. C., Noort, W., Bertozzi, A., Weidinger, G., Posthuma, G., Grün, D., van der Laarse, W. J., Klumperman, J., … Bakkers, J. (2019). Single-cell analysis uncovers that metabolic reprogramming by ErbB2 signaling is essential for cardiomyocyte proliferation in the regenerating heart. *ELife*, 8, e50163. https://doi.org/10.7554/eLife.50163

Horckmans, M., Ring, L., Duchene, J., Santovito, D., Schloss, M. J., Drechsler, M., Weber, C., Soehnlein, O., & Steffens, S. (2017). Neutrophils orchestrate post-myocardial infarction healing by polarizing macrophages towards a reparative phenotype. *European Heart Journal*, 38(3), 187–197. https://doi.org/10.1093/eurheartj/ehw002

Hu, B., Lelek, S., Spanjaard, B., El-Sammak, H., Simões, M. G., Mintcheva, J., Aliee, H., Schäfer, R., Meyer, A. M., Theis, F., Stainier, D. Y. R., Panáková, D., & Junker, J. P. (2022a). Origin and function of activated fibroblast states during zebrafish heart regeneration. *Nature Genetics*, 54(8), 1227–1237. https://doi.org/10.1038/s41588-022-01129-5

Hu, B., Lelek, S., Spanjaard, B., El-Sammak, H., Simões, M. G., Mintcheva, J., Aliee, H., Schäfer, R., Meyer, A. M., Theis, F., Stainier, D. Y. R., Panáková, D., & Junker, J. P. (2022b). Origin and function of activated fibroblast states during zebrafish heart

regeneration. *Nature Genetics*, 54(8), Article 8. https://doi.org/10.1038/s41588-022-01129-5

Huang, S., & Frangogiannis, N. G. (2018). Anti-inflammatory therapies in myocardial infarction: Failures, hopes and challenges. *British Journal of Pharmacology*, 175(9), 1377–1400. https://doi.org/10.1111/bph.14155

Huang, W.-C., Yang, C.-C., Chen, I.-H., Liu, Y.-M. L., Chang, S.-J., & Chuang, Y.-J. (2013). Treatment of Glucocorticoids Inhibited Early Immune Responses and Impaired Cardiac Repair in Adult Zebrafish. *PLoS One*, 8(6), e66613. https://doi.org/10.1371/journal.pone.0066613

Hui, S. P., Sheng, D. Z., Sugimoto, K., Gonzalez-Rajal, A., Nakagawa, S., Hesselson, D., & Kikuchi, K. (2017). Zebrafish Regulatory T Cells Mediate Organ-Specific Regenerative Programs. *Developmental Cell*, 43(6), 659-672.e5. https://doi.org/10.1016/j.devcel.2017.11.010

Hynes, R. O. (2009). Extracellular matrix: Not just pretty fibrils. *Science* (New York, N.Y.), 326(5957), 1216–1219. https://doi.org/10.1126/science.1176009

Ieda, M., Fu, J.-D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B. G., & Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell*, 142(3), 375–386. https://doi.org/10.1016/j.cell.2010.07.002

Ikeda, S., & Sadoshima, J. (2016). Regulation of myocardial cell growth and death by the hippo pathway. *Circulation Journal: Official Journal of the Japanese Circulation Society*, 80(7), 1511–1519. https://doi.org/10.1253/circj.CJ-16-0476

Illingworth, C., M. (1974). Trapped fingers and amputated finger tips in children. *Journal of Pediatric Surgery*, 9(6), 853–858.

Imanaka-Yoshida, K., Hiroe, M., & Yoshida, T. (2004). Interaction between cell and extracellular matrix in heart disease: multiple roles of tenascin-C in tissue remodeling. *Histology and histopathology*, *19*(2), 517–525. https://doi.org/10.14670/HH-19.517

Ito, T., Sawada, R., Fujiwara, Y., Seyama, Y., & Tsuchiya, T. (2007). FGF-2 suppresses cellular senescence of human mesenchymal stem cells by down-regulation of TGF-β2. *Biochemical and Biophysical Research Communications*, 359(1), 108–114. https://doi.org/10.1016/j.bbrc.2007.05.067

Itou, J., Kawakami, H., Burgoyne, T., & Kawakami, Y. (2012). Life-long preservation of the regenerative capacity in the fin and heart in zebrafish. *Biology Open,* 1(8), 739–746. https://doi.org/10.1242/bio.20121057

Jacot, J. G., Kita-Matsuo, H., Wei, K. A., Chen, H. S. V., Omens, J. H., Mercola, M., & Mcculloch, A. D. (2010). Cardiac myocyte force development during differentiation and maturation. *Annals of the New York Academy of Sciences*, 1188, 121–127. https://doi.org/10.1111/j.1749-6632.2009.05091.x

Johnston, A. P. W., Yuzwa, S. A., Carr, M. J., Mahmud, N., Storer, M. A., Krause, M. P., Jones, K., Paul, S., Kaplan, D. R., & Miller, F. D. (2016). Dedifferentiated Schwann cell precursors secreting paracrine factors are required for regeneration of the mammalian digit tip. *Cell Stem Cell*, 19(4), 433–448. https://doi.org/10.1016/j.stem.2016.06.002

Jopling, C., Boue, S., & Izpisua Belmonte, J. C. (2011). Dedifferentiation, transdifferentiation and reprogramming: Three routes to regeneration. *Nature Reviews Molecular Cell Biology*, 12(2), 79–89. https://doi.org/10.1038/nrm3043

Kawaguchi, M., Takahashi, M., Hata, T., Kashima, Y., Usui, F., Morimoto, H., Izawa, A., Takahashi, Y., Masumoto, J., Koyama, J., Hongo, M., Noda, T., Nakayama, J., Sagara, J., Taniguchi, S., & Ikeda, U. (2011). Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. *Circulation*, 123(6), 594–604. https://doi.org/10.1161/CIRCULATIONAHA.110.982777

Kawamura, M., Miyagawa, S., Miki, K., Saito, A., Fukushima, S., Higuchi, T., Kawamura, T., Kuratani, T., Daimon, T., Shimizu, T., Okano, T., & Sawa, Y. (2012). Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiomyocyte sheets in a porcine ischemic cardiomyopathy model. *Circulation*, 126(11 Suppl 1), S29-37. https://doi.org/10.1161/CIRCULATIONAHA.111.084343

Kawasumi, A., Sagawa, N., Hayashi, S., Yokoyama, H., & Tamura, K. (2012). Wound Healing in Mammals and Amphibians: Toward Limb Regeneration in Mammals. In E. Heber-Katz & D. L. Stocum (Eds.), *New Perspectives in Regeneration* (Vol. 367, pp. 33–49). Springer Berlin Heidelberg. https://doi.org/10.1007/82_2012_305

Kierdorf, U., Kierdorf, H., & Szuwart, T. (2007). Deer antler regeneration: Cells, concepts, and controversies. *Journal of Morphology*, 268(8), 726–738. https://doi.org/10.1002/jmor.10546

Kikuchi, K. (2015). Dedifferentiation, transdifferentiation, and proliferation: mechanisms underlying cardiac muscle regeneration in zebrafish. *Current Pathobiology Reports*, 3(1), 81–88. https://doi.org/10.1007/s40139-015-0063-5

Kikuchi, K., Holdway, J. E., Werdich, A. A., Anderson, R. M., Fang, Y., Egnaczyk, G. F., Evans, T., Macrae, C. A., Stainier, D. Y. R., & Poss, K. D. (2010). Primary contribution to zebrafish heart regeneration by *gata4(+)* cardiomyocytes. *Nature*, 464(7288), 601–605. https://doi.org/10.1038/nature08804

Kimura, W., Nakada, Y., & Sadek, H. A. (2017). Hypoxia-induced myocardial regeneration. *Journal of Applied Physiology*, 123(6), 1676–1681. https://doi.org/10.1152/japplphysiol.00328.2017

King, M. W., Neff, A. W., & Mescher, A. L. (2012). The developing *Xenopus* limb as a model for studies on the balance between inflammation and regeneration. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, 295(10), 1552–1561. https://doi.org/10.1002/ar.22443

Konstantinides, N., & Averof, M. (2014). A common cellular basis for muscle regeneration in arthropods and vertebrates. *Science* (New York, N.Y.), 343(6172), 788–791. https://doi.org/10.1126/science.1243529

Kostakopoulou, K., Vogel, A., Brickell, P., & Tickle, C. (1996). 'Regeneration' of wing bud stumps of chick embryos and reactivation of *Msx-1* and *Shh* expression in response to FGF-4 and ridge signals. *Mechanisms of Development*, 55(2), 119–131. https://doi.org/10.1016/0925-4773(95)00492-0

Kovacheva, E. L., Sinha Hikim, A. P., Shen, R., Sinha, I., & Sinha-Hikim, I. (2010). Testosterone supplementation reverses sarcopenia in aging through regulation of Myostatin, c-Jun NH2-Terminal Kinase, Notch, and Akt signaling pathways. *Endocrinology*, 151(2), 628–638. https://doi.org/10.1210/en.2009-1177

Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H. H., & Tanaka, E. M. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature*, 460(7251), 60–65. https://doi.org/10.1038/nature08152

Kujawski, S., Lin, W., Kitte, F., Börmel, M., Fuchs, S., Arulmozhivarman, G., Vogt, S., Theil, D., Zhang, Y., & Antos, C. L. (2014). Calcineurin regulates coordinated outgrowth of zebrafish regenerating fins. *Developmental Cell*, 28(5), 573–587. https://doi.org/10.1016/j.devcel.2014.01.019

Kumar, A., & Brockes, J. P. (2012). Nerve dependence in tissue, organ, and appendage regeneration. *Trends in Neurosciences*, 35(11), 691–699. https://doi.org/10.1016/j.tins.2012.08.003

Lai, A. G., & Aboobaker, A. A. (2018). EvoRegen in animals: Time to uncover deep conservation or convergence of adult stem cell evolution and regenerative processes. *Developmental Biology*, 433(2), 118–131. https://doi.org/10.1016/j.ydbio.2017.10.010

Lai, S.-L., Marín-Juez, R., Moura, P. L., Kuenne, C., Lai, J. K. H., Tsedeke, A. T., Guenther, S., Looso, M., & Stainier, D. Y. (2017). Reciprocal analyses in zebrafish and medaka reveal that harnessing the immune response promotes cardiac regeneration. *ELife*, 6, e25605. https://doi.org/10.7554/eLife.25605

Lai, S.-L., Marín-Juez, R., & Stainier, D. Y. R. (2019). Immune responses in cardiac repair and regeneration: A comparative point of view. *Cellular and Molecular Life Sciences*, 76(7), 1365–1380. https://doi.org/10.1007/s00018-018-2995-5

Lavine, K. J., Epelman, S., Uchida, K., Weber, K. J., Nichols, C. G., Schilling, J. D., Ornitz, D. M., Randolph, G. J., & Mann, D. L. (2014). Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. *Proceedings of the National Academy of Sciences of the United States of America*, 111(45), 16029–16034. https://doi.org/10.1073/pnas.1406508111

Leask, A. (2010). Potential therapeutic targets for cardiac fibrosis: TGFbeta, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. *Circulation Research*, 106(11), 1675–1680. https://doi.org/10.1161/CIRCRESAHA.110.217737

Lehoczky, J. A., Robert, B., & Tabin, C. J. (2011). Mouse digit tip regeneration is mediated by fate-restricted progenitor cells. *Proceedings of the National Academy of Sciences of the United States of America*, 108(51), 20609–20614. https://doi.org/10.1073/pnas.1118017108

Lehoczky, J. A., & Tabin, C. J. (2015). *Lgr6* marks nail stem cells and is required for digit tip regeneration. *Proceedings of the National Academy of Sciences*, 112(43), 13249–13254. https://doi.org/10.1073/pnas.1518874112

Lehtinen, M. K., Yuan, Z., Boag, P. R., Yang, Y., Villén, J., Becker, E. B. E., DiBacco, S., de la Iglesia, N., Gygi, S., Blackwell, T. K., & Bonni, A. (2006). A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell*, 125(5), 987–1001. https://doi.org/10.1016/j.cell.2006.03.046

Li, C., Zhao, H., Liu, Z., & McMahon, C. (2014). Deer antler – A novel model for studying organ regeneration in mammals. *The International Journal of Biochemistry & Cell Biology*, 56, 111–122. https://doi.org/10.1016/j.biocel.2014.07.007

Li, J., Yang, K. Y., Tam, R. C. Y., Chan, V. W., Lan, H. Y., Hori, S., Zhou, B., & Lui, K. O. (2019). Regulatory T-cells regulate neonatal heart regeneration by potentiating cardiomyocyte proliferation in a paracrine manner. *Theranostics*, 9(15), 4324–4341. https://doi.org/10.7150/thno.32734

Li, Y., He, L., Huang, X., Bhaloo, S. I., Zhao, H., Zhang, S., Pu, W., Tian, X., Li, Y., Liu, Q., Yu, W., Zhang, L., Liu, X., Liu, K., Tang, J., Zhang, H., Cai, D., Ralf, A. H., Xu, Q., … Zhou, B. (2018). Genetic lineage tracing of nonmyocyte population by dual recombinases. *Circulation*, 138(8), 793–805. https://doi.org/10.1161/CIRCULATIONAHA.118.034250

Liao, S., Dong, W., Lv, L., Guo, H., Yang, J., Zhao, H., Huang, R., Yuan, Z., Chen, Y., Feng, S., Zheng, X., Huang, J., Huang, W., Qi, X., & Cai, D. (2017). Heart regeneration in adult *Xenopus tropicalis* after apical resection. *Cell & Bioscience*, 7(1), 70. https://doi.org/10.1186/s13578-017-0199-6

Liau, B., Christoforou, N., Leong, K., & Bursac, N. (2011). Pluripotent stem cell-derived cardiac tissue patch with advanced structure and function. *Biomaterials*, 32(35), 9180–9187. https://doi.org/10.1016/j.biomaterials.2011.08.050

Liau, B., Jackman, C. P., Li, Y., & Bursac, N. (2017). Developmental stage-dependent effects of cardiac fibroblasts on function of stem cell-derived engineered cardiac tissues. *Scientific Reports*, 7(1), 42290. https://doi.org/10.1038/srep42290

Lopaschuk, G. D., Collins-Nakai, R. L., & Itoi, T. (1992). Developmental changes in energy substrate use by the heart. *Cardiovascular Research*, 26(12), 1172–1180. https://doi.org/10.1093/cvr/26.12.1172

Love, N. R., Chen, Y., Ishibashi, S., Kritsiligkou, P., Lea, R., Koh, Y., Gallop, J. L., Dorey, K., & Amaya, E. (2013). Amputation-induced reactive oxygen species are required for successful Xenopus tadpole tail regeneration. *Nature Cell Biology*, 15(2), 222–228. https://doi.org/10.1038/ncb2659

Lynch, K. M., & Ahsan, T. (2013). Modulating the physical microenvironment to study regenerative processes *in vitro* using cells from mouse phalangeal elements. *Tissue Engineering Part A*, 19(11–12), 1406–1415. https://doi.org/10.1089/ten.tea.2012.0503

Lynch, K. M., & Ahsan, T. (2014). Correlating the effects of bone morphogenic protein to secreted soluble factors from fibroblasts and mesenchymal stem cells in regulating regenerative processes *in vitro*. *Tissue Engineering Part A*, 20(23–24), 3122–3129. https://doi.org/10.1089/ten.tea.2014.0278

Magadum, A., Singh, N., Kurian, A. A., Munir, I., Mehmood, T., Brown, K., Sharkar, M. T. K., Chepurko, E., Sassi, Y., Oh, J. G., Lee, P., Santos, C. X., Gaziel-Sovran, A., Zhang, G., Cai, C.-L., Kho, C., Mayr, M., Shah, A. M., Hajjar, R. J., & Zangi, L. (2020). Pkm2 regulates cardiomyocyte cell cycle and promotes cardiac regeneration. *Circulation*, 141(15), 1249–1265. https://doi.org/10.1161/CIRCULATIONAHA.119.043067

Mahmoud, A. I., O'Meara, C. C., Gemberling, M., Zhao, L., Bryant, D. M., Zheng, R., Gannon, J. B., Cai, L., Choi, W.-Y., Egnaczyk, G. F., Burns, C. E., Burns, C. G., MacRae, C. A., Poss, K. D., & Lee, R. T. (2015). Nerves regulate cardiomyocyte proliferation and heart regeneration. *Developmental Cell*, 34(4), 387–399. https://doi.org/10.1016/j.devcel.2015.06.017

Maillet, M., van Berlo, J. H., & Molkentin, J. D. (2013). Molecular basis of physiological heart growth: Fundamental concepts and new players. *Nature Reviews Molecular Cell Biology*, 14(1), 38–48. https://doi.org/10.1038/nrm3495

Makarieva, A. M., Gorshkov, V. G., Li, B.-L., Chown, S. L., Reich, P. B., & Gavrilov, V. M. (2008). Mean mass-specific metabolic rates are strikingly similar across life's major domains: Evidence for life's metabolic optimum. *Proceedings of the National Academy of Sciences of the United States of America*, 105(44), 16994–16999. https://doi.org/10.1073/pnas.0802148105

Maliken, B. D., & Molkentin, J. D. (2018). Undeniable evidence that the adult mammalian heart lacks an endogenous regenerative stem cell. *Circulation*, 138(8), 806–808. https://doi.org/10.1161/CIRCULATIONAHA.118.035186

María Aleksandrovna Vorontsova, & Liosner, L. D. (1960). Asexual propagation and regeneration (Vol. 67). Pergamon Press.

Marshall, L., Girardot, F., Demeneix, B. A., & Coen, L. (2018). Is adult cardiac regeneration absent in *Xenopus laevis* yet present in *Xenopus tropicalis*? *Cell & Bioscience*, 8, 31. https://doi.org/10.1186/s13578-018-0231-5

Marshall, L., Vivien, C., Girardot, F., Péricard, L., Demeneix, B. A., Coen, L., & Chai, N. (2017). Persistent fibrosis, hypertrophy and sarcomere disorganisation after endoscopy-guided heart resection in adult *Xenopus*. *PloS One*, 12(3), e0173418. https://doi.org/10.1371/journal.pone.0173418

Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000prime Reports*, 6, 13. https://doi.org/10.12703/P6-13

McCusker, C., Bryant, S. V., & Gardiner, D. M. (2015). The axolotl limb blastema: Cellular and molecular mechanisms driving blastema formation and limb regeneration in tetrapods. *Regeneration*, 2(2), 54–71. https://doi.org/10.1002/reg2.32

Mescher, A. L., & Neff, A. W. (2005). Regenerative capacity and the developing immune system. *Advances in Biochemical Engineering/Biotechnology*, 93, 39–66. https://doi.org/10.1007/b99966

Min, Y.-L., Jaichander, P., Sanchez-Ortiz, E., Bezprozvannaya, S., Malladi, V. S., Cui, M., Wang, Z., Bassel-Duby, R., Olson, E. N., & Liu, N. (2018). Identification of a multipotent Twist2-expressing cell population in the adult heart. *Proceedings of the National Academy of Sciences*, 115(36). https://doi.org/10.1073/pnas.1800526115

Miteva, K., Pappritz, K., El-Shafeey, M., Dong, F., Ringe, J., Tschöpe, C., & Van Linthout, S. (2017). Mesenchymal stromal cells modulate monocytes trafficking in Coxsackievirus B3-induced myocarditis. *Stem Cells Translational Medicine*, 6(4), 1249–1261. https://doi.org/10.1002/sctm.16-0353

Mocatta, T. J., Pilbrow, A. P., Cameron, V. A., Senthilmohan, R., Frampton, C. M., Richards, A. M., & Winterbourn, C. C. (2007). Plasma concentrations of myeloperoxidase predict mortality after myocardial infarction. *Journal of the American College of Cardiology*, 49(20), 1993–2000. https://doi.org/10.1016/j.jacc.2007.02.040

Mohammad, K. S., & Neufeld, D. A. (2000). Denervation retards but does not prevent toetip regeneration. *Wound Repair and Regeneration: Official Publication of the Wound Healing Society [and] the European Tissue Repair Society*, 8(4), 277–281. https://doi.org/10.1046/j.1524-475x.2000.00277.x

Morgan, T. H. (1901). *Regeneration*. New York: Macmillan.

Morgan, T. H. (1902). The reflexes connected with autotomy in the hermit-crab. *American Journal of Physiology-Legacy Content*, *6*(5), 278–282. https://doi.org/10.1152/ajplegacy.1902.6.5.278

Morikawa, Y., Heallen, T., Leach, J., Xiao, Y., & Martin, J. F. (2017). Dystrophin-glycoprotein complex sequesters Yap to inhibit cardiomyocyte proliferation. *Nature*, 547(7662), 227–231. https://doi.org/10.1038/nature22979

Muneoka, K., Allan, C. H., Yang, X., Lee, J., & Han, M. (2008). Mammalian regeneration and regenerative medicine. *Birth Defects Research Part C: Embryo Today: Review*s, 84(4), 265–280. https://doi.org/10.1002/bdrc.20137

Muntean, D. M., Sturza, A., Dănilă, M. D., Borza, C., Duicu, O. M., & Mornoș, C. (2016). The role of mitochondrial reactive oxygen species in cardiovascular injury and protective strategies. *Oxidative Medicine and Cellular Longevity*, 2016, e8254942. https://doi.org/10.1155/2016/8254942

Murao, H., Yamamoto, K., Matsuda, S., & Akiyama, H. (2013). Periosteal cells are a major source of soft callus in bone fracture. *Journal of Bone and Mineral Metabolism*, 31(4), 390–398. https://doi.org/10.1007/s00774-013-0429-x

Murphy, E., & Steenbergen, C. (2008). Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiological Reviews*, 88(2), 581–609. https://doi.org/10.1152/physrev.00024.2007

Murry, C. E., Reinecke, H., & Pabon, L. M. (2006). Regeneration gaps: Observations on stem cells and cardiac repair. *Journal of the American College of Cardiology*, 47(9), 1777–1785. https://doi.org/10.1016/j.jacc.2006.02.002

Nahrendorf, M., Swirski, F. K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J.-L., Libby, P., Weissleder, R., & Pittet, M. J. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *The Journal of Experimental Medicine*, 204(12), 3037–3047. https://doi.org/10.1084/jem.20070885

Nakada, Y., Canseco, D. C., Thet, S., Abdisalaam, S., Asaithamby, A., Santos, C. X., Shah, A. M., Zhang, H., Faber, J. E., Kinter, M. T., Szweda, L. I., Xing, C., Hu, Z., Deberardinis, R. J., Schiattarella, G., Hill, J. A., Oz, O., Lu, Z., Zhang, C. C., … Sadek, H. A. (2017). Hypoxia induces heart regeneration in adult mice. *Nature*, 541(7636), 222–227. https://doi.org/10.1038/nature20173

Neufeld, D. A., & Zhao, W. (1995). Bone regrowth after digit tip amputation in mice is equivalent in adults and neonates. *Wound Repair and Regeneration: Official Publication of the Wound Healing Society [and] the European Tissue Repair Society*, 3(4), 461–466. https://doi.org/10.1046/j.1524-475X.1995.30410.x

Niethammer, P., Grabher, C., Look, A. T., & Mitchison, T. J. (2009). A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature*, 459(7249), Article 7249. https://doi.org/10.1038/nature08119

Noseda, M., Harada, M., McSweeney, S., Leja, T., Belian, E., Stuckey, D. J., Abreu Paiva, M. S., Habib, J., Macaulay, I., de Smith, A. J., al-Beidh, F., Sampson, R., Lumbers, R. T., Rao, P., Harding, S. E., Blakemore, A. I. F., Eirik Jacobsen, S., Barahona, M., & Schneider, M. D. (2015). PDGFRα demarcates the cardiogenic clonogenic Sca1+ stem/progenitor cell in adult murine myocardium. *Nature Communications*, 6(1), Article 1. https://doi.org/10.1038/ncomms7930

Notari, M., Ventura-Rubio, A., Bedford-Guaus, S. J., Jorba, I., Mulero, L., Navajas, D., Martí, M., & Raya, Á. (2018a). The local microenvironment limits the regenerative potential of the mouse neonatal heart. *Science Advances*, 4(5), eaao5553. https://doi.org/10.1126/sciadv.aao5553

Novak, M. L., & Koh, T. J. (2013). Macrophage phenotypes during tissue repair. *Journal of Leukocyte Biology*, 93(6), 875–881. https://doi.org/10.1189/jlb.1012512

Nussbaum, J., Minami, E., Laflamme, M. A., Virag, J. A. I., Ware, C. B., Masino, A., Muskheli, V., Pabon, L., Reinecke, H., & Murry, C. E. (2007). Transplantation of undifferentiated murine embryonic stem cells in the heart: Teratoma formation and immune response. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 21(7), 1345–1357. https://doi.org/10.1096/fj.06-6769com

Odashima, M., Usui, S., Takagi, H., Hong, C., Liu, J., Yokota, M., & Sadoshima, J. (2007). Inhibition of endogenous *Mst1* prevents apoptosis and cardiac dysfunction without affecting cardiac hypertrophy after myocardial infarction. *Circulation Researc*h, 100(9), 1344–1352. https://doi.org/10.1161/01.RES.0000265846.23485.7a

Oh, H., Bradfute, S. B., Gallardo, T. D., Nakamura, T., Gaussin, V., Mishina, Y., Pocius, J., Michael, L. H., Behringer, R. R., Garry, D. J., Entman, M. L., & Schneider, M. D. (2003). Cardiac progenitor cells from adult myocardium: Homing, differentiation, and fusion after infarction. *Proceedings of the National Academy of Sciences*, 100(21), 12313–12318. https://doi.org/10.1073/pnas.2132126100

Pase, L., Layton, J. E., Wittmann, C., Ellett, F., Nowell, C. J., Reyes-Aldasoro, C. C., Varma, S., Rogers, K. L., Hall, C. J., Keightley, M. C., Crosier, P. S., Grabher, C., Heath, J. K., Renshaw, S. A., & Lieschke, G. J. (2012). Neutrophil-delivered myeloperoxidase dampens the hydrogen peroxide burst after tissue wounding in zebrafish. *Current Biology*, 22(19), 1818–1824. https://doi.org/10.1016/j.cub.2012.07.060

Petrie, T. A., Strand, N. S., Tsung-Yang, C., Rabinowitz, J. S., & Moon, R. T. (2014). Macrophages modulate adult zebrafish tail fin regeneration. *Development* (Cambridge, England), 141(13), 2581–2591. https://doi.org/10.1242/dev.098459

