2007

The Immunological Contributions To Heterotopic Ossification Disorders - Insights From Fibrodysplasia Ossificans Progressiva

Michael Richard Convente
University of Pennsylvania, mconvente@gmail.com

The Immunological Contributions To Heterotopic Ossification Disorders - Insights From Fibrodysplasia Ossificans Progressiva

Abstract

The development of pathological bone outside the skeleton, termed heterotopic ossification (HO), is a significant clinical complication that often greatly reduces mobility and diminishes overall quality of life for affected individuals. Patients with fibrodysplasia ossificans progressiva (FOP; OMIM #135100), a genetic disorder of HO in which most affected individuals express a recurrent heterozygous gain-of-function mutation (R206H) in the bone morphogenetic protein (BMP) type I receptor ACVR1/ALK2, develop episodes of HO formation frequently follow injury. Terminal HO formation in FOP occurs following a series of lesion development stages, of which the first recognized is an inflammatory stage associated with immune cell invasion. Of note, an early inflammatory response is a normal response to tissue injury, however in tissues expressing the FOP mutation, the repair program rapidly diverges from a path leading to tissue repair and instead forms ectopic cartilage and bone. I hypothesized that Acvr1R206H enhances the early inflammatory response to injury in FOP and that immune cells promote a permissive microenvironment for the downstream anabolic events that result in HO. Using a conditional knock-in Acvr1R206H mouse model (Acvr1cR206H/+) to investigate the cellular and molecular inflammatory events in FOP Acvr1cR206H/+ and wild-type Acvr1+/+ mice following injury, I determined that the response to tissue injury is similar between cohorts up to 48 hours post-injury, but then diverges toward a prolonged fibroproliferative stage, then to chondrogenic, and osteogenic events in Acvr1cR206H/+ mice. This coincides with a significantly elevated and prolonged pro-inflammatory cytokine expression in vivo and in vitro. I further investigated how modulation of the inflammatory response controls the development of HO in FOP. Induction of the R206H mutation exclusively in immune cells, by whole bone marrow transplant or LysM-Cre-induced myeloid-lineage expression, or selective inhibition of the inflammatory response by Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) neutralizing agents were insufficient to prevent HO formation. However, depletion of mast cells and macrophages from Acvr1cR206H/+ mice dramatically impaired development of HO, highlighting a direct immune cell contribution to HO formation.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Eileen M. Shore

Keywords
ACVR1, Fibrodysplasia Ossificans Progressiva, FOP, Heterotopic Ossification, Immunology, Mast Cells

Subject Categories

Allergy and Immunology | Immunology and Infectious Disease | Medical Immunology | Molecular Biology

THE IMMUNOLOGICAL CONTRIBUTIONS TO HETEROTOPIC OSSIFICATION

DISORDERS – INSIGHTS FROM FIBRODYSPLASIA OSSIFICANS PROGRESSIVA

Michael Richard Convente

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

Supervisor of Dissertation

_____

Eileen M. Shore, Ph.D.

Cali and Weldon Research Professor in FOP

Professor of Orthopaedic Surgery and Genetics

Graduate Group Chairperson

_____

Daniel S. Kessler, Ph.D.

Associate Professor of Cell and Developmental Biology

Dissertation Committee
Mary Mullins, Ph.D. (Chairperson), Professor of Cell and Developmental Biology
Taku Kambayashi, M.D., Ph.D., Associate Professor of Pathology and Laboratory
Medicine
Foteini Mourkioti, Ph.D., Assistant Professor of Orthopaedic Surgery
Susan Volk, Ph.D., V.M.D., Assistant Professor of Small Animal Surgery

THE IMMUNOLOGICAL CONTRIBUTIONS TO HETEROTOPIC OSSIFICATION

DISORDERS – INSIGHTS FROM FIBRODYSPLASIA OSSIFICANS PROGRESSIVA

COPYRIGHT © 2017

Michael Richard Convente

DEDICATION

My mother,

Lisa Kimerer Convente

My father,

Douglas Peter Convente

My identical twin brother,

Matthew Alan Convente

My uncle,

Bruce Kimerer

My grandparents *in memoriam*,

Marilyn Collet Kimerer

Richard Frank Kimerer

Marie Cecilia Convente

Peter Anthony Convente

My friends,

Dr. Robert J. Comito

Dr. Patrick Nosker

Eric Branning

ACKNOWLEDGEMENTS

Most acknowledgements sections are written like an award ceremony acceptance speech; a thoughtful, yet structured list of faculty and family and friends to thank and honor. I instead want to profess my gratitude to the many people who have influenced my life in all facets – familial, social, and scientific – in a more historical context. Everyone I mention in this section has had a significant impact in my life, and let not the order of inclusion make any statement on the weight of their impact. The same can be said for plenty of individuals who may be omitted from this section due to forgetfulness, length considerations, or other regrettable lapses. Consider this the foreword of my scientific and otherwise life autobiography. And if I do end up veering more toward a lengthy acceptance speech, there's no producer nor orchestra to play me off the stage.

My love and curiosity of science started in elementary school, and quickly advanced during middle school and high school years. Many of my teachers provided enrichment and excitement about science, offering many opportunities to learn beyond the classroom curriculum. It's often said that in order to develop your skill sets and achievement to the maximum level, you must engage with people who challenge you. I can say that my dear friend from my hometown of Midland Park, NJ – Dr. Robert J. Comito – is that challenger. Rob is a well-rounded gentleman, a modest man of highest achievement and someone who inspired me to be a better student during our high school and college years. While our peers were playing Family Feud on the back computers, Rob and I were practicing our net ionic equations for the AP Chemistry exam. Don't worry, we still got in a few rounds of Family Feud as well. We continued to Rutgers University together, among

several other great friends from high school, where we further developed our knowledge and excitement for the sciences, as well as our friendship.

Almost immediately upon arriving at Rutgers University, I applied for a work study position, focusing on an assignment related to the life sciences. Students could choose a general area of interest, but positions were assigned without guarantee of desired placement. My placement at the Center of Alcohol Studies in the lab of Dr. Patricia Buckendahl is one of my life's inflection points, those moments that impacted future directions and outcomes when reflected upon years later. Under her direction and those of senior students, I assisted with general lab tasks and became familiar with daily life in lab. During my junior and senior years at Rutgers, I conducted for-credit research on a bone biology project. I owe much of my growth as a scientist to Dr. Buckendahl, whom I have kept in regular contact throughout my graduate school tenure, even meeting up back on campus several times and at ASBMR 2015 in Seattle. Even though Dr. B. is a great mentor to all her students, I think we have a special bond because I'm one of the few students who continued in research instead of attending medical school!

As I entered graduate school here at the University of Pennsylvania, I knew I wanted to continue in the bone biology field. As I browsed the list of faculty who studied bone biology in one way or another, I came across the lab or Dr. Eileen Shore. I scheduled a meeting with her and she was very excited about a new mouse model being developed, and told me it would be better to rotate in her lab in spring 2010. So I did, and after completing my rotation I officially joined the lab in June 2010.

Eileen has been an incredible thesis advisor throughout my tenure in her lab. She has supported me in innumerable ways – guiding my scientific growth and independence, supporting my research intellectually and financially, sending me to multiple research conferences in San Diego, Snowmass, and Seattle, and always being available to discuss new data and any other topic. Eileen has many admirable qualities – she is thoughtful, thorough, knowledgeable, kind, caring, steady. But the one quality that deserves most praise from me is her patience; the patience to support a student for longer than the average graduate school tenure, the patience to support a student who self-admittedly can have highs and lows and depends on her steadiness to manage situations to positive solutions. For all the above and so much more, I offer immeasurable gratitude to Eileen for serving as my advisor during my time in graduate school, and I am excited to see how the lab advances even further over the next years.

All members of the Shore/Kaplan lab, past and present, have welcomed me with professionalism and friendship, to whom I am deeply indebted. Dr. Fred Kaplan, the founder of the FOP lab, has been someone whom I try to emulate, though we all know Dr. Kaplan is one-of-a-kind. He has empowered me to be the best thinker and scientist I can be; he has given me many opportunities to meet with FOP patients and their families to see their uncertainties and fears, but also their boundless hope; he has even increased my fondness for eloquent writing and handwritten letters, as Dr. Kaplan is always carrying with him a stack of papers in which the next big ideas and breakthroughs are emblazoned in ink on those pages.

Dr. Andria Culbert joined the lab one year ahead of me, so we shared a close graduate school journey. She always lent a helpful hand for my experiments, both experimental design planning and sample collection, and was always willing to answer any questions I had (and there were *a lot* of questions) as I grew from a novice student into a veteran. Dr. Salin Chakkalakal has been the wise presence in my lab life, both scientifically and personally. I have always appreciated his perspectives and friendship. My work would not have been possible without his significant contributions, as we share co-authorship on several papers, and for that I am deeply grateful. Dr. Girish Ramaswamy has been a great friend and lab mate throughout my time in the lab. I have always appreciated his powerful scientific mind during lab meetings, and his stellar work ethic. I am excited for him to begin the next phase of his scientific career as I do so myself. Dr. John Fong has been my lab bench neighbor for several years, and we have shared plenty of equipment and conversation. He has always been willing to see some of my preliminary data and offer interpretations and suggestions. And truthfully, he may be happy to have it much quieter in our side of the lab once I leave! Dr. Vitali Lounev and Dr. Haitao Wang have offered much important advice for my project, giving me great ideas and scientific outlooks that I otherwise would have missed. I enjoyed traveling to Boston with Haitao in October 2016 for the FOP Drug Development Forum and having us both share our research to the greater FOP community. Ruth McCarrick-Walmsley, Meiqi Xu, Deyu Zhang, and Bob Caron have been steady helpful hands and minds for the lab. Just as with Andria, I've asked each person countless questions as I shaped my lab experience, and their patience and assistance have

been great. They have all helped with lab experiments, mouse husbandry, and also with our BoneCrushers intramural softball and football teams!

To Will Towler, Lexy Bonomi, and Niambi Brewer – the three graduate students who joined the lab after me – I thank you for your friendship and scientific collaboration. I will miss the baked treats you all bring in, the background music which makes the lab more lively, and all the social fun outside of lab. I hope that I have been a good senior graduate student to you, and I am super excited to see your scientific achievements, present and future, published and with great impact.

I also want to extend gratitude to Dr. Josef Kaplan and Dr. Julia Haupt, two former lab members who I have missed dearly over the past several years. They both provided much fun and scientific input. I particularly enjoyed being lab bench neighbors with Josef during our temporary move to CRB while Stemmler Hall was being renovated (the first time!), and I will always cherish your massive move away party at Tangier Bar with your fellow Aussie expatriates. I definitely plan to visit sooner than later. And the same goes for Julia, to whom I always talked up my brother's self-taught German skills! I can't wait to visit your family in Germany, and hopefully my brother can come as well to speak German with you!

My thesis committee – Dr. Mary Mullins (chairwoman), Dr. Taku Kambayashi, Dr. Susan Volk, and Dr. Foteini Mourkioti – have truly gone above and beyond supporting me throughout this journey. Their professional input on my project, and their personal care for me as an individual, has sustained me through this long process. I thank them for being patient with me as well, as I know I've taken longer to complete this process than the

average student. I especially want to express gratitude to Dr. Taku Kambayashi for being a co-author author on my manuscript, with the help of his former students, Dr. Amanda M. Schmidt Paustian and Dr. EnJun Yang. Without their collective work, my project would not have been possible.

Dr. Dan Perrien of Vanderbilt University has been a great collaborator and friend of our lab. I've enjoyed our conversations at ASBMR 2015 and the FOP Drug Development Forum in 2016. Some of the work in this thesis was done in collaboration with his lab, and it provided key insight into the inflammatory mechanisms of FOP. I hope that our labs' collaboration will strengthen even further in the future.

Growing up in New Jersey, and attending Rutgers University (which is in New Jersey) for college has gifted me many lasting friendships. There are far too many friends to list, but I want to especially recognize Brian Turner, Tyler Goodwin, and all of my Chi Psi Brothers. Three Brothers who have been especially impactful in my life are Dr. Patrick Nosker, Eric Branning, and Eric Solomon. We have shared so many amazing experiences (like going to CES four years in a row!), stories, moments of happiness and moments of sadness, been roommates here in Philadelphia, and we have made it through resolved as ever. I am very much looking forward to seeing how our lives progress as we enter our 30s (and one of us is already there…).

I have equally as many meaningful friendships from my time at Penn. My first graduate school roommates – Ben Ediger, Sudil Mahendra, and Claire O'Leary – were integral in my transition from undergraduate to graduate school. We are all still great friends many years later. Christie Helfer Gifford, Samantha Falk, Julie Horowitz, Matt

Harms, Amy DeMicco, Leksa Nall, Dave Frederick, Lindsey Wingert, Robert Plasschaert, Tanya Corman, Jason Diaz, and so, so many more. We've had amazing times together, dance parties, ski trips, beach trips, hiking trips, even a few weddings. I also want to recognize my Penn City Planning roommates – Matthew Moran, Chris Cummings, Josh Wagner, as well as Penn MVP student Michael Hogan – for keeping my middle graduate school years lively and providing a group of peers to talk with about things other than science.

Of most importance to me is my family. My mother, Lisa Kimerer Convente, and my father, Douglas Convente, have always been supportive and empowering of my efforts, working extra jobs and extra hours to help pay for college. They helped enrich my sense of adventure, curiosity, fun, and work ethic. My grandparents are no longer with us, but they provided much to my educational upbringing, especially Grandpa Kimerer, who always emailed me cool science links while at Rutgers University. The same goes for my uncle Bruce Kimerer. As many know, I am very nostalgic, so I have been reading all of my family Rutgers emails over the past weeks. And finally, my identical twin brother Matt, my only sibling of whom we share so much (obviously including our DNA sequence, which we proved by 23andMe!). I've been very proud of your accomplishments, your work ethic, your constant desire to learn more and teach yourself new skills, and your travel endeavors. I'm excited to be finally be joining the workforce along your side and can't wait to own our 30s (and many, many more decades) together. TeamTwin.

ABSTRACT

THE IMMUNOLOGICAL CONTRIBUTIONS TO HETEROTOPIC OSSIFICATION DISORDERS – INSIGHTS FROM FIBRODYSPLASIA OSSIFICANS PROGRESSIVA

Michael Richard Convente

Eileen M. Shore

The development of pathological bone outside the skeleton, termed heterotopic ossification (HO), is a significant clinical complication that often greatly reduces mobility and diminishes overall quality of life for affected individuals. Patients with fibrodysplasia ossificans progressiva (FOP; OMIM #135100), a genetic disorder of HO in which most affected individuals express a recurrent heterozygous mutation (R206H) in the bone morphogenetic protein (BMP) type I receptor ACVR1/ALK2, develop episodes of HO formation frequently follow injury. Terminal HO formation in FOP occurs following a series of lesion development stages, of which the first recognized is an inflammatory stage associated with immune cell invasion. Of note, an early inflammatory response is a normal response to tissue injury, however in tissues expressing the FOP mutation, the repair program rapidly diverges from a path leading to tissue repair and instead forms ectopic cartilage and bone. I hypothesized that $Acvr1^{R206H}$ enhances the early inflammatory response to injury in FOP and that immune cells promote a permissive microenvironment for the downstream anabolic events that result in HO. Using a conditional knock-in

Acvr1^{R206H} mouse model (*Acvr1*$^{cR206H/+}$) to investigate the cellular and molecular inflammatory events in FOP *Acvr1*$^{cR206H/+}$ and wild-type *Acvr1*$^{+/+}$ mice following injury, I determined that the response to tissue injury is similar between cohorts up to 48 hours post-injury, but then diverges toward a prolonged fibroproliferative stage, then to chondrogenic, and osteogenic events in *Acvr1*$^{cR206H/+}$ mice. This coincides with a significantly elevated and prolonged pro-inflammatory cytokine expression *in vivo* and *in vitro*. I further investigated how modulation of the inflammatory response controls the development of HO in FOP. Induction of the R206H mutation exclusively in immune cells, by whole bone marrow transplant or *LysM*-Cre-induced myeloid-lineage expression, or selective inhibition of the inflammatory response by Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) neutralizing agents were insufficient to prevent HO formation. However, depletion of mast cells and macrophages from *Acvr1*$^{cR206H/+}$ mice dramatically impaired development of HO, highlighting a direct immune cell contribution to HO formation.

TABLE OF CONTENTS

List of Figures and Tables

Chapter Three

Chapter One

Introduction

1.1 Bone Morphogenetic Protein (BMP) signaling

1.1.1 TGF-β/BMP superfamily ligands and receptors

Bone morphogenetic proteins (BMPs) are a subset of extracellular signaling ligands belonging to the transforming growth factor-beta (TGF-β) superfamily [Miyazono et al. 2010]. The TGF-β/BMP superfamily is composed of more than thirty signaling ligands, including TGF-βs, BMPs, activins, inhibins, growth differentiation factors (GDFs), nodal, myostatin, and anti-Müllerian hormone [Miyazawa et al. 2002, Derynck et al. 2003]. BMPs represent the largest subset of the TGF-β/BMP superfamily, with over twenty ligands identified [Bragdon et al. 2011]. BMPs were initially discovered as factors that stimulate production of new bone in demineralized bone matrix implants *in vivo* [Urist 1965]. BMPs and other TGF-β/BMP superfamily ligands have substantial roles in numerous biological systems, including stem cell renewal and differentiation, embryological development, immune cell development, iron homeostasis, skeletal muscle development and regeneration, cancer, and others [Elliott et al. 2005, Babitt et al. 2006, Watabe et al. 2009, Wu et al. 2009, Wu et al. 2012, Sartori et al. 2013, Hager-Theodorides et al. 2014, Martinez et al. 2015]. BMPs have a unique and crucial role during the earliest stages of embryological development. BMPs are required for patterning the dorso-ventral axis in both invertebrates and vertebrates [Little et al. 2006, Tucker et al. 2008, Ramel et al. 2013]. Later in vertebrate development, BMPs are required for all stages of endochondral long bone formation - mesenchymal condensation, chondrogenesis, and osteogenesis [Lim et al. 2015, Salazar et al. 2016]. In the adult, BMPs are involved in skeletal homeostasis and normal bone turnover [Wu et al. 2016], as well as in many other biological systems [Babitt

et al. 2006, Shi et al. 2011, Choi et al. 2012, Wu et al. 2012, Brazil et al. 2015, Sartori et al. 2015, Salazar et al. 2016].

The TGF-β/BMP superfamily receptors are grouped into two classes of transmembrane serine/threonine kinases, type I and type II [Shi et al. 2003, Miyazono et al. 2010] (Figure 1.1). Type I consists of seven receptors (ALK1/ACVR1L, ALK2/ACVR1, ALK3/BMPR1A, ALK4/ACVR1B, ALK5/TGFβR1, ALK6/BMPR1B, and ALK7/ACVR1C). Type II consists of four receptors (ACTR2A, ACTR2B, BMPR2, TGFβR2). The type I and type II receptors that preferentially mediate signaling in response to BMP ligands are ALK1, ALK2, ALK3, ALK6, ACTR2A, ACTR2B, and BMPR2; ALK4, ALK5, ALK7, and TGFβR2 mediate TGF-β signaling. Type I receptors contain several conserved domains – an extracellular ligand binding domain, a transmembrane domain, a glycine/serine (GS) rich domain, and a protein kinase domain [Yadin et al. 2016]. Type II receptor structure is highly similar to type I receptors, except for an absent GS domain. BMPs bind as heterodimers to two type I extracellular ligand binding domains and facilitate recruitment of two type II receptors to form a tetrameric receptor signaling complex [Wrana et al. 1992]. Following BMP dimer binding and receptor complex formation, the constitutively active type II receptor kinase domain, now in proximity of the adjacent type I receptor via a conformational shift, phosphorylates serine residues within GS domain of adjacent type I receptors [Wrana et al. 1992]. This transphosphorylation event activates the kinase domain of the type I receptors, allowing for subsequent phosphorylation of downstream canonical and non-canonical signaling proteins [Salazar et al. 2016].

BMP signaling is tightly controlled by multiple antagonist proteins that act both extracellularly and intracellularly (Figure 1.1). The availability of BMP ligands is controlled by extracellular antagonists, including Noggin, Chordin, and Gremlin, that bind and sequester free BMPs to prevent their binding to receptors [Brazil et al. 2015]. Additionally, the decoy BMP and Activin Membrane Bound Inhibitor (BAMBI) receptor can bind BMPs and limit availability of ligand dimers for propagation of faithful BMP signaling [Brazil et al. 2015]. Intracellular antagonism of BMP signaling functions through an inhibitory mechanism that prevents promiscuous receptor activation in the absence of ligand. FKBP12 binds to type I BMP and TGF-β receptors in a leucine/proline motif within the GS domain and stabilizes the inactive confirmation of type I receptors. Upon ligand binding, FKBP12 dissociates from the receptor complex and allows signaling to advance [Wang et al. 1996, Chaikuad et al. 2012, Yadin et al. 2016].

1.1.2 Canonical and non-canonical signaling pathways

TGF-β/BMP superfamily ligands can propagate signaling through canonical (Smad-dependent) and non-canonical (Smad-independent) mechanisms to regulate gene transcription (Figure 1.1). Pathway activation is dependent on available receptors and ligands, which have stronger binding affinities for preferred BMP or TGF-β receptor partners.

Canonical TGF-β/BMP signaling is mediated by a consortium of Smad proteins that are classified into three groups: regulatory Smads (R-Smads), common Smad (co-Smad), and inhibitory Smads (I-Smads). Five mammalian R-Smads participate in the

canonical signaling pathway. Smad1, Smad5, and Smad8 serve as signaling mediators induced primarily by BMPs, whereas Smad2 and Smad3 serve as signaling mediators induced primarily by TGF-βs and activins. The TGF-β and BMP signaling branches converge with the utilization of the co-Smad Smad4 by all R-Smads. Smad6 and Smad7 function as I-Smads through Smad-receptor or Smad-Smad interactions that inhibit proper signaling [Katagiri et al. 2016].

The R-Smads contain three domains: the highly-conserved DNA-binding "Mad-homology 1" (MH1) and type I receptor-activated MH2 domains, and a variable linker domain. The MH2 domain has a conserved C-terminal motif, Ser-X-Ser, that is phosphorylated by the activated type I receptor. Phosphorylation of R-Smad by the activated type I receptor induces translocation into the nucleus and Smad4 recognition of the phosphorylated R-Smad pSer-X-pSer motif. R-Smads bind to a specific 5'-GTCT-3' DNA motif known as the Smad-binding element (SBE) through their MH1 domain [Shi et al. 1998]. Many Smad-responsive promoters contain one or more SBEs within their promoter sequence. The core R-Smad-Smad4 complex is most commonly a hetero-trimeric complex composed of two phosphorylated R-Smads and one Smad4 protein. However, due to relatively low DNA binding affinity, incorporation of different DNA-binding co-factors to the nuclear R-Smad-Smad4 complex via interactions with the "Smad4 activation domain" (SAD) present on Smad4 allows for enhanced DNA binding and gene target specificity. Many TGF-β/BMP responsive gene promoters contain SBEs and adjacent cognate co-factor binding motifs for targeted gene expression [Massagué et al. 2005].

Smad6 and Smad7 are I-Smads that function to inhibit TGF-β/BMP signaling. Both Smad6 and Smad7 are structurally similar to the R-Smads, however they lack a DNA-binding MH1 domain and a C-terminal Ser-X-Ser motif. Smad6 targets BMP signaling by functioning as a competitive inhibitor preventing phosphorylated Smad1 from binding to Smad4 [Hata et al. 1998]. Smad7 interacts stably with activated BMP and TGF-β and functions as a competitive inhibitor of R-Smad phosphorylation [Hayashi et al. 1997, Nakao et al. 1997].

TGF-β/BMP signaling can also be mediated by multiple non-canonical, Smad-independent mechanisms. Receptor complex activation can propagate signaling through downstream signaling pathways such as extracellular signal-regulated kinase (ERK), p38 map kinase, C-jun N-terminal kinase (JNK), and nuclear factor kappa beta (NF-κB). All are thought to be mediated by TGF-β activated kinase 1 (TAK1). Additional non-canonical signaling pathways include phosphoinositide 3-kinase (PI3K) and Protein Kinase A (PKA), the latter of which operates through cyclic AMP effector signaling [Taylor et al. 2004, Bragdon et al. 2011].

1.2 The immune system and its role in injury repair

1.2.1 Evolution and overview of the immune system

The immune system is a coordinated cellular and molecular system that first evolved as a defense against infectious species [Cooper et al. 2006, Dzik 2010, Flajnik et al. 2010, Horvath et al. 2010, Buchmann 2014, Rath et al. 2015]. The earliest appearance of a primitive immune system in prokaryotes was the evolution of the Clustered Regularly

Interspaced Short Palindromic Repeats (CRISPR) DNA region, first observed in *Escherichia coli*. In combination with CRISPR-associated (Cas) genes, the CRISPR/Cas system protects prokaryotes from viral genetic elements through a targeted process that results in permanent identification and destruction of foreign sequences [Horvath et al. 2010, Rath et al. 2015].