Pfister, O., Mouquet, F., Jain, M., Summer, R., Helmes, M., Fine, A., Colucci, W. S., & Liao, R. (2005). CD31 − but not CD31 + cardiac side population cells exhibit functional cardiomyogenic differentiation. *Circulation Research*, 97(1), 52–61. https://doi.org/10.1161/01.RES.0000173297.53793.fa

Piao, L., Fang, Y.-H., Kubler, M. M., Donnino, M. W., & Sharp, W. W. (2017). Enhanced pyruvate dehydrogenase activity improves cardiac outcomes in a murine model of cardiac arrest. *PLoS ONE,* 12(9), e0185046. https://doi.org/10.1371/journal.pone.0185046

Pinto, A. R., Ilinykh, A., Ivey, M. J., Kuwabara, J. T., D'Antoni, M. L., Debuque, R., Chandran, A., Wang, L., Arora, K., Rosenthal, N. A., & Tallquist, M. D. (2016). Revisiting cardiac cellular composition. *Circulation Research*, 118(3), 400–409. https://doi.org/10.1161/CIRCRESAHA.115.307778

Pittenger, M. F., & Martin, B. J. (2004). Mesenchymal stem cells and their potential as cardiac therapeutics. *Circulation Research*, 95(1), 9–20. https://doi.org/10.1161/01.RES.0000135902.99383.6f

Plikus, M. V., Wang, X., Sinha, S., Forte, E., Thompson, S. M., Herzog, E. L., Driskell, R. R., Rosenthal, N., Biernaskie, J., & Horsley, V. (2021). Fibroblasts: Origins, definitions, and functions in health and disease. *Cell*, 184(15), 3852–3872. https://doi.org/10.1016/j.cell.2021.06.024

Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N., & Sadek, H. A. (2011). Transient regenerative potential of the neonatal mouse heart. *Science* (New York, N.Y.), 331(6020), 1078–1080. https://doi.org/10.1126/science.1200708

Porrello, E. R., Mahmoud, A. I., Simpson, E., Johnson, B. A., Grinsfelder, D., Canseco, D., Mammen, P. P., Rothermel, B. A., Olson, E. N., & Sadek, H. A. (2013). Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. *Proceedings of the National Academy of Sciences of the United States of America*, 110(1), 187–192. https://doi.org/10.1073/pnas.1208863110

Pouly, J., Bruneval, P., Mandet, C., Proksch, S., Peyrard, S., Amrein, C., Bousseaux, V., Guillemain, R., Deloche, A., Fabiani, J.-N., & Menasché, P. (2008). Cardiac stem cells in the real world. *The Journal of Thoracic and Cardiovascular Surgery*, 135(3), 673–678. https://doi.org/10.1016/j.jtcvs.2007.10.024

Prall, O. W. J., Menon, M. K., Solloway, M. J., Watanabe, Y., Zaffran, S., Bajolle, F., Biben, C., McBride, J. J., Robertson, B. R., Chaulet, H., Stennard, F. A., Wise, N., Schaft, D., Wolstein, O., Furtado, M. B., Shiratori, H., Chien, K. R., Hamada, H., Black, B. L., … Harvey, R. P. (2007). An *Nkx2-5/Bmp2/Smad1* negative feedback loop controls heart progenitor specification and proliferation. *Cell*, 128(5), 947–959. https://doi.org/10.1016/j.cell.2007.01.042

Protze, S., Khattak, S., Poulet, C., Lindemann, D., Tanaka, E. M., & Ravens, U. (2012). A new approach to transcription factor screening for reprogramming of fibroblasts to cardiomyocyte-like cells. *Journal of Molecular and Cellular Cardiology*, 53(3), 323–332. https://doi.org/10.1016/j.yjmcc.2012.04.010

Puente, B. N., Kimura, W., Muralidhar, S. A., Moon, J., Amatruda, J. F., Phelps, K. L., Grinsfelder, D., Rothermel, B. A., Chen, R., Garcia, J. A., Santos, C. X., Thet, S., Mori, E., Kinter, M. T., Rindler, P. M., Zacchigna, S., Mukherjee, S., Chen, D. J., Mahmoud, A. I., … Sadek, H. A. (2014). The oxygen rich postnatal environment induces cardiomyocyte cell cycle arrest through DNA damage response. *Cell*, 157(3), 565–579. https://doi.org/10.1016/j.cell.2014.03.032

Ramjee, V., Li, D., Manderfield, L. J., Liu, F., Engleka, K. A., Aghajanian, H., Rodell, C. B., Lu, W., Ho, V., Wang, T., Li, L., Singh, A., Cibi, D. M., Burdick, J. A., Singh, M. K., Jain, R., & Epstein, J. A. (2017). Epicardial YAP/TAZ orchestrate an immunosuppressive response following myocardial infarction. *The Journal of Clinical Investigation*, 127(3), 899–911. https://doi.org/10.1172/JCI88759

Réaumur, R. A. F. (1712). Sur les diverses reproductions qui se font dans les Écrevisses, les Omards, les Crabes, etc., et entr'autres sur celles de leurs jambes et de leurs écailles. *Mem. Acad. Roy. Sci*., 223–245.

Reddien, P. W., Bermange, A. L., Murfitt, K. J., Jennings, J. R., & Sánchez Alvarado, A. (2005). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. *Developmental Cell*, 8(5), 635–649. https://doi.org/10.1016/j.devcel.2005.02.014

Richardson, M. K., & Chipman, A. D. (2003). Developmental constraints in a comparative framework: A test case using variations in phalanx number during amniote evolution. *Journal of Experimental Zoology Part B, Molecular and Developmental Evolution*, 296(1), 8–22. https://doi.org/10.1002/jez.b.13

Rinkevich, Y., Lindau, P., Ueno, H., Longaker, M. T., & Weissman, I. L. (2011). Germ-layer and lineage-restricted stem/progenitors regenerate the mouse digit tip. *Nature*, 476(7361), 409–413. https://doi.org/10.1038/nature10346

Rinkevich, Y., Montoro, D. T., Muhonen, E., Walmsley, G. G., Lo, D., Hasegawa, M., Januszyk, M., Connolly, A. J., Weissman, I. L., & Longaker, M. T. (2014). Clonal analysis reveals nerve-dependent and independent roles on mammalian hind limb tissue maintenance and regeneration. *Proceedings of the National Academy of Sciences of the United States of America*, 111(27), 9846–9851. https://doi.org/10.1073/pnas.1410097111

Ruckh, J. M., Zhao, J.-W., Shadrach, J. L., van Wijngaarden, P., Rao, T. N., Wagers, A. J., & Franklin, R. J. M. (2012). Rejuvenation of regeneration in the aging central nervous system. *Cell Stem Cell*, 10(1), 96–103. https://doi.org/10.1016/j.stem.2011.11.019

Sadoshima, J., & Weiss, J. N. (2014). Cardiac fibroblasts: The good, the bad, the ugly, the beautiful. *Journal of Molecular and Cellular Cardiology*, 70, 1. https://doi.org/10.1016/j.yjmcc.2014.03.009

Said, S., Parke, W., & Neufeld, D. A. (2004). Vascular supplies differ in regenerating and nonregenerating amputated rodent digits. *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology*, 278A(1), 443–449. https://doi.org/10.1002/ar.a.20034

Sammarco, M. C., Simkin, J., Fassler, D., Cammack, A. J., Wilson, A., Van Meter, K., & Muneoka, K. (2014). Endogenous bone regeneration is dependent upon a dynamic oxygen event. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, 29(11), 2336–2345. https://doi.org/10.1002/jbmr.2261

Sánchez-Iranzo, H., Galardi-Castilla, M., Sanz-Morejón, A., González-Rosa, J. M., Costa, R., Ernst, A., Sainz de Aja, J., Langa, X., & Mercader, N. (2018). Transient fibrosis resolves via fibroblast inactivation in the regenerating zebrafish heart. *Proceedings of the National Academy of Sciences*, 115(16), 4188–4193. https://doi.org/10.1073/pnas.1716713115

Sande-Melón, M., Marques, I. J., Galardi-Castilla, M., Langa, X., Pérez-López, M., Botos, M.-A., Sánchez-Iranzo, H., Guzmán-Martínez, G., Ferreira Francisco, D. M., Pavlinic, D., Benes, V., Bruggmann, R., & Mercader, N. (2019). Adult *sox10*+ cardiomyocytes contribute to myocardial regeneration in the zebrafish. *Cell Reports*, 29(4), 1041-1054.e5. https://doi.org/10.1016/j.celrep.2019.09.041

Sandoval-Guzmán, T., Wang, H., Khattak, S., Schuez, M., Roensch, K., Nacu, E., Tazaki, A., Joven, A., Tanaka, E. M., & Simon, A. (2014). Fundamental differences in dedifferentiation and stem cell recruitment during skeletal muscle regeneration in two salamander species. *Cell Stem Cell*, 14(2), 174–187. https://doi.org/10.1016/j.stem.2013.11.007

Sattler, S., Fairchild, P., Watt, F. M., Rosenthal, N., & Harding, S. E. (2017). The adaptive immune response to cardiac injury—The true roadblock to effective regenerative

therapies? *Npj Regenerative Medicine*, 2(1), Article 1. https://doi.org/10.1038/s41536-017-0022-3

Schmuck, E. G., Mulligan, J. D., Ertel, R. L., Kouris, N. A., Ogle, B. M., Raval, A. N., & Saupe, K. W. (2014). Cardiac fibroblast-derived 3D extracellular matrix seeded with mesenchymal stem cells as a novel device to transfer cells to the ischemic myocardium. *Cardiovascular Engineering and Technology*, 5(1), 119–131. https://doi.org/10.1007/s13239-013-0167-1

Seifert, A. W., Kiama, S. G., Seifert, M. G., Goheen, J. R., Palmer, T. M., & Maden, M. (2012). Skin shedding and tissue regeneration in African spiny mice (*Acomys*). *Nature*, 489(7417), Article 7417. https://doi.org/10.1038/nature11499

Seifert, A. W., Monaghan, J. R., Voss, S. R., & Maden, M. (2012). Skin regeneration in adult axolotls: A blueprint for scar-free healing in vertebrates. *PloS One,* 7(4), e32875. https://doi.org/10.1371/journal.pone.0032875

Seifert, A. W., & Muneoka, K. (2018). The blastema and epimorphic regeneration in mammals. *Developmental Biology*, 433(2), 190–199. https://doi.org/10.1016/j.ydbio.2017.08.007

Seifert, A. W., & Voss, S. R. (2013). Revisiting the relationship between regenerative ability and aging. BMC Biology, 11, 2. https://doi.org/10.1186/1741-7007-11-2

Semenza, G. L. (2010). Vascular responses to hypoxia and ischemia. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 30(4), 648–652. https://doi.org/10.1161/ATVBAHA.108.181644

Sensiate, L. A., & Marques-Souza, H. (2019). Bone growth as the main determinant of mouse digit tip regeneration after amputation. *Scientific Reports*, 9(1), 9720. https://doi.org/10.1038/s41598-019-45521-4

Shao, D., Zhai, P., Del Re, D. P., Sciarretta, S., Yabuta, N., Nojima, H., Lim, D.-S., Pan, D., & Sadoshima, J. (2014). A functional interaction between Hippo-YAP signaling and *FoxO1* mediates the oxidative stress response. *Nature Communications*, 5, 3315. https://doi.org/10.1038/ncomms4315

Shiba, Y., Gomibuchi, T., Seto, T., Wada, Y., Ichimura, H., Tanaka, Y., Ogasawara, T., Okada, K., Shiba, N., Sakamoto, K., Ido, D., Shiina, T., Ohkura, M., Nakai, J., Uno, N., Kazuki, Y., Oshimura, M., Minami, I., & Ikeda, U. (2016). Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. *Nature*, 538(7625), 388–391. https://doi.org/10.1038/nature19815

Shiraishi, M., Shintani, Y., Shintani, Y., Ishida, H., Saba, R., Yamaguchi, A., Adachi, H., Yashiro, K., & Suzuki, K. (2016). Alternatively activated macrophages determine repair of the infarcted adult murine heart. *The Journal of Clinical Investigatio*n, 126(6), 2151–2166. https://doi.org/10.1172/JCI85782

Signer, R. A. J., & Morrison, S. J. (2013). Mechanisms that regulate stem cell aging and life span. *Cell Stem Cell*, 12(2), 152–165. https://doi.org/10.1016/j.stem.2013.01.001

Simkin, J., Sammarco, M. C., Marrero, L., Dawson, L. A., Yan, M., Tucker, C., Cammack, A., & Muneoka, K. (2017a). Macrophages are required to coordinate mouse digit tip regeneration. *Development* (Cambridge, England), 144(21), 3907–3916. https://doi.org/10.1242/dev.150086

Simkin, J., Gawriluk, T. R., Gensel, J. C., & Seifert, A. W. (2017b). Macrophages are necessary for epimorphic regeneration in African spiny mice. *ELife,* 6, e24623. https://doi.org/10.7554/eLife.24623

Simkin, J., Sammarco, M. C., Dawson, L. A., Tucker, C., Taylor, L. J., Van Meter, K., & Muneoka, K. (2015a). Epidermal closure regulates histolysis during mammalian (*Mus*) digit regeneration. *Regeneration* (Oxford, England), 2(3), 106–119. https://doi.org/10.1002/reg2.34

Simkin, J., Sammarco, M. C., Dawson, L. A., Schanes, P. P., Yu, L., & Muneoka, K. (2015b). The mammalian blastema: Regeneration at our fingertips. *Regeneration*, 2(3), 93–105. https://doi.org/10.1002/reg2.36

Simões, F. C., Cahill, T. J., Kenyon, A., Gavriouchkina, D., Vieira, J. M., Sun, X., Pezzolla, D., Ravaud, C., Masmanian, E., Weinberger, M., Mayes, S., Lemieux, M. E., Barnette, D. N., Gunadasa-Rohling, M., Williams, R. M., Greaves, D. R., Trinh, L. A., Fraser, S. E., Dallas, S. L., … Riley, P. R. (2020). Macrophages directly contribute collagen to scar formation during zebrafish heart regeneration and mouse heart repair. *Nature Communications*, 11(1), 600. https://doi.org/10.1038/s41467-019-14263-2

Singam, N. S. V., Fine, C., & Fleg, J. L. (2020). Cardiac changes associated with vascular aging. *Clinical Cardiology*, 43(2), 92–98. https://doi.org/10.1002/clc.23313

Singh, P., Goode, T., Dean, A., Awad, S. S., & Darlington, G. J. (2011). Elevated interferon gamma signaling contributes to impaired regeneration in the aged liver. *The Journals of Gerontology: Series A*, 66A(9), 944–956. https://doi.org/10.1093/gerona/glr094

Sinigaglia, C., Alié, A., & Tiozzo, S. (2022). The Hazards of Regeneration: From Morgan's Legacy to Evo-Devo. *Methods in Molecular Biology* (Clifton, N.j.), 2450, 3–25. https://doi.org/10.1007/978-1-0716-2172-1_1

Soehnlein, O., & Lindbom, L. (2010). Phagocyte partnership during the onset and resolution of inflammation. *Nature Reviews Immunology*, 10(6), Article 6. https://doi.org/10.1038/nri2779

Song, K., Nam, Y.-J., Luo, X., Qi, X., Tan, W., Huang, G. N., Acharya, A., Smith, C. L., Tallquist, M. D., Neilson, E. G., Hill, J. A., Bassel-Duby, R., & Olson, E. N. (2012). Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature*, 485(7400), 599–604. https://doi.org/10.1038/nature11139

Soonpaa, M. H., Kim, K. K., Pajak, L., Franklin, M., & Field, L. J. (1996). Cardiomyocyte DNA synthesis and binucleation during murine development. *The American Journal of Physiology*, 271(5 Pt 2), H2183-2189. https://doi.org/10.1152/ajpheart.1996.271.5.H2183

Soonpaa, M. H., Lafontant, P. J., Reuter, S., Scherschel, J. A., Srour, E. F., Zaruba, M.-M., Rubart-von der Lohe, M., & Field, L. J. (2018). Absence of cardiomyocyte differentiation following transplantation of adult cardiac-resident Sca-1+ cells into infarcted mouse hearts. *Circulation*, 138(25), 2963–2966. https://doi.org/10.1161/CIRCULATIONAHA.118.035391

Staller, P., Sulitkova, J., Lisztwan, J., Moch, H., Oakeley, E. J., & Krek, W. (2003). Chemokine receptor CXCR4 downregulated by von Hippel–Lindau tumour suppressor pVHL. *Nature*, 425(6955), 307–311. https://doi.org/10.1038/nature01874

Stocum, D. L., & Thoms, S. D. (1984). Retinoic-acid-induced pattern completion in regenerating double anterior limbs of urodeles. *Journal of Experimental Zoology*, 232(2), 207–215. https://doi.org/10.1002/jez.1402320208

Sugden, M. C., Langdown, M. L., Harris, R. A., & Holness, M. J. (2000). Expression and regulation of pyruvate dehydrogenase kinase isoforms in the developing rat heart and in adulthood: Role of thyroid hormone status and lipid supply. *Biochemical Journal*, 352(Pt 3), 731–738.

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126(4), 663–676. https://doi.org/10.1016/j.cell.2006.07.024

Takeo, M., Chou, W. C., Sun, Q., Lee, W., Rabbani, P., Loomis, C., Taketo, M. M., & Ito, M. (2013). Wnt activation in nail epithelium couples nail growth to digit regeneration. *Nature*, 499(7457), 228–232. https://doi.org/10.1038/nature12214

Tang, W., Martik, M. L., Li, Y., & Bronner, M. E. (2019). Cardiac neural crest contributes to cardiomyocytes in amniotes and heart regeneration in zebrafish. *ELife*, 8, e47929. https://doi.org/10.7554/eLife.47929

Taylor, G. P., Anderson, R., Reginelli, A. D., & Muneoka, K. (1994). FGF-2 induces regeneration of the chick limb bud. *Developmental Biology*, 163(1), 282–284. https://doi.org/10.1006/dbio.1994.1144

Thakker, R., & Yang, P. (2014). Mesenchymal stem cell therapy for cardiac repair. *Current Treatment Options in Cardiovascular Medicin*e, 16(7), 323. https://doi.org/10.1007/s11936-014-0323-4

Thomas, M. V., & Puleo, D. A. (2011). Infection, inflammation, and bone regeneration. *Journal of Dental Research*, 90(9), 1052–1061. https://doi.org/10.1177/0022034510393967

Thornton, C. S. (1957). The effect of apical cap removal on limb regeneration in *Amblystoma* larvae. *The Journal of Experimental Zoology*, 134(2), 357–381. https://doi.org/10.1002/jez.1401340209

Tourneux, P., Libert, J.-P., Ghyselen, L., Léké, A., Delanaud, S., Dégrugilliers, L., & Bach, V. (2009). [Heat exchanges and thermoregulation in the neonate]. *Archives De Pediatrie: Organe Officiel De La Societe Francaise De Pediatrie*, 16(7), 1057–1062. https://doi.org/10.1016/j.arcped.2009.03.014

Tuncay, O. C., Ho, D., & Barker, M. K. (1994). Oxygen tension regulates osteoblast function. *American Journal of Orthodontics and Dentofacial Orthopedics: Official Publication of the American Association of Orthodontists, Its Constituent Societies, and the American Board of Orthodontics*, 105(5), 457–463. https://doi.org/10.1016/S0889-5406(94)70006-0

Tuomainen, T., & Tavi, P. (2017). The role of cardiac energy metabolism in cardiac hypertrophy and failure. *Experimental Cell Research*, 360(1), 12–18. https://doi.org/10.1016/j.yexcr.2017.03.052

Trembley, A. (1744). *Memoires pour servir a l'histoire d'un genre de polypes d'eau douce a bras en forme de cornes*. Leiden, The Netherlands: J. & H. Verbeek.

Uchida, S., De Gaspari, P., Kostin, S., Jenniches, K., Kilic, A., Izumiya, Y., Shiojima, I., grosse Kreymborg, K., Renz, H., Walsh, K., & Braun, T. (2013). *Sca1*-derived cells are a source of myocardial renewal in the murine adult heart. *Stem Cell Reports*, 1(5), 397–410. https://doi.org/10.1016/j.stemcr.2013.09.004

Vagnozzi, R. J., Maillet, M., Sargent, M. A., Khalil, H., Johansen, A. K., Schwanekamp, J. A., York, A. J., Huang, V., Nahrendorf, M., Sadayappan, S., & Molkentin, J. D. (2020). An acute immune response underlies the benefit of cardiac stem cell therapy. *Nature*, 577(7790), 405–409. https://doi.org/10.1038/s41586-019-1802-2

van Berlo, J. H., Kanisicak, O., Maillet, M., Vagnozzi, R. J., Karch, J., Lin, S.-C. J., Middleton, R. C., Marbán, E., & Molkentin, J. D. (2014). *c-kit*+ cells minimally contribute cardiomyocytes to the heart. *Nature*, 509(7500), 337–341. https://doi.org/10.1038/nature13309

VanDusen, N. J., & Firulli, A. B. (2012). *Twist* factor regulation of non-cardiomyocyte cell lineages in the developing heart. *Differentiation; Research in Biological Diversity*, 84(1), 79–88. https://doi.org/10.1016/j.diff.2012.03.002

Velagaleti, R. S., Larson, M. G., Enserro, D., Song, R. J., & Vasan, R. S. (2020). Clinical course after a first episode of heart failure: Insights from the Framingham Heart Study. *European Journal of Heart Failure*, 22(10), 1768–1776. https://doi.org/10.1002/ejhf.1918

Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Südhof, T. C., & Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. *Nature*, 463(7284), 1035–1041. https://doi.org/10.1038/nature08797

Villeda, S. A., Luo, J., Mosher, K. I., Zou, B., Britschgi, M., Bieri, G., Stan, T. M., Fainberg, N., Ding, Z., Eggel, A., Lucin, K. M., Czirr, E., Park, J.-S., Couillard-Després, S., Aigner, L., Li, G., Peskind, E. R., Kaye, J. A., Quinn, J. F., … Wyss-Coray, T. (2011). The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature*, 477(7362), Article 7362. https://doi.org/10.1038/nature10357

Vite, A., Zhang, C., Yi, R., Emms, S., & Radice, G. L. (2018). α-Catenin-dependent cytoskeletal tension controls *Yap* activity in the heart. *Development*, 145(5), dev149823. https://doi.org/10.1242/dev.149823

von Gise, A., Lin, Z., Schlegelmilch, K., Honor, L. B., Pan, G. M., Buck, J. N., Ma, Q., Ishiwata, T., Zhou, B., Camargo, F. D., & Pu, W. T. (2012). YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proceedings of the National Academy of Sciences of the United States of America*, 109(7), 2394–2399. https://doi.org/10.1073/pnas.1116136109

Wagner, G. P. (2007). The developmental genetics of homology. *Nature Reviews. Genetics*, 8(6), 473–479. https://doi.org/10.1038/nrg2099

Wang, G., Miyahara, Y., Guo, Z., Khattar, M., Stepkowski, S. M., & Chen, W. (2010). "Default" generation of neonatal regulatory T cells. *The Journal of Immunology*, 185(1), 71–78. https://doi.org/10.4049/jimmunol.0903806

Wang, H., & Simon, A. (2016). Skeletal muscle dedifferentiation during salamander limb regeneration. *Current Opinion in Genetics & Development*, 40, 108–112. https://doi.org/10.1016/j.gde.2016.06.013

Wang, J., Chen, H., Seth, A., & McCulloch, C. A. (2003). Mechanical force regulation of myofibroblast differentiation in cardiac fibroblasts. *American Journal of Physiology: Heart and Circulatory Physiology*, 285(5), H1871-1881. https://doi.org/10.1152/ajpheart.00387.2003

Wang, J., Karra, R., Dickson, A. L., & Poss, K. D. (2013). Fibronectin is deposited by injury-activated epicardial cells and is necessary for zebrafish heart regeneration. *Developmental Biology*, 382(2), 427–435. https://doi.org/10.1016/j.ydbio.2013.08.012

Wang, J., Liu, S., Heallen, T., & Martin, J. F. (2018). The Hippo pathway in the heart: Pivotal roles in development, disease, and regeneration. *Nature Reviews Cardiology*, 15(11), Article 11. https://doi.org/10.1038/s41569-018-0063-3

Wang, Y., Yao, F., Wang, L., Li, Z., Ren, Z., Li, D., Zhang, M., Han, L., Wang, S., Zhou, B., & Wang, L. (2020). Single-cell analysis of murine fibroblasts identifies neonatal to adult switching that regulates cardiomyocyte maturation. *Nature Communications*, 11(1), Article 1. https://doi.org/10.1038/s41467-020-16204-w

Wang, Y.-T., Tseng, T.-L., Kuo, Y.-C., Yu, J.-K., Su, Y.-H., Poss, K. D., & Chen, C.-H. (2019a). Genetic reprogramming of positional memory in a regenerating appendage. *Current Biology*, 29(24), 4193-4207.e4. https://doi.org/10.1016/j.cub.2019.10.038

Wehman, B., Sharma, S., Pietris, N., Mishra, R., Siddiqui, O. T., Bigham, G., Li, T., Aiello, E., Murthi, S., Pittenger, M., Griffith, B., & Kaushal, S. (2016). Mesenchymal stem cells preserve neonatal right ventricular function in a porcine model of pressure overload. *American Journal of Physiology. Heart and Circulatory Physiology,* 310(11), H1816-1826. https://doi.org/10.1152/ajpheart.00955.2015

Weirather, J., Hofmann, U. D. W., Beyersdorf, N., Ramos, G. C., Vogel, B., Frey, A., Ertl, G., Kerkau, T., & Frantz, S. (2014). *Foxp3*+ CD4+ T cells improve healing after myocardial infarction by modulating monocyte/macrophage differentiation. *Circulation Research*, 115(1), 55–67. https://doi.org/10.1161/CIRCRESAHA.115.303895

Weismann, August. *Das Keimplasma: eine Theorie der Vererbung*, 1892. Translated by W. Newton Parker and Harriet Rönnfeldt as *The Germ-Plasm: a Theory of Heredity*. New York: Scribner, 1893. http://dx.doi.org/10.5962/bhl.title.25196

White, I. A., Gordon, J., Balkan, W., & Hare, J. M. (2015). Sympathetic reinnervation is required for mammalian cardiac regeneration. *Circulation Research*, 117(12), 990–994. https://doi.org/10.1161/CIRCRESAHA.115.307465

Wisneski, J. A., Gertz, E. W., Neese, R. A., Gruenke, L. D., Morris, D. L., & Craig, J. C. (1985). Metabolic fate of extracted glucose in normal human myocardium. *The Journal of Clinical Investigation*, 76(5), 1819–1827. https://doi.org/10.1172/JCI112174

Wittlieb, J., Khalturin, K., Lohmann, J. U., Anton-Erxleben, F., & Bosch, T. C. G. (2006). Transgenic Hydra allow in vivo tracking of individual stem cells during morphogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 103(16), 6208–6211. https://doi.org/10.1073/pnas.0510163103

Wu, Y., Wang, K., Karapetyan, A., Fernando, W. A., Simkin, J., Han, M., Rugg, E. L., & Muneoka, K. (2013). Connective tissue fibroblast properties are position-dependent during mouse digit tip regeneration. *PLoS ONE*, 8(1), e54764. https://doi.org/10.1371/journal.pone.0054764

Xin, M., Kim, Y., Sutherland, L. B., Murakami, M., Qi, X., McAnally, J., Porrello, E. R., Mahmoud, A. I., Tan, W., Shelton, J. M., Richardson, J. A., Sadek, H. A., Bassel-Duby, R., & Olson, E. N. (2013). Hippo pathway effector *Yap* promotes cardiac

regeneration. *Proceedings of the National Academy of Sciences of the United States of America*, 110(34), 13839–13844. https://doi.org/10.1073/pnas.1313192110

Yahalom-Ronen, Y., Rajchman, D., Sarig, R., Geiger, B., & Tzahor, E. (2015). Reduced matrix rigidity promotes neonatal cardiomyocyte dedifferentiation, proliferation and clonal expansion. *ELife*, *4*, e07455. https://doi.org/10.7554/eLife.07455

Yamamoto, S., Yang, G., Zablocki, D., Liu, J., Hong, C., Kim, S.-J., Soler, S., Odashima, M., Thaisz, J., Yehia, G., Molina, C. A., Yatani, A., Vatner, D. E., Vatner, S. F., & Sadoshima, J. (2003). Activation of *Mst1* causes dilated cardiomyopathy by stimulating apoptosis without compensatory ventricular myocyte hypertrophy. *The Journal of Clinical Investigation*, 111(10), 1463–1474. https://doi.org/10.1172/JCI17459

Yan, B., Han, P., Pan, L., Lu, W., Xiong, J., Zhang, M., Zhang, W., Li, L., & Wen, Z. (2014). IL-1β and reactive oxygen species differentially regulate neutrophil directional migration and Basal random motility in a zebrafish injury-induced inflammation model. *Journal of Immunology* (Baltimore, Md.: 1950), 192(12), 5998–6008. https://doi.org/10.4049/jimmunol.1301645

Yan, X., Anzai, A., Katsumata, Y., Matsuhashi, T., Ito, K., Endo, J., Yamamoto, T., Takeshima, A., Shinmura, K., Shen, W., Fukuda, K., & Sano, M. (2013). Temporal dynamics of cardiac immune cell accumulation following acute myocardial infarction. *Journal of Molecular and Cellular Cardiology*, 62, 24–35. https://doi.org/10.1016/j.yjmcc.2013.04.023

Yu, L., Dawson, L. A., Yan, M., Zimmel, K., Lin, Y.-L., Dolan, C. P., Han, M., & Muneoka, K. (2019). BMP9 stimulates joint regeneration at digit amputation wounds in mice. *Nature Communications*, 10, 424. https://doi.org/10.1038/s41467-018-08278-4

Yu, L., Han, M., Yan, M., Lee, E.-C., Lee, J., & Muneoka, K. (2010). BMP signaling induces digit regeneration in neonatal mice. *Development* (Cambridge, England), 137(4), 551–559. https://doi.org/10.1242/dev.042424

Yu, L., Han, M., Yan, M., Lee, J., & Muneoka, K. (2012). BMP2 induces segment-specific skeletal regeneration from digit and limb amputations by establishing a new endochondral ossification center. *Developmental Biology*, 372(2), 263–273. https://doi.org/10.1016/j.ydbio.2012.09.021

Yu, L., Yan, M., Simkin, J., Ketcham, P. D., Leininger, E., Han, M., & Muneoka, K. (2014). Angiogenesis is inhibitory for mammalian digit regeneration. *Regeneration*, 1(3), 33–46. https://doi.org/10.1002/reg2.24

Zahm, A., Bucaro, M., Srinivas, V., Shapiro, I. M., & Adams, C. S. (2008). Oxygen tension regulatates preosteocyte maturation and mineralization. *Bone*, 43(1), 25–31. https://doi.org/10.1016/j.bone.2008.03.010

Zaruba, M.-M., Soonpaa, M., Reuter, S., & Field, L. J. (2010). Cardiomyogenic potential of *c-kit*+ expressing cells derived from neonatal and adult mouse hearts. *Circulation*, 121(18), 1992–2000. https://doi.org/10.1161/CIRCULATIONAHA.109.909093

Zhang, D., Shadrin, I., Lam, J., Xian, H.-Q., Snodgrass, R., & Bursac, N. (2013). Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes. *Biomaterials*, 34(23), 5813–5820. https://doi.org/10.1016/j.biomaterials.2013.04.026

Zhang, L., Sultana, N., Yan, J., Yang, F., Chen, F., Chepurko, E., Yang, F.-C., Du, Q., Zangi, L., Xu, M., Bu, L., & Cai, C.-L. (2018). Cardiac *Sca-1*+ cells are not intrinsic stem cells for myocardial development, renewal, and repair. *Circulation*, 138(25), 2919–2930. https://doi.org/10.1161/circulationaha.118.035200

Zhao, Y., Londono, P., Cao, Y., Sharpe, E. J., Proenza, C., O'Rourke, R., Jones, K. L., Jeong, M. Y., Walker, L. A., Buttrick, P. M., McKinsey, T. A., & Song, K. (2015). High-efficiency reprogramming of fibroblasts into cardiomyocytes requires suppression of pro-fibrotic signalling. *Nature Communications*, 6(1), Article 1. https://doi.org/10.1038/ncomms9243

Zhou, H., Dickson, M. E., Kim, M. S., Bassel-Duby, R., & Olson, E. N. (2015). Akt1/protein kinase B enhances transcriptional reprogramming of fibroblasts to functional cardiomyocytes. *Proceedings of the National Academy of Sciences*, 112(38), 11864–11869. https://doi.org/10.1073/pnas.1516237112

Zhu, W., Zhang, E., Zhao, M., Chong, Z., Fan, C., Tang, Y., Hunter, J. D., Borovjagin, A. V., Walcott, G. P., Chen, J. Y., Qin, G., & Zhang, J. (2018). Regenerative potential of neonatal porcine hearts. *Circulation*, 138(24), 2809–2816. https://doi.org/10.1161/CIRCULATIONAHA.118.034886

Chapter 2

A CONSERVED STRATEGY FOR INDUCING APPENDAGE REGENERATION IN MOON JELLYFISH, *DROSOPHILA*, AND MICE

Adapted from:
Abrams, M.J., **Tan, F.H.**, Li, Y., Basinger, T., Heithe, M.L., Sarma, A.A, Lee, I.T., Condiotte, Z.J., Raffiee, M., Dabiri J.O., Gold D.A., Goentoro, L. (2021). "A conserved strategy for inducing appendage regeneration in moon jellyfish, *Drosophila*, and mice". eLife. **10**:e65092. https://doi.org/10.7554/eLife.65092.

ABSTRACT

Can limb regeneration be induced? Few have pursued this question, and an evolutionarily conserved strategy has yet to emerge. This study reports a strategy for inducing regenerative response in appendages, which works across three species that span the animal phylogeny. In Cnidaria, the frequency of appendage regeneration in the moon jellyfish Aurelia was increased by feeding with the amino acid L-leucine and the growth hormone insulin. In insects, the same strategy induced tibia regeneration in adult *Drosophila*. Finally, in mammals, L-leucine and sucrose administration induced digit regeneration in adult mice, including dramatically from mid-phalangeal amputation. The conserved effect of L-leucine and insulin/sugar suggests a key role for energetic parameters in regeneration induction. The simplicity by which nutrient supplementation can induce appendage regeneration provides a testable hypothesis across animals.

INTRODUCTION

In contrast to humans' poor ability to regenerate, the animal world is filled with seemingly Homeric tales: a creature that regrows when halved or a whole animal growing from a small body piece. Two views have historically prevailed as to why some animals regenerate

better than others (Goss, 1992). Some biologists, including Charles Darwin and August Weismann, hold that regeneration is an adaptive property of a specific organ (Polezhaev, 1972). For instance, some lobsters may evolve the ability to regenerate claws because they often lose them in fights and food foraging. Other biologists, including Thomas Morgan, hold that regeneration is not an evolved trait of a particular organ, but inherent in all organisms (Morgan, 1901). Regeneration evolving for a particular organ versus regeneration being organismally inherent is an important distinction, as the latter suggests that the lack of regeneration is not due to the trait never having evolved, but rather due to inactivation—and may therefore be induced. In support of Morgan's view, studies in past decades have converged on one striking insight: many animal phyla have at least one or more species that regenerate body parts (Sánchez Alvarado, 2000; Bely and Nyberg, 2010). Further, even in poorly regenerative lineages, many embryonic and larval stages can regenerate. In regenerating animals, conserved molecular events (e.g., Cary et al., 2019, Kawakami et al., 2006) and regeneration-responsive enhancers (Wang et al., 2020) were identified. Although the hypothesis of convergent evolution cannot be fully excluded (e.g., Lai and Aboobaker, 2018), these findings begin to build the case that the ability to regenerate may be ancestral (Sánchez Alvarado, 2000; Bely and Nyberg, 2010). Regeneration being possibly ancestral begs the question: is there a conserved mechanism to activate regenerative state?

This study explored how, and whether, limbs can be made to regenerate in animals that do not normally show limb regeneration. In adult frogs, studies from the early 20th century and few recent ones have induced various degrees of outgrowth in the limb using strategies including repeated trauma, electrical stimulation, local progesterone delivery, progenitor cell implantation, and *Wnt* activation (Carlson, 2007; Lin et al., 2013; Kawakami et al., 2006; Herrera-Rincon et al., 2018). *Wnt* activation restored limb development in chick embryos (Kawakami et al., 2006), but there are no reports of postnatal regeneration induction. In salamanders, a wound site that normally just heals can be induced to grow a limb by supplying nerve connection and skin graft from the contralateral limb (Endo et al., 2004), or by delivery of Fgf2, 8, and Bmp2 to the wound site followed by retinoic acid (Vieira et al., 2019). In neonatal and adult mouse digits, a model for exploring limb regeneration in mammals, bone outgrowth, or joint-like structure can be induced via local

implantation of Bmp2 (bone) or Bmp9 (joint; Yu et al., 2019). Thus far, different strategies gain traction in different species, and a common denominator appears elusive.

However, across animal phylogeny, some physiological features show interesting correlation with regenerative ability (Hariharan et al., 2016; Vivien et al., 2016; Sousounis et al., 2014). First, regeneration especially in vertebrates tends to decrease with age, with juveniles and larvae more likely to regenerate than adults. For instance, the mammalian heart rapidly loses the ability to regenerate after birth and anurans cease to regenerate limbs upon metamorphosis. Second, animals that continue to grow throughout life tend to also regenerate. For instance, most annelids continue adding body segments and regenerate well, a striking exception of which is leeches that make exactly 32 segments and one of the few annelids that do not regenerate body segments (Rouse, 1998). Consistent with the notion of regeneration as ancestral, indeterminate growth is thought of as the ancestral state (Hariharan et al., 2016). Finally, a broad correlate of regenerative ability across animal phylogeny is thermal regulation. Poikilotherms, which include most invertebrates, fish, reptiles, and amphibians, tend to have greater regenerative abilities than homeotherms—birds and mammals are animal lineages with poorest regeneration. These physiological correlates, taken together, are united by the notion of energy expenditure. The transition from juvenile to adult is a period of intense energy usage, continued growth is generally underlined by sustained anabolic processes, and regulating body temperature is energetically expensive compared to allowing for fluctuation. Regeneration itself entails activation of anabolic processes to rebuild lost tissues (Hirose et al., 2014; Naviaux et al., 2009; Malandraki-Miller et al., 2018; Takayama et al., 2018). These physiological correlates thus raise the notion of a key role of energetics in the evolution of regeneration in animals. Specifically, we wondered whether energy inputs can promote regenerative state. In this study, we demonstrate that nutrient supplementation can induce regenerative response in appendage and limb across three vastly divergent species.

RESULTS

Leucine and sucrose induce regeneration in mouse digit

The ability of leucine and insulin to induce regenerative response in *Drosophila* limb and *Aurelia* appendage motivated testing in vertebrates. One sign that limb regeneration may be feasible in humans is that fingertips regenerate (Illingworth, 1974). The mammalian model for studying limb regeneration is the house mouse, *Mus musculus*, which like humans, regenerates digit tips. Although proximal regions of digits do not regenerate, increasing evidence suggests that they have inherent regenerative capacity. In adult mice, implanting developmental signals in amputated digits led to specific tissue induction, that is, bone growth with Bmp4 or joint-like structure with Bmp9 (Yu et al., 2019). In neonates, reactivation of the embryonic gene *lin28* led to distal phalange regrowth (Shyh-Chang et al., 2013). Thus, while patterned phalange regeneration can be induced in newborns, induction in adults so far involves a fine-tuned stimulation, for example, to elongate bone and then make joint, Bmp4 was first administered followed by Bmp9 in a timed manner. Motivated by the findings in *Aurelia* and *Drosophila*, we tested if leucine and insulin administration could induce a self-organized regeneration in adult mice.

We performed amputation on the hindpaw (Figure 1a), on digits 2 and 4, leaving the middle digit three as an internal control (Figure 1b). To perform non-regenerating amputation, a clear morphological marker is the nail, which is associated with the distal phalange (P3). Amputation that removes <30% of P3 length, that cuts within the nail, readily regenerates, whereas amputation that removes >60% of P3 length, corresponding to removing almost the entire visible nail, does not regenerate (Figure 1c; Chamberlain et al., 2017; Lehoczky et al., 2011). We therefore performed amputations entirely proximal to the visible nail—giving, within the precision of our amputation, a range of cut across somewhere between the proximal P3 and the distal middle phalange (P2) (Figure 1d)—a range that is well below the regenerating tip region. Note additional morphological markers that lie within the non-regenerating region: the os hole (‘o’ in Figure 1c), where vasculatures and nerves enter P3,

the bone marrow cavity (‘m’ in Figure 6c), and the sesamoid bone (‘s’ in Figure 1c) adjacent to P2.

The digit portion removed was immediately fixed to determine the precise plane of amputation. The amputated mice were either provided with water as usual (control) or water supplemented with leucine and sucrose (treated) (Figure 1e). Both groups were monitored for 7–8 weeks. Sucrose was used because insulin is proteolytically digested in the mammalian gut. The sucrose doses used are lower or the administration duration is shorter than those shown to induce insulin resistance (Cao et al., 2007; Togo et al., 2019). We verified that control and treated mice had comparable initial weights (35.1±0.6 vs. 34.1±1.1 g, p=0.402, Student’s t-test), and that as expected from amino acid and sugar supplementation, treated mice gained more weight over the experimental duration (4.5±1.0 vs. 7.8±1.0 g, p=0.028, Student’s t-test).

As expected for amputation proximal to the nail, no regeneration was observed in the control mice (N=34 digits, 17 mice). Amputated digits healed and re-epithelialized the wound as expected (Figure 1f). Skeletal staining shows blunt-ended digit stumps (Figure 1i) and in many instances, as expected, dramatic histolysis, a phenomenon where bone recedes further from the amputation plane (Figure 1—figure supplement 1; Chamberlain et al., 2017). By contrast, 18.8% of the treated digits (N=48 digits, 24 mice) showed various extents of regenerative response (Figure 1—figure supplement 1). The increase in regeneration frequency due to the treatment is statistically significant (95% CI [8, 30%], p=0.0019,**, Student’s t-test).

We observed, as in *Aurelia* and *Drosophila*, an unpatterned response (Figure 1—figure supplement 1), wherein skeletal staining reveals excessive bone mass around the digit stump, similarly to what was observed in some cases with BMP stimulation (Yu et al., 2019). However, we also observed patterned responses (Figure 1—figure supplement 2). The most dramatic regenerative response was observed in two digits (Figure 1g–h). In one digit, an almost complete regrowth of the distal phalange and the nail was observed (Figure 1g). Skeletal staining of the portion removed from this digit (Figure 1j) shows that it was amputated at the proximal P3 transecting the os hole. By 7 weeks, skeletal staining of the

regrown digit (Figure 1j) shows that the P3 bone was almost completely regrown. The regrown P3 shows trabecular appearance that is similar in general structure but not identical to the original P3. Another dramatic response was observed from another digit, which began reforming the nail by 7 weeks (Figure 1h). Skeletal staining of the portion removed from this digit shows that it was amputated across the P2 bone, removing the entire epiphyseal cap along with the sesamoid bone (Figure 1k). Skeletal staining of the regenerating digit shows that the epiphyseal cap was regrown, along with its associated sesamoid bone. Moreover, articulating from the regenerated P2 appears to be the beginning of the next phalangeal bone (arrow, Figure 1k). To our knowledge, the regenerative response observed in these digits represents the most dramatic extent of self-organized mammalian digit regeneration reported thus far. Distal phalange regeneration in adults has not been reported, while interphalangeal joint formation from a P2 amputation has been achieved only through sequential Bmp administration (Yu et al., 2019) and there has been no documentation of the regrowth of the sesamoid bone.

DISCUSSION

In this study, amputations were performed on *Aurelia* appendage, *Drosophila* limb, and mouse digit. None of these animals are known to regenerate robustly (*Aurelia*) if at all (*Drosophila* and mouse) from these amputations. Upon administration of L-leucine and sugar/insulin, dramatic regenerative response was observed in all systems. The conserved effect of nutrient supplementation across three species that span more than 500 million years of evolutionary divergence suggests energetic parameters as ancestral regulators of regeneration activation in animals.

While we did not test the regenerative effect of hypoxia beyond *Aurelia*, it is notable that in mice hypoxia coaxes cardiomyocytes to re-enter cell cycle (Kimura et al., 2015) and activate HIF1α promotes healing of ear hole punch injury (Zhang et al., 2015). Notably in *Aurelia*, the amputation bisected through the body, and more than appendage was in fact regenerated, for example, circular muscle in the body is regrown. Thus, nutrient supplementation may have regenerative effect in body parts beyond appendage.

The diverse physiologies of animals across phylogeny may seem difficult to reconcile with a conserved regulation of regeneration activation, especially in the view of regeneration as recapitulation of development. Growing a jellyfish appendage is different from building a fly leg or making a mouse digit. However, there is another way of looking at regeneration as a part of tissue plasticity (Galliot and Ghila, 2010). In this view of regeneration, upstream from tissue-specific morphogenesis is a conserved regulation of cell growth and proliferation. In support of this idea, early steps in regeneration across species and organs rely one way or another on proliferation by stem cells or differentiated cells re-entering cell cycle (Cox et al., 2019). We propose that in animals that poorly regenerate, high nutrient input turns on growth and anabolic states that promote tissue rebuilding upon injury.

That regenerative response can be induced seemingly blurs the boundary between regenerating versus non-regenerating animals, because the factors identified in the study are not exotic. Variations in amino acids, carbohydrates, and oxygen levels are conditions that the animals can plausibly encounter in nature. These observations highlight two potential insights into regeneration. First, regeneration is environmentally dependent. An animal would stop at wound healing under low-energy conditions and regenerate in energy-replete conditions. In this view, for the animals examined in this study, the typical laboratory conditions may simply not be conducive to regeneration. Alternatively, the interpretation we favor, what we observed is inherent regeneration, which can be activated with broad environmental factors. We favor this interpretation because the regenerative response was unusually variable. The variability stands in stark contrast to the robust regeneration in, for example, axolotl, planaria, or hydra. Whereas wild-type processes tend to be robust, mutations produce phenotypes that are sensitive to variations in physiological parameters. Thus, just like mutant phenotypes show varying penetrance and expressivity, the variable regenerative response speaks to us as a fundamental consequence of activating a latent biological module. In this interpretation, the ordinariness of the activators suggests ancestral regeneration as part of a response to broad environmental stimuli. It would be interesting next to identify individual differences that contribute to varying propensities to mount regenerative response.

In particular, the conserved effects of nutrient supplementation suggest that regeneration might have originally been a part of growth response to abundant environments. No nutrient dependence has been observed in highly regenerating animal models such as planaria, hydra, and axolotl. Environment-dependent plasticity, however, is pervasive in development, physiology, behavior, and phenology (West-Eberhard, 2003; Moczek et al., 2011). We therefore conjecture that environment-dependent plasticity may have characterized the ancestral form of regeneration. In this conjecture, present regenerating lineages might have decoupled the linkage with environmental input and genetically assimilated regenerative response—because regeneration is adaptive or coupled to a strongly selected process, for example, reproduction. In parallel, non- or poorly regenerating animals might have also weakened the linkage with environmental input, but to silence the regenerative response. This predicts an ancient form of a robustly regenerative animal (like planaria, hydra, and axolotl) that tunes its regeneration frequency to nutrient abundance. Such plasticity has been reported in the basal lineage Ctenophora (Bading et al., 2017).

In conclusion, this study suggests that an inherent ability for appendage regeneration is retained in non-regenerating animals and can be unlocked with a conserved strategy. The treatments across species were not exactly identical, and correspondingly there might be differences in the precise molecular mechanisms—in spite of which they could be applied across species in a predictive manner. In line with our findings, the role of nutrients in promoting regeneration was reported in yet another species (in the *Xenopus tadpoles*, Williams et al., 2021). While the observed regenerative response is not perfect, this motivates further investigation into potentially more promoting factors or the possibility of combining broad promoting factors with species- or tissue-specific morphogenetic regulators. Reiterating Spallanzani's hope, Marcus Singer supposed half a century ago that '... every organ has the power to regrow lying latent within it, needing only the appropriate "useful dispositions" to bring it out (Singer, 1958).' The surprise, in hindsight, is the simplicity by which the regenerative state can be promoted with *ad libitum* amino acid and sugar supplementation. This simplicity demonstrates a much broader possibility of organismal regeneration, and can help accelerate progress in regeneration induction across animals.

MATERIALS AND METHODS

Mus musculus

All studies comply with relevant ethical regulations for animal testing and research, and received ethical approval by the Institutional Animal Care and Use Committees at the California Institute of Technology.

Strain

Adult female (3–6 months old) wild-type CD1 mice (Charles River Laboratories strain 022) were used for all regeneration studies.

Regeneration experiments

Digit amputation was performed following the established protocol in the field (Simkin et al., 2013). Mice were anesthetized with 1–5% isoflurane (in oxygen) in an induction chamber, followed by maintenance on a nosecone. The mouse was positioned on its belly with its hind paws outstretched and the ventral side of the paw facing upwards. Sustained-Release Buprenorphine was administered (Buprenorphine SR LAB) at 0.5 mg/kg subcutaneously as an analgesic. Blood flow to the hindlimb was stemmed by tying a rubber band around the ankle and clamping it with a hemostat. All surgical procedures were carried out under a Zeiss Stemi 305 dissection microscope. An initial incision, parallel to the position of foot, was made through the ventral fat pad using Vannas spring scissors (World Precision Instruments, 14003). The length of this incision was determined by the amount of ventral skin needed to seal the digit amputation wound completely. The ventral skin freed in the initial incision was peeled back using surgical forceps, and a no. 10 scalpel (Sklar, 06-3110) was used to amputate and bisect the digit completely through the second or third phalange. Digits 2 and 4 on the right hind paw were operated on in this fashion, while digit three remained unamputated as an internal control. The amputation wound was immediately closed with the ventral skin flap and sealed with GLUture (Zoetis, Kalamazoo, MI). Amputated portions were immediately fixed as control for skeletal staining. Dissolved 1.5% L-leucine (USP grade, VWR E811), 1.5% L-glutamine (USP

grade, Sigma-Aldrich G8540), and 4–10% sucrose (AR ACS grade, Avantor 8360) in drinking water was administered to mice in the experimental group ad libitum after amputation. Control mice were given untreated drinking water. Drinking water was refreshed weekly for both control and experimental groups, and treated water was made fresh on the day that drinking water was replaced. The amputated digit stumps were photographed weekly for 7–8 weeks, at which time the digits were dissected for skeletal staining.

Mouse digit dissection and skeletal staining

Mice were euthanized and digits 2, 3, and 4 were removed with a no. 10 scalpel (Sklar, 06-3110) through the first phalange. Excess skin and flesh were removed with spring scissors (Fine Science Tools, 91500-09) and fine dissecting forceps (Fine Science Tools, 11254-20). All digits analyzed by whole-mount skeletal stains were prepared with a standard alizarin red and alcian blue staining protocol (McLeod, 1980). Digits were dehydrated in 95% ethanol for 1 day, and incubated in staining solution (0.005% alizarin red (Beantown Chemical, BT144735), 0.015% alcian blue (Acros Organics, AC40046-0100), 5 % acetic acid, 60 % ethanol) for 1 day at 37 °C. Tissue was cleared in 2 % potassium hydroxide at room temperature for 1 day, 1% potassium hydroxide for 1 day, and then taken through an increasing glycerol series (25%, 50%, 75%, and 100%). The stained samples were imaged on Zeiss AxioZoom.V16 stereo zoom microscope with a Zeiss AxioCam 503 color camera or a Zeiss Stemi 305 dissection microscope with an iPhone six camera.