Evolution of a more sophisticated "self vs. non-self" recognition system occurred simultaneously with emergence of increasingly complex multicellular eukaryotes during the Cambrian explosion starting 542 million years ago [Buchmann 2014]. This mechanism depended on evolution of appropriate molecules and cells that perform two functions in sequence: detection of non-self pathogens, and phagocytic lysis of identified pathogens. Recognition of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) and viral genetic elements, first evolved in multicellular sponges with the appearance of LPS-binding receptors. The emergence of intracellular receptors that can detect pathogens and foreign DNA, such as the nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, or "NLRs"), further enhanced the ability of organisms to detect pathogens, including viruses, fungi, parasites, and their associated PAMP molecules [Takeuchi et al. 2010, Buchmann 2014]. Evolution of cells with phagocytic function occurred concurrent with the appearance of recognition receptors, with early multi-cellular species in the Porifera and Cnidaria phyla containing phagocytic amebocytes [Dzik 2010]. Cells with equivalent function are found throughout the invertebrates, with more sophisticated and diverse cell types, such as macrophages and granulocytes, first appearing in sea stars, sea urchins, tunicates, and others [Rhodes et al.

1982, Crivellato et al. 2010]. This system, classified today as innate immunity, relies on early detection of conserved PAMPs to trigger a first-wave inflammatory response to limit pathogen invasion [Akira et al. 2006].

A more targeted immune response, dependent on diversification of evolved antigen-receptor genes to initially and permanently recognize unique non-self molecules, defines the adaptive immune system. The primordial adaptive immune system was first observed in jawless invertebrates belonging to the Agnatha superclass of the Chordata phylum, which includes lampreys and hagfish among its members [Cooper et al. 2006]. Jawless fish possess a primitive mechanism for generating genetic diversity required for mounting antigen-specific immune responses. This mechanism depends upon use of variant leucine-rich repeats (LRRs) to form variable lymphocyte receptors (VLRs), generating clonal specificity through somatic recombination [Litman et al. 2010]. The appearance of an adaptive immune system most closely resembling its modern composition in mammals was first observed in jawed vertebrates, specifically jawed fish [Cooper et al. 2006]. Detection of unique antigens required concurrent evolution of several genes and gene loci – the variable-diversity-joining rearrangement (VDJ) locus, the major histocompatibility complex (MHC) locus, and the set of recombination-activating genes (RAGs) [Flajnik et al. 2010]. In combination, all three components provided the parts and machinery for functional and lasting defense against unique antigens. A new diverse class of immune cells termed lymphocytes evolved in parallel and utilized the above genetic components to mount robust and efficient attacks against pathogens [Flajnik et al. 2010].

The classic antigen recognition and clonal expansion of lymphocytes described above remains the foundation of adaptive immunity in modern vertebrates.

Hematopoietic stem cells (HSCs) are a multipotent, self-renewing cell population that resides in the bone marrow niche [Zon 2008]. HSCs are able to generate all transient and terminally differentiated immune cells [Benveniste et al. 2003], which are classified into two main groups: myeloid lineage and lymphoid lineage (Figure 1.2). HSCs are defined by a triple-labeling identification by the Lineage (Lin), stem cells antigen-1 (Sca-1), and c-Kit proteins; HSCs are Lin^-, $Sca-1^+$, $c-Kit^+$. HSCs are subdivided into two populations: a long-term (LT-HSC) population that can self-renew or asymmetrically divide to produce the other, a short-term (ST-HSC) population that retains multipotency while undergoing robust proliferation and differentiation toward the numerous transient and terminal immune cell lineages. ST-HSCs can differentiate into common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs), which serve as multipotent progenitor populations for all myeloid and lymphoid lineages, respectively [Challen et al. 2009]. Cells derived from CMPs compose the innate immune system, and cells derived from CLPs compose the adaptive immune system.

1.2.2 Innate immunity

The innate immune system functions as a first line of defense against infectious species, utilizing several families of pattern recognition receptors such as toll-like receptors (TLRs) or NLRs to provide a short-term barrier against pathogen invasion [Mogensen 2009]. Cells of the innate immune system, such as neutrophils and macrophages, possess

the ability to attach to and digest pathogens, assisting with clearance of active infection. In species with a functional antigen-mediated immune system, innate immune cells also play a major role in presentation of digested pathogenic particles, activating and inducing clonal expansion of adaptive immune cells.

Receptors and molecular pathways of innate immunity

Cells of the innate immune system, also known as leukocytes, express several families of receptors and signaling molecules that perform two functions in sequence: recognition of non-self pathogens, and presentation of antigens to cells of the adaptive immune system. Both of these functions use the complement system, which is composed of receptors and soluble proteins that function as a surveillance system against pathogens. Complement proteins include pattern recognition proteins like C1q, proteases that regulate the complement signaling cascade, opsonization proteins that increase detection of foreign species by host immune cells, and receptors that bind to opsonized pathogens to induce phagocytic destruction. The complement system is often the first to recognize pathogens and also functions to alert other immune cells to an ongoing infection [Ricklin et al. 2010]. Cells of the innate immune system also express a comprehensive panel of pattern recognition receptors that recognize conserved pathogen moieties, including lipopolysaccharides, peptidoglycans, flagellin, and others [Buchmann 2014]. Receptor families like the TLRs and NLRs alert the host of an active infection and induce inflammatory factor expression that amplifies the host immune response.

10

The TLR family of pattern recognition receptors was initially discovered in *Drosophila melanogaster* and soon after confirmed to be expressed in vertebrates [Hansson et al. 2005]. TLRs recognize conserved moieties and genetic elements from pathogens, including LPS, double-stranded RNA, and bacterial flagella, among others [Kawai et al. 2010]. TLRs are classified into two branches: cell-surface receptors and intracellular receptors. TLR1, TLR2, TLR4, TLR5, and TLR6 reside on the cell surface and detect PAMPs that primarily compose bacterial and fungi membrane moieties, such as lipids and lipoproteins. TLR3, TLR7, TLR8, TLR9, and TLR10 reside intracellularly within endosomes and detect microbial nucleic acids. All TLRs signal through the intracellular effector protein MyD88, except for TLR3, which signals downstream through the TIR-domain-containing adapter-inducing interferon-β (TRIF) protein. Upon moiety binding, receptor activation, and signal propagation, the TLR family upregulates inflammatory signaling proteins, such as NF-κB, that potentiate a host inflammatory response [Kawai et al. 2010].

The NLR and TLR families of pattern recognition receptors identify similar conserved pathogen moieties; however, NLRs propagate signaling through a TLR-independent mechanism. NLRs primarily reside in the cytosol, although low cell-surface expression has been detected. NOD1 and NOD2 are the two major NLRs and both recognize peptidoglycan motifs present in bacterial cell walls [Shaw et al. 2008]. In addition to their pattern recognition function, NLRs can form inflammasomes, protein complexes composed of NLRs and other scaffold proteins, that activate caspase-1, a key step for processing pro-inflammatory cytokines [Martinon et al. 2002].

11

Origin of innate immune cells

All myeloid lineage cells of the innate immune system are derived from a clonogenic common myeloid progenitor (CMP) [Akashi et al. 2000]. CMPs can differentiate into two distinct transient progenitor populations: megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs). The MEP branch differentiates into all cells of the erythropoietic lineage, including erythrocytes, megakaryocytes, and platelets, and is not considered to have substantial inflammatory function. The GMP branch differentiates into granulocytes, which includes neutrophils and mast cells, as well as monocyte/macrophage lineages [Kolaczkowska et al. 2013, Dahlin et al. 2014].

Neutrophils

Neutrophils are the most abundant leukocyte in the blood and are the first responding cells during active pathogen infection [Witko-Sarsat et al. 2000]. Neutrophils defend against pathogen infection through several mechanisms, including phagocytosis, oxidative bursts, and neutrophil extracellular traps (NETs) [Brinkmann et al. 2004, de Oliveira et al. 2016]. The lifespan of neutrophils in circulation is relatively short, ranging from eight to twelve hours. Neutrophils in circulation migrate to localized sites of infection upon detection of cytokines and other inflammatory factors released by tissue-resident cells. Neutrophil migration from blood vessels to infection sites, termed extravasation, involves a cascade of events including cell rolling, adhesion, and transmigration through vessel endothelial cells [Phillipson et al. 2011]. Upon reaching a site of active infection,

neutrophils attack foreign species and secrete numerous pro-inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6, among others, amplifying the local immune response [Witko-Sarsat et al. 2000, Kaplanski et al. 2003].

Monocytes/macrophages

Monocytes are leukocytes that participate in phagocytosis of pathogens. During active infection, monocytes are recruited to the site of pathogen invasion and convert into macrophages or dendritic cells [Ginhoux et al. 2014]. Monocytes survey the biological landscape via passage through the circulatory system, with reserve populations residing in the spleen primed for deployment [Swirski et al. 2009]. Macrophages derived from monocytes phagocytose pathogens and contribute to acute inflammatory events that defend against infection. Tissue resident macrophages, such as Kupffer cells, alveolar macrophages, and microglia, reside in numerous organs and provide local protection against pathogens and other functions. These cells, however, are derived from the embryonic yolk sac and are distinct from monocyte-derived macrophage populations [Epelman et al. 2014].

Macrophages that respond to infection or injury are broadly grouped into two branches: the pro-inflammatory M1 macrophages and the anti-inflammatory M2 macrophages. M1 macrophages are first-responder macrophages that produce and secrete numerous pro-inflammatory cytokines and chemokines to sustain an ongoing acute inflammatory reaction. M1 macrophages communicate with adaptive immune cells via inflammatory factors including IFN-γ and TNF-α, leading to cytotoxic attacks pathogens

and damaged tissue. Following the initial pro-inflammatory response, M1 macrophages can class-switch into anti-inflammatory M2 macrophages, which participate in wound repair and final pathogen clearance [Italiani et al. 2014].

Mast cells

Mast cells are tissue resident granulocytes distributed throughout the vascularized tissues and serosal cavity. Mast cells were originally recognized for their roles in allergy response and anaphylaxis [Bischoff 2007]; however, they have recently been appreciated for having diverse biological roles, including wound repair, scar formation, fibrosis, pain pathophysiology, angiogenesis, and ectopic bone formation [Kalesnikoff et al. 2008, Kan et al. 2011, Thevenot et al. 2011, Gri et al. 2012, Frieri et al. 2013, Vincent et al. 2013, Douaiher et al. 2014, Convente et al. 2015]. Mast cells are the major inflammatory cell involved in allergenic responses, mediated by Immunoglobulin E (IgE) antibody response. During active bouts of allergy, IgE class antibodies bind to $Fc_{\varepsilon}RI_{\alpha}$ receptors present on the cell surface of mast cells, triggering an intracellular signaling cascade that results in degranulation and release of histamine, proteases, cytokines, and chemokines [Kalesnikoff et al. 2008, Crivellato et al. 2010]. Non-IgE-mediated stimulation of mast cells, induced by neuropeptides like Substance P, can also stimulate mast cell degranulation and release of inflammatory factors [Azzolina et al. 2003, O'Connor et al. 2004, Galli et al. 2005].

Dendritic cells

Dendritic cells are defined as professional antigen presenting cells (APCs) that help direct the adaptive immune response. Classic dendritic cells are derived from CMPs and are thought to be appropriately classified as myeloid lineage cells, however some dendritic cells have also been identified having a CLP origin. Dendritic cells express MHC Class I and Class II receptors, which contribute to antigen presentation, however a subset of dendritic cells can also express putative T cell receptors like CD4 and CD8 [Merad et al. 2013, Mildner et al. 2014]. Given their dual roles as APCs and T cell co-stimulatory cells, dendritic cells are considered the interface between innate and adaptive immunity.

1.2.3 Adaptive immunity

The adaptive immune system provides long term defense against pathogens and other non-self particles, utilizing somatic gene rearrangement mechanisms to generate diverse and clonally expressed antigen receptors [Boehm 2011]. Cells of the adaptive immune system, such as T cells and B cells, possess the ability to recognize specific antigens and mount robust antigen-specific immune responses through use of T cell receptors (TCRs) and B cell receptors (BCRs). The adaptive immune system is unique in its ability to detect and respond to secondary waves of pathogen invasion derived from a set of mechanisms commonly termed as "memory" [Boehm 2011].

The Variable-Diversity-Joining V(D)J locus and assembly of TCRs and immunoglobulins

Cells of the adaptive immune system, known as lymphocytes, initiate a functional antigen-mediated immune response through mechanisms that generate antigen-specific receptors that bind only to a single putative antigen. The variable-diversity-joining – also known as V(D)J – locus is a complex region of genes segments that serve as the foundation for TCR and immunoglobulin formation. During differentiation of T and B cells, single V, (D), and J segments from the V(D)J locus are randomly recombined to form a unique cell-surface receptor [Schatz et al. 2011]. This process depends on a consortium of enzymes that work in tandem to faithfully complete receptor construction. The recombination-activating genes (RAG1 and RAG2) function as endonucleases and recombinases that carry out faithful V(D)J recombination [Oettinger et al. 1990]. RAG1 and RAG2 are required for development of mature T and B cells, as mutations in either gene are known to cause severe immunodeficiency disorders [Notarangelo et al. 2016]. Additional receptor diversity is introduced by the terminal deoxynucleotidyl transferase (TdT) enzyme. TdT is a specialized DNA polymerase that adds two-to-five random N-nucleotides to linked V(D)J sites during recombination, substantially increasing genetic diversity capacity of formed receptors [Yamtich et al. 2010].

Origin of adaptive immune cells

All lymphoid lineage cells of the adaptive immune system are derived from a clonogenic common lymphoid progenitor (CLP) [Kondo et al. 1997]. CLPs can differentiate into three distinct populations – T cells, B cells, and natural killer cells. Both

T cells and B cells differentiate through a series of transient progenitor cells known as pro- and pre-stages that are accompanied with generation of their V(D)J-derived cell-surface receptors used for antigen-mediated immune function.

T cells

T cells are derived from HSCs but mature within the thymus [Zuniga-Pflucker 2004]. Multiple subpopulations of T cells exist, including helper T cells, cytotoxic (killer) T cells, and regulatory T cells, among others, that are defined by varied function and cell-surface receptor expression [Broere et al. 2011]. The earliest T cell progenitors, also known as thymocytes, are double-negative for CD4 and CD8 receptors and migrate from the bone marrow to the thymus, where they undergo multiple checkpoints and differentiation stages, including two antigen evaluation checkpoints known as positive and negative selection [Starr et al. 2003], until they reach their mature state. Positive selection preferentially selects for developing thymocytes that can interact with MHC-presented antigens. At this stage, developing thymocytes express both CD4 and CD8 receptors. Developing thymocytes within an accepted range of peptide binding affinities receive a Wnt-dependent survival signal [Ioannidis et al. 2001] and proceed to negative selection, which ensures that thymocytes do not bind to self-peptides. Thymocytes that fail this checkpoint are removed by apoptosis [Starr et al. 2003]. Upon passing all developmental checkpoints, thymocytes are directed down a CD4 or CD8 lineage depending on their TCR binding affinity to MHC Class I or Class II peptides, respectively, where they are now defined as mature T cells.

T cells circulate throughout the body in a naïve, unstimulated state until they encounter a putative antigen. Upon antigen binding, T cells require an additional co-stimulatory signal mediated through the CD28 receptor for full activation [Chen et al. 2013]. Once activated, the antigen-specific T cell clonally expands by several orders of magnitude and mediates a cytotoxic mechanism against pathogens expressing the putative antigen [Broere et al. 2011].

B cells

B cells are lymphocytes that act in humoral immunity by functioning as antibody-producing cells. B cells undergo a similar developmental pathway as T cells, including V(D)J recombination of the immunoglobulin portion of the BCR and a positive/negative selection process [Pieper et al. 2013]. Naïve mature B cells express unique cell-surface immunoglobulin receptors mimicking the structure of an antibody. Upon binding of putative antigen, the unique B cell is activated, clonally expands, and differentiates into a plasma cell that secretes soluble antibodies against the antigen. A residual memory B cell subpopulation remains to recognize pathogen secondary responses and secrete antibodies accordingly [LeBien et al. 2008].

Natural killer (NK) cells

Natural killer (NK) cells are cytotoxic cells derived from CLPs [Kondo et al. 1997]. However, unlike $CD8^+$ cytotoxic T cells, which require antigen-specific binding to initiate destruction mechanisms, NK cells are able to identify and destroy pathogens in absence of

any antigen-mediated process [Vivier et al. 2011]. Notably, NK cells are able to detect and destroy unhealthy host "self" cells, hence their eponym. Similar to dendritic cells, NK cells function as intermediaries between the innate and adaptive immune systems.

1.2.4 Inflammatory contribution to injury and wound repair

A second, yet equally important function of the immune system is the ability to respond to tissue injury. The panel of pattern recognition receptors (PRRs) utilized for pathogen detection, such as TLRs and NLRs, also can bind to damage-associated molecular patterns (DAMPs) that are released from damaged cells following injury [Takeuchi et al. 2010]. The DAMP family of molecules is extensive, composed of numerous proteoglycan, glycosaminoglycan, glycoprotein, nucleic acid, and inorganic phosphate molecules [Schaefer 2014]. DAMPs can be released by an injury force in tandem with cellular and nuclear membrane rupture, however DAMPs may also be released following early innate immunity responses to pathogens, such as neutrophil oxidative bursts that may indiscriminately damage host tissue [Brinkmann et al. 2004, Rubartelli et al. 2007, de Oliveira et al. 2016].

The inflammatory response during tissue injury and wound repair can be grouped into two main phases – a pro-inflammatory phase that coincides with cytokine, chemokine, and immune cell propagation that functions to clear damaged tissue and resultant pathogen infection; and an anti-inflammatory phase that dampens the pro-inflammatory response and recruits progenitors cells to regenerate damaged tissue.

The cascade of pro-inflammatory and anti-inflammatory events in skeletal muscle injury and regeneration have been well characterized [Chargé et al. 2004, Philippou et al. 2012]. Neutrophils are the earliest responding cells to skeletal muscle injury, functioning to digest damaged tissue and protect against pathogen invasion from open wounds [Philippou et al. 2012]. Neutrophils secrete numerous chemokines and cytokines, such as CXCL8, IL-8, TNF-α, IL-1β, and IL-6 [Witko-Sarsat et al. 2000, Kaplanski et al. 2003, de Oliveira et al. 2016], that recruit additional immune cells to the site of injury. Classically-activated M1 macrophages, derived from migratory monocytes, are next to respond to injury. These cells phagocytose damaged tissue, secrete TNF-α and nitric oxide (NO), and perform anti-microbial functions [Murray et al. 2011]. As clearance of damaged tissue nears its completion, the pro-inflammatory response is dampened and replaced with an anti-inflammatory response that coincides with the transition of M1 to M2 macrophages [Arnold et al. 2007, Laskin et al. 2011]. M2 macrophages secrete anti-inflammatory proteins and growth factors, including IL-4, IL-10, TGF-β1, and platelet-derived growth factor (PDGF) to support myofibroblast differentiation and eventual restoration of damaged skeletal muscle [Murray et al. 2011].

1.3 Regulation of the immune system by BMP pathway signaling

Immune cells communicate primarily via inflammatory chemokine and cytokine pathway networks. However, the BMP signaling pathway also has significant roles in the development, recruitment, stimulation, and cross-talk of progenitor and terminally differentiated immune cell populations [Chen et al. 2016]. Adult human HSCs express

BMP type I receptors and BMP ligands, and maintain robust repopulation capacity when cultured with high BMP4 concentrations, indicating a significant role for BMP signaling in HSC self renewal [Bhatia et al. 1999]. Smad4 is required for HSC self-renewal, further demonstrating the importance of BMP/TGF-β signaling in HSC biology [Karlsson et al. 2007]. In addition to its role in HSC self-renewal, BMP signaling also directs cell fate of HSCs toward myeloid or lymphoid lineages [Crisan et al. 2015].

1.3.1 BMP signaling in the innate immune system

BMP ligands function as chemoattractant factors to recruit several innate immune cell populations to sites of infection or injury. Injection of recombinant human BMP-2B (later renamed BMP4) was demonstrated to induce monocyte migration in an *in vitro* assay [Cunningham et al. 1992]. Additionally, BMP4 was shown to recruit monocytes in pro-atherogenic conditions [Simoes Sato et al. 2014], and BMP6 was shown to induce neutrophil migration into ovaries [Akiyama et al. 2014]. In human dendritic cells, BMP signaling promotes maturation from monocyte precursors, induces IL-8 and TNF-α secretion, and enhances T cell stimulatory capacity [Martinez et al. 2011]. BMP signaling also potentiates the inflammatory state of other innate immune cells, inducing IL-1β and NO production in macrophages and Substance P expression in mast cells [Kwon et al. 2009, Salisbury et al. 2011].

1.3.2 BMP signaling in the adaptive immune system

The BMP signaling pathway is active in multiple adaptive immune cells. BMP2 and BMP4 are crucial signaling ligands for progression of CD4$^-$/CD8$^-$ double-negative T cell precursors to CD4$^+$/CD8$^+$ double-positive cells [Hager-Theodorides et al. 2014]. In mature CD4 T cells, BMP receptors are upregulated during TCR antigen binding, and BMP signaling is required for production of the cell survival and homeostasis signals IL-7, CXCR4, and CCR9 [Martinez et al. 2015]. In contrast, BMP signaling was shown to decrease TH17 helper T cell and regulatory T cell differentiation, suggesting that not all T cell subsets respond identically to the BMP signaling pathway [Yoshioka et al. 2012]. BMPs generally have a suppressive effect on B cells, inhibiting IgM, IgG, and IgA production in naïve and memory B cells, although the effect was ligand-specific [Huse et al. 2011].

1.4 Fibrodysplasia ossificans progressiva

1.4.1 Clinical features

Fibrodysplasia ossificans progressiva (FOP) is clinically defined by two characteristic features: 1) progressive formation of heterotopic ossification (HO), and 2) congenital malformations of the great toe (clinically termed "hallux valgus") (Figure 1.3).

At birth, the only observable phenotype of FOP is the presence of hallux valgus, a deformity of the great toe that is present in nearly all FOP patients [Kaplan et al. 2009]. As the child ages, appearance of HO usually manifests within the first decade of life. HO develops progressively within soft connective tissues, including skeletal muscle, tendon,

and ligament, in a characteristic pattern that first impacts the cranial, axial, and dorsal regions, including the head, neck, shoulders, and back [Pignolo et al. 2016]. HO in FOP is often induced by tissue injury events, including intramuscular vaccination, contusion, blunt force trauma, and surgical removal of ectopic bone [Lanchoney et al. 1995, Glaser et al. 1998, Scarlett et al. 2004]; however, HO can also form in the absence of overt injury [Cohen et al. 1993]. As HO progresses, patient mobility is increasingly diminished as joints ankylose and ectopic bone extends across adjacent tissues [Smith 1998, Kaplan et al. 2004, Pignolo et al. 2016].

FOP patients frequently present with abnormalities in addition to the two classical features. Over half of patients with FOP have conductive hearing impairment, cervical spine fusions of the facet joints between C2 and C7, short, broad femoral necks, and tibial osteochondromas [Kaplan et al. 2005, Schaffer et al. 2005, Deirmengian et al. 2008]. Craniofacial alterations, including reduced mandible and low-set ears, have also been observed [Hammond et al. 2012]. Some FOP patients report multiple neurological complications, including recurrent severe headaches, neuropathic pain, and recurrent seizures [Kitterman et al. 2012]. Later in life, patients commonly develop thoracic insufficiency syndrome (TIS), a life-threatening cardiopulmonary condition that can cause pneumonia and right-sided heart failure, and is a common cause of death in FOP [Kaplan et al. 2010].

1.4.2 Histopathology of FOP lesions

Following a conclusive diagnosis of FOP, biopsies are not recommended, as the accompanying tissue injury stimulates new HO formation at site of collection [Pignolo et al. 2016]. However, biopsies collected prior to diagnosis of FOP have revealed the histological sequence of catabolic and anabolic events throughout lesion development [Kaplan et al. 1993, Gannon et al. 1998, Gannon et al. 2001, Glaser et al. 2003, Hegyi et al. 2003] (Figure 1.4). The earliest stage of lesion development involves robust invasion of neutrophils, monocytes/macrophages, mast cells, and lymphocytes that accompany extensive connective tissue destruction [Gannon et al. 1998, Gannon et al. 2001, Kaplan et al. 2005]. Subsequently, fibroproliferative cells mark the transition from the catabolic to anabolic phase, which occurs with concomitant with angiogenesis [Gannon et al. 1998, Glaser et al. 2003]. Lineage tracing studies (Lounev 2009; Medici et al) suggest that the fibroproliferative cells transition to endochondral ossification with characteristic cartilage morphology and extracellular matrix composition [Kaplan et al. 1993, Gannon et al. 1998, Glaser et al. 2003]. A final osteogenesis stage occurs, resulting in mature heterotopic bone that may contain an active bone marrow niche [Kaplan et al. 1993]. The origin of cells that contribute to HO remains incompletely defined. Tie-2, Mx1, and Scx have been used as markers to identify lineages that contribute to HO development *in vivo* [Lounev et al. 2009, Wosczyna et al. 2012, Agarwal et al. 2016, Dey et al. 2016]. Of note, cells of hematopoietic or vascular smooth muscle origin were not represented in the fibroproliferative, chondrogenic, or osteogenic stages of HO [Kaplan et al. 2007, Lounev et al. 2009].

1.4.3 Causative mutations in ACVR1 in FOP

In 2006, *ACVR1* was identified as the mutated gene in FOP [Shore et al. 2006]. *ACVR1* encodes for the BMP type I receptor Activin-like kinase 2 (ALK2). FOP is inherited in an autosomal dominant pattern, although most cases of FOP arise from sporadic mutations with no associated family history [Shore et al. 2010]. Greater than 95% of patients express a specific heterozygous missense mutation, an arginine to histidine amino acid residue change at codon 206 (R206H) caused by a single nucleotide substitution (c.617G>A) (Figure 1.5) [Shore et al. 2006, Kaplan et al. 2009]. The R206H mutation is located within the GS domain of ALK2 and is predicted to impair salt bridge formation with a tertiary-adjacent aspartate residue at codon 269 [Groppe et al. 2007], reducing binding affinity for FKBP12, an inhibitory protein that stabilizes the inactive conformation of the receptor [Huse et al. 1999, Shen et al. 2009, Chaikuad et al. 2012].

Additional causative mutations in *ACVR1* have been identified in patients with FOP in combination with atypical features and/or more severe or mild FOP characteristics [Convente et al. 2017]. All identified classic and variant FOP mutations are single nucleotide substitutions within the GS or protein kinase domains of ALK2, except for a three-nucleotide deletion spanning two codons that results in replacement of a single amino acid residue (P197-F198 del ins L) (Figure 1.5) [Kaplan et al. 2009].

1.4.4 ACVR1^{R206H} causes enhanced BMP signaling activity

In vitro studies using cells derived from human patients and various animal models have demonstrated elevated BMP pathway signaling caused by the ACVR1 R206H

25

mutation. Patient-derived lymphoblastoid cell lines (LCLs) exhibited enhanced phosphorylated-p38 MAPK levels and BMP target gene expression in the presence of BMP4 ligand [Fiori et al. 2006]. Upregulation of canonical BMP signaling by ALK2^{R206H} was first observed using FOP patient-derived stem cells from human exfoliated deciduous teeth (SHED) [Billings et al. 2008]. Ligand-independent and ligand-dependent activation of ALK2^{R206H} was also observed in SHED cells, and confirmed in chick limb bud micromass cultures and zebrafish embryonic development assays [Billings et al. 2008, Shen et al. 2009]. Increased signaling has also been observed in over-expression C2C12 (mouse myoblast precursor line), COS-7 (monkey kidney cell line), and MC3T3-E1 (mouse pre-osteoblast cell line) experiments, indicating the enhanced activity of ALK2^{R206H} is not cell specific [Fukuda et al. 2009, Shen et al. 2009]. Notably, increased signaling conferred by ALK2^{R206H} is not prevented by addition of extracellular BMP antagonists, such as noggin, further suggesting that the enhanced signaling activity is in part ligand-independent [Shen et al. 2009].

The observed enhanced signaling activity in ALK2^{R206H} is consistent with predictive structural modeling data [Groppe et al. 2007, Chaikuad et al. 2012]. The crystal structure of ALK2 [Chaikuad et al. 2012] indicates that FOP mutations may alter interactions that stabilize the inactive state of the kinase domain, leading to inappropriate activation. Furthermore, the ACVR1 R206H mutation is predicted to reduce binding of FKBP12, an intracellular regulatory protein that binds the GS domain of BMP/TGF-β type I receptors to prevent leaky receptor activation in absence of ligand binding [Wang et al. 1996, Groppe et al. 2007]. Co-immunoprecipitation and size exclusion high-performance

26

liquid chromatography (HPLC) experiments have shown reduced FKBP12 binding affinity

to ALK2^{R206H} compared to wild-type ALK2 [Shen et al. 2009, Song et al. 2010, van Dinther

et al. 2010, Groppe et al. 2011], matching structural homology predictions.

1.4.5 Conditional-on knock-in mouse model of FOP

The need for high fidelity mouse models is essential to studying FOP, especially in

light of the rarity of biopsy tissue. A knock-in mouse model was generated to carry the

identical nucleotide substitution as in patients, c.617G>A;R206H in exon 5 at the

endogenous *Acvr1* locus [Chakkalakal et al. 2012]. Chimeric founder mice were viable,

however germline transmission pups died perinatally. These mice phenocopied all clinical

features of FOP, including the two characteristic features of disease – progressive HO and

great toe malformation. An unexpected finding from the chimeric model is the presence of

wild-type cells in ectopic bone, suggesting that although the ACVR1 R206H mutation is

necessary to induce the bone formation process, the mutation is not necessary for

differentiation of progenitor cells into cartilage and bone within maturing lesions.

Histopathological analysis also demonstrated that lesion development in the knock-in

mouse model progressed through all catabolic and anabolic stages seen in FOP. This mouse

model was the first to demonstrate that the ACVR1 R206H mutation is sufficient to cause

FOP [Chakkalakal et al. 2012].

In order to study FOP etiology throughout neonatal development and adulthood, a

conditional-on knock-in *Acvr1^{R206H}* mouse was generated [Hatsell et al. 2015]. This mouse

contains an engineered gene construct at the endogenous *Acvr1* locus with the human wild-

type exon 5 followed by downstream mouse R206H exon 5 in anti-sense orientation, with adjacent intron sequence and engineered LoxP sites in between. Upon Cre-recombinase-mediated recombination, the human wild-type exon is deleted and the mouse R206H exon is flipped into sense orientation, generating a functional $Acvr1^{R206H}$ allele (Figure 1.6). The Acvr1^{R206H} allele can be induced globally, or in specific cell populations induced by promoter-driven Cre recombinase expression. Several studies have used these mice for *in vitro* and *in vivo* experiments [Hatsell et al. 2015, Chakkalakal et al. 2016, Dey et al. 2016].

1.5 Project summary

Much has been learned about cellular and molecular mechanisms driving FOP pathology since the discovery of the gene mutation in 2006. Clinical evaluation, in combination with mouse model data, has vastly expanded the knowledge of disease, especially for the later chondrogenesis and osteogenesis stages. Previous work from our lab and others has demonstrated accelerated chondrogenesis as a consequence of the ACVR1 R206H mutation, and the first clinical trials for FOP are currently evaluating drug efficacy at inhibiting this stage of lesion progression. Despite the recent advances in understanding of FOP pathology, an area that remains unclear is the contribution of the immune system to disease initiation and progression. As outlined in the Introduction, BMP signaling is crucial for immune cell development and activation. This suggests that the increased activity conferred by Acvr1^{R206H} would enhance the early inflammatory response stage of FOP disease progression, which occurs in episodes of HO even without an overt injury. In the case of tissue injury, an early inflammatory response is seen in both FOP

28

patients and unaffected individuals, yet the final outcome of the injury repair program is strikingly dissimilar, as FOP patients develop ectopic cartilage and bone in place of faithful tissue regeneration. Elucidating how the ACVR1 R206H mutation and the inflammatory response direct the divergence of repair events has not been determined and is a goal of this project.

I investigated two primary research questions for this project: 1) to determine the effect of $Acvr1^{R206H}$ on the cellular and molecular inflammatory response to tissue injury, and 2) to determine whether modulation of the immune system can control HO formation. The experimental results presented in this dissertation demonstrate an active role for $Acvr1^{R206H}$ in driving an elevated inflammatory response in FOP, and that inhibition of the immune system can impair HO development in an FOP genetic background.
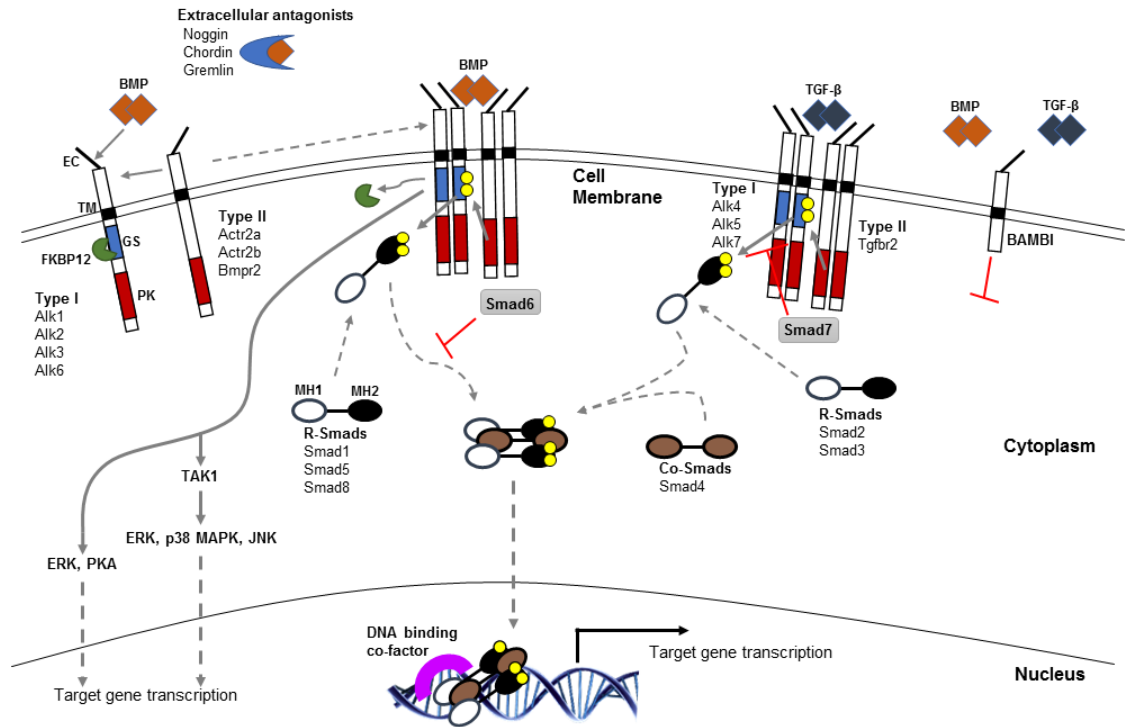
Figure 1.1: BMP and TGF-β signaling pathways
BMP and TGF-β signaling pathways (canonical and non-canonical) are shown. Extracellular, membrane-bound, and intracellular antagonists are included. Abbreviations: EC = extracellular domain; TM = transmembrane domain; GS = glycine/serine domain; PK = protein kinase domain
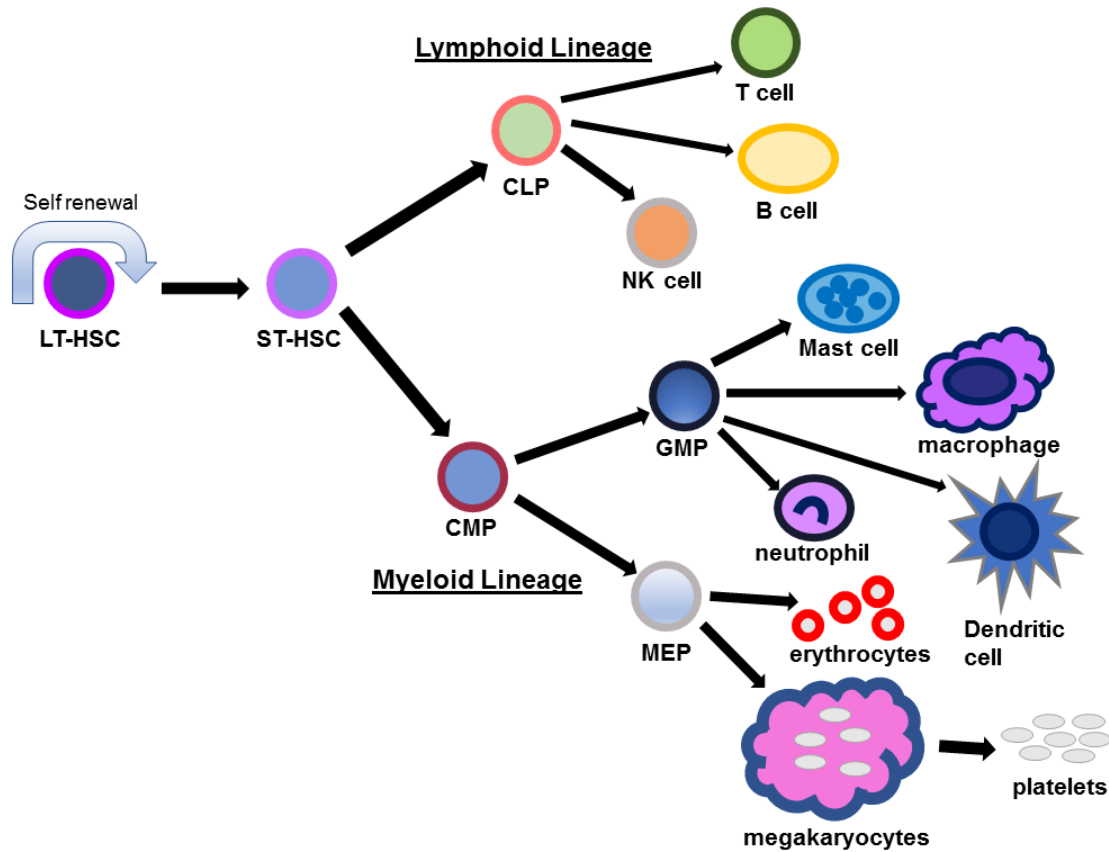
Figure 1.2: Hematopoiesis differentiation chart
Generalized hematopoiesis self-renewal and differentiation chart is shown. LT-HSCs self-renew or asymmetrically divide into ST-HSCs, which proliferate and differentiate into two branches – CLPs or CMPs. All lymphoid- and myeloid-derived precursor and terminally differentiated cells are derived from either CLPs or CMPs, respectively. Abbreviations: HSC = hematopoietic stem cell; LT-HSC = long-term HSC; ST-HSC = short-term HSC; CLP = common lymphoid progenitor; CMP = common myeloid progenitor; GMP = granulocyte/macrophage progenitor; MEP = megakaryocyte/erythrocyte progenitor

31

Figure 1.3: Clinical diagnostic features of FOP
(A) Three-dimensional volume rendering demonstrating progressive HO observed in FOP. (B) Radiograph of feet showing the great toe malformation ("hallux valgus") in FOP.

Figure 1.4: Histopathology of FOP lesion

(A) Schematic representation of lesion progression in FOP. In response to injury, skeletal muscle destruction occurs followed by robust immune cell invasion. As these catabolic events conclude, an anabolic stage begins with recruitment of fibroproliferative progenitor cells that condense and differentiate to chondrocytes, ultimately resulting in mature bone with an active marrow niche. (B) Histological section from $Acvr1^{cR206H/+}$ mouse containing all sections of lesion. (C) Histological section from FOP patient biopsy containing comparable stages of lesion to mouse. Scale bar = 100 μm. Abbreviations: DM = damaged muscle; FP = fibroproliferation; C = chondrocytes; B = bone.
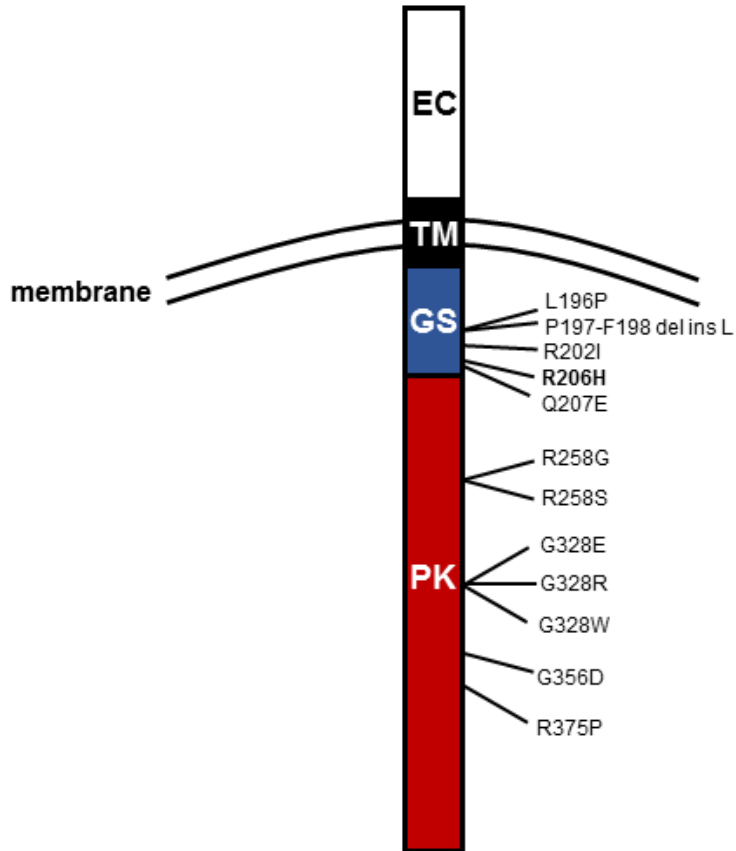
Figure 1.5: ALK2 classical and variant mutations in FOP
ALK2, the BMP type I receptor mutated in FOP, consists of four domains: an extracellular ligand binding domain (EC), a transmembrane domain (TM), a glycine/serine domain (GS), and a protein kinase domain (PK). A total of 12 mutations have been identified, all present within the GS or PK domains. Over 95% of patients possess the classical R206H mutation.

Based on figure from Convente M, Towler O, Stanley A, Brewer N, Allen R, Kaplan F, Shore E. "Chapter 30: Fibrodysplasia (Myositis) Ossificans Progressiva." In: Thakker R, Whyte M, Eisman J, Igarashi T, editors. Genetics of Bone Biology and Skeletal Disease: 2nd Edition (in press). 2: Elsevier; 2017.

A GAACTACTAGATCACTCGTGTACATCAGGAAGTGGCTCCGGTCTTCCTTTCCTGGTACAGAGAACGGTGGCTCGCCAGATAACCCTGTTGGAGTGTGTCG

Acvr1-001 isoform

Acvr1

B *Acvr1*[R206]FlEx **allele**

WT human exon region   R206H mouse exon region   rßgli insert

P   2372   P   2372   FRT

C *Acvr1*[R206H] **allele (post-Cre)**

FRT

100 bp
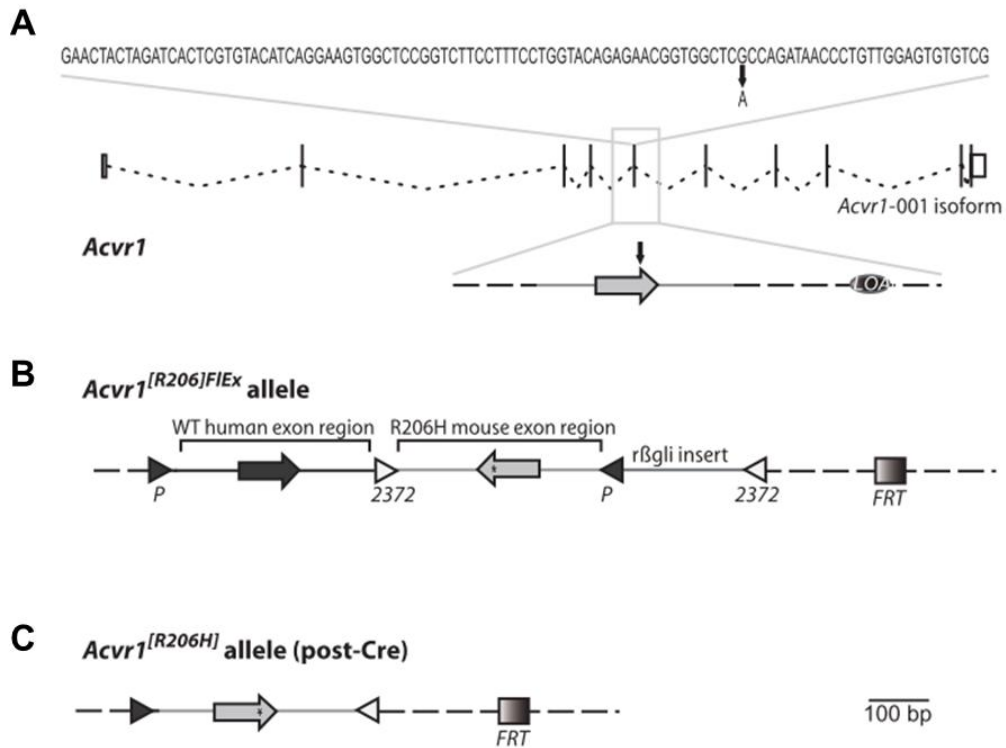
Figure 1.6: Conditional-on knock-in *Acvr1*[R206H] mouse
(A) Sequence of *Acvr1* exon 5, with the nucleotide substitution c.617G>A shown. (B) Structure of the *Acvr1*[R206H]FlEx allele. The wild-type human exon 5 region is in sense orientation, followed downstream by the R206H mouse exon 5 region in antisense orientation; both regions are flanked by two pairs of loxP sites arranged so that the wild-type region is deleted and the R206H region is flipped into sense orientation upon recombination. (C) Structure of R206H allele post-recombination.

Chapter Two

Materials and Methods

2.1 Study Animals

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at University of Pennsylvania.

2.1.1 Knock-in $Acvr1^{R206H/+}$ mouse colony

A conditional knock-in $Acvr1^{R206H/+}$ mouse was previously generated [Hatsell et al. 2015]. Mice were crossed into two different induction systems – *ERT2-Cre* tamoxifen induction, and R26-rtTA; TetO-Cre doxycycline induction.

Our lab obtained B6.129-*Gt(ROSA)26Sor^{tm(cre/ERT2)Tyj}*/J mice from the Jackson Laboratory (Stock #008463; hereafter referred to as "*ERT2-Cre*"). These mice express Cre recombinase under the presence of tamoxifen. *ERT2-Cre* mice were mated with $Acvr1^{cR206H/+}$ mice to generate *ERT2-Cre*; $Acvr1^{cR206H/+}$ mice that express the mutant $Acvr1^{cR206H}$ after administration of tamoxifen (200 μL of 10 mg/mL concentration dissolved in corn oil). This FOP mouse is referred to as *ERT2-Cre*; $Acvr1^{cR206H/+}$ and was only used for our bone marrow transplant experiment.

$Acvr1^{[R206H]FlEx/+}$ mice with mice double transgenic for R26-rtTA and tetO-Cre (heterozygous *Gt(ROSA)26Sor^{tm1(rtTA_M2)Jae}* and hemizygous *Tg(tetO-Cre)1Jaw*; (Jackson Laboratory) were mated to generate doxycycline-inducible global allele expression $Acvr1^{[R206H]FlEx/+}$; *Gt(ROSA)26Sor^{tm1(rtTA_M2)Jae}*; *Tg(tetO-Cre)1Jaw* mice. This FOP mouse is hereafter referred to as $Acvr1^{cR206H/+}$ and was used for all experiments unless otherwise noted.

2.1.2 *c-Kit^{W-sh/W-sh}* mast cell-deficient mouse colony

Our lab obtained heterozygous B6.Cg-*Kit^{W-sh}*/HNihrJaeBsmGlliJ from the Jackson Laboratory (Stock #012861, "White Sash"). To generate mast cell-deficient *Acvr1^{cR206H/+}* mice, I mated *Acvr1^{cR206H/+}* mice with heterozygous White Sash B6.Cg-*Kit^{W-sh}*/HNihrJaeBsmGlliJ) to generate compound heterozygous *Acvr1^{cR206H/+}*; B6.Cg-*Kit^{W-sh}*/HNihrJaeBsmGlliJ mice. Offspring from this parental generation cross were mated to generate homozygous mast cell-deficient *Acvr1^{cR206H/+}*; B6.Cg-*Kit^{W-sh/W-sh}*/HNihrJaeBsmGlliJ mice (hereafter referred to as *Acvr1^{cR206H/+}*; *c-Kit^{W-Sh/W-sh}*).

2.1.3 B6.SJL CD45.1 mice for allogenic bone marrow transplant

B6.SJL CD45.1 mice were obtained from the National Cancer Institute and used as recipients for allogenic bone marrow transplant from CD45.2 donor bone marrow (*ERT2-Cre; Acvr1^{cR206H/+}* or *Acvr1^{+/+}* mice). Donors and recipients expressed different isoforms of CD45 to quantify engraftment and reconstitution of donor bone marrow into recipients.