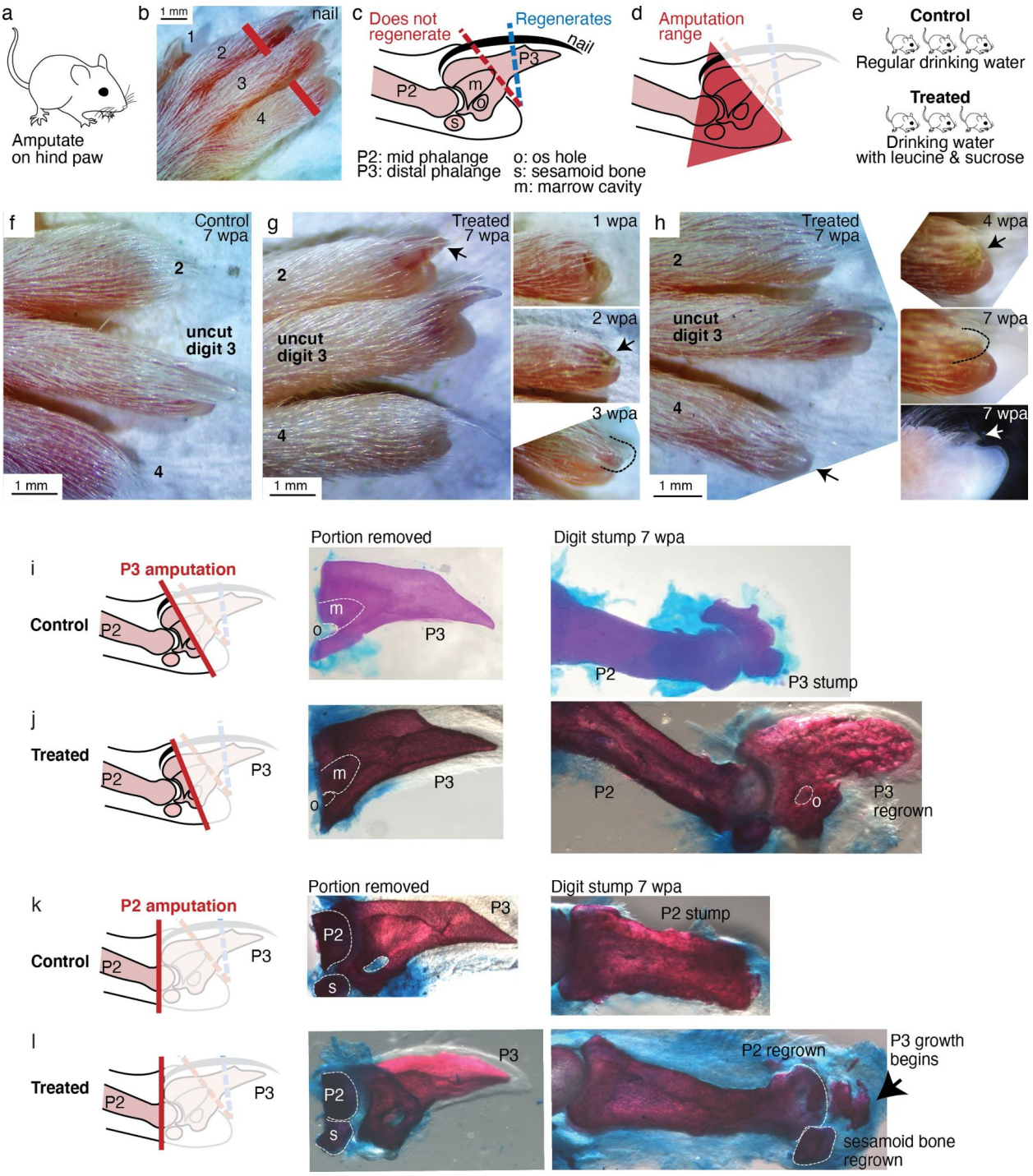
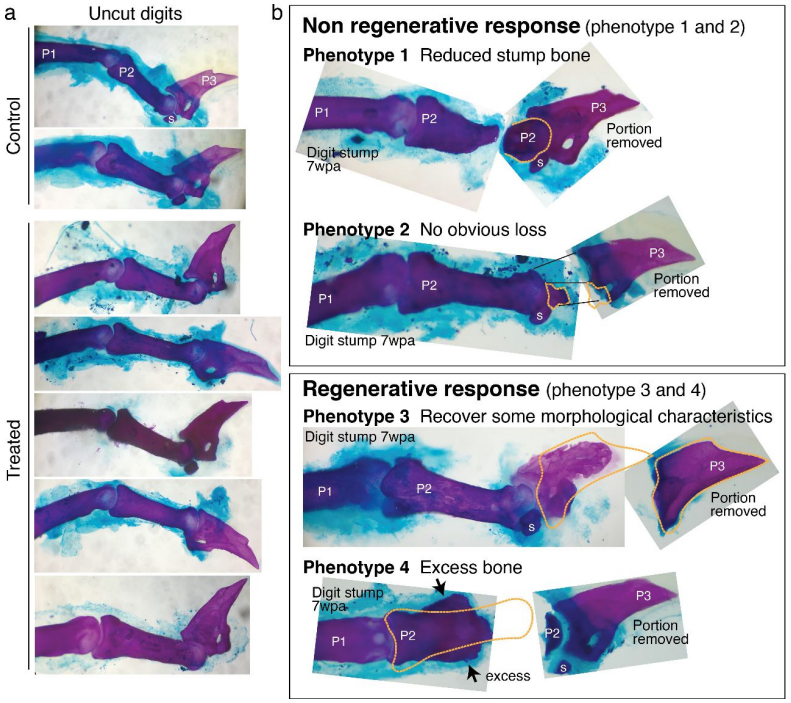


Figure 1 (a–b). Amputation was performed on hindpaws of adult (3–6 months old) mice, on digits 2 and 4, proximal to the nail. (**c**) Schematic of the distal phalange (P3) and middle phalange (P2). Amputations that remove <30% of P3 (blue line) regenerate, whereas amputations that remove >60% of P3 (red line) do not regenerate. Amputations in the intermediate region can occasionally show partial regenerative response. (**d**) Amputations in this study were performed within the red-shaded triangle. (**e**) Amputated mice were

given regular drinking water (control) or drinking water supplemented with 1.5% L-leucine, 1.5% L-glutamine, and 4–10% w/v sucrose (2 exps with 4%, 3 exps with 10%). Drinking water, control and treated, was refreshed weekly. (**f**) A representative paw from the control group. The amputated digits 2 and 4 simply healed the wound and did not regrow the distal phalange. (**g**) In this treated mouse, digit 2 (arrow) regrew the distal phalange and nail. Insets on the right show the digit at earlier time points. At week 1, the amputation site still appeared inflamed. At week 3, the beginning of the nail appears (arrow). At week 3, a clear nail plate was observed. (**h**) In this treated mouse, digit 4 (arrow) regrew and began to show nail reformation by week 4 (top inset, see arrow), that turns into a clear nail plate by week 7 (middle inset), as can be seen more clearly from the side-view darkfield image (bottom inset). (**i–l**) Whole-mount skeletal staining. Dissected digits were stained with Alizarin red, an anionic dye that highly localizes to the bone. Left panels show illustration of the amputation plane, middle panels show skeletal staining of the portions removed, and right panels show skeletal staining of the digit stumps 7 weeks after amputation.



c All digits

Phenotype	Control # digits	Control %	Treated # digits	Treated %
1 - Reduced stump bone	22	64.7	26	54.2
2 - No obvious loss	12	35.3	13	27.1
3 - Recover morphology	**0**	**0.0**	**6**	**12.5**
4 - Excess bone	**0**	**0.0**	**3**	**6.3**
Total	34		48	

d P2 amputation only

Phenotype	Control # digits	Control %	Treated # digits	Treated %
1 - Reduced stump bone	11	84.6	23	65.7
2 - No obvious loss	2	15.4	6	17.1
3 - Recover morphology	**0**	**0.0**	**3**	**8.6**
4 - Excess bone	**0**	**0.0**	**3**	**8.6**
Total	13		35	

P3 amputation only

Phenotype	Control # digits	Control %	Treated # digits	Treated %
1 - Reduced stump bone	11	52.4	3	23.1
2 - No obvious loss	10	47.6	7	53.8
3 - Recover morphology	**0**	**0.0**	**3**	**23.1**
4 - Excess bone	**0**	**0.0**	**0**	**0.0**
Total	21		13	

Figure 1—figure supplement 1 Whole-mount skeletal staining was performed with Alizarin red. wpa: week post-amputation, P1: phalange 1, P2: phalange 2, P3: phalange 3, s: sesamoid bone. (**a**) Skeletal staining of unamputated digits (digit 3) from control and treated groups show no obvious differences in uncut digits due to the treatment. (**b**) Skeletal staining of digits stumps at 7 wpa and the original portion removed from the digits. Some digit stumps show no change or appear to have undergone histolysis (Chamberlain et al., 2017) resulting in reduced bone mass (phenotypes 1 and 2). Some digit stumps show

regenerative response, either recovery of some morphological characteristics (phenotype 3, detailed more in Figure 1—figure supplement 2) or excess, ectopic bone mass (Phenotype 4). We erred on the conservative side in scoring phenotype 3 and 4; when in doubt, digits were classified into phenotype 1 or 2. (**c–e**) Phenotype counts in all digits (**c**), in digits amputated across P2 (**d**), and in digits amputated across P3 or joint (**e**).

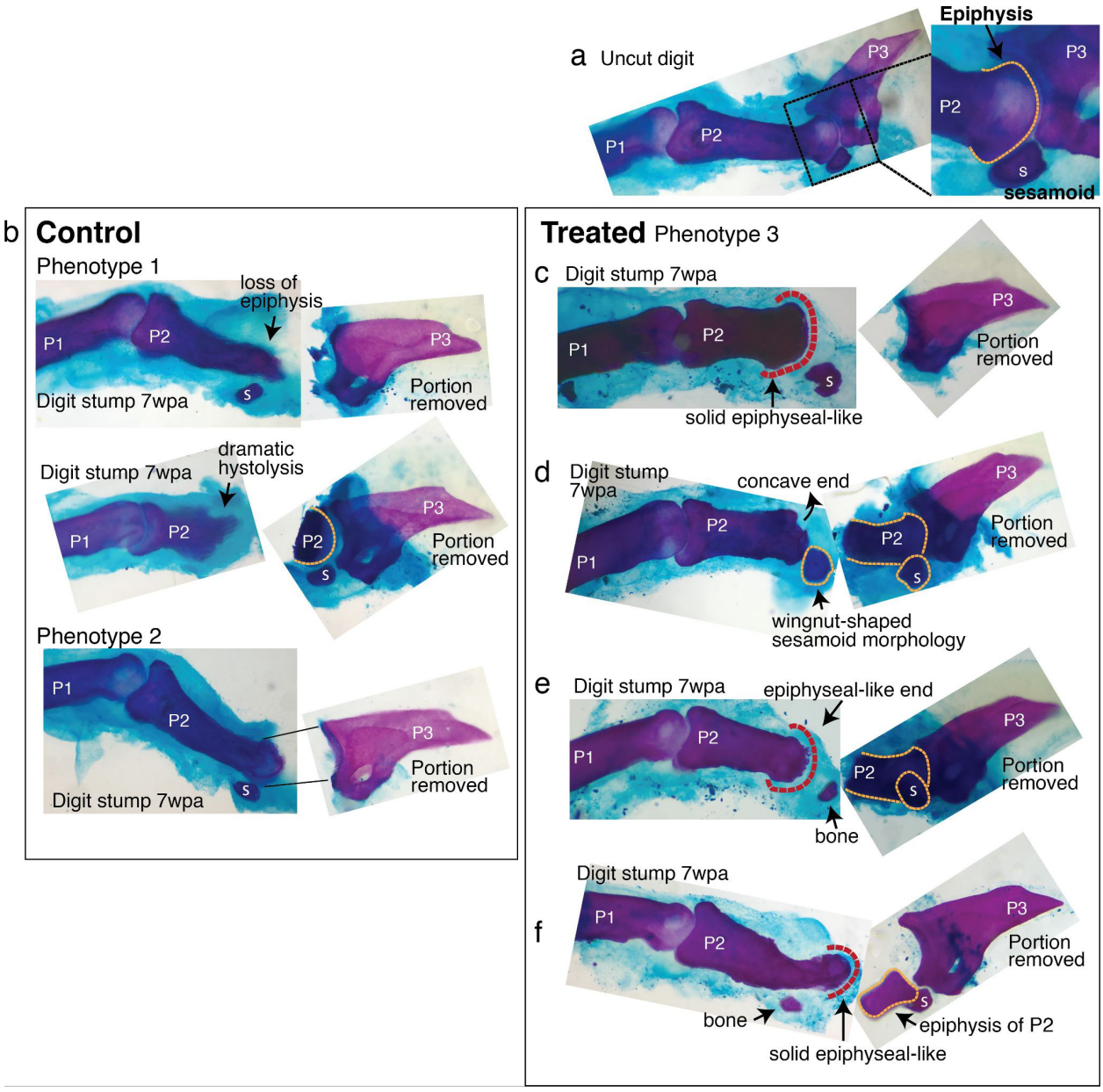


Figure 1—figure supplement 2 Six digit stumps (of total 48 examined) show regenerative response. The most dramatic two are presented in Figure 1. The remaining four are presented here. wpa: week post-amputation, P1: phalange 1, P2: phalange 2, P3: phalange 3, s: sesamoid bone. (**a**) An uncut digit, shown for a comparison. Magnified is the P2/P3 joint area to highlight key morphological markers: the knobby epiphyseal cap of P2 and

the sesamoid bone embedded in the tendon on the flexor side of P2. (**b**) Digit stumps from control mice show either bone stump histolysis (top and middle, phenotype 1) and no visible changes in bone stump (bottom, phenotype 2). (**c–f**) Digit stumps from treated mice that show regenerative response. (**c**) In this digit, the amputation removed all P3 by a cut through the joint. At 7 wpa, the P2 stump is reduced, but recovered the epiphyseal-like end (red dashed line)—marked by solid curved shape, as opposed to irregularly shaped histolyzing bone. (**d**) In this digit, the amputation removed a significant portion of P2 and the sesamoid bone. The P2 stump does not regain an epiphyseal end (the end is concave and irregular). However, the sesamoid bone is reformed, as identified by its location on the flexor side of P2 and wingnut shape (Wirtschafter and Tsujimura, 1961) under the microscope. The recovery of sesamoid bone is non-trivial, as digit sesamoids form in juxtaposition to the condensing phalange, detaching from the phalange by formation of a cartilaginous joint (Eyal et al., 2019). (**e**) In this digit, the amputation removed a significant portion of P2 and the sesamoid bone. At 7 wpa, the P2 stump appears to be reforming an epiphyseal, rounded end (red dashed line). There is a small bone distal to P2, whose curvature articulates with the P2 end, but there are not enough morphological characters to identify the bone. (**f**) In this digit, the amputation removed the epiphyseal cap of P2 and the sesamoid bone. The P2 stump appears to have lost some mass, but reforms an epiphyseal-like end (red dashed line). There is an additional small bone located where the sesamoid bone should be, but lacks sufficient morphological characters to identify.

BIBLIOGRAPHY

Alvarado, A. S. (2000). Regeneration in the metazoans: Why does it happen? *BioEssays*, *22*(6), 578–590. https://doi.org/10.1002/(SICI)1521-1878(200006)22:6<578::AIDBIES11>3.0.CO;2-#

Bading, K. T., Kaehlert, S., Chi, X., Jaspers, C., Martindale, M. Q., & Javidpour, J. (2017). Food availability drives plastic self-repair response in a basal metazoan- case study on the ctenophore Mnemiopsis leidyi A. Agassiz 1865. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-16346-w

Bely, A. E., & Nyberg, K. G. (2010). Evolution of animal regeneration: Re-emergence of a field. *Trends in Ecology & Evolution*, *25*(3), 161–170. https://doi.org/10.1016/j.tree.2009.08.005

Cao, D., Lu, H., Lewis, T. L., & Li, L. (2007). Intake of sucrose-sweetened water induces insulin resistance and exacerbates memory deficits and amyloidosis in a transgenic mouse model of Alzheimer disease. *Journal of Biological Chemistry*, *282*(50), 36275–36282. https://doi.org/10.1074/jbc.M703561200

Carlson, B., M. (2007). *Principles of Regenerative Biology*. Elsevier.

Chamberlain, C. S., Jeffery, J. J., Leiferman, E. M., Yildirim, T., Sun, X., Baer, G. S., Murphy, W. L., & Vanderby, R. (2017). Level-specific amputations and resulting regenerative outcomes in the mouse distal phalanx. *Wound Repair and Regeneration : Official Publication of the Wound Healing Society [and] the European Tissue Repair Society*, *25*(3), 443–453. https://doi.org/10.1111/wrr.12544

Cox, B. D., Yun, M. H., & Poss, K. D. (2019). Can laboratory model systems instruct human limb regeneration? *Development*, *146*(20), dev181016. https://doi.org/10.1242/dev.181016

Endo, T., Bryant, S. V., & Gardiner, D. M. (2004). A stepwise model system for limb regeneration. *Developmental Biology*, 11.

Eyal, S., Rubin, S., Krief, S., Levin, L., & Zelzer, E. (2019). Common cellular origin and diverging developmental programs for different sesamoid bones. *Development*, *146*(4), dev167452. https://doi.org/10.1242/dev.167452

Galliot, B., & Ghila, L. (2010). Cell plasticity in homeostasis and regeneration. *Molecular Reproduction and Development*, *77*(10), 837–855. https://doi.org/10.1002/mrd.21206

Goss, R. J. (1992). The evolution of regeneration: Adaptive or inherent? *Journal of Theoretical Biology*, *159*(2), 241–260. https://doi.org/10.1016/S0022-5193(05)80704-0

Hariharan, I. K., Wake, D. B., & Wake, M. H. (2016). Indeterminate growth: could it represent the ancestral condition? *Cold Spring Harbor Perspectives in Biology*, *8*(2). https://doi.org/10.1101/cshperspect.a019174

Herrera-Rincon, C., Golding, A. S., Moran, K. M., Harrison, C., Martyniuk, C. J., Guay, J. A., Zaltsman, J., Carabello, H., Kaplan, D. L., & Levin, M. (2018). Brief local application of progesterone via a wearable bioreactor induces long-term regenerative response in adult Xenopus hindlimb. *Cell Reports*, *25*(6), 1593-1609.e7. https://doi.org/10.1016/j.celrep.2018.10.010

Hirose, K., Shiomi, T., Hozumi, S., & Kikuchi, Y. (2014). Mechanistic target of rapamycin complex 1 signaling regulates cell proliferation, cell survival, and differentiation in regenerating zebrafish fins. *BMC Developmental Biology*, *14*(1). https://doi.org/10.1186/s12861-014-0042-9

Illingworth, C., M. (1974). Trapped fingers and amputated finger tips in children. *Journal of Pediatric Surgery*, *9*(6), 853–858.

Kawakami, Y., Rodriguez Esteban, C., Raya, M., Kawakami, H., Martí, M., Dubova, I., & Izpisúa Belmonte, J. C. (2006). Wnt/β-catenin signaling regulates vertebrate limb regeneration. *Genes & Development*, *20*(23), 3232–3237. https://doi.org/10.1101/gad.1475106

Kimura, W., Xiao, F., Canseco, D. C., Muralidhar, S., Thet, S., Zhang, H. M., Abderrahman, Y., Chen, R., Garcia, J. A., Shelton, J. M., Richardson, J. A., Ashour, A. M., Asaithamby, A., Liang, H., Xing, C., Lu, Z., Zhang, C. C., & Sadek, H. A. (2015). Hypoxia fate mapping identifies cycling cardiomyocytes in the adult heart. *Nature*, *523*(7559), 226–230. https://doi.org/10.1038/nature14582

Lehoczky, J. A., Robert, B., & Tabin, C. J. (2011). Mouse digit tip regeneration is mediated by fate-restricted progenitor cells. *Proceedings of the National Academy of Sciences*, *108*(51), 20609–20614.https://doi.org/10.1073/pnas.1118017108

Lin, G., Chen, Y., & Slack, J. M. W. (2013). Imparting regenerative capacity to limbs by progenitor cell transplantation. *Developmental Cell*, *24*(1), 41–51. https://doi.org/10.1016/j.devcel.2012.11.017

McLeod MJ (1980) Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. Teratology 22:299e301.

Malandraki-Miller, S., Lopez, C. A., Al-Siddiqi, H., & Carr, C. A. (2018). Changing Metabolism in Differentiating Cardiac Progenitor Cells—Can Stem Cells Become Metabolically Flexible Cardiomyocytes? *Frontiers in Cardiovascular Medicine*, *5*. https://doi.org/10.3389/fcvm.2018.00119

Moczek, A. P., Sultan, S., Foster, S., Ledón-Rettig, C., Dworkin, I., Nijhout, H. F., Abouheif, E., & Pfennig, D. W. (2011). The role of developmental plasticity in evolutionary innovation. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1719), 2705–2713. https://doi.org/10.1098/rspb.2011.0971

Morgan, T. H. (1901). *Regeneration*. Macmillan and Co., Ltd.

Naviaux, R. K., Le, T. P., Bedelbaeva, K., Leferovich, J., Gourevitch, D., Sachadyn, P., Zhang, X.-M., Clark, L., & Heber-Katz, E. (2009). Retained Features of Embryonic Metabolism in the Adult MRL Mouse. *Molecular Genetics and Metabolism*, *96*(3), 133–144. https://doi.org/10.1016/j.ymgme.2008.11.164

Polezhaev, L. V. (1972). *Loss and restoration of regenerative capacity in tissues and organs of animals* (Y. Halperin, Trans.). Harvard University Press.

Simkin, J., Han, M., Yu, L., Yan, M., & Muneoka, K. (2013). The Mouse Digit Tip: From Wound Healing to Regeneration. In R. G. Gourdie & T. A. Myers (Eds.), *Wound Regeneration and Repair* (Vol. 1037, pp. 419–435). Humana Press. https://doi.org/10.1007/978-1-62703-505-7_24

Singer, M. (1958). The Regeneration of Body Parts. *Scientific American*, *199*(4), 79–94.

Sousonis, K., Baddour, J. A., & Tsonis, P. A. (2014). Aging and regeneration in vertebrates. In *Current topics in developmental biology* (Vol. 108, pp. 217–246). Academic Press.

Togo, J., Hu, S., Li, M., Niu, C., & Speakman, J. R. (2019). Impact of dietary sucrose on adiposity and glucose homeostasis in C57BL/6J mice depends on mode of ingestion: Liquid or solid. *Molecular Metabolism*, *27*, 22–32. https://doi.org/10.1016/j.molmet.2019.05.010

Vieira, W. A., Wells, K. M., Raymond, M. J., De Souza, L., Garcia, E., & McCusker, C. D. (2019). FGF, BMP, and RA signaling are sufficient for the induction of complete limb regeneration from non-regenerating wounds on Ambystoma mexicanum limbs. *Developmental Biology*, *451*(2), 146–157. https://doi.org/10.1016/j.ydbio.2019.04.008

Vivien, C. J., Hudson, J. E., & Porrello, E. R. (2016). Evolution, comparative biology and ontogeny of vertebrate heart regeneration. *Npj Regenerative Medicine*, *1*(1), 16012. https://doi.org/10.1038/npjregenmed.2016.12

Wang, W., Hu, C.-K., Zeng, A., Alegre, D., Hu, D., Gotting, K., Ortega Granillo, A., Wang, Y., Robb, S., Schnittker, R., Zhang, S., Alegre, D., Li, H., Ross, E., Zhang, N., Brunet, A., & Sánchez Alvarado, A. (2020). Changes in regeneration-responsive enhancers shape regenerative capacities in vertebrates. *Science*, *369*(6508), eaaz3090. https://doi.org/10.1126/science.aaz3090

West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*. Oxford University Press.

Wirtschafter, Z. T., & Tsujimura, J. K. (1961). The sesamoid bones in long-evans strain rats. *The Anatomical Record*, *141*(3), 195–204. https://doi.org/10.1002/ar.1091410304

Yu, L., Dawson, L. A., Yan, M., Zimmel, K., Lin, Y.-L., Dolan, C. P., Han, M., & Muneoka, K. (2019). BMP9 stimulates joint regeneration at digit amputation wounds in mice. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-018-08278-4

Zhang, Y., Strehin, I., Bedelbaeva, K., Gourevitch, D., Clark, L., Leferovich, J., Messersmith, P.B., & Heber-Katz, E. (2015). Drug-induced regeneration in adult mice. *Science Translational Medicine*, *7*(290), 290ra92.https://doi.org/10.1126/scitranslmed.3010228

Chapter 3

CORRELATING CHANGES IN THE MOUSE CARDIAC NEURAL CREST WITH NEONATAL HEART REGENERATION

ABSTRACT

The cardiac neural crest is a subpopulation of the multipotent neural crest stem cell population that migrates during development to form structures in the heart such as the outflow tract, valves, and arteries. Recent lineage tracing work has shown that cardiac crest derivatives additionally contribute to the myocardium in vertebrate models such as zebrafish and chick and can contribute to new myocardium in the regenerating zebrafish heart. However, cardiac neural crest cell (CNCC) contribution to mouse myocardium is still contested, and it remains unclear if they participate in neonatal mouse regeneration, an ability which is limited to the first week of postnatal life. Our transcriptomic analyses from both bulk and single-cell RNA sequencing suggest that CNCC derivatives possess a distinct molecular profile from their non-cardiac crest counterparts, which also undergoes a significant shift as the heart matures from P1 to >P7. We find that >P7 cardiac crest derivatives upregulate genes related to immune response, growth, and cytoskeletal structure, with marked changes in ECM-receptor signaling compared to their P1 counterparts. Additionally, we find further evidence for CNCC-derived cardiomyocytes in the mouse. Specifically, re-analysis of single cell data in both embryonic and postnatal stages, with their numbers peaking in late embryonic stages followed by a sharp decline postnatally. Taken together, our results show that CNCC derivatives in the mouse heart may be particularly responsive to the changing physiology undergone during cardiac maturation, and suggest that they may be an ideal target for interventions intended to improve mammalian heart regeneration.

INTRODUCTION

The neural crest is a unique population of multipotent, migratory stem cells, characterized by their ability to differentiate into diverse derivatives, with contributions to the cardiovascular system, craniofacial skeleton, and peripheral nervous system. Distinct subpopulations of neural crest cells are found along the body axis, one of which is the cardiac neural crest. Cardiac neural crest cells (CNCCs) are known to form crucial structures in the heart, with cardiovascular defects such as persistent truncus arteriosus and the misalignment of the pharyngeal arch arteries attributed to defects in CNCC migration, survival, or differentiation (Kirby et al., 1983; Bookman et al., 1987).

The diverse derivatives of CNCCs in the heart have led many to investigate the full spectrum of CNCC contributions to cardiac development. Classically, quail-chick chimeric transplants were used to determine CNCC contributions to the heart, while more recent attempts utilize LacZ or fluorescent reporters to lineage trace CNCCs as they migrate from the neural tube to the embryonic heart. The findings of lineage tracing experiments suggest that CNCCs contribute to the smooth muscle walls of arterial vasculature (Jiang et al., 2000; Plein et al., 2015), the septum and valves that separate the atrial and ventricular compartments of the heart (Kirby et al., 1983; Nakamura et al., 2006), and neuronal and glial derivatives that mediate the parasympathetic innervation of the heart (Hildreth et al., 2008; Végh et al., 2016). More recently, work using retroviral labeling in chick and *Wnt1-Cre2* reporter lines in mice suggest that CNCCs may also contribute to ventricular myocardium, a feature once thought to be limited to non-amniotic vertebrates such as zebrafish (Sato and Yost, 2003; Tang et al., 2019).