2.1.4 *LysM*-Cre mouse colony

LysM (also known as *Lyz2*) encodes the sequence for the Lysozyme M protein, a glycoside hydrolase enzyme that is expressed in myeloid lineage cells, including monocytes and macrophages [Cross et al. 1988, Faust et al. 2000]. Our lab obtained B6.129P2-*Lyz2^{tm1(cre)Ifo}*/J mice from the Jackson Laboratory (Stock #004781; hereafter referred to as *LysM*-Cre) To induce expression of *Acvr1^{cR206H}* exclusively in myeloid

lineage cells, I mated heterozygous $Acvr1^{cR206H/+}$ with heterozygous $LysM$-Cre/+ mice to generate $LysM$-Cre/+; $Acvr1^{cR206H/+}$ mice.

2.2 PCR Genotyping

2.2.1 Extraction of genomic DNA

Tail snips were collected from 3 week mice and processed using the KAPA Express Extract Kit (KAPA Biosystems). Tissue was placed in 100 μL digestion buffer (88 μL PCR grade water 10 μL KAPA Express Extract Buffer, 2 μL KAPA Express Extract Enzyme (1 U/μL) and lysed at 75°C for 10 minutes, and inactivated at 95°C for 5 minutes.

2.2.2 Preparation of PCR reaction

A master-mix containing 12.5 μL KAPA2X Robust HotStart ReadyMix, 1.0 μL of forward and reverse primer solution (200 μM final concentration), 1.0 μL genomic DNA, and 10.5 μL PCR grade water (total volume of 25 μL) was made for each reaction.

2.2.3 PCR protocol for genotyping doxycycline-inducible $Acvr1^{cR206H/+}$ mice

$Acvr1^{cR206H/+}$ mice require expression of three transgenes for doxycycline-inducible expression of the $Acvr1^{cR206H}$ allele - R26-rtTA, tetO-Cre, and $Acvr1^{[R206H]FlEx}$. Expression of all three amplicons for a single mouse indicates a positive $Acvr1^{cR206H/+}$ animal. Primer pair sequences targeting each transgene, thermocycler method, and agarose gel composition are listed in Table 2.1.

2.2.4 PCR protocol for genotyping tamoxifen-inducible *ERT2-Cre; Acvr1$^{cR206H/+}$* mice

   Acvr1$^{cR206H/+}$ mice require expression of two transgenes for tamoxifen-inducible expression of the *Acvr1^{cR206H}* allele – *ERT2-Cre* and *Acvr1$^{[R206H]FlEx}$*. Expression of both amplicons for a single mouse indicates a positive *ERT2-Cre; Acvr1$^{cR206H/+}$* animal. Primer pair sequences targeting each transgene, thermocycler method, and agarose gel composition are listed in Table 2.2.

2.2.5 PCR protocol to confirm *Acvr1^{cR206H}* recombination and expression

   Expression of the *Acvr1^{cR206H}* allele is confirmed by detecting presence of amplicon 32 bp greater in length compared to unrecombined allele from genomic DNA sample post-induction. Primer pair sequences, thermocycler method, and agarose gel composition are listed in Table 2.3.

2.2.6 PCR protocol for genotyping mast cell-deficient *c-Kit$^{W-Sh/W-sh}$* mice

   c-Kit$^{W-Sh/W-sh}$ mice require homozygous expression of the mutant *c-Kit^{W-Sh}* allele for full mast cell deficiency. Primer pair sequences, thermocycler method, and agarose gel composition are listed in Table 2.4. For additional genotype confirmation, in a C57BL/6J background, *c-Kit$^{W-Sh/+}$* mice have a patch of white fur around the abdomen and back; *c-Kit$^{W-Sh/W-sh}$* mice have entirely white fur [Grimbaldeston et al. 2005].

2.2.7 PCR protocol for genotyping *LysM*-Cre mice

Heterzygous and hemizygous expression of *LysM*-Cre and *Acvr1$^{cR206H/+}$*, respectively, will activate the *Acvr1^{cR206H}* allele exclusively in myeloid lineage populations. Primer pair sequences, thermocycler method, and agarose gel composition are listed in Table 2.5.

2.3 Cross-sectional injury experimental design

Skeletal muscles of *Acvr1$^{cR206H/+}$* and *Acvr1$^{+/+}$* mice (4 weeks of age) were injured by injecting 50 µL of 20 µM cardiotoxin from *Naja mossambica mossambica* (Sigma-Aldrich) into hamstring muscles of the hind limbs. Mice were euthanized by CO_2 asphyxiation and whole hind limbs were collected at days 0, 1, 2, 3, 4, 5, 6, 7, 10, and 14 post-injection. Day 0 samples were collected without cardiotoxin injection. *Acvr1$^{cR206H/+}$* mice were placed on a doxycycline chow diet (625 mg/kg doxycycline chow) for 5 days prior to cardiotoxin injection to induce mutant gene expression.

2.4 Isolation and culture of primary murine immune cells

2.4.1 Isolation of murine bone marrow

Whole bone marrow was collected from femurs and tibias of *Acvr1$^{cR206H/+}$* and *Acvr1$^{+/+}$* mice. Skeletal muscle was removed from femurs and tibias, and the bones were cut at the diaphyseal midshaft, with approximately 0.5 mm of each epiphysis removed. 10 mL D-MEM was aspirated through each hollow cortical bone piece using a 27G needle into a 15 mL conical tube. Cells were spun and plated fresh in appropriate

41

maturation/growth medium, or resuspended in 95% : 5% ratio; FBS : DMSO freeze medium and kept for long term storage in liquid nitrogen.

2.4.2 Maturation of primary murine mast cells from whole bone marrow

Whole bone marrow was cultured in suspension for 6 weeks in mast cell maturation medium (RPMI 1640, 10% FBS, 1X Penicillin/Streptomycin, 25 mM HEPES, 1X MEM Non-Essential Amino Acids, 1X GlutaMAX, 100 mM Sodium pyruvate, 50 μM β-mercaptoethanol) with added recombinant murine Stem Cell Factor (Peprotech; 15 ng/mL final concentration) and recombinant murine Interleukin (IL)-3 (Peprotech; 12 ng/mL final concentration) [Jamur et al. 2011]. Mast cell purity was determined via flow cytometry and gating for c-kit receptor/CD117 and $Fc_\varepsilon RI_\alpha$ receptor double-positive cells [Weller et al. 2005].

2.4.3 Isolation of primary murine macrophages by thioglycollate elicitation

To obtain primary murine macrophages, 1.5 mL aged 4.0% thioglycolate solution (Sigma-Aldrich) was injected intraperitoneally into $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ mice [Zhang et al. 2008]. After 5 days, mice were euthanized by CO_2 asphyxiation and peritoneal exudate was collected using 10 mL PBS. Cells were centrifuged at 300 x G for 5 minutes and plated in 100 cm plates in macrophage growth medium (RPMI 1640, 5% FBS, 10 mM HEPES, 1X Penicillin/Streptomycin, 1X GlutaMAX, 23.3 mM Sodium bicarbonate, 50 μM β-mercaptoethanol).

2.5 BMP ligand and inflammatory stimulation of immune cells

2.5.1 BMP ligand stimulation of immune cells

1×10^6 mast cells or 5×10^5 macrophages were plated in Tyrode's solution for overnight or 2 hours, respectively, and then treated with increasing concentrations of recombinant human BMP4 for 1 hour.

2.5.2 Mast cell inflammatory stimulation with Substance P

1×10^6 cells were plated in 1 mL Tyrode's solution overnight, and then stimulated with 100 μM Substance P (Sigma-Aldrich) plus 15 ng/mL of recombinant human BMP4 for 1 hour.

2.5.2 Mast cell degranulation assay

Mast cell degranulation was assayed using a Mast Cell Degranulation Assay Kit (EMD Millipore). 1.5×10^6 cells were plated in 1 mL Tyrode's solution overnight, and then stimulated with 100 μM Substance P, plus 15 ng/mL of recombinant human BMP4 for 1 hour. Cells were spun and 180 μL of conditioned medium was added to 20 μL tryptase substrate in a 96 well plate for 2 hours in an incubator at 37°C and 5% CO_2. The assay detects the chromophore p-nitroanaline (pNA) after tryptase-mediated cleavage of the labeled substrate tosyl-gly-pro-lys-pNA. The free pNA is quantified using a microplate reader at a wavelength of 405 nm.

2.5.2 Macrophage inflammatory stimulation with lipopolysaccharides

For macrophage inflammatory stimulation, 5 X 10^5 cells were plated in 1 mL Tyrode's solution for 2 hours, and then stimulated with 1 ng/mL lipopolysaccharides (Sigma-Aldrich) plus 15 ng/mL of recombinant human BMP4 for 1 hour.

2.6 Gene and protein expression analysis

2.6.1 Gene expression analysis

RNA was isolated from mast cells and macrophages using TRIzol (Thermo Fisher Scientific) and quantified using a Nanodrop instrument. 1.0 μg cDNA was synthesized using High Capacity RNA-to-cDNA reagents (Applied Biosystems). Real-time quantitative PCR reactions contained forward/reverse primers (0.37 μM), cDNA (1:5 dilution), and 1X Fast SYBR Green PCR Master Mix (Applied Biosystems) for a final volume of 13 μL; each sample was analyzed in triplicate. Target gene mRNAs were quantified from standard curves and normalized to *Gapdh*. Forward and reverse primers are listed in Table 2.6.

2.6.2 Protein expression analysis

Total cell protein was recovered using RIPA lysis and extraction buffer (Thermo Fisher Scientific) containing Halt Protease and Halt Phosphatase Inhibitor Cocktails (Thermo Fisher Scientific) and quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 30 μg protein were electrophoresed through 4-12% Bolt Bis-Tris polyacrylamide gels (Thermo Fisher Scientific) and transferred to nitrocellulose

44

membranes using a Bio-Rad Trans Blot Turbo instrument (Bio-Rad). Membranes were blocked in 5% Bovine Serum Albumin and incubated with primary antibody against Phosphorylated Smad 1/5/8 (Cell Signaling Technology, 1:750 dilution) at 4°C overnight. Membranes were then incubated with primary antibody against GAPDH (Cell Signaling Technology, 1:5000 dilution) at room temperature for 1 hour. Bound primary antibodies were detected with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, 1:10,000 dilution) at room temperature for 1 hour. Detected proteins were imaged with WesternSure PREMIUM Chemiluminescent Substrate (LI-COR) using a LI-COR C-DiGit Blot Scanner, and quantified using LI-COR Image Studio software.

2.7 *In vivo* heterotopic ossification experiments

2.7.1 Injury-induced heterotopic ossification assay

Skeletal muscles of *Acvr1$^{cR206H/+}$* and *Acvr1$^{+/+}$* mice (4 weeks of age) were injured by injecting 50 μL of 20 μM cardiotoxin from *Naja mossambica mossambica* (Sigma-Aldrich) into hamstring muscles of the hind limbs. Heterotopic ossification develops by 14 days post-injection with 100% consistency.

2.7.2 Targeted depletion of mast cells and macrophages *in vivo*

Three immunodeficient *Acvr1$^{cR206H/+}$* transgenic mice were generated: mast cell-deficient *Acvr1$^{cR206H/+}$*; *c-Kit$^{W-Sh/W-sh}$* mice, a macrophage-depleted *Acvr1$^{cR206H/+}$*; Clo. mice, and combined mast cell-deficient and macrophage-depleted *Acvr1$^{cR206H/+}$*; *c-Kit^{W-}*

Sh/W-sh; Clo. mice. Heterotopic ossification was initiated by injury as in 2.7.1, except that duration of experiment was 17 days post-injury.

2.7.3 Clodronate-liposome mediated macrophage depletion

To deplete macrophages from mouse cohorts [van Rooijen et al. 1984, Van Rooijen 1989], clodronate-liposomes (100 µL / 10 g bodyweight) were injected intraperitoneally using a 27G needle into appropriate mouse cohorts. Clodronate-liposomes were obtained from the lab of Dr. Nico van Rooijen (http://www.clodronateliposomes.com, The Netherlands).

2.7.4 Bone marrow transplant of *Acvr1$^{cR206H/+}$* bone marrow into recipient mice

Whole bone marrow from donor mice was collected from femurs and tibias. Recipient mice were irradiated with 1000 rads, allowed to recover, and then injected with 5×10^6 bone marrow cells by tail vein injection. Engraftment of donor bone marrow and immune cell reconstitution occurred for 8 weeks. Donor mice expressed the CD45.2 isoform, recipient mice expressed the CD45.1 isoform; this allows for detection of engraftment and reconstitution by flow cytometry.

2.7.5 Injection of neutralizing agents as a method for inhibiting heterotopic ossification development

To neutralize the pro-inflammatory cytokines TNF-α and IL-6, I injected etanercept (targets TNF-α), mouse-anti-IL-6 antibody, or etanercept and mouse-anti-IL-6-antibody

via retro-orbital delivery into *Acvr1*^{cR206H/+} mice. The first experiment utilized the following dosing regimens: etanercept (4.0 mg/kg, 3X week; targets TNF-α), mouse-anti-IL-6 antibody (150 mg per mouse, 3X week) or etanercept and mouse-anti-IL-6-antibody (previous concentrations). The second experiment utilized the following dosing regimens: etanercept (4 mg/kg, 2X week; 2 mg/kg, 2X week; 4 mg/kg, 3X week).

2.7.6 Retro-orbital blood collection for detection of serum cytokines

Mice were anesthetized by 3% isoflurane gas. Blood (no more than 150 µL) was collected by retro-orbital bleeding using heparinized micro-hematocrit capillary tubes (Fisher Scientific). After conclusion of blood collection, mice were blotted with gauze to stop bleeding, returned to their cage and monitored for several minutes to ensure return to normal activity. Blood was collected at Day 0, Day 7, and Day 14 during course of two-week experiment; if blood collection and antibody injection days overlapped, blood was collected before antibody injection. Collected blood was centrifuged in Microvette Capillary Blood EDTA collection tubes (Kent Scientific) at 14 X G for 20 minutes at 4° C. Serum was collected and stored in separate tubes at -80°C before analysis.

2.7.7 Micro Computed Tomography (µCT) analysis

High-resolution, cross-sectional images of hind limbs were obtained using a VivaCT 40 (Scanco Medical AG) at a source voltage of 55 kV, a source current of 145 µA, and an isotropic voxel size of 19.0 µm. A three-dimensional (3D) rendering was reconstructed using Scanco microCT V6.1 software. Thresholding values for heterotopic

ossification detection ranged from 240 – 1,000 Hounsfield units. Detected heterotopic ossification was quantified using Scanco microCT V6.1 software.

2.8 Flow Cytometry

2.8.1 Confirming primary murine mast cell purity

Mast cells were incubated with c-Kit-APC (BioLegend, 1:150 dilution) and $Fc_{\varepsilon}RI_{\alpha}$-PE (BioLegend, 1:150 dilution) antibodies for 30 minutes and then run on a BD FACSCalibur instrument (BD Biosciences). Double-positive cells expressing c-Kit and $Fc_{\varepsilon}RI_{\alpha}$ receptors were gated as mast cells.

2.8.2 Confirming clodronate-liposome-mediated macrophage depletion

Whole bone marrow was collected at sacrifice from clodronate-liposome-injected cohorts and stained with F4/80-PE (BD Pharmingen), CD11b-PerCP/Cy5.5 (BD Pharmingen), and MHC Class II-APC antibodies (BD Pharmingen). Cells were run on a BD FACSCanto instrument (BD Biosciences). Triple-positive cells expressing F4/80, CD11b, and MHC Class II receptors were gated as macrophages.

2.8.3 Confirming allogenic hematopoetic engraftment and immune cell reconstitution

Whole bone marrow and spleens were collected from bone marrow transplant recipient mice and analyzed by flow cytometry to determine engraftment of donor CD45.1 bone marrow and reconstitution of five immune cell populations: B cells, CD4 T cells, CD8 T Cells, neutrophils, and natural killer cells. Cell expressing the following receptors

48

were used to detect listed immune cell populations and gated as indicated: CD45.1+, CD45.2+, CD19+ (B cells), CD3e+ / CD4+ (CD4 T cells), CD3e+ / CD8a+ (CD8 T cells), CD11b+ / Gr-1+ (neutrophils), CD3e- / CD49b+ / NK1.1+ (natural killer cells).

2.9 Histological analysis

2.9.1 Sample preparation

Hind limb samples were collected at sacrifice and fixed in 4% paraformaldehyde for 24 hours. Samples were decalcified in Immunocal (Decal Chemical Corporation) for 3 days, embedded in paraffin blocks, and sectioned serially at 5 μm.

2.9.2 Gross morphology histological stain

Deparaffinized sections were stained with combined Alcian Blue / Orange G / Hematoxylin / Eosin stain to detect glycosaminoglycans (GAG), bone, nucleated cells, and skeletal muscle, respectively.

2.9.3 Mast cell detection via combined eosinophil and mast cell (C.E.M.) stain

Deparaffinized sections were stained with C.E.M. stain (American Master Tech) to identify mast cells.

2.9.4 Immunohistochemstry

Deparaffinized sections were treated for antigen retrieval with 10 mM Sodium-Citrate buffer (pH 6.0) at 95°C for 20 minutes (for cytokine and chemokine detection) or

Digest-All 2 Trypsin (Thermo Fisher Scientific) at 37°C for 10 minutes (for immune cell detection). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide solution. Sections were blocked using Background Buster (Innovex Biosciences) and incubated with primary antibodies overnight at 4°C. Sections were then incubated with appropriate host secondary antibody and developed using SuperPicture Polymer DAB Detection Kit (Thermo Fisher Scientific) and counterstained with haematoxylin. Primary antibodies used were: Phosphorylated Smad 1/5/8 (Cell Signaling, 1:50 dilution), Myeloperoxidase (Abcam, 1:200 dilution), F4/80 (Abcam, 1:500 dilution), CD3 (Abcam, 1:50 dilution), TNF-α (Abcam, 1:100 dilution), IL-6 (Abcam, 1:400 dilution), IL-1β (Abcam, 1:400 dilution), MCP-1 (Abcam, 1:200 dilution), IL-13 (Abcam, 1:400 dilution), Activin A (LS Bio, 1:400 dilution).

2.10 Statistical analysis

Data obtained were analyzed statistically using GraphPad Prism 7 software (unpaired, two-sided, equal variance Student's t test; two-way ANOVA with Sidak's multiple comparisons test; two-way repeated measures ANOVA with Holm-Sidak's multiple comparisons test; one-way ANOVA with Tukey's multiple comparisons test); values are expressed as the mean ± SEM. Statistical significance was $p < 0.05$.

PCR protocol for genotyping doxycycline-inducible $Acvr1^{cR206H/+}$ mice		
	R26-rtTA	**TetO-Cre**
Forward primer	5'-GCGAAGAGTTTGTCCTCAACC-3' (ES3356)	5'-ATTCTCCCACCGTCAGTACG-3' (ES2856)
Reverse primer	5'-AAAGTCGCTCTGAGTTGTTAT-3' (ES3357)	5'-CGTTTTCTGAGCATACCTGGA-3' (ES2857)
Denature	95°C; 15 seconds	95°C; 15 seconds
Anneal	60°C; 15 seconds	60°C; 15 seconds
Extension	72°C; 15 seconds	72°C; 15 seconds
Cycles	35	35
Agarose Gel	2.0%	2.0%
Amplicon length	350 bp	500 bp

	$Acvr1^{[R206H]FlEx}$
Forward primer	5'-AACCAACATTGCCTGCTGCCCA-3' (ES3350) 5'-GCGCGTTAGCTTAGCTCTGT-3' (ES3748)
Reverse primer	5'-GCAGCCTCGTGGATTCACGCT-3' (ES3351) 5'-ACTCACATGAAGGGCAGCAA-3' (ES3750)
Denature	94°C; 30 seconds
Anneal	65°C; 45 seconds
Extension	72°C; 35 seconds
Cycles	35
Agarose Gel	2.0%
Amplicon length	650 bp (ES3350/3351) or 280 bp (ES3748/3750)

Table 2.1: PCR protocol for genotyping doxycycline-inducible $Acvr1^{cR206H/+}$ mice

	ERT2-Cre mutant	ERT2-Cre wild-type
PCR protocol for genotyping tamoxifen-inducible *ERT2*-Cre;*Acvr1^{cR206H/+}* mice		
Forward primer	5'-AAAGTCGCTCTGAGTTGTTAT-3' (ES3718)	5'-AAAGTCGCTCTGAGTTGTTAT-3' (ES3718)
Reverse primer	5'-CCTGATCCTGGCAATTTCG-3' (ES3720)	5'-GGAGCGGGAGAAATGGATATG-3' (ES3719)
Denature	95°C; 60 seconds	95°C; 60 seconds
Anneal	58°C; 60 seconds	58°C; 60 seconds
Extension	72°C; 60 seconds	72°C; 60 seconds
Cycles	35	35
Agarose Gel	2.0%	2.0%
Amplicon length	825 bp	650 bp

	Acvr1^{[R206H]FlEx}
Forward primer	5'-AACCAACATTGCCTGCTGCCCA-3' (ES3350)
Reverse primer	5'-GCAGCCTCGTGGATTCACGCT-3' (ES3351)
Denature	94°C; 30 seconds
Anneal	65°C; 45 seconds
Extension	72°C; 35 seconds
Cycles	35
Agarose Gel	2.0%
Amplicon length	650 bp

Table 2.2: PCR protocol for genotyping tamoxifen-inducible *ERT2*-Cre;*Acvr1^{cR206H/+}* mice

PCR protocol for detecting recombination of *Acvr1^cR206H^* allele	
	*Acvr1^cR206H^* **recombination**
Forward primer	5'-TGTATTGCAGGACGCTGAAG-3' (ES2935)
Reverse primer	5'-CCCCTGAAGTGGAATAACCA-3' (ES2936)
Denature	94°C; 30 seconds
Anneal	55°C; 45 seconds
Extension	72°C; 35 seconds
Cycles	35
Agarose Gel	4.0%
Amplicon length	Recombination = 370 bp and 336 bp No recombination = 336 bp

Table 2.3: PCR protocol for detecting recombination of *Acvr1^cR206H^* allele

PCR protocol for genotyping mast cell-deficient *c-Kit*[W-Sh/W-sh] mice		
	c-Kit[W-Sh] **mutant**	*c-Kit* **wild-type**
Forward primer	5'-AGGCTTGCAGCGCATTAT-3' (ES3755)	5'-TTTGCACGTGCTAGTTACAC-3' (ES3753)
Reverse primer	5'-GAGGATTCATAGTTGTTCAATGTCC-3' (ES3756)	5'-TTAAGATGGCACCCTGCTG-3' (ES3754)
Denature	95°C; 30 seconds	94°C; 60 seconds
Anneal	57°C; 60 seconds	57°C; 60 seconds
Extension	72°C; 60 seconds	72°C; 60 seconds
Cycles	35	35
Agarose Gel	2.0%	2.0%
Amplicon length	165 bp	650 bp

Table 2.4: PCR protocol for genotyping mast cell-deficient *c-Kit*[W-Sh/W-sh] mice

PCR protocol for genotyping *LysM*-Cre mice	
	LysM-Cre
Forward primer	5'-CCCAGAAATGCCAGATTACG-3' (ES4018)
Reverse primer	5'-CTTGGGCTGCCAGAATTTCTC-3' (ES4019)
Denature	95°C; 15 seconds
Anneal	60°C; 15 seconds
Extension	72°C; 15 seconds
Cycles	35
Agarose Gel	2.0%
Amplicon length	700 bp

Table 2.5: PCR protocol for genotyping *LysM*-Cre mice

Target	Full Transcript Name	Forward Primer	Reverse Primer	Stock Number
Actr2a	Activin receptor type 2a	GAACATGATGGAGGCGGCAGCAG	GACCCCGTCCAATCAGCTCCA	ES3318/3319
Actr2b	Activin receptor type 2b	CCTACTCAAGACCCAGGACCACC	GAGCAACTGGGCTTTCCAGACAC	ES3320/3321
Acvr1	Activin A receptor type 1	TGGTACAGAGAACGGTGGCT	ACTTCTCCATACCGGCCCTTC	ES3213/3217
Acvrl1	Activin A receptor Like type 1	ACACCCACCATCCCTAACC	ACCAGCACTCTCTCATCATCTG	ES3306/3307
Acvr1b	Activin A receptor type 1B	GCGGCGGTTACTATGGCGGA	CGCACACAGCAGAGCCTGGAT	ES3310/3311
Acvr1c	Activin A receptor type 1C	GCTCTGGGACCCCGAAGCCTTG	ACACACACACTTCAGTCCTGCCGCA	ES3316/3317
Bmpr1a	Bone morphogenetic protein receptor type 1A	AGCGATGAATGTCTTCGAGCAGTT	TCAAAGCTGTGAGTCTGGAGGCT	ES3308/3309
Bmpr1b	Bone morphogenetic protein receptor type 1B	CCTCCCTCTGCTGGTCCAAAGGA	CCTCGCCATAGCGGCCTTTTCC	ES3314/3315
Bmpr2	Bone morphogenetic protein receptor type 2	GAACATGATGGAGGCGGCAGCAG	GACCCCGTCCAATCAGCTCCA	ES3322/3323
Il1b	Interleukin 1 Beta	GCCAAGCTTCCTTGTGCAAGTGT	GCCCTTCATCTTTTGGGGTCCG	ES3443/3444
Il6	Interleukin 6	TCGGAGGCTTAATTACACATGTT	TGCCATTGCACAACTCTTTTCT	ES3479/3480
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	CAAGGTCATCCATGACAACTTT	GGCCATCCACAGTCTTCTGG	ES2452/2453
Tgfb1	Transforming growth factor beta 1	GGACACACAGTACAGCAAGGTCC	CTCCACCTTGGGCTTGCGAC	ES3439/3440
Tgfbr1	Transforming growth factor beta 2	GGCAGAGCTGTGAGGCCTTGAGA	ATGCCTTCCTGTTGGCTGAGTTGTG	ES3312/3313
Tgfbr2	Transforming growth factor beta receptor 2	CGCACGTTCCCAAGTCGGATGT	TCGCTGGCCATGACATCACTGT	ES3465/3466
Tnf	Tumor Necrosis Factor	CCCACGTCGTAGCAAACCAC	AGGTACAACCCATCGGCTGGC	ES3449/3450

Table 2.6: Quantitative real-time RT-PCR primers
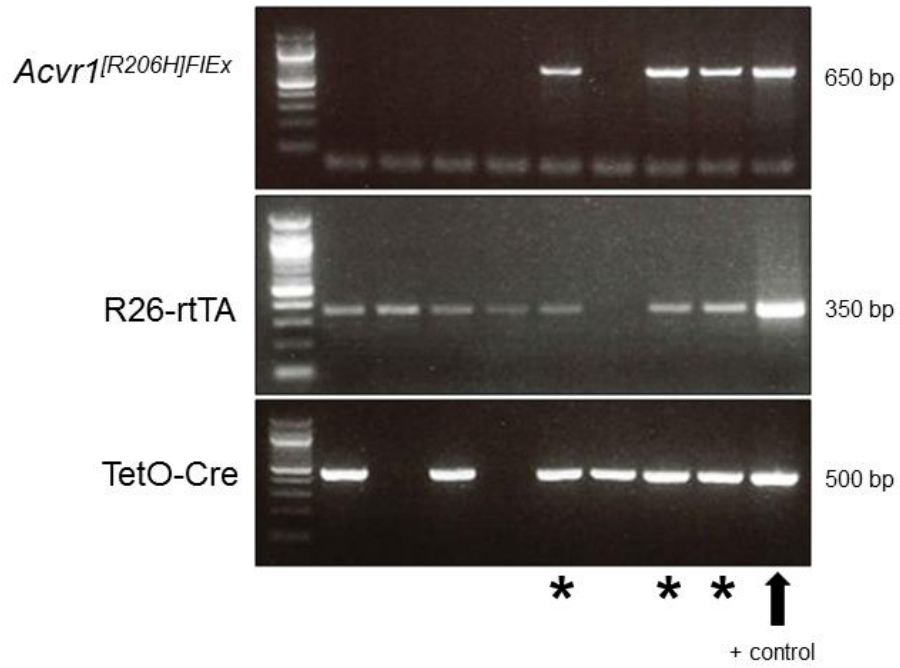
Figure 2.1: Genotyping of $Acvr1^{cR206H/+}$ mice by PCR

DNA isolated from mouse tail snips was amplified with primers specific to $Acvr1^{[R206H]FlEx}$, R26-rtTA, and TetO-Cre. Lower bands <100 bp in $Acvr1^{[R206H]FlEx}$ gel are non-specific primer dimer amplicons and do not reflect genotype. Lanes marked with an asterisk indicate $Acvr1^{cR206H/+}$ mice.
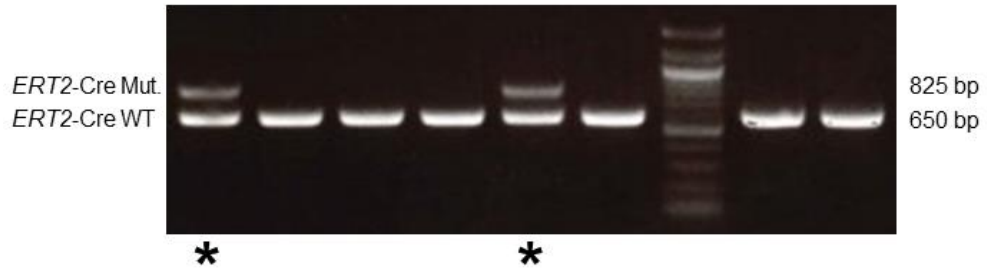
Figure 2.2: Genotyping of *ERT2*-Cre mice by PCR
DNA isolated from mouse tail snips was amplified with primers specific to *ERT2*-Cre. Lanes marked with an asterisk indicate *ERT2*-Cre mice.
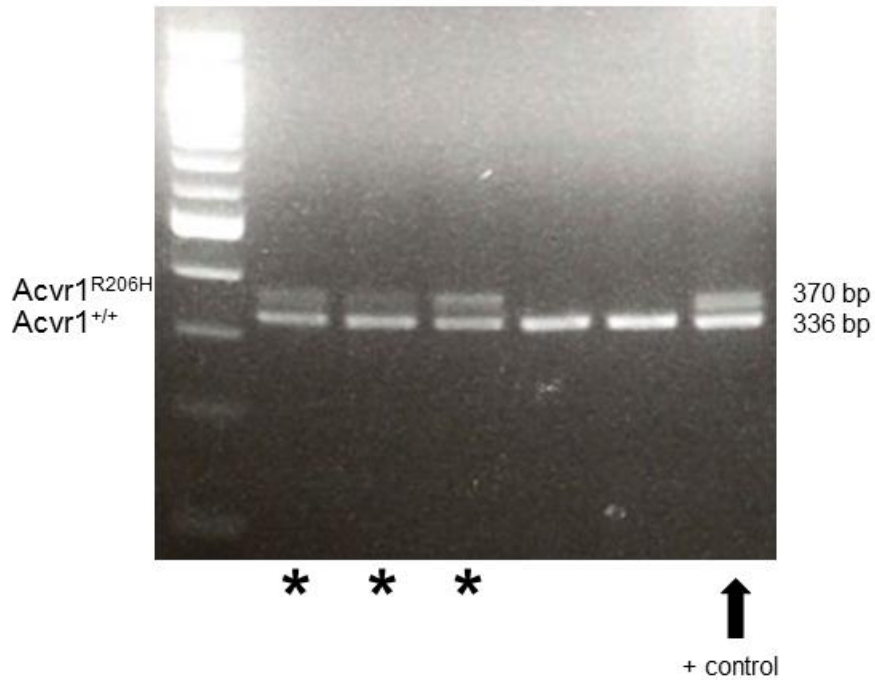
Figure 2.3: Detection of recombined and expressed *Acvr1^cR206H* allele in mice by PCR
DNA isolated from mouse tail snips was amplified with primers specific to recombination of *Acvr1^[R206H]FlEx*
amplicon (370 bp) and *Acvr1^+/+* control amplicon (336 bp), indicating active *Acvr1^cR206H* allele expression.
Lanes marked with an asterisk indicate recombined DNA and active *Acvr1^cR206H* allele expression.

59
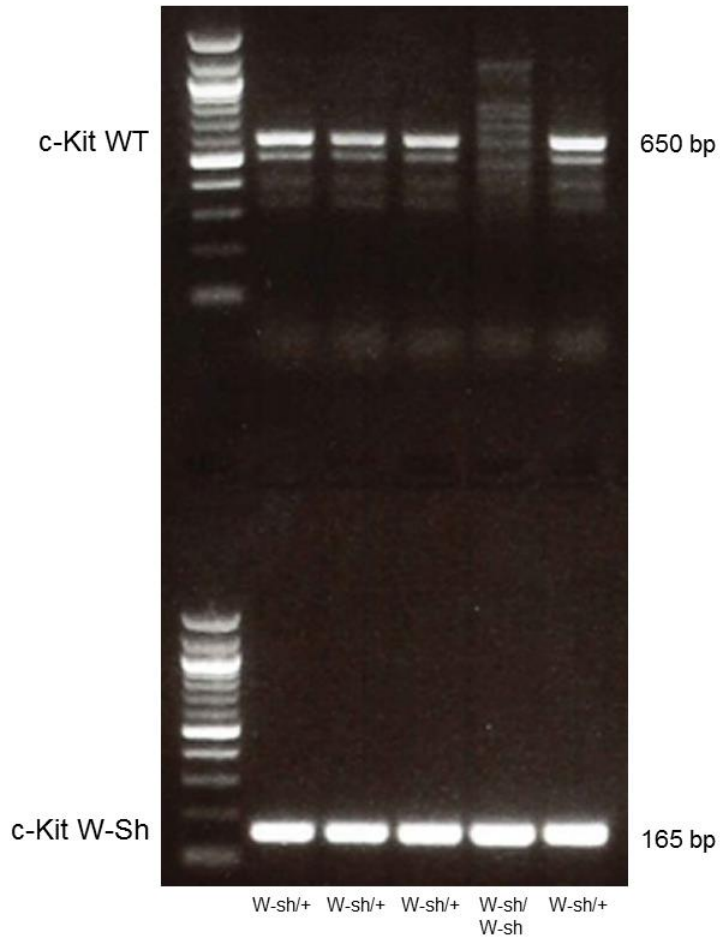
Figure 2.4: Genotyping of *c-Kit^W-sh/W-sh* mice by PCR
DNA isolated from mouse tail snips was amplified with primers specific to *c-Kit^W-sh* and *c-Kit^+* alleles. Lanes are marked with mouse genotypes. Non-specific bands for the *c-Kit^+* PCR do not reflect genotype.
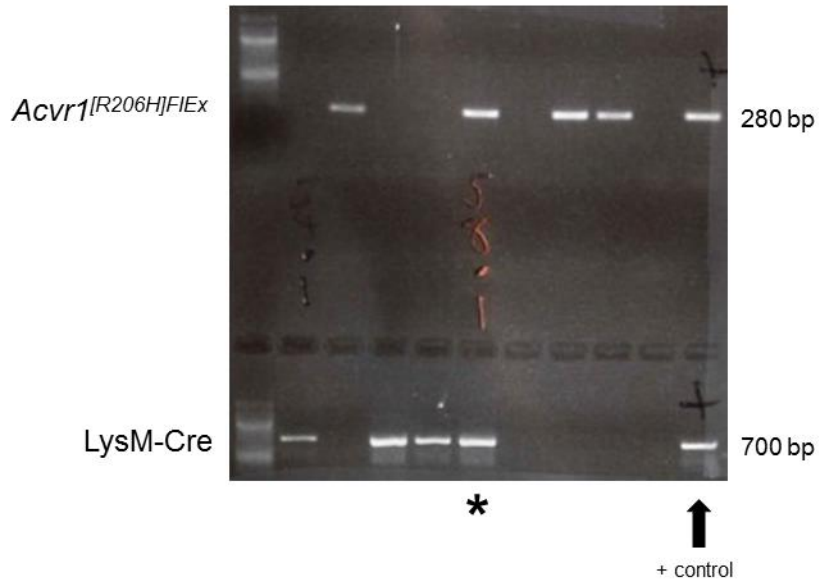
Figure 2.5: Genotyping of *LysM*-Cre;*Acvr1*[R206H]FlEx mice by PCR
DNA isolated from mouse tail snips was amplified with primers specific to *LysM*-Cre and *Acvr1*[R206H]FlEx alleles. Lanes marked with an asterisk indicate *LysM*-Cre;*Acvr1*[R206H]FlEx mice.

Chapter Three

Acvr1^{R206H} amplifies and prolongs the cellular and molecular inflammatory response to injury

3.1 Summary

An early inflammatory response is observed in all HO episodes occuring in FOP, whether induced by tissue trauma or without overt injury. In either scenario, patients often report swellings, tissue stiffness, sensations of warmth, and pain – termed "flare-ups" – that indicate the onset of localized disease. Although some flare-ups resolve without progression to HO, many continue through the characterized catabolic and anabolic events of lesion formation. The presence of immune cells has been noted in biopsies from human patients, however the limited availability of samples has hindered further *in vivo* investigation. Given the role of BMP signaling in hematopoietic development and immune cell activation, I hypothesized that the ACVR1 R206H mutation amplifies the inflammatory response to tissue injury, establishing a supportive microenvironment for the subsequent fibroliferative, chondrogenic, and osteogenic stages that result in terminal HO and mature bone tissue.

I used a recently available conditional-on knock-in *Acvr1^{R206H}* mouse (hereafter referred to as *Acvr1$^{cR206H/+}$*) to investigate the cellular and molecular inflammatory response to injury by examining levels of secreted inflammatory factors. I show that *Acvr1^{cR206H}* causes early divergence from the normal skeletal muscle repair program, however, surprisingly, the earliest events are histologically indistinguishable between *Acvr1$^{cR206H/+}$* and control *Acvr1$^{+/+}$* mice during the first 48-hours post-injury. As lesions progress, the cellular and molecular inflammatory responses are not only amplified in *Acvr1$^{cR206H/+}$* mice, but also persist over time. At the cellular level, *Acvr1$^{cR206H/+}$* expression in primary mast cells potentiated their inflammatory activation. These results suggest that

the ACVR1 R206H mutation produces an inflammatory response to tissue injury that mimics chronic inflammation.

3.2 Introduction

3.2.1 The inflammatory response to tissue injury in FOP

Patient biopsies show tissue destruction and an influx of immune cells at the earliest stage of lesion progression in FOP [Kaplan et al. 1993]. Histological analysis revealed the presence of neutrophils, macrophages, mast cells, and lymphocytes [Kaplan et al. 1993, Gannon et al. 2001, Kaplan et al. 2005]. Mast cell involvement in FOP patient lesions was particularly remarkable, with mast cell density reaching peak levels unseen in any other inflammatory myopathy [Gannon et al. 2001]. The same early inflammatory response (Figure 1.4) has been shown in chimeric $Alk2^{R206H/+}$ mice, the first FOP mouse model expressing the Acvr1 R206H mutation [Chakkalakal et al. 2012]. It is important to note, however, that immune cell invasion is a characteristic feature of the normal tissue injury program, and the early response is qualitatively similar to what occurs in FOP [Chargé et al. 2004, Bentzinger et al. 2013].

Following normal tissue injury by cardiotoxin (CTX), a snake venom commonly used to study skeletal muscle damage and repair [Couteaux et al. 1988], a cascade of early inflammatory events activates a localized immune response. In response to tissue damage, tissue resident macrophages and mast cells detect DAMPs and release pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and Activin A, that recruit neutrophils to the localized site of injury [Galli et al. 2011, Hedger et al. 2011]. Neutrophils are the first migratory responding cell to tissue damage and further potentiate the ongoing inflammatory response [Phillipson et al. 2011, Kolaczkowska et al. 2013] via secretion of their own TNF-α, IL-1β, and IL-8 [Witko-Sarsat et al. 2000]. Monocytes and mast cell

progenitors then migrate to the injury site and differentiate into macrophages and mature mast cells. These mature immune cells clear damaged debris and secrete chemokines necessary for recruitment of fibroproliferative cells that act as scaffold intermediaries while the skeletal muscle regeneration program augments [Murray et al. 2011, Duchesne et al. 2013, Wynn et al. 2013]. Within 1-2 weeks, skeletal muscle is fully restored through a well-characterized regeneration program initiated by muscle stem cell activation [Karalaki et al. 2009, Bentzinger et al. 2013].

The normal skeletal muscle injury and repair program establishes a baseline cellular and molecular response that can be used to compare to injured Acvr1 R206H tissue. Despite ongoing interest in the early inflammatory events during FOP lesion formation, few studies have directly quantified any immune cell populations in lesion tissue [Gannon et al. 2001, Chakkalakal et al. 2016], and none have investigated the molecular inflammatory response. Our conditionl-on knock-in $Acvr1^{cR206H/+}$ mouse model recapitulates features of FOP [Hatsell et al. 2015, Chakkalakal et al. 2016] and is a key tool that can be used to investigate the inflammatory response to injury. These mice reproducibly develop HO two weeks following CTX injection, establishing a reliable and genetically high fidelity model to study the inflammatory response in FOP.

3.2.2 BMP pathway control of inflammatory activation

The BMP signaling pathway has well-defined roles in hematopoesis and inflammatory activation of numerous immune cell populations [Starr et al. 2003, Karlsson et al. 2007, Huse et al. 2011, Yoshioka et al. 2012] (also see Chapter 1.3). In the clinic, use

of recombinant BMPs as treatment for fractures is commonly associated with inflammation, soft tissue edema, and radiculitis [MacDonald et al. 2010, Lee et al. 2012, Villavicencio et al. 2016]. Notably, edema is a common feature of flare-ups as reported by FOP patients, and often precedes the catabolic lesion events [Pignolo et al. 2016]. Soft tissue swelling has also been observed in chimeric $Alk2^{R206H/+}$ mice [Chakkalakal et al. 2012]. Current therapies for FOP, while palliative, predominantly target edema, pain, and other inflammatory symptoms [Kaplan et al. 2016, Pignolo et al. 2016], although the pursuit for therapeutics that pause or even reverse symptoms is ongoing. Previous observations suggest that BMP signaling has distinct cellular and molecular functions within the immune system, and given the R206H mutation present in FOP, the dysregulated BMP signaling may be an appealing therapeutic target.

3.3 Results

3.3.1 The Acvr1 R206H mutation causes early divergence from the normal skeletal muscle repair program

I conducted a cross-sectional injury study to investigate the histological events throughout lesion development in $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ mice, specifically focusing on the cellular and molecular inflammatory response. To induce conditional expression of the $Acvr1^{cR206H}$ allele, I administered doxycycline to $Acvr1^{cR206H/+}$ mice for five days prior to injury; this amount of time is sufficient to induce Cre-recombinase-mediated recombination of the $Acvr1^{cR206H}$ allele (Figure 2.3). Hamstring muscles of $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ mice were injured by CTX injection, and sacrificed at various time points over two-weeks; day 0 samples (without injury) were collected (Figure 3.1).

Uninjured $Acvr1^{cR206H/+}$ skeletal muscle has normal skeletal muscle morphology (Figure 3.2A). The early tissue destruction and immune cell influx events are indistinguishable between $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ lesion through 2 days post-injury (days 1-2; Figure 3.2B, C). As the lesions progress, mutant tissue exhibits robust immune cell invasion (days 3-4; Figure 3.3A, B) and progressive accumulation of glycosaminoglycan (GAG), an extracellular matrix component associated with chondrogenesis [San Antonio et al. 1987] (days 5-6; Figure 3.3C, D). In contrast, the immune cell invasion in control tissue peaks at day four, but then clears from the injury site.

By day 7 post-injury, $Acvr1^{+/+}$ lesions exhibited further immune cell clearance and presence of regenerating muscle (Figure 3.4A), however $Acvr1^{cR206H/+}$ lesions showed a

persistence of fibroproliferative cells together with ectopic pre-hypertrophic chondrocytes (Figure 3.4A). By day 10, immune cells were absent from $Acvr1^{+/+}$ tissue, with skeletal muscle repair reaching near completion at day 14 (Figure 3.4B, C). Myocentric nuclei, which denote ongoing skeletal muscle repair, were observed in late-stage $Acvr1^{+/+}$ tissue (Figure 3.5). By contrast, heterotopic endochondral ossification had significantly progressed from day 6 to day 7 (Figure 3.4A) in $Acvr1^{cR206H/+}$ tissue, with abundant hypertrophic chondrocytes and bone matrix present at day 10 (Figure 3.4C) and more mature heterotopic bone at day 14 (Figure 3.4C). These results suggest that the ACVR1 R206H mutation disrupts the normal skeletal muscle repair program, driving heterotopic endochondral ossification in place of normal skeletal muscle regeneration.

3.3.2 BMP signaling is upregulated in $Acvr1^{cR206H/+}$ post-traumatic lesions

Upregulated BMP signaling has been observed in late-stage lesions from our previous chimeric $Alk2^{R206H/+}$ mouse model, as detected by enhanced levels of nuclear phosphorylated-Smad 1/5/8 (p-Smad 1/5/8) protein in tissue [Chakkalakal et al. 2012]. I investigated the levels of p-Smad 1/5/8 by immunohistochemistry (IHC) throughout lesion progression in our $Acvr1^{cR206H/+}$ mouse model. Levels of p-Smad 1/5/8 were higher in $Acvr1^{cR206H/+}$ mice compared with controls at all stages examined - the early tissue destruction stage, intermediate fibroproliferative stage, and late ectopic cartilage and bone stage (Figure 3.6). p-Smad 1/5/8 was detected within the cell nuclei, consistent with the expected localization of p-Smad 1/5/8 protein following BMP pathway activation (Figure 1.1). Elevated p-Smad1/5/8 protein was most prominent in the nuclei of fibroproliferative

cells and pre-hypertrophic chondrocytes, consistent with accelerated chondrogenesis resulting from the ACVR1 R206H mutation [Culbert et al. 2014].

Because the *Acvr1cR206H/+* mouse model globally expresses the R206H mutant allele, I examined BMP signaling levels at other tissue sites. I investigated the levels of p-Smad 1/5/8 protein at the endogenous knee joint growth plate and femoral bone marrow niche and detected higher p-Smad1/5/8 expression at both sites in *Acvr1cR206H/+* mice (Figure 3.7AB), confirming that activation of the conditional *Acvr1cR206H* allele occurs widely and is active in cell types known to be responsive to BMP signaling. Enhanced BMP pathway signaling in the bone marrow is of particular interest since bone marrow is the primary adult hematopoietic tissue and source of hematopoietic stem cells from which mature immune cell populations are derived [Zhao et al. 2012] (Figure 1.2).

3.3.3 The cellular and molecular pro-inflammatory response is amplified and prolonged in *Acvr1cR206H/+* mice following tissue injury

Previous studies using patient biopsies and FOP mouse models observed that multiple immune cell types are present in developing HO lesions, with most attention given to cells of the innate immune system such as neutrophils, macrophages, and mast cells [Gannon et al. 1998, Gannon et al. 2001, Kaplan et al. 2005, Chakkalakal et al. 2012, Chakkalakal et al. 2016]. However, these studies were limited to the earliest stages of lesion progression and most did not quantify immune cells nor investigate cytokine and chemokine contributions. To examine the immunological contributions to HO development in FOP in detail, I investigated the cellular and molecular inflammatory

70

response to skeletal muscle injury through lesion progression to HO formation. I first quantified cell density of four immune cells known to reside within lesion tissue – neutrophils, macrophages, mast cells, and T cells [Chakkalakal et al. 2012].

Neutrophils are recruited within hours after tissue injury in response to damage-associated molecular pattern (DAMP) pathway activation [Phillipson et al. 2011] and then release proteases that degrade damaged tissue in preparation for clearance by macrophages [Tidball 2005]. Neutrophil numbers were significantly elevated in early $Acvr1^{cR206H/+}$ lesions compared to $Acvr1^{+/+}$ (Figure 3.8A), reaching peak density at day 2 and persisting at higher levels at day 4.

Macrophages rapidly respond to neutrophils, phagocytosing damaged tissue and debris generated by neutrophils and secreting cytokines and chemokines that recruit progenitor cells to facilitate wound healing [Laskin et al. 2011]. Macrophages were significantly increased in early- and intermediate-stage (days 2-6) $Acvr1^{cR206H/+}$ lesions compared to $Acvr1^{+/+}$ (Figure 3.8B). While numbers of macrophages in control tissue returned to pre-injury levels by day 6, these cells persisted in $Acvr1^{cR206H/+}$ lesions.

Mast cells contribute to diverse biological processes, including wound repair, fibrosis, pain pathophysiology, and allergy [Bischoff 2007, Thevenot et al. 2011, Heron et al. 2013, Douaiher et al. 2014], and have been previously reported in biopsy samples from FOP patients [Gannon et al. 2001] and in FOP mouse models [Chakkalakal et al. 2012, Chakkalakal et al. 2016]. Consistent with previous studies, I determined that mast cell density was significantly greater in $Acvr1^{cR206H/+}$ lesions, and that elevated levels of mast

71

cells became evident by day 4 following injury and persisted at high levels through day 14 and heterotopic bone formation (Figure 3.8C).

In addition to cells of the innate immune system, T cells have also been reported in FOP lesions [Gannon et al. 1998, Chakkalakal et al. 2012], and a BMP4 over-expression mouse model that lacked mature lymphocytes formed reduced HO [Kan et al. 2009]. At day 3 post-injury, T cell density was more than 2-fold higher in $Acvr1^{cR206H/+}$ lesions compared to $Acvr1^{+/+}$, similarly to myeloid lineage cells, and higher numbers of T cells persisted through the progression to heterotopic bone (Figure 3.8D).