Despite the striking multipotency of neural crest cells, there is strong evidence showing that they are heterogenous in that respect. They possess differing developmental potentials—from relatively plastic to committed precursors—that typically become more restricted over the course of development. In light of the CNCC contribution to myocardium in mice, neonatal mouse heart regeneration presents an intriguing case study to investigate the physiological impacts of developing CNCCs. The mouse heart is able to

regenerate up till neonates are a week old, though marked decreases in regenerative ability are seen by postnatal days 3 and 4, with complete loss typically occurring by day 7 (Porello et al., 2011; Notari et al., 2018). Recent work also established a role for CNCCs in zebrafish heart regeneration, further suggesting that changes in CNCCs may be involved in the retention or loss of heart regenerative ability (Sande-Melon et al., 2019; Tang et al., 2019).

To determine how changes in the CNCC specifically may affect cardiac physiology, we perform RNA-seq to compare both CNCC and non-CNCC derivatives in the mouse heart at postnatal day 1 and day 8. Furthermore, we also re-analyze recent data generated from single cell RNA-seq of CNCC derivatives from embryonic and postnatal stages to examine the developmental trajectories of cell lineages and infer how changes within specific cell types might impact heart physiology. Understanding the changing molecular landscape of developing CNCCs might highlight mechanisms through which regeneration loss occurs, and by extension, suggest future avenues through which the regenerative competency of mammalian hearts could be improved.

RESULTS

Transcriptomic changes in the neonatal mouse cardiac neural crest

To compare molecular changes within the cardiac neural crest over the first week of postnatal development, we profiled changes in gene expression using using hearts derived from the *Wnt1-Cre2;Rosa26-tdTomato* mouse model, in which cardiac neural crest cells are labeled with cytoplasmic RFP. We dissected and dissociated hearts from P1 and P8 mice into single cell suspensions, then performed FAC-sorting to isolate *Wnt1:tdTomato*+ cells from *tdTomato*- cells. RNA-sequencing (RNA-seq) was used to compare the transcriptomic profile of *Wnt1*+ cells and *Wnt1*- cells both within and across ages (Fig. 1A).

Hierarchical clustering (Fig. 1C) based on the transcript expression of the 14 samples (P1 n=3 hearts, 6 positive/negative samples, P8=4 hearts, 8 positive/negative samples) reveals that samples generally cluster by age and if they were *Wnt1*+ or *Wnt1*-. At P1, the samples appear to cluster based on the differences between *Wnt1*+ and *Wnt1*- samples. In contrast,

at P8, both *Wnt1*+ and *Wnt1*- samples display relatively similar molecular profiles to one another. However, they segregate into *Wnt1*+ and *Wnt1*- clusters based on their dissimilarity to P1 samples. *Wnt1*+ P8 samples appear to have a more distinct molecular profile from their P1 counterparts than *Wnt1*- P8 samples. This finding suggests that changes in the molecular profile of the neonatal heart could be driven by changes in *Wnt1*+ cells from P1 to P8.

As *Wnt1*+ cells underwent more marked molecular shifts from P1 to P8, we obtained differentially-expressed genes by comparing *Wnt1*+ populations to their *Wnt1*- negative counterparts, and Wnt1+ populations from each age to one another. Differentially expressed genes were then clustered by their relative expression across comparisons—*Wnt1*+ P8 cells (P8+) versus *Wnt1*- P8 cells (P8-), *Wnt1*+ P8 cells versus *Wnt1*+ P1 cells (P1+), and *Wnt1*+ P1 cells versus *Wnt1*- P1 cells (P1-) (Fig. 1D).

This clustering analysis identified 6 modules of genes—modules 1 and 2 were generally upregulated in P1+ cells and downregulated in P8+, while modules 3, 4 and 5 were generally upregulated in P8+ cells and downregulated in P1+ cells. Module 6 was generally upregulated in P8 hearts relative to P1 hearts, likely representing genes that are differentially expressed primarily due to age and not differences within or between *Wnt1*+ or *Wnt1*- populations. Comparative gene ontology (GO) analysis showed that pathways upregulated in P8+ cells can be broadly grouped as growth or immune related. Growth related processes such as myogenesis and oxidative phosphorylation were upregulated in all P8 vs P1+ cells, while immune or stress related processes such as inflammatory response, complement pathway and Il6 JAK-STAT3 signaling were specifically upregulated in P8+ vs P1+ cells. Prolonged inflammation is typically associated with poorer regenerative outcomes, as it is associated with fibrotic remodeling, higher levels of cell death, and adverse cardiac remodeling in mice (Dobaczewski et al., 2010; Cochain et al., 2012).

Single cell analysis of P1 and P7 CNCC-derived populations

As our results suggested that the biological processes mediated by *Wnt1+* cells undergo distinct changes over the first week of development, we sought to identify specific cell types or populations that might drive this shift. We used publicly available single-cell RNA-seq raw data of *Wnt1+* CNCCs derived from *Wnt1-Cre;Rosa26-tdTomato* mouse hearts as published in Chen et al. 2021. We performed novel analyses on the P1 and P7 dataset, representing 9084 CNCC-derived cells. The unsupervised clustering of CNCCs using Leiden clustering (Traag et al., 2019) across the two stages yielded 21-cell clusters, representing 8-cell lineages (Fig. 2A, Fig. 2C).

In accordance with the findings in Chen et al., 2021, the majority of CNCCs represented two lineages: vascular smooth muscle cells (VSMCs), marked by the canonical VSMC marker *Myh11* (Sinha et al., 2014) and the VSMC progenitor marker *Cxcl12* (Liu et al., 2019), and cells of mesenchymal origin (MES) marked by *Pdgfra* and *Lum* (Farahani and Xaymardan, 2015; van Kuijk et al., 2023). Likewise, other cell types among the CNCC derivatives that could be identified by previously established markers included pericytes (c11, *P2ry14* and *Vtn*) (Skelly et al., 2018; Farbehi et al., 2019), melanocytes (c18, *Dct* and *Mlana*) (Mica et al., 2013), endothelial cells (c19, *Pecam1* and *Cdh5*) (Lother et al., 2018), as well as neurons (c20, *Th* and *Phox2b*) (Ernsberger et al., 2000) and Schwann cell precursors (c8 and c12, *Gfra3* and *Mpz*) (Franzén et al., 2019; Hörner et al., 2022) (Fig. 2B, Fig. 2C).

Using cell type specific gene markers, MES lineage cells can be further identified as specific subpopulations of fibroblast or fibroblast-like cells (Fig 2D). Two fibroblast clusters (c2 and c13) express the epithelial fibroblast (EpFB) marker *Tcf21* (Tallquist and Molkentin, 2017), while another (c7) expresses the Wnt inhibitors *Dkk3, Wif1 a*nd *Tbx20*. This reflects a previously identified cardiac fibroblast termed WntX fibroblasts (WntX FB), a population which distinctly expressed inhibitors of canonical and non-canonical Wnt signaling (Farbehi et al., 2019). Fibroblast-like cells included a population of myofibroblasts (Myofb, c15), which express both fibroblast markers (*Vim, Col1a1*) and muscle contractility markers (*Acta2, Tagln, Pdgfrb, Postn*) at high levels (Henderson et al.,

2013; Kanisicak et al., 2016), and a population of mesenchymal stromal cells (MSC, c3) that expresses the stemness marker *Ly6a* (Noseda et al., 2015).

The VSMC populations recovered in our data appear to be able to be differentiated on the basis of maturity, as molecular markers that indicate specific points on the VSMC developmental trajectory are expressed in different clusters (Fig. 2E). The VSMC progenitor marker *Cxcl12* is most highly expressed in VSMC c1 and c6, while the immature VSMC markers for stemness and proliferative ability, *Lgr6* and *Rgs5* (Litviňuková et al., 2020), are expressed in c5. The canonical VSMC marker *Myh11* is expressed at its highest levels in c0, which indicates that those clusters are the most mature VSMCs. Recent work studying VSMC phenotypes has also revealed that VSMCs are both more phenotypically diverse and plastic than previously thought. VSMCs were thought to adopt either a differentiated contractile or de-differentiated synthetic phenotype, where synthetic VSMC characterized by the loss of smooth muscle markers, an increase in proliferation and migration, and an increase in the synthesis of extracellular matrix components (Liu and Gomez, 2019). VSMCs appear to adopt this phenotypic switch under pathological conditions, as VSMCs present in vascular injury or atherosclerosis often lose expression of their contractile gene marker repertoire (Allahverdian et al., 2018; Wamhoff et al., 2004). However, more recent work has further characterized VSMC phenotypes, including observations of mesenchymal-like, fibroblast-like, osteoblast-like and macrophage-like molecular profiles and the ability to readily switch between states or occupy transitional states (Cao et al., 2022). Within our analysis, most VSMC clusters appear to express genes associated with a fibroblast-like and a contractile phenotype (Fig. 2E). As the VSMC clusters seem to differentiate partly based on age, we visualized the UMAP embedding colored by age to distinguish which cells belonged to P1 or P7 and compared it to the UMAP as colored by cell type (Fig. 2F). As expected, the identified cell types were not equally distributed between P1 and P7 timepoints (Fig. 2G). This implies that the phenotypic differences between these populations may reflect key postnatal developmental changes in CNCC-derived cells as the heart physiologically matures.

Computational analysis of CNCC-derived cardiomyocytes in the postnatal heart

One notable divergence in our findings from Chen et al., 2021 is the recovery of a distinct cardiomyocyte cluster in our analysis as Chen et al. do not find support for the direct contribution of CNCC-derived cells to myocardium. vergence in our findings is the recovery of a distinct cardiomyocyte cluster. The existence of CNCC-derived cardiomyocytes in mice has been contested in the literature thus far, but we believe the results of this re-analysis supports previous fate mapping of CNCC cells of *Wnt1-Cre* mice, providing additional confirmation of the crest contribution to the myocardium in amniotes.

Correlation analysis between Leiden clusters results in clusters belonging to common lineages, such as VSMC and mesenchymal lineages, grouping together. However, the putative CM cluster (Leiden, c17) shows little correlation with other clusters, distinguishing it as a discrete cell type. Comparing highly differentially expressed genes (DEGs) in the putative CM cluster with all other clusters yields genes specifically expressed in myocardium (*Tnni3, Tnnt2, Actc1*), as well as genes associated with cardiac muscle function, such as those mediating mitochondrial respiration (*Cox6a2, Cox8b*), cardiac contractility (*Tnnc1, Pln, Mybpc3*) and metabolism (*Ckm, Fabp3*) (Fig. 3B). Searching the top 500 DEGs using the Enrichr interface (Chen et al., 2013; Kuleshov et al., 2016) against a Tabula Muris, a mouse single cell annotated database (The Tabula Muris Consortium et al., 2018), also identifies the cluster as cardiomyocytes (Fig. 3C). Comparing gene expression of common genes associated with myocardial physiology across lineages, including those regulating contractile muscle, Ca^{2+} ion cycling and cardiac metabolism (Guo and Pu, 2020; Wang et al., 2020), revealed that these genes are distinctly highly expressed only in the putative CMs, and not within other cell lineages (Fig. 3D).

The discrepancy in these analyses might be due to limitations of experimental design or differences in computational analyses. We detected a relatively low number of CMs— 70 cells in total, with 54 at P1 and 16 at P7. As mature cardiomyocytes are large, binucleated cells, they might not be effectively captured using single-cell fluidic methods, especially at later stages. In their computational analyses, Chen et al. also regress out a proportion of mitochondrial gene expression. While this is common practice to mitigate uninteresting

sources of variation, it might also remove salient biological covariates (Luecken and Theis, 2019). Cardiomyocyte mitochondrial gene expression is relevant to biological function due to their high energetic needs, and filtering may have removed too many features for cardiomyocytes to form a discrete cluster.

With evidence of postnatal contributions of CNCCs to CMs, we returned to Chen et al.'s full dataset, which contains stages E10.5-E.14.5, E17.5 in addition to P1 and P7. We sought to investigate if putative CMs could also be recovered from embryonic stages, and if so, when they emerged. Unsupervised clustering of the full dataset returned a discrete CM cluster which contained cells across all stages (Fig 3E and E'). Comparing the expression over common CM (*Tnnt2, Tnni3, Tnni1, Myl2, Myh6, Myh7*) (Wang et al., 2020) and earlier CM (*Actc1, Gja1*) (Llucià-Valldeperas et al., 2014) markers across all clusters returned in all data showed that the putative CMs exclusively (Fig. 3F). Intriguingly, the number of CNCC-derived CMs shows an increase over the course of embryonic development, peaking at E17.5 (Fig. 3G). However, they appear to decrease sharply at the time of birth, as there are far fewer cells recorded by P1 (Fig. 3G). This raises the possibility that regenerative ability is already markedly decreased at the time of birth, perhaps as an incidental consequence of cardiac maturation. This is supported by myocardial lineage tracing in embryonic and neonatal mice, as the proportion of proliferating CMs was markedly reduced even at P1 when compared to embryonic development (Sereti et al., 2018). While the typical regenerative window for neonatal mice is usually cited as a week, more recent work suggests that this window might be as short as 3 days, with P2 hearts already showing signs of regenerative decline compared to those at P1 (Notari et al., 2018).

Unsupervised re-clustering of CM cells only across all 7 stages returns 8 cell clusters (Fig. 3H). Using the Enrichr interface to search these genes against gene ontology databases, these 8 clusters broadly represent 7 biological processes (Fig. 3J). These biological processes are not evenly distributed across age and appear to reflect the changing molecular and physiological profile of the maturing heart. Following the numbers of CMs grouped by biological process shows the sharpest declines over developmental time in CM genes regulating mitotic cell cycle and DNA damage repair as well as glycolysis, while those

showing the least declines are related to oxidative phosphorylation and striated muscle contraction (Fig. 3I, Fig. 3J). Additionally, between P1 and P7 only, the most marked decline is seen in genes related to mitotic cell cycle and cytoskeletal properties (Fig. 3K). These physiological changes reflect well-documented changes in mammalian CMs during development, including a metabolic shift from glycolysis to oxidative phosphorylation, as well as a declining ability to repair DNA damage, which has been associated with decreased proliferation (Puente et al., 2014; Nakada et al., 2017, Lalowski et al., 2018). Furthermore, it highlights the questions of how and why embryonic CMs lose their proliferative ability. This has previously been attributed to a number of factors accompanying cardiac maturation. Cardiac ECM becomes more stiff as it matures postnatally, with decreased matrix stiffness encouraging CM de-differentiation and proliferation *in vitro* and *in vivo* (Yahalom-Ronen et al., 2015; Notari et al., 2018). Other work suggests that CM proliferative ability may be correlated with cell cycle gene upregulation, independent of age (Sereti et al., 2018)—this may point to the influence of nonmyocyte cell types, with other cell types such as cardiac fibroblasts secreting pro-maturation factors that promote CM maturation (Wang et al., 2020).

Maturing CNCC derivatives reflect a distinct shift in the molecular profile of the postnatal heart

As our bulk RNA-seq data and the CM analysis suggests, the P8+ population of CNCC derivatives are markedly different from P1+ cells. This was supported by our single-cell analysis of the data, as comparing UMAP visualizations by cell type and age showed that cell types were segregated by age (Fig. 2F and Fig. 2G). Counts of cell types divided by age reveal that certain cell types belong almost entirely to P1 samples, including VSMC 2, VSMC 3 and MSCs, while others such as VSMC 1, EpFB and Myofb conversely belong almost entirely to P7 samples (Fig. 4A). Comparing the percent change of each cell type relative to the total number of cells in both samples, VSMC 1 and VSMC 3 cells show the greatest increase and decrease, respectively, with VSMC 1 cells increasing by 19.86% and VSMC 3 cells decreasing by 18.53% (Fig. 4B). The almost inverse nature of this increase and decrease from P1 to P7 suggested that these populations are part of a developmental

trajectory for CNCC-derived cells. This was also supported by gene markers for VSMC developmental states, as VSMC 1 expressed higher levels of mature VSMC marker *Myh11* and lower levels of progenitor marker *Cxcl12*, while the opposite is true of VSMC 3. In addition to VSMC 1 and VSMC 3, other populations that show a significant shift in their relative composition belong to VSMC and MES lineages (Fig. 4C). VSMC 2 and MSCs markedly decrease while EpFBs increase from P1 to P7. Proliferating VSMCs show a noticeable decrease while still maintaining cells in both P1 and P7 samples, suggesting that they might represent a transitional state, undergoing changes that reflect the changing physiology of maturing VSMCs in the heart.

To confirm that these clusters represent the postnatal developmental trajectory of CNCC-derived VSMCs, we performed RNA velocity analysis using a dynamical model (Bergen et al., 2020) on VSMC and mesenchymal lineage cells, excluding other lineages as well as the documented pericyte-VSMC transition (Fig. 4D and Fig. 4E). Latent time analysis identified one origin point within the mesenchymal stromal cell (MSC) cluster, and one terminal point in the VSMC 1 cluster, supporting the conclusion that VSMC 1 represents the most mature VSMC cells in the neonatal heart (Fig. 4F). Using the directional information from the dynamical model, we used CellRank (Lange et al., 2022) to visualize macrostates, which assign cells to a specific lineage trajectory. We recovered two cellular trajectories for VSMC clusters. (Fig. 4G). Adding to the trajectory suggested by the latent time analysis, the macrostates seem to illustrate the developmental trajectory at P1 and at P7 respectively. Like the latent time analysis, the trajectories originate in MSC cluster, with the P1 trajectory terminating in the VSMC 2 cluster and the P7 trajectory terminating in the VSMC 1 cluster.

To understand how maturing VSMCs may impact the biology of the postnatal heart, we performed pathway enrichment analysis for the top 1000 differentially expressed genes for each VSMC cluster. Interestingly, the differing enriched terms observed in the bulk RNA-seq analysis between P8 and P1 samples are not mirrored by the single-cell analysis. However, the differences between P8+ and P1+ samples are most closely recapitulated by

pathways identified in the VSMC 2 cluster, with immune, stress and growth-related pathways being enriched (Fig. 4H).

When comparing the transcriptomic profile of samples in our bulk RNA-seq data, Wnt1+ cells are noticeably much more different at P8 and P1 than their *Wnt1*- counterparts are from one another (Fig. 1B). Additionally, P1+ cells are also more similar to P8- cells than to P8+ cells. (Fig. 1B). The finer resolution of single-cell data provides more insight into the latter observation: *Wnt1*+ cell populations in the P1 heart can already be delineated by differing levels of maturity as seen with VSMC 2 and VSMC 3 populations. Taken together, our findings suggest that *Wnt1*+ populations undergo noticeable molecular changes over the first week of development, and their postnatal maturation, which is already detectable at P1, occurs more rapidly than that of their *Wnt1*- counterparts. Thus, the maturation of CNCC-derived cell populations may drive a distinct shift in the molecular profile of the neonatal heart, representing the leading edge of molecular changes that in turn could contribute to the differential biological properties of the heart at P1 and >P7.

Potential impacts of maturing CNCC derivatives on cardiac physiology

Next, we asked if maturing CNCC derivatives might have an impact on cardiomyocyte biology. Using CellPhoneDB to perform ligand-receptor analysis, we identified statistically significant interactions between all cell populations and cardiomyocytes. Examining the cell populations that displayed a noticeable shift in proportions from P1 to P7 (MSCs, VSMC 3, VSMC 2, Prolif. VSMCs, EpFBs, VSMC 1), we identified ligand-receptor pairs that were only expressed in cell types predominantly found in P1 vs. P7 hearts (Fig. 5B).

Quantifying the number of interactions from involved cell types revealed that MSCs and EpFBs had the highest number of interactions in predominantly P1 and predominantly P7 cell types, respectively (Fig. 5A). Broadly classifying signaling pathways across P1 and P7 cell types shows that ECM-related signaling comprises a higher proportion of signaling in P7 interactions as compared to P1, but interactions related to cell adhesion and motility represent a higher proportion of interactions in P1 (Fig. 5C). A GO biological process term

enrichment analysis supports the significance of ECM and cell adhesion-related processes in CNCC derivatives, as it returned terms related to extracellular matrix organization and receptor protein tyrosine kinase signaling, which often regulates cytoskeletal modifications that accompany changes in cell adhesion dynamics (Fig. 5D).

One major signaling pathway that regulates the cardiac ECM as well as cell adhesion dynamics is the TGF-β pathway. Performing pathway activity inference against the PROGENy showed that *TGF-β* activity was highest in VSMC populations, and most active in VSMC 2, followed by VSMC 1 cells. *TGF-β* is known to have pleiotropic effects in heart regeneration— while it is known to be a fibrogenic mediator of pathological cardiac and vascular fibrosis, inhibiting *TGF-β* signaling abolished zebrafish heart regeneration, implying *TGF-β*-mediated fibrosis is an essential component of the regenerative response (Chablais and Jaźwińska, 2012). Similarly, *Smad3* KO in mouse myofibroblasts after myocardial infarction resulted in higher mortality due to impaired scar formation and rupture (Kong et al., 2018). This highlights that fibrotic scar formation is not necessarily detrimental and may be necessary for regeneration to take place.

However, one feature that might distinguish P1 and P7 hearts is the composition of cardiac ECM that is produced upon activation of *TGF-β* signaling. Examining the expression of ECM components returned by the ligand-receptor analysis, we see that ECM components differ across age. Most noticeably, *Fn1* and *Col12a1*are more highly expressed in P1 cell types (outlined in pink, Fig. 5F), while *Col15a1* and *Lamc1* are more highly expressed in P7 cell types (outlined in yellow, Fig. 5F). This suggests cardiac ECM compositions that diverge in both their biomechanical properties and potentially their effect on cardiomyocytes. Notably, collagen type XII and fibronectin are major components of the transient scar formed in zebrafish hearts, with collagen type XII partially co-localizing with fibronectin and the de-adhesive matrix protein tenascin C (Marro et al., 2016). Ultrastructural analyses of *Col15a1* null mice revealed that type XV collagen was required to structurally stabilize skeletal muscle, as its interaction with basement membrane components such as laminin resulted in decreased cell adhesion and more degenerative muscle fibers—but also more regenerative fibers (Eklund et al., 2001). ECM/β1 integrin signaling from cardiac fibroblasts is known to regulate myocardial proliferation, both

through paracrine mechanisms and through affecting the stiffness of the cardiac ECM (Ieda et al., 2009; Notari et al., 2018; Wang et al., 2020). Taken together, it appears that P7 components contribute to higher stiffness and more robust cytoskeletal organization, while P1 components form a more pliable matrix that encourages cellular motility. This may have ramifications for cardiomyocytes as myocardial cell cycling has been explicitly linked to matrix biomechanical properties, with stiffer substrates encouraging differentiating and cell cycle exit, and softer substrates promoting continued proliferation (Yahalom-Ronen et al., 2015).

DISCUSSION

Heart regeneration is a carefully coordinated process. It relies on interactions between multiple cell types, both myocytes and non-myocytes alike, and can be affected by changes in systemic metabolism as well as cues from the local microenvironment. The CNCC is thus an intriguing population to examine in this context: with its diverse derivatives and highly responsive nature, it has the potential to affect cardiac regenerative outcomes through multiple avenues. In this study, we compare the transcriptional changes in the mouse heart over the first week of life in both *Wnt1*+ CNCC derivatives and the rest of the heart. We find that *Wnt1*+ cells at P8 show a marked dissimilarity to their *Wnt1*- counterparts, a dissimilarity that is far less pronounced at P1. Together with our computational re-analysis of *Wnt1*+ populations at P1 and P7 at a single cell resolution, we find that these changes are related to an enrichment for growth, immune response, and cytoskeletal genes. The additional resolution provided by single-cell analyses showed that the cell types contained are largely of VSMC or mesenchymal lineages, with most cell types from both lineages being identified as fibroblasts or possessing a fibroblast-like molecular profile. CNCC derivatives are known to facilitate heart regeneration by creating a supportive environment for regeneration, such as by contributing to cardiac adrenergic function in the regenerating heart (Tamura et al., 2016). This demonstrates the important role of non-myocyte cells in cardiac regeneration—crosstalk with cardiac fibroblasts has received much attention in recent work. Fibroblasts secreted factors are known to trigger cardiomyocyte hypertrophy (Bang et al., 2014), interact with resident macrophages to influence monocyte and neutrophil recruitment (Anzai et al., 2017; Sandanger et al., 2013),

as well as determine cardiac ECM composition (Ieda et al., 2009; Williams et al., 2014) — factors that influence the immune response dynamics and mechanical properties of the heart, both significant contributors to regenerative outcome (Aurora et al., 2014; Dolejsi et al., 2022; Yahalom-Ronen et al., 2015; Notari et al., 2018). Notably, IL-6 signaling, which was identified as upregulated in P8+ cells, has been shown to promote CM hypertrophy and fibroblast proliferation, which often leads to adverse cardiac remodeling and fibrosis (Fredj et al., 2005; Bageghni et al., 2018; Nuamnaichati et al., 2018).

Our data suggests that *Wnt1*+ cells undergo a greater shift in molecular profile over the course of early postnatal development than *Wnt1*- cells. While this is interpreted as reflecting a more rapid maturation of the *Wnt1*+ population, this might also suggest that *Wnt1*+ cells are more responsive to external maturation cues, such as shift in cardiac metabolism or increased oxidative stress. It would therefore be of interest to test if this responsiveness is maintained in later postnatal stages, and if manipulating external conditions to those of an earlier state might in turn influence *Wnt1*+ cells to adopt a younger molecular profile. VSMCs and fibroblasts are remarkably plastic cell types, with the demonstrated capability to both adopt different phenotypes in response to changes in environmental and molecular cues both i*n vivo* and *in vitro* (Sorokin et al., 2020; Engel and Ardehali, 2018) and are relatively amenable to being transdifferentiated into different cell types by external interventions (Ieda et al., 2010; Song et al., 2012; Hong et al., 2017). This suggests that the rapid molecular shift observed in CNCC derivatives is perhaps more reflective of an adaptable and responsive cellular profile, rather than an irreversible course of maturation.

While the results of our single-cell analyses are largely concordant with previous analyses of CNCC derivatives (Chen et al., 2021), we recover additional support for CNCC contribution to the myocardium. It is generally accepted from lineage tracing studies in zebrafish and chick that CNCCs differentiate into cardiomyocytes (Sato and Yost, 2003; Abdul-Wajid et al., 2018, Tang et al., 2019), but evidence of cardiomyocyte contribution in the mouse has remained contested (George et al., 2020). Our computational analysis of cardiomyocytes across embryonic and postnatal stages not only supports their existence throughout development, but it also recapitulates the specific dynamics observed in a

population of cardiomyocytes derived from *Isl1*+ CNCCs (Hatzistergos et al., 2020). As described in Hatzistergos et al., we similarly find that CNCC-derived cardiomyocytes peak in late embryonic development and then rapidly decline postnatally, with relatively few remaining at the time of birth. Our analysis of gene ontology terms associated with clusters of cardiomyocytes tracked over developmental age also seems to support general trends associated with cardiac maturity. Established changes in maturing cardiac physiology, such as the shift from glycolysis or the declining ability to fix DNA damage, may thus be responsible for the poor representation of CNCC-derived cardiomyocytes postnatally.

As suggested by the results of our bulk RNA-seq analysis, tracking the developmental trajectory of CNCC derivatives with RNA velocity in single cell analyses shows that the maturation of *Wnt1*+ cells from P1 to P7. Our ligand-receptor analysis reveals the changing signaling milieu of the maturing heart, with notable changes in ECM-receptor and cell adhesion dynamics interactions across age. ECM/ β1 integrin signaling from cardiac fibroblasts has directly been linked to myocardial cell cycling, as immature CMs express higher levels of receptors for collagen and fibronectin, α1 and α5 integrins, while mature cardiomyocytes primarily express laminin receptor α6 and α7 integrins (Ieda et al., 2009). Our results show that, in line with the changing expression of receptors, CNCC derivatives express higher levels of *Fn1* and *Lamc1* at P1 and P7, respectively. Specifically, culturing CMs on laminin and fibronectin-coated plates resulted in CM hypertrophy and proliferation, respectively (Ieda et al., 2009). α5β1 integrin, which is the receptor for fibronectin, is additionally known to activate growth-related signaling by associating with EGF receptors (Moro et al., 1998), which could further promote proliferative signaling in P1 cardiomyocytes. Differences in cellular organization and structure could also be significant in an injury context. The sarcomeric disorganization observed in *Col15a1* null mice not only encouraged regeneration after injury, but it also resulted in higher inflammation as neutrophils were able to infiltrate the cellular milieu more easily. Immune cell infiltration dynamics can be consequential to regenerative outcome, as comparative studies in zebrafish and the non-regenerative medaka showed that delayed neutrophil recruitment and clearance observed in the medaka heart impaired its ability to regenerate (Lai et al., 2017).

The cardiac neural crest and its derivatives represents a unique population of cells within the mammalian heart that could be targeted to improve or restore regenerative outcomes in adulthood. Insights from both its myocardial and non-myocardial lineages across developmental time suggest many compelling directions for future research to take. Myocardial derivatives could be compared across embryonic and postnatal stages to pinpoint the mechanisms through which they lose their proliferative ability—and perhaps if this ability could be restored. The many nonmyocyte, fibroblast-like CNCC derivatives also hold much potential in terms of creating a pro-regenerative cardiac environment, either through better understanding of their crosstalk with other crucial cell types such as those modulating the immune response, or by manipulating molecular signals from older cells to resemble those of younger ones. The diversity of CNCC derivatives thus makes the cardiac neural crest a promising subject of study in mammalian heart regeneration, providing numerous and possibly mutually reinforcing opportunities for interventions that might help us restore the full regenerative potential of the heart.

MATERIALS AND METHODS

Mice

All studies comply with relevant ethical regulations for animal testing and research and received ethical approval by the Institutional Animal Care and Use Committees at the California Institute of Technology. *Wnt1-Cre2;Rosa26tdTomato* mice were obtained by crossing *Wnt1-Cre2* transgenic mice (Jax Labs, 22501) with *Rosa26tdTomato* reporter line mice (Jax Labs, 7914).

Bulk RNA sequencing of *Wnt1*+ and *Wnt1*- cells from postnatal hearts

Tissue collection, cell sorting, and sequencing

For each replicate (P1, n=3; P8, n=4), hearts were collected from *Wnt1-Cre2;R26tdTomato* mice and dissected , homogenized and dissociated into a single cell suspension. *Wnt1-tdTomato* positive and *Wnt1-tdTomato* negative were collected by FAC-sorting on a BD Biosciences FACS Aria Fusion Cell Sorter. cDNA from *tdTomato*-positive and negative cells were prepared using SMART-seq Ultra Low Input RNA Kit V4 (Takara) according to the manufacturer's protocol. Sequencing libraries were built according to Illumina Standard Protocols and sequenced using an Illumina HiSeq2500 sequencer at the Millard and Muriel Jacobs Genetics and Genomics Laboratory (California Institute of Technology, Pasadena, CA).

Bulk RNA-seq analysis

kallisto v.0.44.0 (Bray et al., 2016) was used to generate an index from mouse reference transcriptome (Ensembl GRCm39, release 106). 30 million, 50bp single ended reads from *tdTomato*-positive and negative cells in each replicate were pseudoaligned to the reference transcriptome using the kallisto quant function. The transcript counts were summarized to gene level and converted into edgeR and DESeq2 objects using tximport (Soneson et al., 2015). Spearmann correlation between samples and Pearson correlation between normalized gene counts was performed using the hclust function from the R stats package. Differential gene analysis was performed with DESeq2 (Love et al., 2014), with only genes meeting a cutoff of p-adjusted < 0.001, a log2FC >1 and average cpm >1 used retained.

Hierarchical clustering was carried out on DEGs returning k=6 clusters, and a heatmap of normalized gene counts was generated using Heatmap2. To find which genes were differentially expressed between P8+ and P8-, P8+ and P1+, P8+ and P1- and P1+ and P1- groups, we used edgeR (Robinson et al., 2010) to perform a one-way ANOVA-like test using glmQLFTest for each cluster of genes, returning genes that were upregulated in each comparison.

Computational analysis of single-cell data from *Wnt1*+ postnatal cardiac cells

Data Availability

Raw sequencing reads in FASTQ format for P1 and P7 hearts were acquired from the Sequencing Read Archive under the accession numbers SRR10065152 and SRR10065151, respectively. Raw sequencing reads for E10.5, E11.5, E12.5, E13.5 and E17.5 hearts were acquired from the Sequencing Read Archive under the accession numbers SRR10065157, SRR10065156, SRR10065155,SRR10065154 and SRR10065153, respectively.

Single cell RNA-seq sample barcode processing and UMI counting

The software wrapper kb-python was run to execute the kallisto | bustools pipeline for analyzing single cell RNA-seq data (Bray et al., 2016; Melsted et al., 2021). The function kb ref was used to build a mouse transcriptome index to pseudoalign reads. The lamanno workflow was selected for analysis of the postnatal data to include both exonic and intronic transcripts in the index. The function kb count was then used to pseudoalign the reads to the index and quantify UMIs to generate a gene-barcode matrix as an annotated data file (h5ad) for each run. The mouse transcriptome and GTF version used was Ensembl GRCm39, release 106. Software versions used were kb-python v0.27.2, a wrapper for kallisto v0.46.0 and bustools v0.39.1.

Data cleaning, normalization and scaling

The matrices from each run for the postnatal stages only and all combined data were each concatenated into a single h5ad file. The origin of each row in both the postnatal dataset and the combined dataset was preserved. The Scanpy toolkit v.1.9.3 (Wolf et al., 2018)

was used to filter and pre-process the gene-barcode matrix. A knee plot was used to filter cells and genes by a threshold, removing cells with less than 1500 UMI counts for the postnatal data and under 1800 UMI counts for the combined data, as well as genes with 0 UMI counts. Genes likely arising from random noise were removed by filtering out genes with counts in fewer than 3 cells. Quality control was performed by removing outliers and cells with high mitochondrial gene content, removing cells with under 200 genes, over 6000 genes or above 0.1% mitochondrial content for the postnatal data, and under 200 genes, over 7000 genes or above 0.1% mitochondrial content for the combined data. The data were then normalized to 10,000 reads per cell so counts were comparable between cells, and the raw counts replaced with their logarithm.

Dimensional reduction and clustering of cells

Highly variable genes were determined in Scanpy and subjected to linear dimensional reduction using principal component analysis (PCA). The first 30 principal components of the PCA were used to construct a neighborhood graph of the cells (n neighbors= 30) and embedded in 2-dimensional space using uniform manifold approximation and projection (UMAP) (Becht et al., 2019). The neighborhood graph of cells was clustered using the Leiden clustering algorithm (Traag et al., 2019). Batch correction was not applied to the postnatal dataset as in our analyses of all data, P1 and P7 data did not display noticeable batch effects, and batch correction on only P1 and P7 datasets resulted in the loss of cell populations previously confirmed in published literature.

Differential expression analysis and cell lineage identification

The genetic signature for each cluster was determined using the Wilcoxon rank-sum test with Bonferroni correction (corrected P-value set to <0.05) in Scanpy's _rankgenesgroups_ function. The same function was also used to compare differentially expressed genes between specific clusters.

Analysis of CM data across all stages

The barcodes for the Leiden cluster representing putative CMs were extracted from the concatenated matrix of raw counts. The data were then re-normalized to 10,000 reads per

cell so counts were comparable between cells, and the raw counts replaced with their logarithm. Batch correction was applied to the CM data using Scanorama (v.1.7.3) (Hie et al., 2019). We only used genes selected as highly variable in 2 or more batches. The batch-corrected dataset was integrated with scanorama.integrate_scanpy_. The batch-corrected data was then re-clustered using the first 30 principal components of the PCA were used to construct a neighborhood graph (n neighbors= 15) using the Leiden clustering algorithm (Traag et al., 2019). The cells were embedded in 2-dimensional space using uniform manifold approximation and projection (UMAP) (Becht et al., 2019).

The genetic signature for each cluster was determined using the Wilcoxon rank-sum test with Bonferroni correction (corrected P-value set to <0.05) in Scanpy's _rankgenesgroups_ function. The same function was also used to compare differentially expressed genes between specific clusters.

RNA velocity analysis

RNA velocity analysis on the postnatal dataset was performed using scVelo 0.2.2 (Bergen et al., 2020). The first and second order moments were calculated using the first 30 PCA components. Velocities were then estimated with the scvelo.tl.velocity function and velocity graphs built using the scvelo.tl.velocitygraph function. Genes with different transcriptional dynamics in each cluster were identified using scvelo.tl.rankvelocitygenes. The full splicing kinetics, including the transcription rate, splicing rate and degradation rate, were obtained with scvelo.tl.recoverdynamics. The latent time was inferred with the function scvelo.tl.latenttime.

Cell trajectory analysis

The RNA velocity data was used to compute cell developmental trajectories using CellRank (Lange et al., 2022). Macrostates were calculated with the Generalized Perron Cluster Cluster Analysis (GPCCA). Once terminal states were determined, fate probabilities for each cell were computed with compute_absorption_probabilities.

Interaction analysis

We used CellPhoneDB (v.4.0.0) (Garcia-Alonso et al., 2022) to analyze interactions between cell types after converting gene names to their human orthologs. Using v.4.0.1 of the database, we employed the statistical analysis method in CellPhoneDB, which returns interactions where the mean expression of interacting proteins displays significant cell state specificity. We set a threshold of 10% of cells within a cell type expressing a gene for it to be included in the analysis and set the number of random iterations to use to determine significance to 1000.

Pathway and transcription factor activity inference analysis

The weighted mean method in the decoupleR R package (Badia-i-Mompel et al., 2022) was used to infer regulatory activities, using the PROGENy database (Schubert et al., 2018) for pathway analysis. Geyser plots and expression heatmaps were generated from the normalized activity scores using SCpubr (v1.1.2) (Blanco-Carmona, 2022).

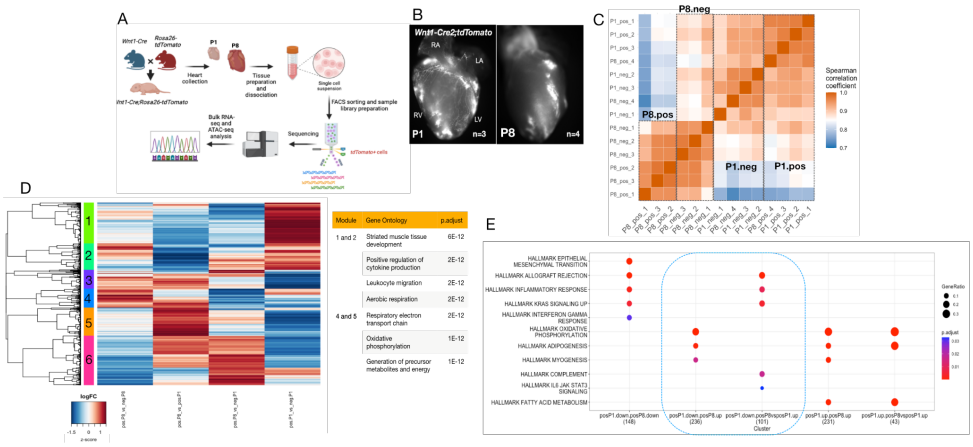


Figure 2 (A) Schematic of RNA-seq experimental design and sample collection (B)Whole *Wnt1-Cre;tdTomato* hearts at P1 and P8. CNCC derivatives labeled by *tdTomato*. RA: Right atrium LA: Left atrium RV: Right ventricle LV: Left ventricle (C) Spearman correlation plot of samples (D) Heatmap of all differentially expressed genes by log10 fold-change across all comparative groups. Selected top enriched GO terms for modules of genes enriched in modules 1 and 2 (P1+ vs. P1-) and modules 4 and 5 (P8+ vs. P8- and P8+ vs. P1+). (E) Pathway enrichment analysis using the formula interface of compareCluster in clusterProfiler, comparing and contrasting DEGs across individual comparisons (adjusted p-value <0.05). In the formula design, DEGs with logfc > 0 in P1+ vs. P1- samples are labeled posP1.up, and DEGs with logfc > 0 in both P8+ vs. P1+ and P8+ vs P8- are labeled posP8.up. DEGs with logfc < 0 meeting the same conditions are labeled posP1.down or posP8 down, respectively. DEGs with logfc > 0 in only one P8+ condition are indicated with the specific comparison.

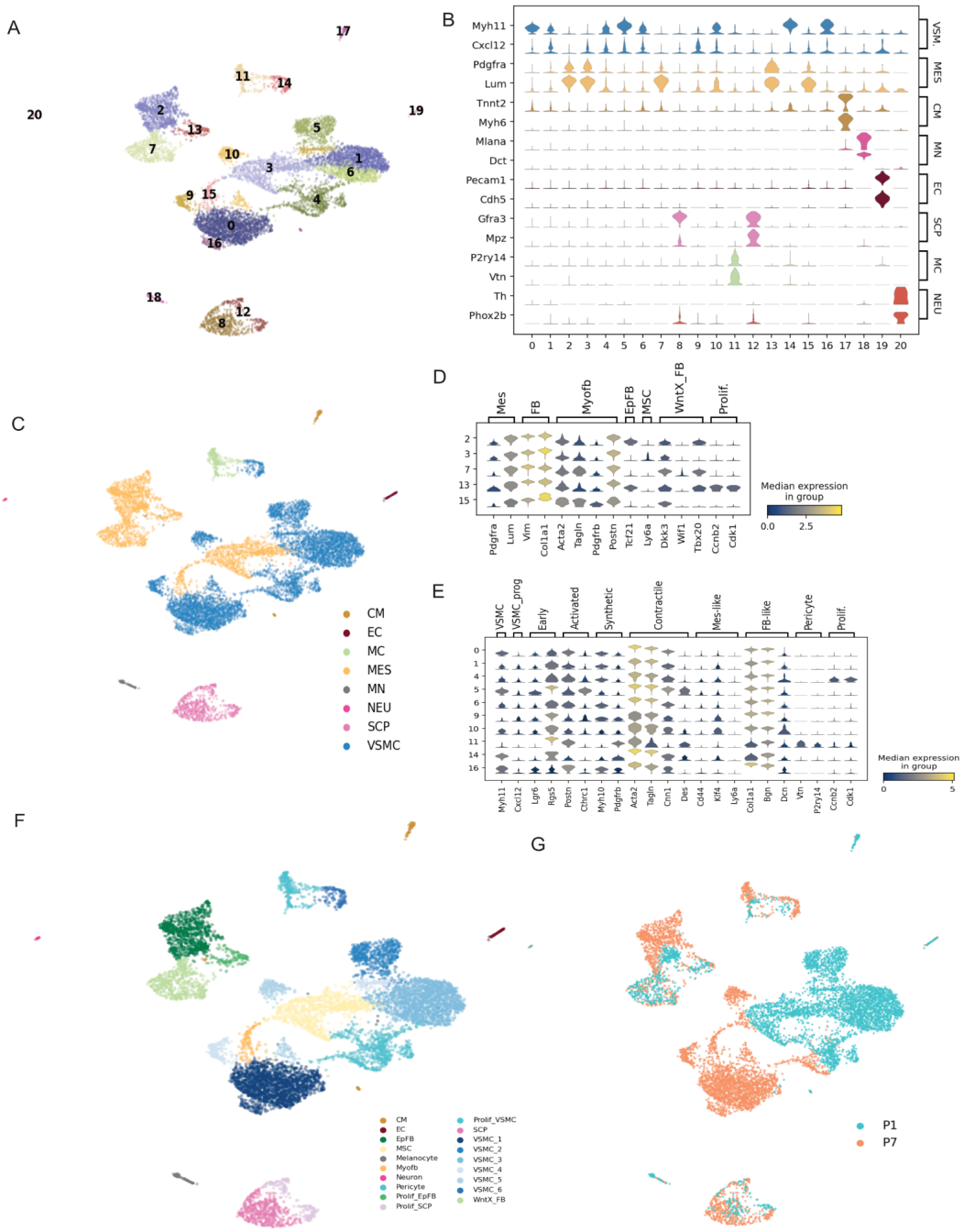


Figure 3 (A) Single-cell transcriptomes of 9084 CNCC derivatives projected onto a two-dimensional UMAP embedding using Leiden clustering. (B) Scaled expression of established marker genes for major cell lineages VSM.: Vascular smooth muscle MES: mesenchymal CM: Cardiomyocyte MN: Melanocyte EC: Endothelial cell SCP: Schwann cell precursor MC: Mural cell NEU: Neuron (C) UMAP with clusters grouped and colored by cell lineages to demonstrate the major cell lineages represented in the data. Each major lineage is represented by one color. (D)

Expression of established gene markers for VSMC subpopulations and phenotypes within CNCC-derived VSMCs (E) Expression of established gene markers for cell types of mesenchymal origin within CNCC-derived mesenchymal cell populations (F) UMAP colored by distinct cell populations identified within each lineage. (G) UMAP of cell populations colored by age

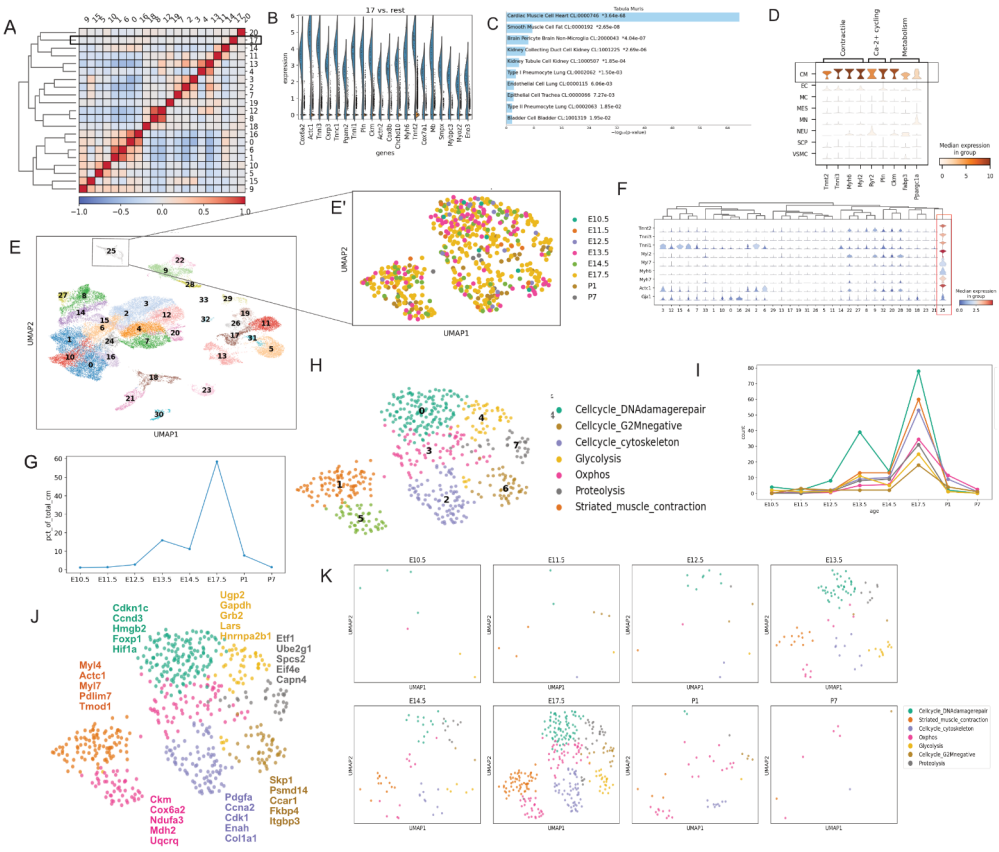


Figure 4 (A) Matrix of Pearson correlation scores between Leiden clusters. c17 (outlined in black), the putative CM cluster, appears to be highly distinct from other clusters. (B) Most differentially-expressed genes in c17 as compared to all other clusters. Genes recovered include established markers for CMs. (C) Top 500 DEGs in putative CM cluster searched against Tabula Muris. Ranked by -log10(p-value) for most significant overlap with databases. Actual p-value shown next to each result. (D) Expression of genes commonly involved in CM-mediated physiological processes in putative CM as compared to all other cell lineages (outlined in black) (E) UMAP of Leiden clusters from combined data across all stages (E') CM cluster recovered from all data colored by age (F) Expression of CM and CM progenitor markers in putative CM cluster (c24, outlined in red) compared to all other clusters recovered from combined data (G) Percent of total CMs found in each

developmental stage (H) UMAP of re-clustered CM cells grouped by Leiden clustering (I) Number of CMs over developmental time, divided into representative biological processes. Leiden c3 and c5 were combined into a single cluster expressing oxidative phosphorylation genes (colored fuschia) (J) Representative DEGs from each cluster, colored by biological process (K) Timeline of CMs colored by biological process, demonstrating the sequence of appearance and order of decline of each cluster.