A maximal inflammatory response depends on the synthesis and secretion of pro-inflammatory cytokines and chemokines that amplify and sustain an ongoing immune response and act as chemoattractant molecules for progenitor cells that participate in tissue repair [Ishida et al. 2007, Fong et al. 2011, Halova et al. 2012, Turner et al. 2014]. To determine whether the elevated and prolonged immune cell response in $Acvr1^{cR206H/+}$ lesions (Figure 3.8) is accompanied by general or specific differences in pro-inflammatory cytokines and chemokines, I examined expression in early-, intermediate-, and late-stage $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ lesions. The pro-inflammatory cytokines TNFα, IL-1β, and IL-6 were detected at higher levels in $Acvr1^{cR206H/+}$ lesions compared to $Acvr1^{+/+}$ throughout lesion progression (Figure 3.9 and 3.10). TNFα and IL-6 were robustly present and detected as early as day 2 post-injury in $Acvr1^{cR206H/+}$ lesions (Figure 3.9AB), while IL-1β more slightly increased (Figure 3.10). These cytokines were detected throughout the injured tissues.

Monocyte chemoattractant protein-1 (MCP-1) is a chemokine abundantly produced by white adipose tissue [Fasshauer et al. 2015] that functions to recruit monocytes and other immune cells [Deshmane et al. 2009], and was previously proposed as a predictive biomarker for blast injury patients who developed HO [Evans et al. 2012]. In both $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ lesions, MCP-1 was mainly localized to white adipose adjacent to skeletal muscle, with generally low detection in the lesions throughout their progression (Figure 3.11A).

IL-13 is a pro-inflammatory cytokine that is expressed in many fibrosis pathologies [Wynn et al. 2012, Borthwick et al. 2013] and, like MCP-1, was suggested as a predictive biomarker for blast injury patients who developed HO [Forsberg et al. 2014]. IL-13 expression was relatively low and equivalent in $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ lesions (Figure 3.11B).

Activin A is a TGF-β superfamily ligand involved in inflammatory stimulation and regulation of several immune cell populations [Aleman-Muench et al. 2012]. Recently, Activin A was reported to aberrantly activate BMP pathway signaling through $Acvr1^{R206H}$ and contribute to HO progression in FOP [Hatsell et al. 2015]. I detected higher Activin A expression in intermediate- and late-stage $Acvr1^{cR206H/+}$ lesions relative to controls (Figure 3.12A), with Activin A expression in fibroproliferative regions as well as in ectopic chondrocytes (Figure 3.12B).

These results suggest that although the inflammatory response is substantially upregulated in $Acvr1^{cR206H/+}$ mice, the response is not a total upregulation of all inflammatory mediators.

73

3.3.4 Increased BMP signaling conferred by $Acvr1^{cR206H/+}$ in mast cells enhances inflammatory activation

The ACVR1 R206H mutation confers increased BMP pathway signaling in numerous cell types, including fibroblastic cells such as patient-derived SHED cells and mouse-derived MEFs [Culbert et al. 2014, Wang et al. 2016]. Expression of $Acvr1^{R206H}$ potentiates cell-autonomous differentiation programs of these tri-potent mesenchymal cells, accelerating their differentiation toward chondrogenic and osteogenic fates. However, despite the cell-autonomous effects of the ACVR1 R206H mutation in these mesenchymal cells, the mutation alone does not appear to be sufficient to promote spontaneous cell differentiation [Culbert et al. 2014], as is supported by the episodic nature of HO formation in FOP patients. Rather, an additional stimulus appears to trigger onset of bone formation. I considered that immune cells could play such a role and investigated whether increased BMP signaling conferred by $Acvr1^{cR206H}$ in immune cells could upregulate their inflammatory activation.

To examine the effect of enhanced BMP signaling conferred by the ACVR1 R206H mutation on the inflammatory potential of immune cells, I examined primary murine mast cells and macrophages *in vitro*. I collected primary mast cells by harvesting whole bone marrow from $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ mice and culturing cells in suspension for 6 weeks in mast cell maturation medium. Mast cell purity was verified by flow cytometry, gating for c-kit receptor/CD117 and Fc$_\varepsilon$RI$_\alpha$ receptor double-positive cells [Weller et al. 2005] (Figure 3.13). Macrophages were collected by intraperitoneal injection of 1.5 mL aged 4.0% thioglycolate solution into $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ mice [Zhang et al. 2008]. After

5 days, peritoneal exudate was collected from euthanized mice and plated in macrophage growth medium.

I examined BMP pathway signaling competency in mast cells and macrophages, detecting expression of a broad panel of BMP/TGF-β type I and II receptor mRNAs, including *Acvr1*, the type I receptor mutated in FOP [Shore et al. 2006], in both cell types (Figure 3.14A). BMP pathway activity, quantified by p-Smad1/5/8 protein levels, was increased in both mast cells and macrophages expressing the *Acvr1^{cR206H}* allele compared with *Acvr1$^{+/+}$* (Figure 3.14B, C, D) with relative levels of p-Smad1/5/8 between *Acvr1$^{cR206H/+}$* and *Acvr1$^{+/+}$* similar to that reported previously [Culbert et al. 2014].

BMP pathway activation has been previously reported to enhance the inflammatory state of multiple immune cell populations [Hong et al. 2009, Lee et al. 2011, Martinez et al. 2011, Martinez et al. 2015]. To investigate whether expression of the *Acvr1^{cR206H}* allele in mast cells and macrophages confers enhanced inflammatory signaling, I examined pro-inflammatory cytokine expression. *Acvr1$^{cR206H/+}$* mast cells exhibited significantly elevated mRNA expression of TNFα and IL-6 and a trend toward elevated IL-1β compared with *Acvr1$^{+/+}$* mast cells (Figure 3.15A). No significant differences in cytokine expression between *Acvr1$^{cR206H/+}$* and *Acvr1$^{+/+}$* macrophages were detected (Figure 3.15B).

Degranulation is a directed response to inflammatory stimulation in granulocytes, including mast cells [Kulka et al. 2008], releasing numerous inflammatory mediators, including TNFα and other cytokines [Moon et al. 2014]. Quantification of degranulation showed elevated activity in *Acvr1$^{cR206H/+}$* mast cells compared to controls (Figure 3.15C).

75

These data reveal an elevated and prolonged pro-inflammatory response in *Acvr1*$^{cR206H/+}$ tissues in response to injury, as well as in *Acvr1*$^{cR206H/+}$ mast cells, with higher expression of multiple, but not all, pro-inflammatory cytokines and chemokines investigated.

3.4 Discussion

3.4.1 *Acvr1^{R206H}* disrupts the normal skeletal muscle regeneration program, favoring heterotopic endochondral ossification

Skeletal muscle regeneration is a well-characterized process that faithfully restores tissue to its pre-injury state. Shortly after tissue injury and immune cell influx, a skeletal muscle-resident stem cell population known as muscle stem cells (or satellite cells) is activated from its quiescent state and begins the muscle regeneration process [Wang et al. 2012]. Muscle stem cells are identified through their expression of Pax-7, and are indispensible for adult skeletal muscle regeneration [Sambasivan et al. 2011]. Following muscle stem cell activation, clonal expansion and migration to the injury site occurs, after which a self-renewing pool of quiescent muscle stem cells is restored, with the remaining daughter cells cells continuing the regeneration process assisted by myogenic regulatory factors (MRFs) [Schultz et al. 1985]. The pool of proliferated muscle stem cells is now referred to as myogenic precursor cells (MPCs) that are characterized by upregulation of two MRFs, Myf5 and MyoD [Cooper et al. 1999]. Expansion of MPCs is followed by their upregulation of MRF4 and myogenin, the later of which is associated with terminal differentiation of myoblasts into myocytes and fusion of these cells to new or existing fibers [Karalaki et al. 2009]. The final result is regenerated skeletal muscle that is indistinguishable from its pre-injury state [Bentzinger et al. 2013].

As observed in Figures 3.3, and 3.4, the normal skeletal muscle regenerative program in the *Acvr1$^{cR206H/+}$* group is disrupted, instead replaced by the metamorphosis of damaged tissue with fibroproliferative cells and eventual ectopic cartilage and bone. In

contrast, the repair mechanism is fully functional in the $Acvr1^{+/+}$ group, with the cellular events matching the previously established regeneration timeline (Figures 3.2, 3.3, 3.4, 3.5) [Bentzinger et al. 2013]. It is known that elevated BMP signaling prevents myogenic differentiation of satellite cells [Ono et al. 2011], suggesting a negative feedback mechanism that limits skeletal muscle regenerative capacity may exist in FOP. Additionally, MEF cells expressing $Acvr1^{R206H}$ were shown to be primed toward chondrogenic differentiation in vitro, [Culbert et al. 2014], which may suggest a similar mechanism in vivo that may compensate for any regenerative deficiency existing in muscle stem cells or their subsequent myogenic populations. Strikingly, despite the progressive divergence in response to injury between $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ cohorts, the tissue morphology is equivalent up to 48 hours post-injury (Figure 3.2). This suggests that the early repair stages of damaged tissue turnover, mediated in part by active caspase-3 [Chakkalakal et al. 2012], may be unaffected by the ACVR1 R206H mutation. However, an alternative possibility is that inflammatory gene expression changes as a consequence of the R206H shortly after tissue injury, but histological changes in tissue composition are not observable until 2-3 days post-injury. This can be addressed using the same cross-sectional injury protocol as indicated in Figure 3.1. Injured skeletal muscle could be collected and digested, and immune cells could be quantified and collected using fluorescence-activated cell sorting (FACS). Collected immune cell populations could then be investigated for inflammatory gene expression by qRT-PCR or microarray, as well as inflammatory factor secretory potential by ELISA.

3.4.2 The inflammatory response following skeletal muscle injury in an *Acvr1^{R206H}* background mimics chronic inflammation

An important question addressed in our experiments is how does *Acvr1^{R206H}* affect the cellular and molecular inflammatory response to tissue injury. An inflammatory phase is part of the normal tissue injury response, with influx of immune cells and upregulation of pro-inflammatory cytokines and chemokines observed [Tidball 2005, Philippou et al. 2012]. Neutrophils, macrophages, and mast cells all release pro-inflammatory cytokines that promote clearance of damaged muscle and upregulate growth factors that promote regenerative events. Neutrophils release nitric oxide (NO), which aids in digesting injured tissue for subsequent phagocytosis by macrophages [Nguyen et al. 2003]. Neutrophils, macrophages, mast cells, and damaged muscle fibers all release TNFα, IL-1β, and IL-6 [Smith et al. 2008], pro-inflammatory factors that are involved in the destruction of skeletal muscle following injury. Many of these factors are linked in inflammatory networks that cyclically upregulate each other and potentiate the pro-inflammatory response [Panzer et al. 1993, Gallucci et al. 1998]. However, eventually the pro-inflammatory response is dampened and is replaced by anti-inflammatory cytokines, including IL-4, IL-10, and TGF-β, factors that promote tissue regeneration [Philippou et al. 2012].

The key cellular event that initiates the anti-inflammatory response during tissue injury is the transition from classically-activated M1 macrophages, which are involved in pathogen and damaged tissue phagocytosis, to anti-inflammatory M2 macrophages, which promote tissue healing [Laskin et al. 2011, Murray et al. 2011]. M2 macrophages secrete known anti-inflammatory factors, including IL-4, IL-10, IL-13, and TGF-β, as well as

respond to these same factors [Gordon 2003, Arnold et al. 2007, Barron et al. 2011]. In concert with IL-4 and IL-10 activity that promotes myoblast fusion and muscle regeneration [Horsley et al. 2003, Arnold et al. 2007], M2 macrophages also secrete a consortium of growth factors, including platelet-derived growth factor (PDGF) [Murray et al. 2011], that facilitate tissue repair. Failing to transition to an M2 macrophage state impairs wound healing via a TNFα mechanism [Sindrilaru et al. 2011]. Overall, proper skeletal muscle regeneration depends on appropriate balance and timing of the M1 and M2 macrophage response [Arnold et al. 2007].

The density of neutrophils, macrophages, mast cells, and T cells was significantly increased in the $Acvr1^{cR206H/+}$ cohort following injury, compared to $Acvr1^{+/+}$, and macrophage and mast cell density remained elevated as the lesion progressed (Figure 3.8). These data were the first to quantify the density of multiple immune cells throughout lesion progression, an important finding that supports the hypothesis of an elevated immune response in FOP. However, there are some limitations with this experiment, as well as additional approaches that could further improve the dataset. Quantification of immune cells was performed via IHC of paraffin-embedded histological sections and manual cell counting. This approach is not easily conducive to multiple cell labeling, which is important for identifying immune cell subtypes, such as CD4 vs. CD8 T cells. Using cryosections to avoid paraffin auto-fluorescence image artifacts, allows for multiple cell marker labeling by different fluoro-tagged antibodies. For even greater immune cell subtype identification and quantification, injured skeletal muscle can be collected and digested, after which immune cells can be quantified using multi-laser flow cytometry and

multiple cell labeling that is required to indentify certain immune cell populations (such as double-positive c-Kit and $Fc_\varepsilon RI_\alpha$ for mast cells [Weller et al. 2005]).

Although neutrophil density was robustly increased in early $Acvr1^{cR206H/+}$ lesions compared to $Acvr1^{+/+}$ lesions, the first time point investigated for injured mice was two days post-injury. Neutrophils are known to respond rapidly to injury, with first populations appearing as early as one hour post-injury [Tidball 2005]. Additional experiments examining neutrophil density at earlier time points following injury, such as 3, 6, 12, and 24 hours post-injury, will provide further clarification of the neutrophil response in early FOP lesions.

Mast cell density was equivalent between cohorts up to 3 days post-injury, after which the numbers of these cells were significantly elevated in $Acvr1^{cR206H/+}$ lesions (Figure 3.8). This trend matches mast cell quantification of early- and intermediate-stage human biopsy samples [Gannon et al. 2001]; however, mast cell density was observed to decrease in late-stage human samples, while we found mast cells remained elevated in mouse samples through day 4-14 time points (Figure 3.8C). TNFα is a known mast cell chemoattractant factor [Halova et al. 2012], suggesting that elevated TNFα present in $Acvr1^{cR206H/+}$ lesions may enhance mast cell recruitment to the site of injury.

Given the role of BMP signaling in hematopoietic development and immune cell activation, I hypothesized that the ACVR1 R206H mutation amplifies the inflammatory response to tissue injury, establishing an appropriate microenvironment for the subsequent fibroliferative, chondrogenic, and osteogenic stages that result in terminal HO. The robust increase in TNFα, IL-1β, and IL-6 levels *in vivo* and *in vitro* in an $Acvr1^{cR206H/+}$ background

suggests a setting that mimics chronic inflammation, maintaining a microenvironment conducive to continued tissue destruction at the expense of skeletal muscle regeneration. These cytokines are involved in the normal tissue injury response, which is replicated in our *Acvr1$^{+/+}$* cohort (Figures 3.10 and 3.11); however, they are amplified in the *Acvr1$^{cR206H/+}$* cohort and persist for much longer in the injury lesion. IL-1β inhibits satellite cell differentiation, IL-6 may precede myofiber rupture, and TNFα induced muscle wasting and prevented muscle regeneration in a chronic pulmonary inflammation mouse model [Allen et al. 1989, Tomiya et al. 2004, Langen et al. 2006]. These same cytokines also enhance proliferation of recruited fibroblasts [Mast et al. 1996] and macrophage secretion of matrix metalloproteinase (MMP) enzymes [Murray et al. 2011] that contribute to HO formation [Rodenberg et al. 2011, Davis et al. 2016]. TNFα has been shown to promote osteogenic differentiation of human mesenchymal stem cells via the NF-κB [Hess et al. 2009]. Furthermore, fracture healing was significantly impaired in separate TNF receptor and IL-6 knock-out mice, demonstrating major roles for these cytokines in the bone formation process [Gerstenfeld et al. 2003, Yang et al. 2007]. Our data and supporting references suggest that prolonged expression of pro-inflammatory cytokines may actively suppress muscle repair and regeneration.

I also identified an elevated pro-inflammatory response in *Acvr1$^{cR206H/+}$* mast cells (Figure 3.15), as mRNA expression of TNFα and IL-6 are significantly upregulated, with a trend toward increased IL-1β expression. Although mast cell density is significantly increased in *Acvr1$^{cR206H/+}$* lesions (Figure 3.8C), the massive increase in pro-inflammatory

82

cytokine levels observed *in vivo* is likely caused by multiple cell types secreting these factors.

I examined Activin A expression *in vivo* and demonstrated increased levels in intermediate- and late-stage $Acvr1^{cR206H/+}$ lesions (Figure 3.12A). Presence of Activin A in $Acvr1^{cR206H/+}$ lesions, particularly within fibroproliferative cells and chondrocytes (Figure 3.12B) is notable given the recent finding that Activin A can induce HO formation through a gain-of-function sensitivity conferred by the ACVR1 R206H mutation [Hatsell et al. 2015, Hino et al. 2015]. Activin A skews macrophage polarization toward pro-inflammatory M1 populations [Sierra-Filardi et al. 2011] and recruits mast cell progenitors to sites of inflammation [Funaba 2003], suggesting it may have broader pro-inflammatory functions in FOP beyond the specific $Acvr1^{R206H}$ receptor sensitivity.

Although the pro-inflammatory response induced by $Acvr1^{cR206H}$ is comprehensive and robust, not all inflammatory factors are upregulated. MCP-1 and IL-13 expression was relatively mild and equivalent in $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ lesions (Figure 3.11). IL-13 is expressed in many fibrosis pathologies [Kaviratne et al. 2004, Wynn 2008, Wynn et al. 2012], suggesting that although there is a substantial fibroproliferative response in FOP, the disease should not be classified as fibrosis.

Overall, a plentiful body of literature suggests that a prolonged pro-inflammatory response following tissue injury suppresses proper regeneration. In context with our results showing an amplified and prolonged inflammatory response in $Acvr1^{cR206H/+}$ mice post-injury, this strongly suggests that the ACVR1 R206H mutation induces a chronic

inflammatory state following injury that establishes a permissive microenvironment for the

subsequent heterotopic endochondral ossification events.
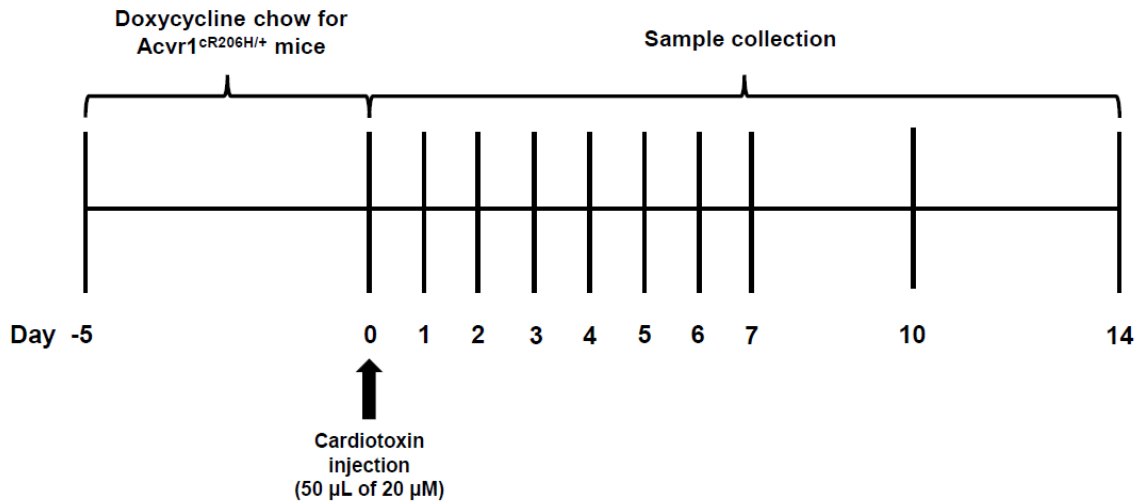
Figure 3.1: Cross-sectional injury study experimental design
Acvr1^{cR206H/+} mice were placed on doxycycline chow (625 mg / kg) for five days prior to injury to induce expression of the conditional *Acvr1^{cR206H}* allele. Hamstring muscles of *Acvr1^{cR206H/+}* and *Acvr1^{+/+}* mice were injected with cardiotoxin (50 μL of 20 μM concentration) and samples were collected at day 0 (uninjected controls); and days 1, 2, 3, 4, 5, 6, 7, 10 and 14 post-injury. n = 4 for each genotype, per day.

Figure panel from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^{R206H}* Mouse Model of Fibrodysplasia Ossificans Progressiva"
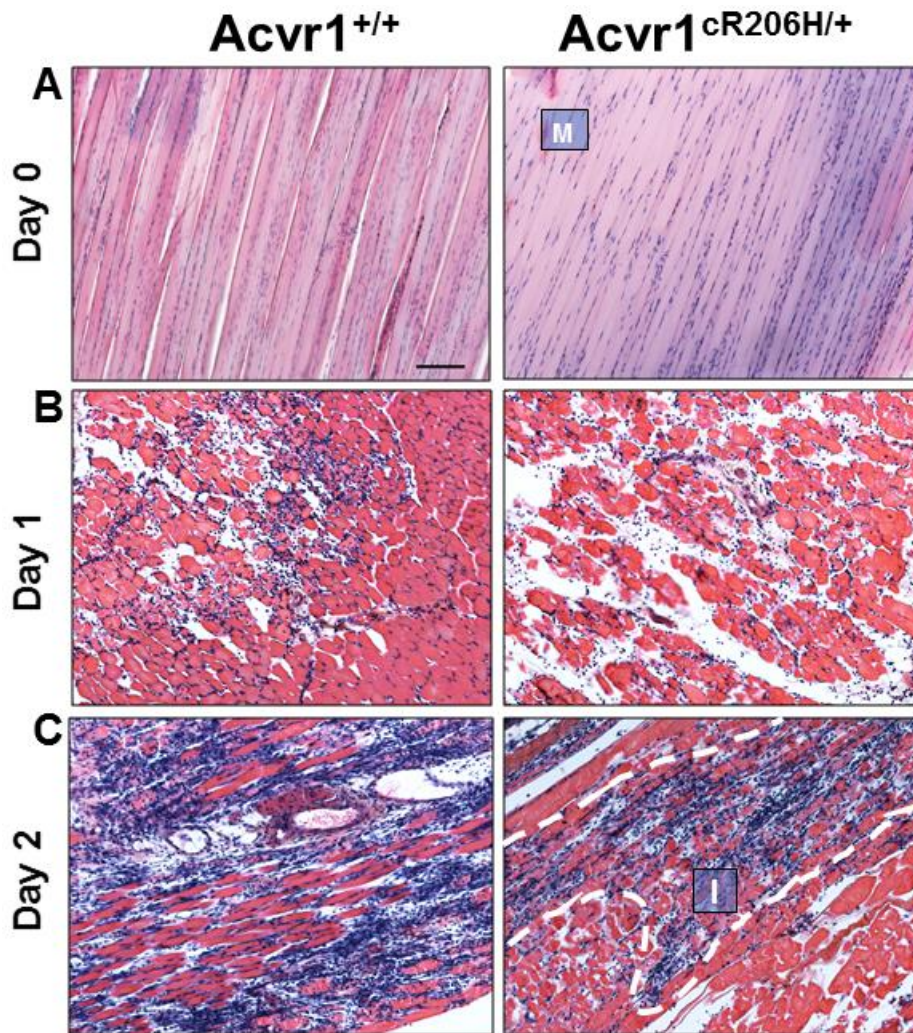
Figure 3.2: The early response to injury in *Acvr1^cR206H/+* and *Acvr1^+/+* mice is indistinguishable
Skeletal muscle tissue sections from *Acvr1^cR206H/+* and *Acvr1^+/+* mice from indicated days following cardiotoxin injury were detected for cartilage (Alcian Blue), bone (Orange G), cell nuclei/immune cells (Haematoxylin), and skeletal muscle (Eosin). (A) Skeletal muscle histology is similar in mutant and control mice before injury (day 0). (B, C) Skeletal muscle degradation and increasingly abundant immune cells are present by days 1-2 in both mutant and control mice. Scale bar = 100 μm for all images. Dotted lines indicate tissue areas as noted. n = 4 for each genotype, per day. Representative images are shown. Abbreviations: M = skeletal muscle, I = immune infiltration