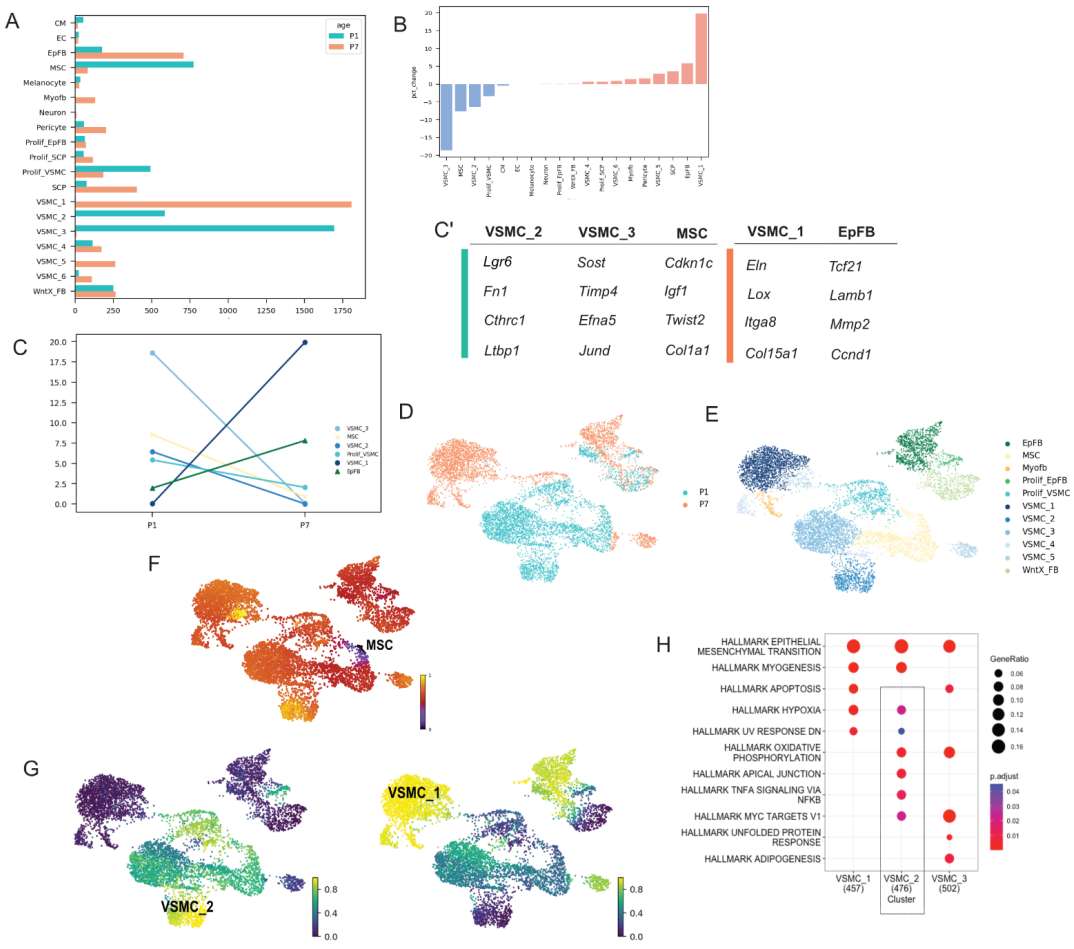


Figure 5 (A) Number of cells in each cell type present at P1 and P7 (B) Percent change of cell types from P1 to P7 relative to the total number of cells in both stages (C) Percent change of cell types that show the most change from P1 to P7. Markers indicate cell lineage and color indicates cell types (C') Selected DEGs from each cluster. Clusters bound by the turquoise line show the sharpest decline from P1 to P7, while clusters bound by the orange line show the highest increase from P1 to P7.(D) UMAP of selected VSMC and MES lineages colored by age (E) UMAP of cell types in selected lineages (F) RNA velocity analysis using latent time identifies a common origin and terminal point within VSMC and

MES populations (G) Macrostates ending in VSMC 2 and VSMC 1 populations, delineating two developmental trajectories for VSMC and MES CNCC-derived cells (H) Pathway enrichment results for VSMC 1, VSMC 2 and VSMC 3 clusters

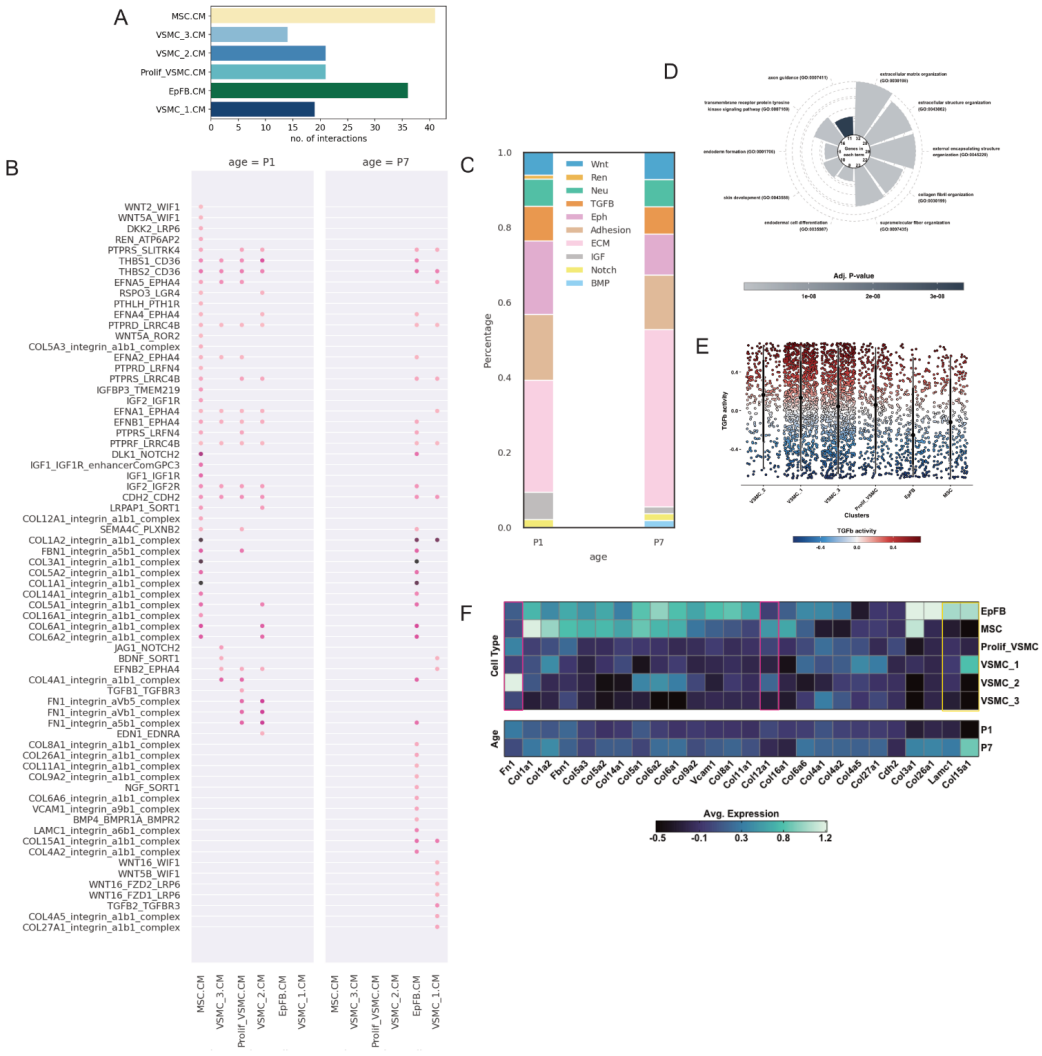


Figure 6. (A) Number of interactions each cell type has with CMs. (B) Statistically significant ligand-receptor pairs recovered from each cell type and CMs across both stages. Ligands originate from non-CM cell types and receptors are found in CMs (C) Relative proportion of ligand-receptor pairs associated with major signaling pathways in P1 and P7 (D) GO term enrichment for all ligand-receptor genes returned by the analysis (D) GO term enrichment for non-ECM component genes returned by the analysis (E) Pathway activity of TGF-β pathway for all cell types (F) Expression heatmap of ECM component genes over cell types and age

BIBLIOGRAPHY

Abdul-Wajid, S., Demarest, B. L., & Yost, H. J. (2018). Loss of embryonic neural crest derived cardiomyocytes causes adult onset hypertrophic cardiomyopathy in zebrafish. *Nature Communications*, *9*(1), Article 1. https://doi.org/10.1038/s41467-018-07054-8

Allahverdian, S., Chaabane, C., Boukais, K., Francis, G. A., & Bochaton-Piallat, M.-L. (2018). Smooth muscle cell fate and plasticity in atherosclerosis. *Cardiovascular Research*, *114*(4), 540–550. https://doi.org/10.1093/cvr/cvy022

Anzai, A., Choi, J. L., He, S., Fenn, A. M., Nairz, M., Rattik, S., McAlpine, C. S., Mindur, J. E., Chan, C. T., Iwamoto, Y., Tricot, B., Wojtkiewicz, G. R., Weissleder, R., Libby, P., Nahrendorf, M., Stone, J. R., Becher, B., & Swirski, F. K. (2017). The infarcted myocardium solicits GM-CSF for the detrimental oversupply of inflammatory leukocytes. *The Journal of Experimental Medicine*, *214*(11), 3293–3310. https://doi.org/10.1084/jem.20170689

Aurora, A. B., Porrello, E. R., Tan, W., Mahmoud, A. I., Hill, J. A., Bassel-Duby, R., Sadek, H. A., & Olson, E. N. (2014). Macrophages are required for neonatal heart regeneration. *The Journal of Clinical Investigation*, *124*(3), 1382–1392. https://doi.org/10.1172/JCI72181

Bageghni, S. A., Hemmings, K. E., Zava, N., Denton, C. P., Porter, K. E., Ainscough, J. F. X., Drinkhill, M. J., & Turner, N. A. (2018). Cardiac fibroblast-specific p38α MAP kinase promotes cardiac hypertrophy via a putative paracrine interleukin-6 signaling mechanism. *The FASEB Journal*, *32*(9), 4941–4954. https://doi.org/10.1096/fj.201701455RR

Bang, C., Batkai, S., Dangwal, S., Gupta, S. K., Foinquinos, A., Holzmann, A., Just, A., Remke, J., Zimmer, K., Zeug, A., Ponimaskin, E., Schmiedl, A., Yin, X., Mayr, M., Halder, R., Fischer, A., Engelhardt, S., Wei, Y., Schober, A., … Thum, T. (2014). Cardiac fibroblast–derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. *The Journal of Clinical Investigation*, *124*(5), 2136–2146. https://doi.org/10.1172/JCI70577

Bergen, V., Lange, M., Peidli, S., Wolf, F. A., & Theis, F. J. (2020). Generalizing RNA velocity to transient cell states through dynamical modeling. *Nature Biotechnology*, *38*(12), Article 12. https://doi.org/10.1038/s41587-020-0591-3

Bookman, D. E., Redmond, M. E., Waldo, K., Davis, H., & Kirby, M. L. (1987). Effect of neural crest ablation on development of the heart and arch arteries in the chick. *American Journal of Anatomy*, *180*(4), 332–341. https://doi.org/10.1002/aja.1001800403

Cao, G., Xuan, X., Hu, J., Zhang, R., Jin, H., & Dong, H. (2022). How vascular smooth muscle cell phenotype switching contributes to vascular disease. *Cell Communication and Signaling : CCS*, *20*, 180. https://doi.org/10.1186/s12964-022-00993-2

Chablais, F., & Jazwinska, A. (2012). The regenerative capacity of the zebrafish heart is dependent on TGFβ signaling. *Development (Cambridge, England)*, *139*(11), 1921–1930. https://doi.org/10.1242/dev.078543

Chen, E. Y., Tan, C. M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G. V., Clark, N. R., & Ma'ayan, A. (2013). Enrichr: Interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics*, *14*, 128. https://doi.org/10.1186/1471-2105-14-128

Chen, W., Liu, X., Li, W., Shen, H., Zeng, Z., Yin, K., Priest, J. R., & Zhou, Z. (2021). Single-cell transcriptomic landscape of cardiac neural crest cell derivatives during development. *EMBO Reports*, *22*(11), e52389. https://doi.org/10.15252/embr.202152389

Cochain, C., Auvynet, C., Poupel, L., Vilar, J., Dumeau, E., Richart, A., Récalde, A., Zouggari, Y., Yin, K. Y. H. W., Bruneval, P., Renault, G., Marchiol, C., Bonnin, P., Lévy, B., Bonecchi, R., Locati, M., Combadière, C., & Silvestre, J.-S. (2012). The Chemokine Decoy Receptor D6 Prevents Excessive Inflammation and Adverse Ventricular Remodeling After Myocardial Infarction. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *32*(9), 2206–2213. https://doi.org/10.1161/ATVBAHA.112.254409

Dobaczewski, M., Bujak, M., Li, N., Gonzalez-Quesada, C., Mendoza, L. H., Wang, X.-F., & Frangogiannis, N. G. (2010). Smad3 signaling critically regulates fibroblast phenotype and function in healing myocardial infarction. *Circulation Research*, *107*(3), 418–428. https://doi.org/10.1161/CIRCRESAHA.109.216101

Dolejsi, T., Delgobo, M., Schuetz, T., Tortola, L., Heinze, K. G., Hofmann, U., Frantz, S., Bauer, A., Ruschitzka, F., Penninger, J. M., Campos Ramos, G., & Haubner, B. J. (2022). Adult T-cells impair neonatal cardiac regeneration. *European Heart Journal*, *43*(28), 2698–2709. https://doi.org/10.1093/eurheartj/ehac153

Eklund, L., Piuhola, J., Komulainen, J., Sormunen, R., Ongvarrasopone, C., Fässler, R., Muona, A., Ilves, M., Ruskoaho, H., Takala, T. E. S., & Pihlajaniemi, T. (2001). Lack of type XV collagen causes a skeletal myopathy and cardiovascular defects in mice. *Proceedings of the National Academy of Sciences*, *98*(3), 1194–1199. https://doi.org/10.1073/pnas.98.3.1194

Engel, J. L., & Ardehali, R. (2018). Direct cardiac reprogramming: progress and promise. *Stem Cells International*, *2018*, 1435746. https://doi.org/10.1155/2018/1435746

Ernsberger, U., Reissmann, E., Mason, I., & Rohrer, H. (2000). The expression of dopamine β-hydroxylase, tyrosine hydroxylase, and Phox2 transcription factors in sympathetic neurons: Evidence for common regulation during noradrenergic induction and diverging regulation later in development. *Mechanisms of Development*, *92*(2), 169–177. https://doi.org/10.1016/S0925-4773(99)00336-6

Farahani, R. M., & Xaymardan, M. (2015). Platelet-derived growth factor receptor alpha as a marker of mesenchymal stem cells in development and stem cell biology. *Stem Cells International*, *2015*, 1–8. https://doi.org/10.1155/2015/362753

Farbehi, N., Patrick, R., Dorison, A., Xaymardan, M., Janbandhu, V., Wystub-Lis, K., Ho, J. W., Nordon, R. E., & Harvey, R. P. (2019). Single-cell expression profiling reveals dynamic flux of cardiac stromal, vascular and immune cells in health and injury. *ELife*, *8*, e43882. https://doi.org/10.7554/eLife.43882

Franzén, O., Gan, L.-M., & Björkegren, J. L. M. (2019). PanglaoDB: A web server for exploration of mouse and human single-cell RNA sequencing data. *Database*, *2019*, baz046. https://doi.org/10.1093/database/baz046

Fredj, S., Bescond, J., Louault, C., Delwail, A., Lecron, J., & Potreau, D. (2005). Role of interleukin-6 in cardiomyocyte/cardiac fibroblast interactions during myocyte hypertrophy and fibroblast proliferation. *Journal of Cellular Physiology*, *204*(2), 428–436. https://doi.org/10.1002/jcp.20307

Garcia-Alonso, L., Lorenzi, V., Mazzeo, C. I., Alves-Lopes, J. P., Roberts, K., Sancho-Serra, C., Engelbert, J., Marečková, M., Gruhn, W. H., Botting, R. A., Li, T., Crespo, B., van Dongen, S., Kiselev, V. Y., Prigmore, E., Herbert, M., Moffett, A., Chédotal, A.,

Bayraktar, O. A., … Vento-Tormo, R. (2022). Single-cell roadmap of human gonadal development. *Nature*, *607*(7919), Article 7919. https://doi.org/10.1038/s41586-022-04918-4

George, R. M., Maldonado-Velez, G., & Firulli, A. B. (2020). The heart of the neural crest: Cardiac neural crest cells in development and regeneration. *Development (Cambridge, England)*, *147*(20), dev188706. https://doi.org/10.1242/dev.188706

Guo, Y., & Pu, W. T. (2020). Cardiomyocyte Maturation. *Circulation Research*, *126*(8), 1086–1106. https://doi.org/10.1161/CIRCRESAHA.119.315862

Hatzistergos, K. E., Durante, M. A., Valasaki, K., Wanschel, A. C. B. A., Harbour, J. W., & Hare, J. M. (2020). A novel cardiomyogenic role for Isl1+ neural crest cells in the inflow tract. *Science Advances*, *6*(49), eaba9950. https://doi.org/10.1126/sciadv.aba9950

Henderson, N. C., Arnold, T. D., Katamura, Y., Giacomini, M. M., Rodriguez, J. D., McCarty, J. H., Pellicoro, A., Raschperger, E., Betsholtz, C., Ruminski, P. G., Griggs, D. W., Prinsen, M. J., Maher, J. J., Iredale, J. P., Lacy-Hulbert, A., Adams, R. H., & Sheppard, D. (2013). Targeting of αv integrin identifies a core molecular pathway that regulates fibrosis in several organs. *Nature Medicine*, *19*(12), Article 12. https://doi.org/10.1038/nm.3282

Hie, B., Bryson, B., & Berger, B. (2019). Efficient integration of heterogeneous single-cell transcriptomes using Scanorama. *Nature Biotechnology*, *37*(6), Article 6. https://doi.org/10.1038/s41587-019-0113-3

Hildreth, V., Webb, S., Bradshaw, L., Brown, N. A., Anderson, R. H., & Henderson, D. J. (2008). Cells migrating from the neural crest contribute to the innervation of the venous pole of the heart. *Journal of Anatomy*, *212*(1), 1–11. https://doi.org/10.1111/j.1469-7580.2007.00833.x

Hong, X., Margariti, A., Le Bras, A., Jacquet, L., Kong, W., Hu, Y., & Xu, Q. (2017). Transdifferentiated human vascular smooth muscle cells are a new potential cell source for endothelial regeneration. *Scientific Reports*, *7*, 5590. https://doi.org/10.1038/s41598-017-05665-7

Hörner, S. J., Couturier, N., Gueiber, D. C., Hafner, M., & Rudolf, R. (2022). Development and in vitro differentiation of Schwann cells. *Cells*, *11*(23), Article 23. https://doi.org/10.3390/cells11233753

Ieda, M., Fu, J.-D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B. G., & Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell*, *142*(3), 375–386. https://doi.org/10.1016/j.cell.2010.07.002

Ieda, M., Tsuchihashi, T., Ivey, K. N., Ross, R. S., Hong, T.-T., Shaw, R. M., & Srivastava, D. (2009). Cardiac fibroblasts regulate myocardial proliferation through β1 integrin signaling. *Developmental Cell*, *16*(2), 233–244. https://doi.org/10.1016/j.devcel.2008.12.007

Jiang, X., Rowitch, D. H., Soriano, P., McMahon, A. P., & Sucov, H. M. (2000). Fate of the mammalian cardiac neural crest. *Development (Cambridge, England)*, *127*(8), 1607–1616. https://doi.org/10.1242/dev.127.8.1607

Kanisicak, O., Khalil, H., Ivey, M. J., Karch, J., Maliken, B. D., Correll, R. N., Brody, M. J., J. Lin, S.-C., Aronow, B. J., Tallquist, M. D., & Molkentin, J. D. (2016). Genetic lineage tracing defines myofibroblast origin and function in the injured heart. *Nature Communications*, *7*, 12260. https://doi.org/10.1038/ncomms12260

Kirby, M. L., Gale, T. F., & Stewart, D. E. (1983). Neural crest cells contribute to normal aorticopulmonary septation. *Science*, *220*(4601), 1059–1061. https://doi.org/10.1126/science.6844926

Kong, P., Shinde, A. V., Su, Y., Russo, I., Chen, B., Saxena, A., Conway, S. J., Graff, J. M., & Frangogiannis, N. G. (2018). Opposing actions of fibroblast and cardiomyocyte Smad3 signaling in the infarcted myocardium. *Circulation*, *137*(7), 707–724. https://doi.org/10.1161/CIRCULATIONAHA.117.029622

Kuleshov, M. V., Jones, M. R., Rouillard, A. D., Fernandez, N. F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S. L., Jagodnik, K. M., Lachmann, A., McDermott, M. G., Monteiro, C. D., Gundersen, G. W., & Ma'ayan, A. (2016). Enrichr: A comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Research*, *44*(W1), W90-97. https://doi.org/10.1093/nar/gkw377

Lai, S.-L., Marín-Juez, R., Moura, P. L., Kuenne, C., Lai, J. K. H., Tsedeke, A. T., Guenther, S., Looso, M., & Stainier, D. Y. (2017). Reciprocal analyses in zebrafish and medaka reveal that harnessing the immune response promotes cardiac regeneration. *ELife*, *6*, e25605. https://doi.org/10.7554/eLife.25605

Lalowski, M. M., Björk, S., Finckenberg, P., Soliymani, R., Tarkia, M., Calza, G., Blokhina, D., Tulokas, S., Kankainen, M., Lakkisto, P., Baumann, M., Kankuri, E., & Mervaala, E. (2018). Characterizing the key metabolic pathways of the neonatal mouse heart using a quantitative combinatorial omics approach. *Frontiers in Physiology*, *9*, 365. https://doi.org/10.3389/fphys.2018.00365

Lange, M., Bergen, V., Klein, M., Setty, M., Reuter, B., Bakhti, M., Lickert, H., Ansari, M., Schniering, J., Schiller, H. B., Pe'er, D., & Theis, F. J. (2022). CellRank for directed single-cell fate mapping. *Nature Methods*, *19*(2), Article 2. https://doi.org/10.1038/s41592-021-01346-6

Litviňuková, M., Talavera-López, C., Maatz, H., Reichart, D., Worth, C. L., Lindberg, E. L., Kanda, M., Polanski, K., Heinig, M., Lee, M., Nadelmann, E. R., Roberts, K., Tuck, L., Fasouli, E. S., DeLaughter, D. M., McDonough, B., Wakimoto, H., Gorham, J. M., Samari, S., … Teichmann, S. A. (2020). Cells of the adult human heart. *Nature*, *588*(7838), 466–472. https://doi.org/10.1038/s41586-020-2797-4

Llucià-Valldeperas, A., Sanchez, B., Soler-Botija, C., Gálvez-Montón, C., Roura, S., Prat-Vidal, C., Perea-Gil, I., Rosell-Ferrer, J., Bragos, R., & Bayes-Genis, A. (2014). Physiological conditioning by electric field stimulation promotes cardiomyogenic gene expression in human cardiomyocyte progenitor cells. *Stem Cell Research & Therapy*, *5*(4), 93. https://doi.org/10.1186/scrt482

Lother, A., Bergemann, S., Deng, L., Moser, M., Bode, C., & Hein, L. (2018). Cardiac endothelial cell transcriptome. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *38*(3), 566–574. https://doi.org/10.1161/ATVBAHA.117.310549

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Luecken, M. D., & Theis, F. J. (2019). Current best practices in single-cell RNA-seq analysis: A tutorial. *Molecular Systems Biology*, *15*(6), e8746. https://doi.org/10.15252/msb.20188746

Mica, Y., Lee, G., Chambers, S. M., Tomishima, M. J., & Studer, L. (2013). Modeling neural crest induction, melanocyte specification, and disease-related pigmentation defects in

hESCs and patient-specific iPSCs. *Cell Reports*, *3*(4), 1140–1152. https://doi.org/10.1016/j.celrep.2013.03.025

Nakada, Y., Canseco, D. C., Thet, S., Abdisalaam, S., Asaithamby, A., Santos, C. X., Shah, A. M., Zhang, H., Faber, J. E., Kinter, M. T., Szweda, L. I., Xing, C., Hu, Z., Deberardinis, R. J., Schiattarella, G., Hill, J. A., Oz, O., Lu, Z., Zhang, C. C., … Sadek, H. A. (2017). Hypoxia induces heart regeneration in adult mice. *Nature*, *541*(7636), 222–227. https://doi.org/10.1038/nature20173

Nakamura, T., Colbert, M. C., & Robbins, J. (2006). Neural crest cells retain multipotential characteristics in the developing valves and label the cardiac conduction system. *Circulation Research*, *98*(12), 1547–1554. https://doi.org/10.1161/01.RES.0000227505.19472.69

Noseda, M., Harada, M., McSweeney, S., Leja, T., Belian, E., Stuckey, D. J., Abreu Paiva, M. S., Habib, J., Macaulay, I., de Smith, A. J., al-Beidh, F., Sampson, R., Lumbers, R. T., Rao, P., Harding, S. E., Blakemore, A. I. F., Eirik Jacobsen, S., Barahona, M., & Schneider, M. D. (2015). PDGFRα demarcates the cardiogenic clonogenic Sca1+ stem/progenitor cell in adult murine myocardium. *Nature Communications*, *6*(1), Article 1. https://doi.org/10.1038/ncomms7930

Notari, M., Ventura-Rubio, A., Bedford-Guaus, S. J., Jorba, I., Mulero, L., Navajas, D., Martí, M., & Raya, Á. (2018). The local microenvironment limits the regenerative potential of the mouse neonatal heart. *Science Advances*, *4*(5), eaao5553. https://doi.org/10.1126/sciadv.aao5553

Nuamnaichati, N., Sato, V. H., Moongkarndi, P., Parichatikanond, W., & Mangmool, S. (2018). Sustained β-AR stimulation induces synthesis and secretion of growth factors in cardiac myocytes that affect on cardiac fibroblast activation. *Life Sciences*, *193*, 257–269. https://doi.org/10.1016/j.lfs.2017.10.034

Plein, A., Fantin, A., & Ruhrberg, C. (2015). Chapter Six—Neural crest cells in cardiovascular development. In P. A. Trainor (Ed.), *Current Topics in Developmental Biology* (Vol. 111, pp. 183–200). Academic Press. https://doi.org/10.1016/bs.ctdb.2014.11.006

Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N., & Sadek, H. A. (2011). Transient regenerative potential of the neonatal mouse heart. *Science (New York, N.Y.)*, *331*(6020), 1078–1080. https://doi.org/10.1126/science.1200708

Puente, B. N., Kimura, W., Muralidhar, S. A., Moon, J., Amatruda, J. F., Phelps, K. L., Grinsfelder, D., Rothermel, B. A., Chen, R., Garcia, J. A., Santos, C. X., Thet, S., Mori, E., Kinter, M. T., Rindler, P. M., Zacchigna, S., Mukherjee, S., Chen, D. J., Mahmoud, A. I., … Sadek, H. A. (2014). The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell*, *157*(3), 565–579. https://doi.org/10.1016/j.cell.2014.03.032

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)*, *26*(1), 139–140. https://doi.org/10.1093/bioinformatics/btp616

Sandanger, Ø., Ranheim, T., Vinge, L. E., Bliksøen, M., Alfsnes, K., Finsen, A. V., Dahl, C. P., Askevold, E. T., Florholmen, G., Christensen, G., Fitzgerald, K. A., Lien, E., Valen, G., Espevik, T., Aukrust, P., & Yndestad, A. (2013). The NLRP3 inflammasome is up-regulated in cardiac fibroblasts and mediates myocardial ischaemia–reperfusion injury. *Cardiovascular Research*, *99*(1), 164–174. https://doi.org/10.1093/cvr/cvt091

Sande-Melón, M., Marques, I. J., Galardi-Castilla, M., Langa, X., Pérez-López, M., Botos, M.-A., Sánchez-Iranzo, H., Guzmán-Martínez, G., Ferreira Francisco, D. M., Pavlinic, D., Benes, V., Bruggmann, R., & Mercader, N. (2019). Adult sox10+ cardiomyocytes contribute to myocardial regeneration in the zebrafish. *Cell Reports*, *29*(4), 1041-1054.e5. https://doi.org/10.1016/j.celrep.2019.09.041

Sato, M., & Yost, H. J. (2003). Cardiac neural crest contributes to cardiomyogenesis in zebrafish. *Developmental Biology*, *257*(1), 127–139. https://doi.org/10.1016/s0012-1606(03)00037-x

Schaum, N., Karkanias, J., Neff, N. F., May, A. P., Quake, S. R., Wyss-Coray, T., Darmanis, S., Batson, J., Botvinnik, O., Chen, M. B., Chen, S., Green, F., Jones, R. C., Maynard, A., Penland, L., Pisco, A. O., Sit, R. V., Stanley, G. M., Webber, J. T., … Principal investigators. (2018). Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. *Nature*, *562*(7727), Article 7727. https://doi.org/10.1038/s41586-018-0590-4

Sereti, K.-I., Nguyen, N. B., Kamran, P., Zhao, P., Ranjbarvaziri, S., Park, S., Sabri, S., Engel, J. L., Sung, K., Kulkarni, R. P., Ding, Y., Hsiai, T. K., Plath, K., Ernst, J., Sahoo, D., Mikkola, H. K. A., Iruela-Arispe, M. L., & Ardehali, R. (2018). Analysis of cardiomyocyte clonal expansion during mouse heart development and injury. *Nature Communications*, *9*(1), Article 1. https://doi.org/10.1038/s41467-018-02891-z

Skelly, D. A., Squiers, G. T., McLellan, M. A., Bolisetty, M. T., Robson, P., Rosenthal, N. A., & Pinto, A. R. (2018). Single-cell transcriptional profiling reveals cellular diversity and intercommunication in the mouse heart. *Cell Reports*, *22*(3), 600–610. https://doi.org/10.1016/j.celrep.2017.12.072

Soneson, C., Love, M. I., & Robinson, M. D. (2015). Differential analyses for RNA-seq: Transcript-level estimates improve gene-level inferences. *F1000Research*, *4*, 1521. https://doi.org/10.12688/f1000research.7563.2