Figure panel modified from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^R206H* Mouse Model of Fibrodysplasia Ossificans Progressiva"
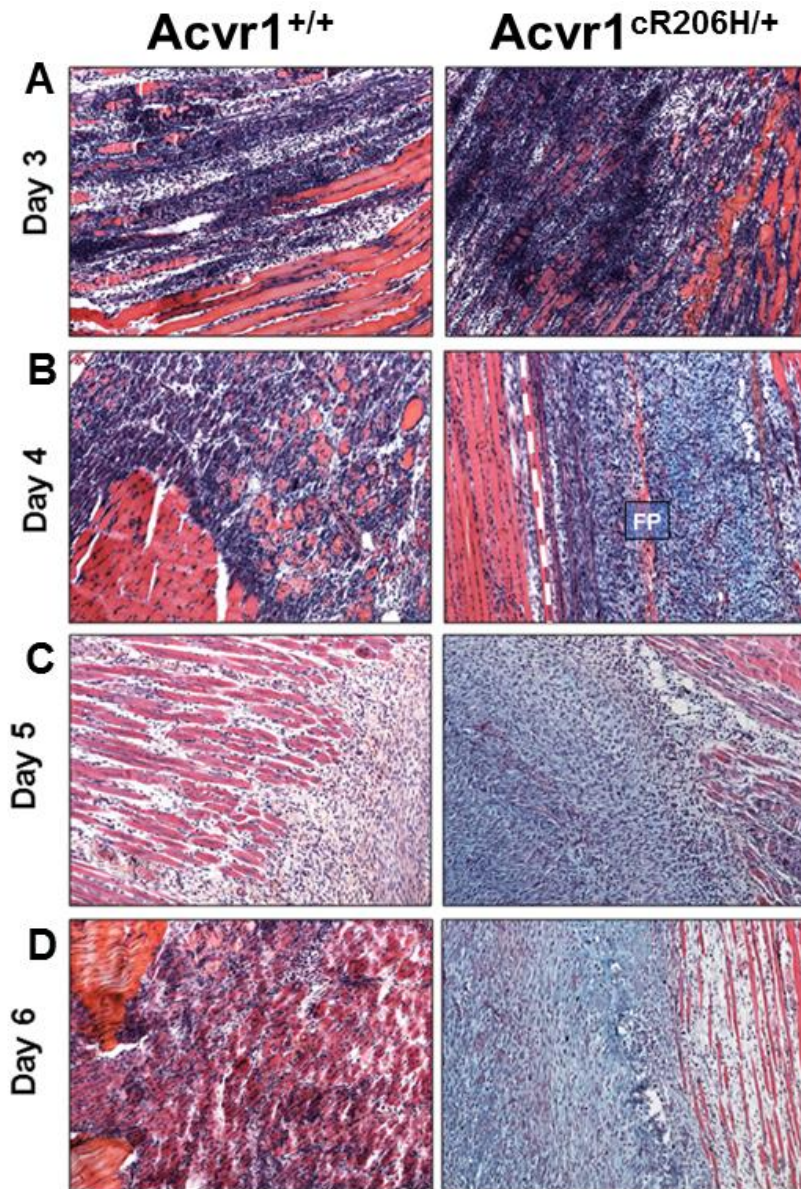
Figure 3.3: Extensive fibroproliferation and glycosaminoglycan accumulation in $Acvr1^{cR206H/+}$ lesions

Skeletal muscle tissue sections from $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ mice from indicated days following cardiotoxin injury were detected for cartilage (Alcian Blue), bone (Orange G), cell nuclei/immune cells (Haematoxylin), and skeletal muscle (Eosin). (A) At day 3, immune cells increased further, but to a greater extent in $Acvr1^{cR206H/+}$ tissue. (B, C, D) In control tissue, muscle repair progresses through the fibroproliferative stage and clearance of immune cells (days 4-6), however GAG proteins are detected (Alcian Blue) in $Acvr1^{cR206H/+}$ tissue. Dotted lines indicate tissue areas as noted. n = 4 for each genotype, per day. Representative images are shown. Abbreviations: FP = fibroproliferation

Figure panel modified from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an $Acvr1^{R206H}$ Mouse Model of Fibrodysplasia Ossificans Progressiva" *JBMR*.
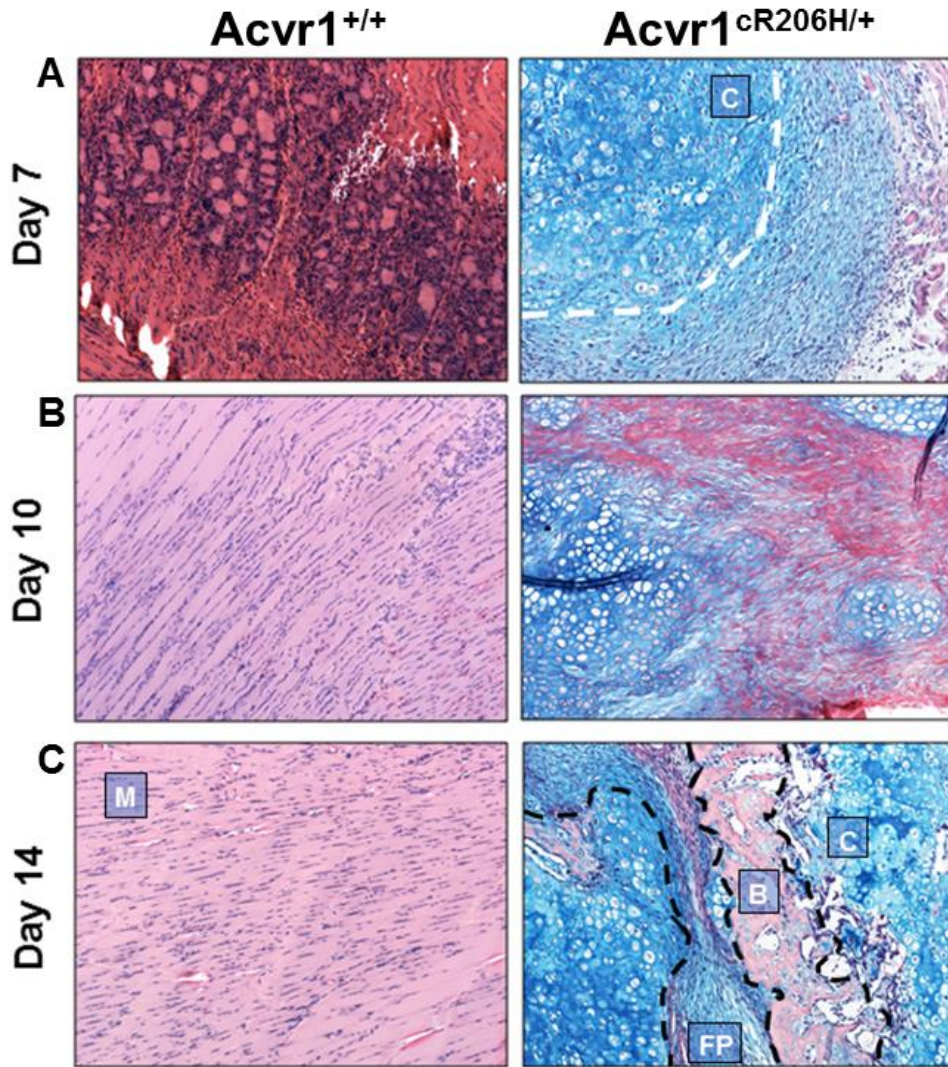
Figure 3.4: Heterotopic endochondral ossification in *Acvr1^{cR206H/+}* lesions

Skeletal muscle tissue sections from *Acvr1^{cR206H/+}* and *Acvr1^{+/+}* mice from indicated days following cardiotoxin injury were detected for cartilage (Alcian Blue), bone (Orange G), cell nuclei/immune cells (Haematoxylin), and skeletal muscle (Eosin). (A, B, C) In control tissue, skeletal muscle repair continues and is completed over days 7-14. (A, B) In *Acvr1^{cR206H/+}* lesions, endochondral ossification proceeds with maturation to hypertrophic chondrocytes and mineralized bone. (C) Ectopic bone with adjacent regions of mature cartilage and remaining fibroproliferation at day 14 is shown. n = 4 for each genotype, per day. Representative images are shown. Dotted lines indicate tissue areas as noted. Abbreviations: M = skeletal muscle, FP = fibroproliferation, C = chondrocytes, B = bone

Figure panel modified from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^{R206H}* Mouse Model of Fibrodysplasia Ossificans Progressiva"
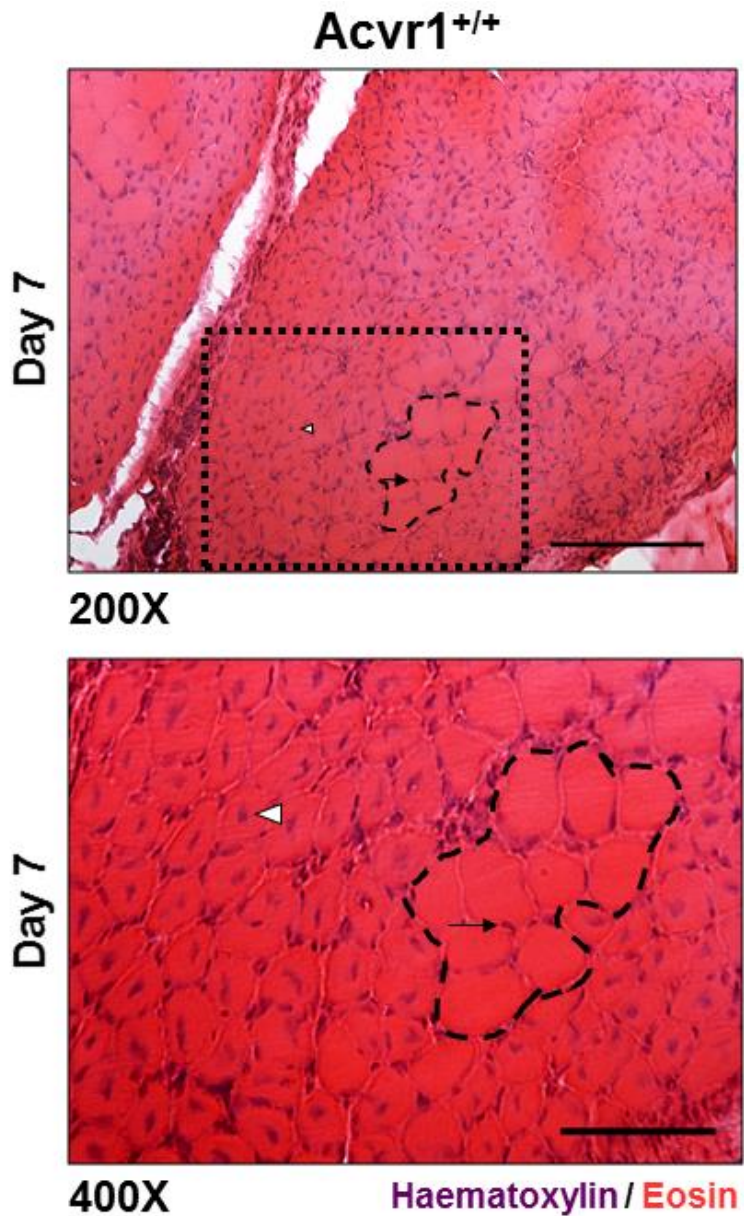
Figure 3.5: Presence of myocentric nuclei indicates ongoing muscle regeneration in *Acvr1*^{+/+} lesions

Histological representative *Acvr1*^{+/+} skeletal muscle section at 200X magnification is shown, with dotted rectangle indicating further magnification at 400X. Myocentric nuclei, indicating ongoing muscle regeneration, is shown (white arrowhead). Note presence of uninjured tissue (freehand dotted region) and normal peripheral myonucleus location (black arrow). Scale bar = 100 μm for 200X, 50 μm for 400X
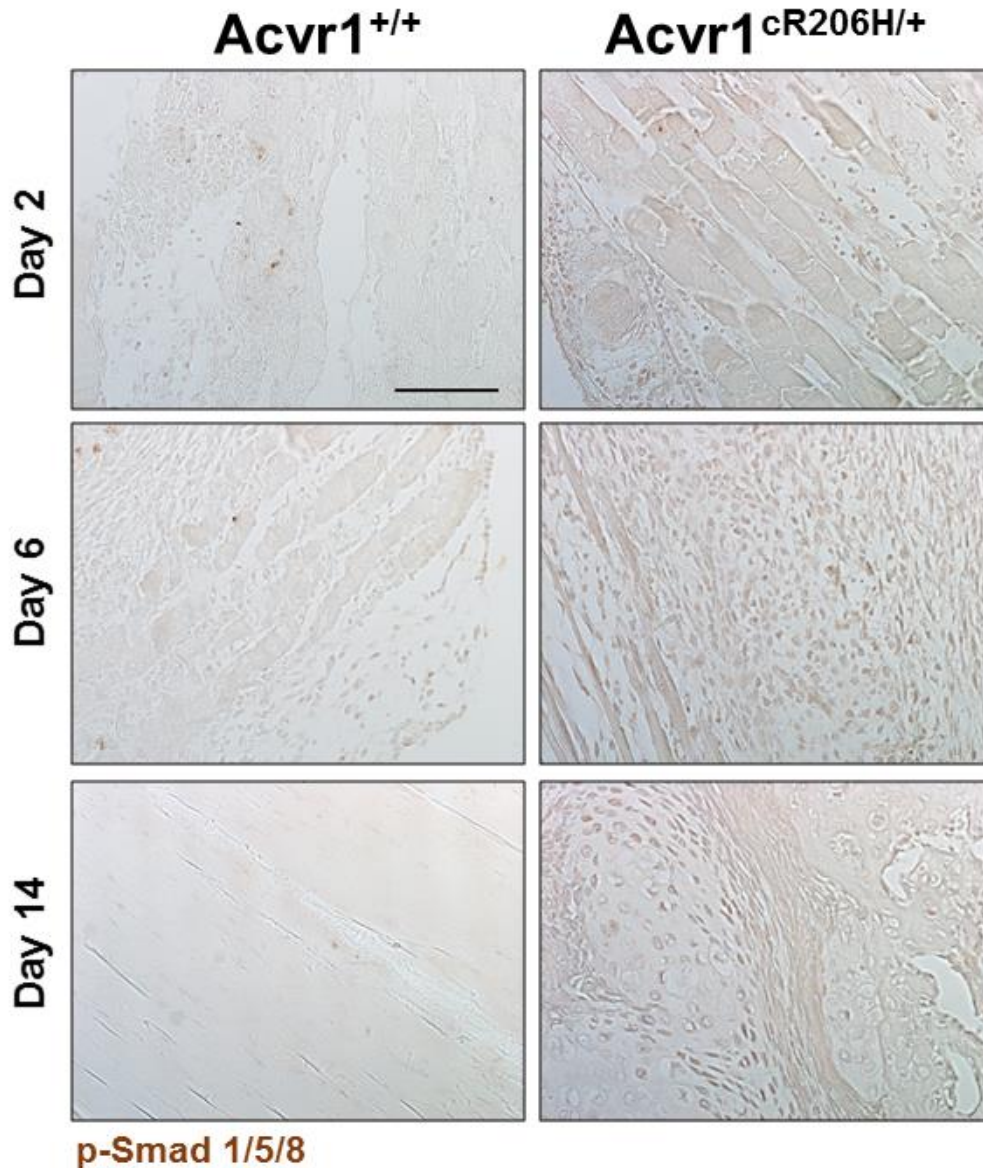
Figure 3.6: BMP signaling is upregulated in *Acvr1^{cR206H/+}* post-traumatic lesions

Sections from early- (day 2), intermediate- (day 6), and late-stage (day 14) *Acvr1^{cR206H/+}* and *Acvr1^{+/+}* lesions were immunostained for expression of p-Smad 1/5/8 protein. No haematoxylin counterstain was performed in order to enhance contrast of nuclear p-Smad 1/5/8 stain. n = 3 for each genotype, per day. Representative images are shown Scale bar = 100 μm for all images

Figure panel from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^{R206H}* Mouse Model of Fibrodysplasia Ossificans Progressiva"
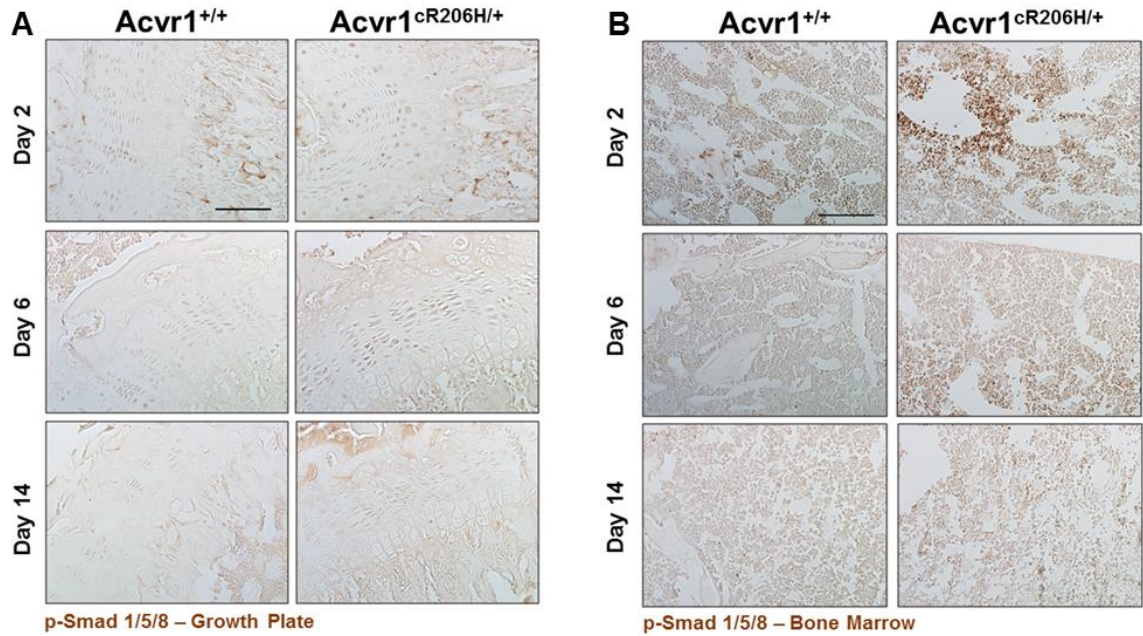
Figure 3.7: BMP signaling is upregulated in the *Acvr1^cR206H/+* **endogenous knee joint growth plate and femoral bone marrow niche**

Sections from early- (day 2), intermediate- (day 6), and late-stage (day 14) *Acvr1^cR206H/+* and *Acvr1^+/+* (A) endogenous knee joint growth plates and (B) femoral bone marrow niches are shown. No haematoxylin counterstain was performed in order to enhance contrast of nuclear p-Smad 1/5/8 stain. n = 3 for each genotype, per day. Representative images are shown. Scale bar = 100 μm for all images

Figure panel from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^R206H* Mouse Model of Fibrodysplasia Ossificans Progressiva"
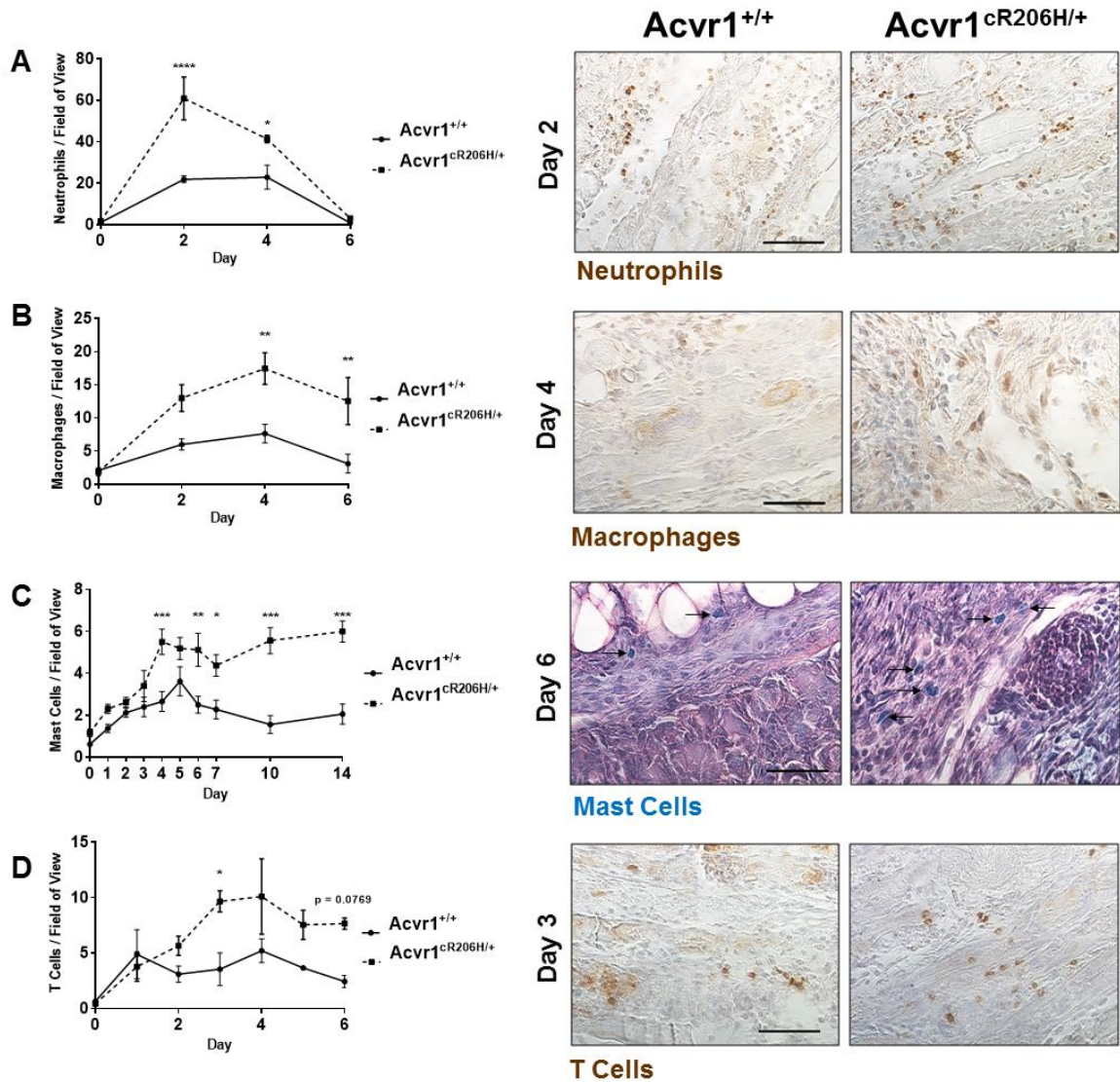
Figure 3.8: Immune cell density is elevated and prolonged in *Acvr1^cR206H/+* lesions.
Specific immune cell populations were detected and quantified in injured *Acvr1^cR206H/+* and *Acvr1^+/+* skeletal muscle over time; early- (day 2), intermediate- (day 6), and late- (day 14) stages after injury are shown. (A) Neutrophils were detected by myeloperoxidase IHC. (B) Macrophages were detected with F4/80 IHC. (C) Mast cells were detected by C.E.M. stain kit, and indicated by black arrows. (D) T cells were detected with CD3 IHC. Cells were quantified from three fields of view per independent sample; n = 3 for neutrophils, macrophages, T cells; n = 4 for mast cells. Representative images are shown. Scale bar = 50 μm. Data shown are mean values ± SEM; two-way ANOVA with Sidak's multiple comparisons test comparing *Acvr1^cR206H/+* versus *Acvr1^+/+* per day was performed; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001

Figure panel from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^R206H* Mouse Model of Fibrodysplasia Ossificans Progressiva"
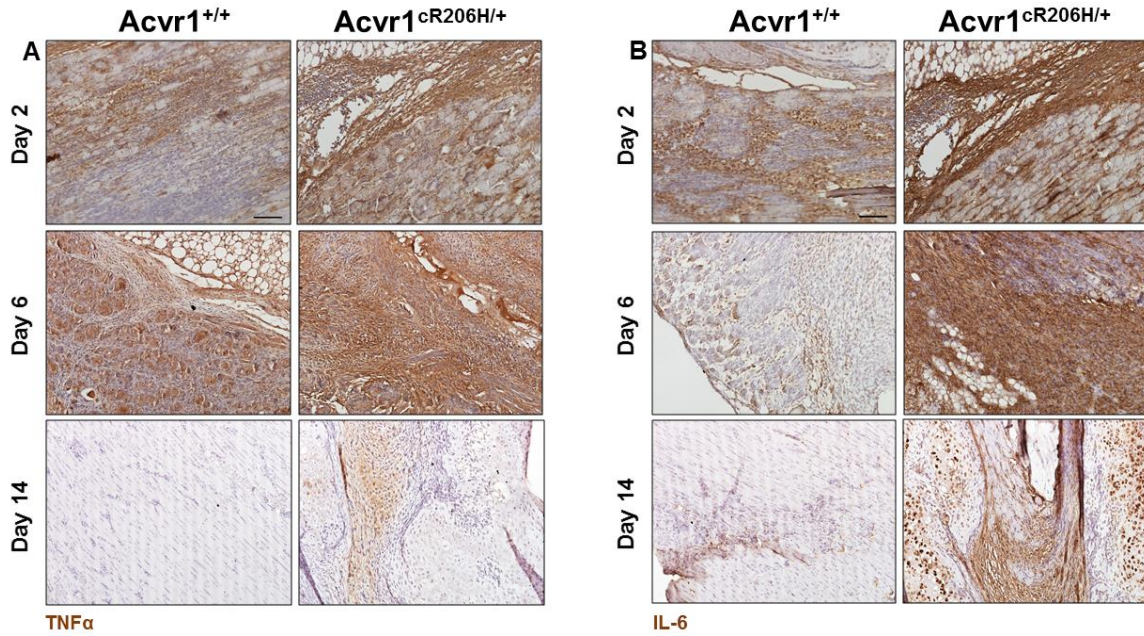
Figure 3.9: TNFα and IL-6 expression is robustly elevated and prolonged in *Acvr1$^{cR206H/+}$* lesions

Specific pro-inflammatory cytokines were detected by immunostaining of injured tissues from early- (day 2), intermediate- (day 6), and late- (day 14) stage *Acvr1$^{cR206H/+}$* and *Acvr1$^{+/+}$* lesions: (A) TNFα, (B) IL-6. n = 3 for each genotype, per day. Representative images are shown. Scale bar = 100 μm for all images.