Song, K., Nam, Y.-J., Luo, X., Qi, X., Tan, W., Huang, G. N., Acharya, A., Smith, C. L., Tallquist, M. D., Neilson, E. G., Hill, J. A., Bassel-Duby, R., & Olson, E. N. (2012). Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature*, *485*(7400), 599–604. https://doi.org/10.1038/nature11139

Sorokin, V., Vickneson, K., Kofidis, T., Woo, C. C., Lin, X. Y., Foo, R., & Shanahan, C. M. (2020). Role of vascular smooth muscle cell plasticity and interactions in vessel wall inflammation. *Frontiers in Immunology*, *11*. https://www.frontiersin.org/articles/10.3389/fimmu.2020.599415

Tallquist, M. D., & Molkentin, J. D. (2017). Redefining the identity of cardiac fibroblasts. *Nature Reviews. Cardiology*, *14*(8), 484–491. https://doi.org/10.1038/nrcardio.2017.57

Tamura, Y., Sano, M., Nakamura, H., Ito, K., Sato, Y., Shinmura, K., Ieda, M., Fujita, J., Kurosawa, H., Ogawa, S., Nakano, S., Matsuzaki, M., & Fukuda, K. (2016). Neural crest-derived resident cardiac cells contribute to the restoration of adrenergic function of transplanted heart in rodent. *Cardiovascular Research*, *109*(3), 350–357. https://doi.org/10.1093/cvr/cvv267

Tang, W., Martik, M. L., Li, Y., & Bronner, M. E. (2019). Cardiac neural crest contributes to cardiomyocytes in amniotes and heart regeneration in zebrafish. *ELife*, *8*, e47929. https://doi.org/10.7554/eLife.47929

Traag, V. A., Waltman, L., & van Eck, N. J. (2019). From Louvain to Leiden: Guaranteeing well-connected communities. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-41695-z

van Kuijk, K., McCracken, I. R., Tillie, R. J. H. A., Asselberghs, S. E. J., Kheder, D. A., Muitjens, S., Jin, H., Taylor, R. S., Wichers Schreur, R., Kuppe, C., Dobie, R., Ramachandran, P., Gijbels, M. J., Temmerman, L., Kirkwoord, P. M., Luyten, J., Li, Y., Noels, H., Goossens, P., … Sluimer, J. C. (2023). Human and murine fibroblast single-cell transcriptomics reveals fibroblast clusters are differentially affected by ageing and serum cholesterol. *Cardiovascular Research*, cvad016. https://doi.org/10.1093/cvr/cvad016

Végh, A. M. D., Duim, S. N., Smits, A. M., Poelmann, R. E., Ten Harkel, A. D. J., DeRuiter, M. C., Goumans, M. J., & Jongbloed, M. R. M. (2016). Part and parcel of the cardiac autonomic nerve system: Unravelling its cellular building blocks during development. *Journal of Cardiovascular Development and Disease*, *3*(3), 28. https://doi.org/10.3390/jcdd3030028

Wamhoff, B. R., Hoofnagle, M. H., Burns, A., Sinha, S., McDonald, O. G., & Owens, G. K. (2004). A G/C element mediates repression of the SM22alpha promoter within phenotypically modulated smooth muscle cells in experimental atherosclerosis. *Circulation Research*, *95*(10), 981–988. https://doi.org/10.1161/01.RES.0000147961.09840.fb

Wang, L., Yu, P., Zhou, B., Song, J., Li, Z., Zhang, M., Guo, G., Wang, Y., Chen, X., Han, L., & Hu, S. (2020). Single-cell reconstruction of the adult human heart during heart failure and recovery reveals the cellular landscape underlying cardiac function. *Nature Cell Biology*, *22*(1), Article 1. https://doi.org/10.1038/s41556-019-0446-7

Williams, C., Quinn, K. P., Georgakoudi, I., & Black, L. D. (2014). Young developmental age cardiac extracellular matrix promotes the expansion of neonatal cardiomyocytes in vitro. *Acta Biomaterialia*, *10*(1), 10.1016/j.actbio.2013.08.037. https://doi.org/10.1016/j.actbio.2013.08.037

Wolf, F. A., Angerer, P., & Theis, F. J. (2018). SCANPY: Large-scale single-cell gene expression data analysis. *Genome Biology*, *19*(1), 15. https://doi.org/10.1186/s13059-017-1382-0

Yahalom-Ronen, Y., Rajchman, D., Sarig, R., Geiger, B., & Tzahor, E. (2015). Reduced matrix rigidity promotes neonatal cardiomyocyte dedifferentiation, proliferation and clonal expansion. *ELife*, *4*, e07455. https://doi.org/10.7554/eLife.07455

Chapter 4

FUTURE DIRECTIONS

Proposal: Investigating the role of CNCC derivatives in mouse heart regeneration

While the role of the CNCC has been studied in non-amniotic vertebrates such as the zebrafish (Sande-Melon et al., 2019, Tang et al., 2019), the CNCC's contribution to mouse heart regeneration is not as well characterized. Though CNCCs facilitated heart regeneration by promoting adrenergic innervation of the heart (Tamura et al., 2016), the endogenous contributions of the CNCC lineage in mammalian heart regeneration have not been studied directly. However, previous work supports the notion that CNCC derivatives do play a significant role in mammalian heart regeneration. *Twist2*+ cells in the heart were proposed as a multipotent cardiac progenitor cell that could participate in regeneration by differentiating into endothelial cells and fibroblasts (Min et al., 2018). *Twist2* is a transcription factor known to play a role in CNCC migration (VanDusen and Firulli, 2012), and previous work, including our analyses, show that CNCCs can differentiate into both endothelial cells and fibroblasts (Chen et al., 2021). Furthermore, another study explicitly identifies *Isl1*+ CNCC derivatives as a progenitor population in the heart, demonstrating that fetal CNCC-derived CMs are highly proliferative (Hatzistergos et al., 2020), with their distribution in the embryonic heart appearing similar to the clonal expansion of trabecular and intraventricular septum (IVS) CMs, which proliferate in clusters that are dispersed throughout the myocardium (Meilhac et al., 2004).

Combined with the characterization of CNCCs conducted in Chapter 3, I believe that this provides strong motivations for studying the role of CNCC derivatives in mouse heart regeneration directly, as valuable insight may be gained from further investigation of both myocardial and non-myocardial lineages of CNCCs. In terms of myocardial cells, it would be valuable to understand if there are differences specifically between CNCC-derived CMs

and their non-CNCC derived counterparts. I propose comparing their proliferation dynamics over the course of embryonic development, and directly comparing the molecular profiles of CMs from each lineage across embryonic stages with bulk RNA sequencing. Secondly, to confirm the involvement of the age-related biological correlates we discuss in Chapter 3, it would be beneficial to conduct single nuclei RNA-sequencing in regenerative and non-regenerative *Wnt1-Cre2;tdTomato* mouse hearts. Lastly, to better understand how CNCC derivatives may facilitate regeneration, I propose a conditional ablation experiment in regenerating P1 mice. To do so, I make use of a combined *Cre* and *(r)tTA* gene driver system where CNCC derivatives in the heart specifically can be targeted and ablated after cryoinjury of the heart. With the precision granted by this conditionally-activated, dual driver system, we can better avoid confounding phenotypes and high mortality rates that may arise from general ablation of all CNCC derivatives, or surgical intervention on already-sickly neonates.

Specific Aim 1: Comparative transcriptional profiling of CNCC-derived *Wnt1+* CMs with non-CNCC derived CMs in embryonic and postnatal hearts

Our results support existing work suggesting that CNCC-derived CMs display distinct dynamics of proliferation and decline over the course of development. In our analyses, CNCC-CMs peak during late embryonic development (E17.5), and display a precipitous decline postnatally. While we recorded 334 CMs at E17.5, only 44 were captured at P1. These results are in concordance with another study that tracked *Isl1+* CNCC derivatives, with fetal CMs undergoing a phase of high proliferation but losing this ability postnatally (Hatzistergos et al., 2020). This is also supported by work showing CM proliferative ability, despite being present at birth, is already markedly reduced compared to proliferation during embryonic development (Sereti et al., 2018). Hatzistergos et al. suggest that this is due to the loss of clonal expansion ability of CNCC-CMs postnatally, hypothesizing that major changes in cardiac physiology, such as ventricular trabeculation and the metabolic shift from glycolysis to fatty acid oxidation could be responsible. It would thus be of interest to investigate the differences between CNCC and non-CNCC derived CMs, with the ultimate goal of understanding why CM proliferative capacity diminishes after birth.

In the context of heart regeneration, pan-myocardial lineage tracing shows that distinct subpopulations of CMs contribute to regenerating hearts in zebrafish and in mice (Gupta et al., 2013, Sereti et al., 2018). These CMs seem to be more able to re-enter the cell cycle and contribute to new myocardium. In the mouse, these CMs, which were quite limited by P1, had higher levels of cell cycle genes and early cardiac development transcription factors (*Gata4, Nkx2-5, Tbx18*), and lower levels of structural proteins associated with mature cardiomyocytes (*Myh6, Ttn, Myl4, Flnc*) (Sereti et al., 2018). This is concordant with transcriptomic profiling of CNCC-CMs in the regenerating zebrafish heart, as zebrafish CNCC-CMs retain the ability to proliferate, and reactivate an embryonic neural crest molecular profile (Tang et al., 2019)

First, it would be beneficial to establish if CNCC-CMs are more proliferative during embryonic development than non-CNCC CMs. We could quantify the numbers of proliferating CMs conducting BrdU pulse-chase experiments in *Wnt1-Cre2;tdTomato* mice, followed by staining with a cardiomyocyte marker such as anti-αMHC and wheat germ agglutinin to visualize cell outlines. $BrdU^{+}$*Wnt1*$^{+}$$\alpha MHC^{+}$ cells could be quantified and compared to both the total number of BrdU+ cells and $BrdU^{+}$*Wnt1*$^{-}$$\alpha MHC^{+}$ cells at multiple points in embryonic development, starting at E9.5, as our analyses show that CNCC-CMs are present as early as E10.5. Understanding the dynamics of CNCC-CM proliferation might give us better insight into whether their dynamics differ from non-CNCC CMs. Intriguingly, pan-myocardial tracing of CM proliferation in mice showed that proliferative ability globally declined from E9.5, but our analyses of CNCC-CMs specifically show that CNCC-CMs only peak in late embryonic development. This might suggest that CNCC-CMs either retain their proliferative ability for longer during development, or their differential developmental dynamics result in CMs that are comparatively more “immature” than non-CNCC CMs.

Secondly, while acquiring postnatal CNCC-CMs in large enough numbers for transcriptomic study might be challenging, transcriptomic profiling and comparison of embryonic CNCC-CMs and non-CNCC CMs could be feasible at least for bulk RNA-sequencing. Recently developed intersectional reporter systems could be used to identify CNCC-CMs, non-CNCC CMs, and non-myocyte CNCC derivatives, such as the *Rosa26*-

Traffic Light Reporter (*R26-TLR*) dual recombinase line (Liu et al., 2020). The *R26-TLR* reporter is designed with both *rox* and *loxP* stop sites, with expression levels of *ZsGreen* and *tdTomato* being mediated by independent *Cre-lox* and *Dre-rox* recombination events respectively (Fig. 7A). Thus, crossing this reporter line with *Tnni3-Dre* (He et al., 2017) and *Wnt1-Cre2* would eventually result in *Wnt1-Cre2;Tnni3-Dre;R26-TLR* mice with *Tnni3*+ cardiomyocytes expressing *ZsGreen*, *Wnt1*+ CNCC derivatives expressing *tdTomato*, and $Tnni3^{+}Wnt1^{+}$ CNCC-CMs expressing both reporters (Fig. 7B). Evidence of $Tnni3^{+}Wnt1^{+}$ cells would provide additional confirmation of CNCC-CMs, and CMs could then be FAC-sorted and sequenced according to their origin.

Specific Aim 2: Single nuclei transcriptional profiling of cardiac neural crest-derived cells in regenerating postnatal mouse hearts

Using *Wnt1-Cre2* mice, we will selectively label CNCC derivatives in P1 and P8 mouse hearts. We will then isolate ventricular tissue, dissociate and FAC-sort at the single nuclei level to compare molecular changes accompanying the loss of regenerative ability. Though datasets of single-cell RNA-seq results for *Wnt1* cells already exist (Chen et al., 2021, Iwase et al., 2022), the large, multinucleated nature of mature cardiomyocytes means that a significant number of cardiomyocytes would not be captured by DropSeq methods. This may be reflected in the low number of cardiomyocytes at P7 in Chen et al.'s dataset. While CNCC-derived CMs do decrease postnatally, it is vital to ensure that the decreased number we are observing truly reflects a biological decline, and not a technical limitation. Thus, repeating the experiment with a protocol designed to isolate single nuclei from postnatal mice (such as Cui and Olson, 2020), will confirm the true extent of decline from embryonic to postnatal stages.

We will then perform cryoinjury experiments in *Wnt1-Cre2* mice at P1 and P8. Neonatal mice will be anesthetized by hypothermia on ice for 3-5 minutes while insulated in a glove to prevent direct contact to ice. Pups will be placed in the surgical area in a supine position and their limbs and tail will be immobilized with tape. The chest area will be sterilized with an alcohol swab prior to surgery. Iridectomy scissors will be used to make a 1cm transverse incision over the left side of chest at the armpit level. The skin will be held by tweezers to

elevate it from the underlying muscle to facilitate the skin incision. Tweezers will be used to bluntly dissect the intercostal muscles at fifth intercostal space until the chest cavity can be accessed. Gentle pressure will be applied to the pup's torso to expose the heart from the thoracic cavity. A 1.5 mm metal probe in liquid nitrogen (prechilled for 30s) will be applied to the left ventricular anterolateral wall for 2s to create an injury. Sham-operated hearts are only exposed without probe application. Chest will be sutured closed using 7-0 Prolene, including the upper and lower ribs. Pups will be checked for bleeding and remaining blood will be removed with sterile gauze. Skin edges will be closed and the wound sealed by surgical glue.

Ventricular tissue will be collected 3 days post-injury and flow-assisted cytometry will be used to sort CNCC derivatives from the heart. Based on our results, we hypothesize that immune related and cytoskeletal modifications, both in ECM-receptor interactions and cell adhesion and motility, will be differentially regulated. Previous work in mouse heart regeneration also shows that longer term response differences persist: in P1 mice, inflammatory cytokine-related genes are strongly upregulated but only for the first three days post-injury, while P8 mice have a blunted initial response that increases and persists even a week after injury (Whitehead and Engler, 2021). Given how specific temporal dynamics affects contributing factors such as the immune response, repeating these experiments with hearts collected at various time points—e.g., one week and two weeks after injury—might thus provide additional valuable insight.

Specific Aim 3: Effects of genetic ablation of neural crest derived cells on heart regeneration in P1 mice

Previous work suggests that CNCC-derived CMs are not essential for successful development of myocardium, as *Wnt1* gain-of-function (GOF) experiments in mice, which directs CNCC derivatives to non-CM fates, still result in cardiac anatomy that appears grossly ordinary (Hatzistergos et al., 2020), perhaps due to the ability of CMs from other sources being able to compensate. In zebrafish, CNCC-CM ablation similarly does not affect heart development, or even final numbers of CMs, but intriguingly results in

pathological myocardial hypertrophy and decreased regenerative ability (Abdul-Wadjid et al., 2018; Sande-Melon et al., 2019, Tang et al., 2019). These results suggest that CNCC-CMs may possess a higher tendency to build myocardium by proliferation rather than grow via hypertrophy, as most mature CMs do. In regenerative contexts, this tendency might translate to an increased ability to re-enter the cell cycle and become proliferative once again.

It would be thus of interest to investigate if CNCC ablation also results in the same pathological hypertrophy (preliminary observations by Hatzistergos et al., 2020 suggest that *Wnt1*-GOF resulted in trabecular and IVS hypertrophy), and if CNCC ablation affects regeneration in neonatal mice. Simply ablating all *Wnt1*-labeled CNCC derivatives would be likely to result in high rates of mortality, particularly when combined with surgical intervention. Thus, we propose a method of conditional ablation of CNCC derivatives in the neonatal mouse heart. To do so, we will make use of the *TigreDragon-DTA* mouse line (Ahmadzadeh et al., 2020). The *TigreDragon*-DTA line (*Igs7tm2(tetO-tdTomato,-DTAG128D)Rdiez/AljJ*, #34778, Jax Labs) conditionally expresses Diphtheria toxin (DTA), but requires both *Cre* recombinase and *(r)tTA* drivers. *TigreDragon*-DTA mice will first be crossed with *Nkx2.5-Cre* mice, and *Nkx2.5-Cre; TigreDragon*-DTA/+ offspring subsequently crossed to *Wnt1-tTA* mice (*Tg(Wnt1-rtTA2SM2)1Whsu/J,* #26194, Jax Labs) to generate *Wnt1*-tTA/+;*Nkx2.5-Cre;TigreDragon-DTA*/+ mice. To suppress *(r)tTA* activity, 1 mg/mL of doxycycline (Dox) will be added to the drinking water of pregnant and nursing mice. After cryoinjury experiments in P1 pups, Dox will be withdrawn to induce DTA ablation of *Wnt1*+ cells in the heart.

3 weeks post injury, hearts will be collected and processed for histological and immunohistochemical analysis. Masson trichome and H&E staining will be performed on heart sections to determine the extent of fibrotic scarring (stained blue in Masson trichome staining) across the injured area. Relative regenerative success will be determined through measurements and observations of scarring, as well as the expression of myocardial markers such as troponin-I and troponin-T. BrdU pulse-chase experiments will also be carried out, with hearts collected 3-, 7- and 21-days post injury to examine cardiomyocyte

proliferation, which will be assessed through BrdU+ colocalization with a cardiomyocyte specific antibody such as one for αMHC.

Hearts will be examined for signs of hypertrophy by firstly quantifying the percentage of an area in a ventricular section that is covered by αMHC staining compared to non-ablated hearts. In hypertrophic cardiomyopathies, cardiomyocyte numbers remain unchanged, but cardiomyocyte size is significantly increased (Harvey and Leinwald, 2011). The overall number of cardiomyocytes will be determined using DAPI colocalization with αMHC staining, combined with wheat germ agglutinin to determine cell boundaries. Individual cardiomyocyte size will be measured in images of sections, measuring single cardiomyocytes length- and width-wise.

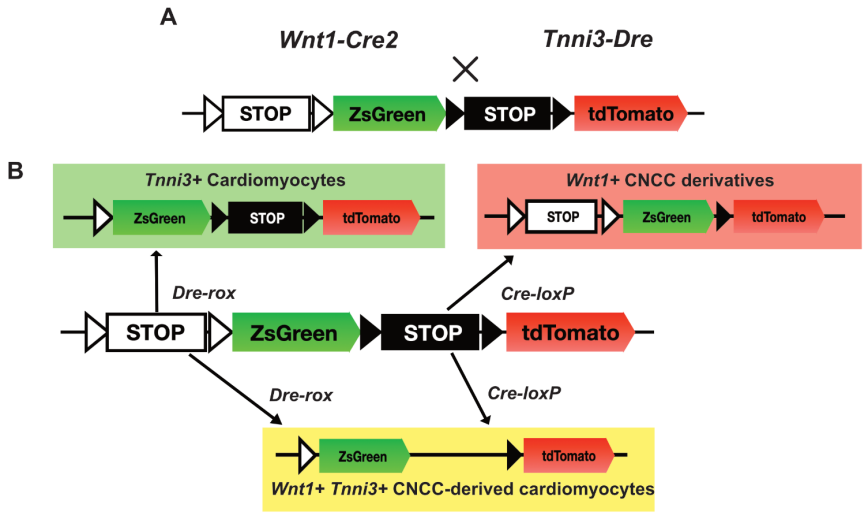


Figure 7. (A) Cross to generate *Wnt1-Cre2;Tnni3-Dre;R26TLR* mice (B) Independent recombination events allows for three separate populations of cells to express different fluorescent reporters. *Dre-rox* recombination with *Tnni3-Dre* results in cardiomyocytes expressing *ZsGreen*, *Cre-loxP* recombination with *Wnt1-Cre2* results in all CNCC-derivatives expressing *tdTomato*, and dual recombination of *Dre* and *Cre* drivers results in CNCC-CMs expressing both *ZsGreen* and *tdTomato.*

BIBLIOGRAPHY

Abdul-Wajid, S., Demarest, B. L., & Yost, H. J. (2018). Loss of embryonic neural crest derived cardiomyocytes causes adult onset hypertrophic cardiomyopathy in zebrafish. *Nature Communications*, *9*, 4603. https://doi.org/10.1038/s41467-018-07054-8

Ahmadzadeh, E., Bayin, N. S., Qu, X., Singh, A., Madisen, L., Stephen, D., Zeng, H., Joyner, A. L., & Rosello-Diez, A. (2020). A collection of genetic mouse lines and related tools for inducible and reversible intersectional mis-expression. *Development (Cambridge, England)*, *147*(10), dev186650. https://doi.org/10.1242/dev.186650

Chen, W., Liu, X., Li, W., Shen, H., Zeng, Z., Yin, K., Priest, J. R., & Zhou, Z. (2021). Single-cell transcriptomic landscape of cardiac neural crest cell derivatives during development. *EMBO Reports*, *22*(11), e52389. https://doi.org/10.15252/embr.202152389

Cui, M., & Olson, E. N. (2020). Protocol for single-nucleus transcriptomics of diploid and tetraploid cardiomyocytes in murine hearts. *STAR Protocols*, *1*(2), 100049. https://doi.org/10.1016/j.xpro.2020.100049

Gupta, V., Gemberling, M., Karra, R., Rosenfeld, G. E., Evans, T., & Poss, K. D. (2013). An injury-responsive *Gata4* program shapes the zebrafish cardiac ventricle. *Current Biology : CB*, *23*(13), 1221–1227. https://doi.org/10.1016/j.cub.2013.05.028

Harvey, P. A., & Leinwand, L. A. (2011). Cellular mechanisms of cardiomyopathy. *The Journal of Cell Biology*, *194*(3), 355–365. https://doi.org/10.1083/jcb.201101100

Hatzistergos, K. E., Durante, M. A., Valasaki, K., Wanschel, A. C. B. A., Harbour, J. W., & Hare, J. M. (2020). A novel cardiomyogenic role for Isl1+ neural crest cells in the inflow tract. *Science Advances*, *6*(49), eaba9950. https://doi.org/10.1126/sciadv.aba9950

He, L., Li, Y., Li, Y., Pu, W., Huang, X., Tian, X., Wang, Y., Zhang, H., Liu, Q., Zhang, L., Zhao, H., Tang, J., Ji, H., Cai, D., Han, Z., Han, Z., Nie, Y., Hu, S., Wang, Q. D., Sun, R., … Zhou, B. (2017). Enhancing the precision of genetic lineage tracing using dual recombinases. *Nature Medicine*, *23*(12), 1488–1498. https://doi.org/10.1038/nm.4437

Iwase, A., Uchijima, Y., Seya, D., Kida, M., Higashiyama, H., Matsui, K., Taguchi, A., Yamamoto, S., Fukuda, S., Nomura, S., Kohro, T., Shukunami, C., Akiyama, H., Seki, M., Suzuki, Y., Wada, Y., Aburatani, H., Kurihara, Y., & Miyagawa-Tomita, S. (n.d.). *Cardiac neural crest lineage diversity and underlying gene regulatory networks revealed by multimodal analysis*. 36.

Liu, K., Tang, M., Jin, H., Liu, Q., He, L., Zhu, H., Liu, X., Han, X., Li, Y., Zhang, L., Tang, J., Pu, W., Lv, Z., Wang, H., Ji, H., & Zhou, B. (2020). Triple-cell lineage tracing by a dual reporter on a single allele. *The Journal of Biological Chemistry*, *295*(3), 690–700. https://doi.org/10.1074/jbc.RA119.011349

Meilhac, S. M., Esner, M., Kerszberg, M., Moss, J. E., & Buckingham, M. E. (2004). Oriented clonal cell growth in the developing mouse myocardium underlies cardiac

morphogenesis. *The Journal of Cell Biology*, *164*(1), 97–109. https://doi.org/10.1083/jcb.200309160

Min, Y.-L., Jaichander, P., Sanchez-Ortiz, E., Bezprozvannaya, S., Malladi, V. S., Cui, M., Wang, Z., Bassel-Duby, R., Olson, E. N., & Liu, N. (2018). Identification of a multipotent *Twist2*-expressing cell population in the adult heart. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(36), E8430–E8439. https://doi.org/10.1073/pnas.1800526115

Sande-Melón, M., Marques, I. J., Galardi-Castilla, M., Langa, X., Pérez-López, M., Botos, M.-A., Sánchez-Iranzo, H., Guzmán-Martínez, G., Ferreira Francisco, D. M., Pavlinic, D., Benes, V., Bruggmann, R., & Mercader, N. (2019). Adult *sox10*+ cardiomyocytes contribute to myocardial regeneration in the zebrafish. *Cell Reports*, *29*(4), 1041-1054.e5. https://doi.org/10.1016/j.celrep.2019.09.041

Sereti, K.-I., Nguyen, N. B., Kamran, P., Zhao, P., Ranjbarvaziri, S., Park, S., Sabri, S., Engel, J. L., Sung, K., Kulkarni, R. P., Ding, Y., Hsiai, T. K., Plath, K., Ernst, J., Sahoo, D., Mikkola, H. K. A., Iruela-Arispe, M. L., & Ardehali, R. (2018). Analysis of cardiomyocyte clonal expansion during mouse heart development and injury. *Nature Communications*, *9*, 754. https://doi.org/10.1038/s41467-018-02891-z

Tamura, Y., Sano, M., Nakamura, H., Ito, K., Sato, Y., Shinmura, K., Ieda, M., Fujita, J., Kurosawa, H., Ogawa, S., Nakano, S., Matsuzaki, M., & Fukuda, K. (2016). Neural crest-derived resident cardiac cells contribute to the restoration of adrenergic function of transplanted heart in rodent. *Cardiovascular Research*, *109*(3), 350–357. https://doi.org/10.1093/cvr/cvv267

VanDusen, N. J., & Firulli, A. B. (2012). Twist factor regulation of non-cardiomyocyte cell lineages in the developing heart. *Differentiation; Research in Biological Diversity*, *84*(1), 79–88. https://doi.org/10.1016/j.diff.2012.03.002

Whitehead, A. J., & Engler, A. J. (2021). Regenerative cross talk between cardiac cells and macrophages. *American Journal of Physiology-Heart and Circulatory Physiology*, *320*(6), H2211–H2221. https://doi.org/10.1152/ajpheart.00056.2021