Figure panel modified from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^{R206H}* Mouse Model of Fibrodysplasia Ossificans Progressiva"
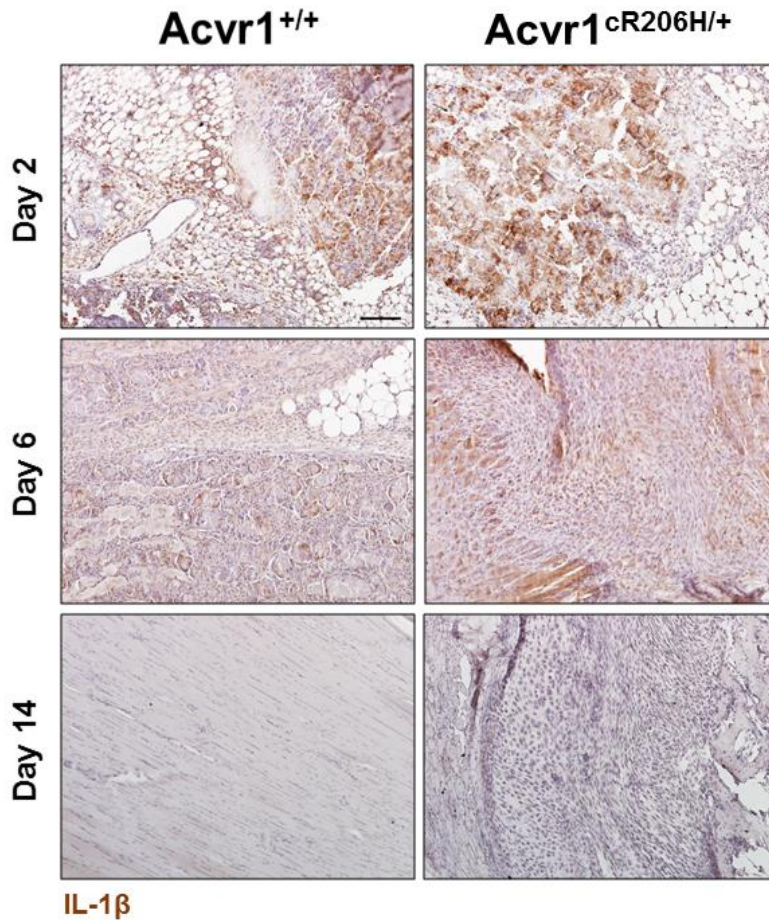
Figure 3.10: IL-1β expression is moderately elevated and prolonged in *Acvr1*^{cR206H/+} lesions

IL-1β was detected by immunostaining of injured tissues from early- (day 2), intermediate- (day 6), and late- (day 14) stage *Acvr1*^{cR206H/+} and *Acvr1*^{+/+} lesions. n = 3 for each genotype, per day. Representative images are shown. Scale bar = 100 μm for all images.

Figure panel modified from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1*^{R206H} Mouse Model of Fibrodysplasia Ossificans Progressiva"

Figure 3.11: Variable MCP-1 and IL-13 expression in *Acvr1$^{cR206H/+}$* and *Acvr1$^{+/+}$* lesions
Sections from early- (day 2), intermediate- (day 6), and late-stage (day 14) *Acvr1$^{cR206H/+}$* and *Acvr1$^{+/+}$* lesions were immunostained for expression of: (A) MCP-1, (B) IL-13. n = 3 for each genotype, per day. Representative images are shown. Scale bar = 100 μm for all images

Figure panel modified from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^{R206H}* Mouse Model of Fibrodysplasia Ossificans Progressiva"
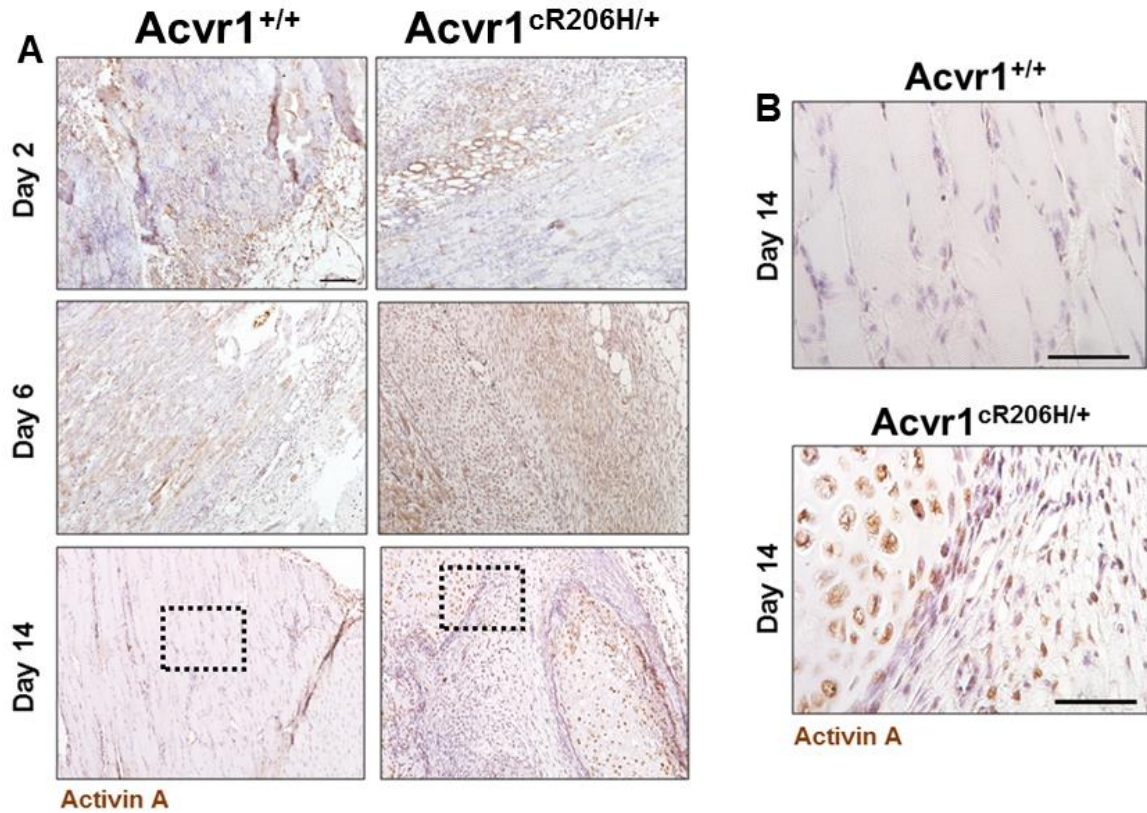
Figure 3.12: Elevated Activin A protein in intermediate- and late-stage *Acvr1$^{cR206H/+}$* lesions
Sections from early- (day 2), intermediate- (day 6), and late-stage (day 14) *Acvr1$^{cR206H/+}$* and *Acvr1$^{+/+}$* lesions were immunostained for expression of: (A) Activin A. (B) Higher magnification insets demonstrating fibroblast and chondrocyte Activin A expression in *Acvr1$^{cR206H/+}$* lesions are shown, indicated by dotted rectangles in (A). n = 3 for each genotype, per day. Representative images are shown. Scale bar = 100 μm for all images in (A); scale bar = 50 μm for all images in (B).

Figure panel modified from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^{R206H}* Mouse Model of Fibrodysplasia Ossificans Progressiva"
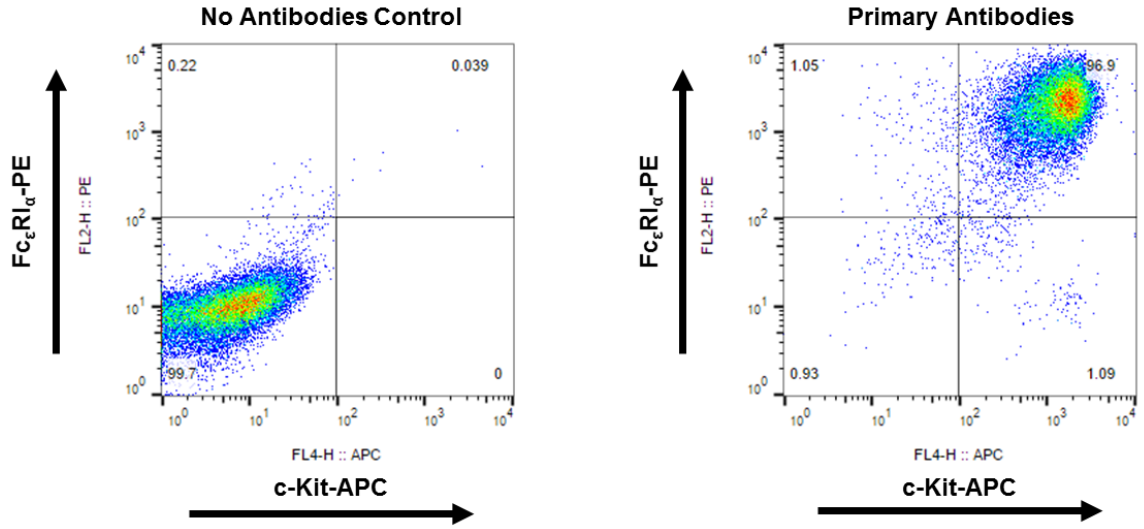
Figure 3.13: Confirmation of primary mast cell purity by flow cytometry
Whole bone marrow was collected from *Acvr1^cR206H/+^* and *Acvr1^+/+^* mice and grown in suspension in mast cell maturation medium for 6 weeks. Mast cells were stained with fluoro-tagged primary antibodies (or "no antibody control") against c-Kit and Fc$_\varepsilon$RI$_\alpha$, cell surface receptors that in combination identify mast cells. Mast cells were gated as double-positive cells (upper right quadrant). Numbers listed are cell percentages per gating.

Figure panel from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^R206H^* Mouse Model of Fibrodysplasia Ossificans Progressiva"
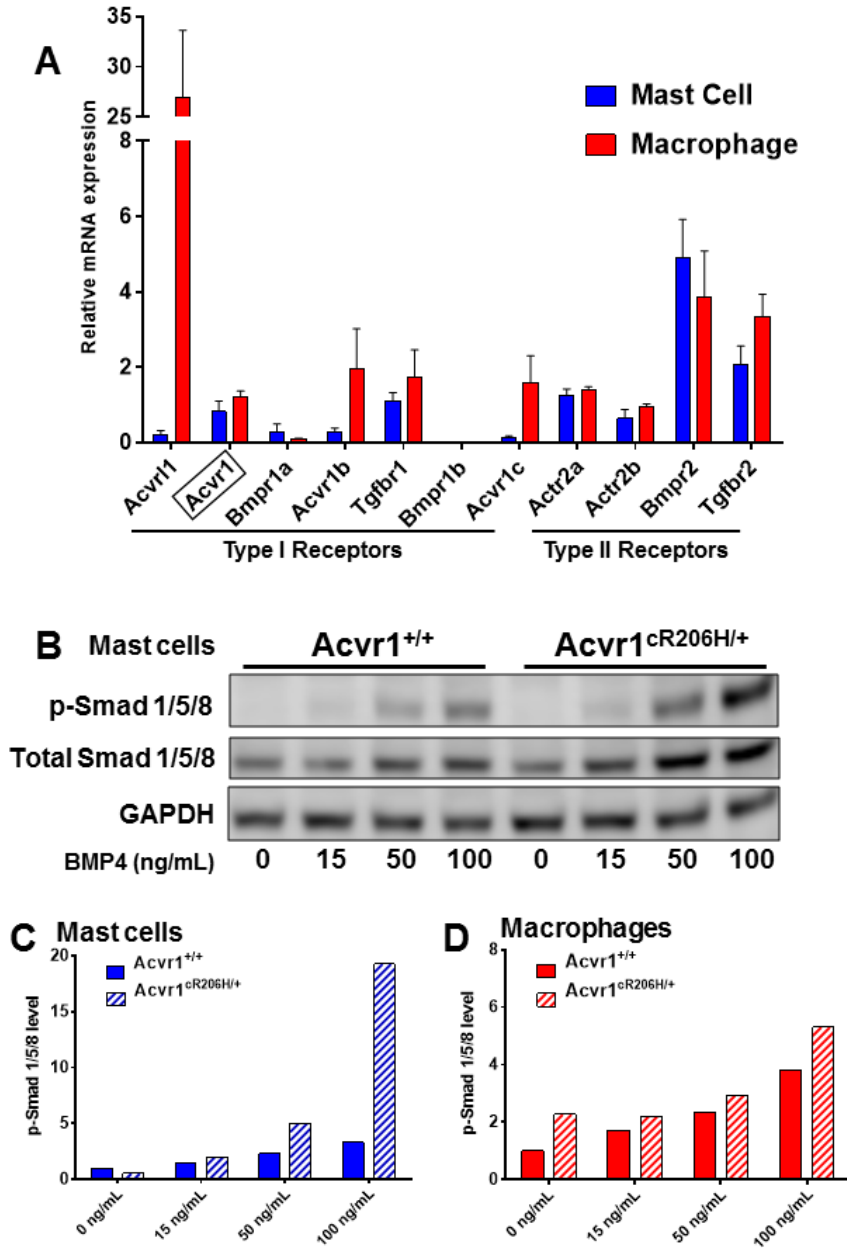
Figure 3.14: BMP signaling is increased in *Acvr1cR206H/+* mast cells and macrophages
(A) Quantification of BMP/TGF-β type I and II receptor mRNAs in Acvr1^{+/+} mast cells and macrophages. Note expression of *Acvr1*, the receptor mutated in FOP (rectangle). (B) Mast cells treated with BMP4 ligand show dose responsive increases in p-Smad1/5/8 protein levels with enhanced pSmad1/5/8 detected in mutant cells. Quantified p-Smad1/5/8 protein relative to untreated control cells is shown. Quantification of (C) mast cell and (D) macrophage p-Smad 1/5/8 levels. n = 4; data shown are mean values ± SEM in A.

Figure panel modified from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1R206H* Mouse Model of Fibrodysplasia Ossificans Progressiva"
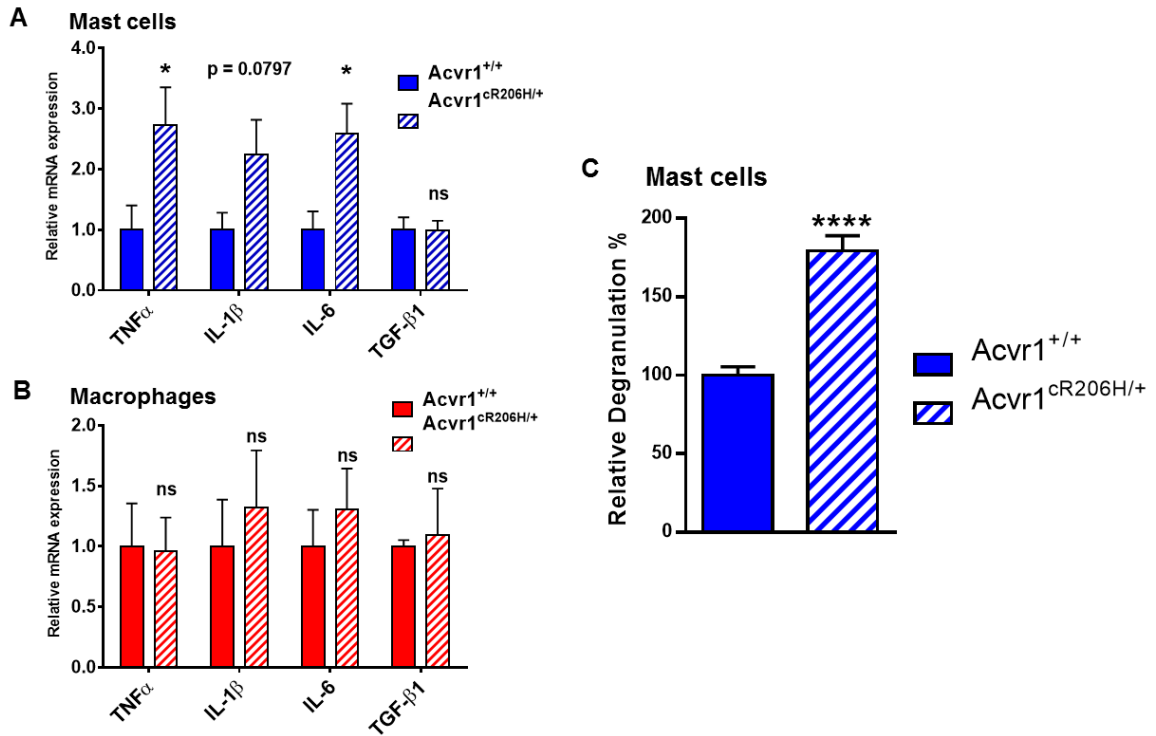
Figure 3.15: Enhanced pro-inflammatory activation in *Acvr1^{cR206H/+}* mast cells

mRNA expression of specific pro-inflammatory cytokines in (A) mast cells and (B) macrophages was detected by qRT-PCR following mast cell treatment with 100 μM Substance P or macrophages treatment with 1 ng/mL lipopolysaccharides, then 15 ng/mL BMP4 for 1 hour. Data were normalized to *Gapdh* and are shown as mean values ± SEM; Student's t-test compared expression in *Acvr1^{cR206H/+}* vs. *Acvr1^{+/+}*; * $p < 0.05$; ns = not significant. n = 6 per gene, per genotype. (C) Mast cells were treated with 100 μM Substance P, plus 15 ng/mL BMP4, for 1 hour. Cells were spun down and conditioned medium was collected and added to tryptase substrate for 2 hours. Free *p*NA, indicating degranulation activity, was quantified using a microplate reader at a wavelength of 405 nm. n = 5; data shown are mean values ± SEM; Student's t-test comparing *Acvr1^{cR206H/+}* versus *Acvr1^{+/+}* was performed; **** $p < 0.0001$
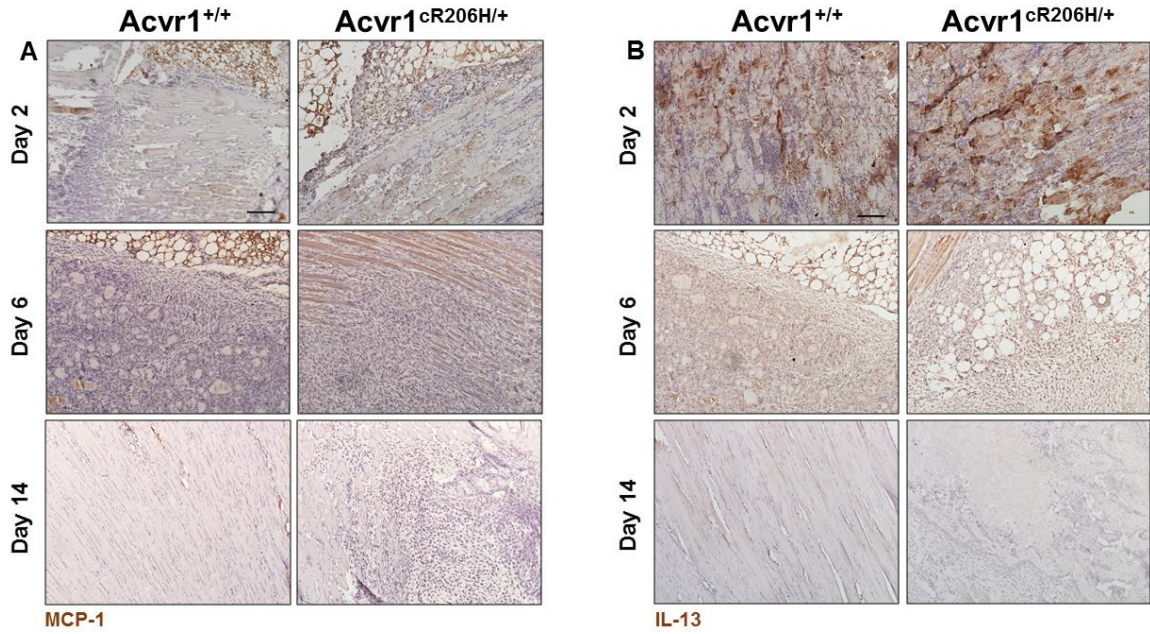
Figure panel modified from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^{R206H}* Mouse Model of Fibrodysplasia Ossificans Progressiva"

Chapter Four

Modulation of the immune system to control heterotopic ossification

4.1 Summary

Current treatments for FOP are mostly ineffective at preventing disease progression to HO. Pre-clinical therapeutics targeting the endochondral ossification stage of FOP have shown promise in mouse models and are under clinical trial investigation; however, there still is an unmet need of effective therapies. The immune system is an appealing therapeutic target for FOP, as inflammation is the earliest stage of disease progression, therefore treating FOP at its earliest phase may provide optimal disease management. A precise understanding of the cellular and molecular inflammatory response, in context with the ACVR1 R206H mutation, is key to developing novel and effective methods of inhibiting HO formation.

I investigated whether expression of $Acvr1^{cR206H}$ restricted to immune cell lineages is sufficient to produce HO in $Acvr1^{+/+}$ mice. I performed two experiments to address this question – a bone marrow transplant (BMT) of $Acvr1^{cR206H/+}$ whole bone marrow into $Acvr1^{+/+}$ recipients, and Cre-recombinase-mediated expression of the $Acvr1^{cR206H}$ allele in myeloid lineage cells via use of the Lysozyme M (*LysM*-Cre) promoter. I additionally investigated whether blocking the cellular and molecular inflammatory response could impair HO formation in $Acvr1^{cR206H/+}$ mice; this was examined via two approaches: administration of two neutralizing agents against pro-inflammatory cytokines, and development of immunodeficient $Acvr1^{cR206H/+}$ mice. Our results suggest that while immune cells expressing $Acvr1^{cR206H}$ are alone insufficient to induce HO formation, depleting mast cells and macrophages dramatically impaired HO development in $Acvr1^{cR206H/+}$ mice, highlighting their necessity for HO formation *in vivo*.

4.2 Introduction

Modulation of the immune system to control disease progression in FOP has been an approach of significant interest. The most noteworthy attempt to treat FOP in this manner involved a single patient who, in addition to FOP, presented with aplastic anemia [Kaplan et al. 2007]. This patient underwent two allogeneic HLA-matched bone marrow transplants (BMT) from his sister, who did not have FOP, as treatment for aplastic anemia. Complete engraftment and reconstitution of donor bone marrow was confirmed by genetic analysis of short tandem repeats. Following successful BMT, the patient developed acute and chronic graft-versus-host disease and was treated with prednisone, cyclosporine, and methotrexate for fourteen years. During this time period, FOP disease progression was halted, with no advancement of HO observed. After discontinuation of the immunosuppressive regimen, the patient's FOP symptoms rapidly rebounded, with new HO developing in previously unaffected areas [Kaplan et al. 2007]. This case report suggested two significant findings: a wild-type immune system in an ACVR1 R206H background is insufficient to prevent disease progression, and suppressing the immune system may be an effective method to halt FOP disease progression.

To further enhance our understanding of the cellular inflammatory contribution to HO, I investigated the converse question: are immune cells expressing the R206H mutation sufficient to develop HO in an $Acvr1^{+/+}$ genetic background? Although previous studies have shown that cells of hematopoietic origin do not directly contribute to the fibroproliferative, chondrogenic, and osteogenic stages of heterotopic endochondral ossification [Kaplan et al. 2007, Kan et al. 2009], I hypothesized that the upregulated

inflammatory activation present in $Acvr1^{cR206H/+}$ mast cells (Figure 3.15A, C) and possibly other immune cell lineages may suggest that hematopoietic lineages alone are sufficient to induce HO formation in FOP. Notably, cells that do not express $Acvr1^{R206H}$ are capable of contributing to the chondrogenic and osteogenic stages of lesion formation [Chakkalakal et al. 2012], suggesting that expression of $Acvr1^{R206H}$ restricted to hematopoietic cells may be able to induce an HO response with the participation of $Acvr1^{+/+}$ fibro-progenitor cells. To investigate the above hypothesis, I performed two experiments: a BMT of $Acvr1^{cR206H/+}$ whole bone marrow into $Acvr1^{+/+}$ recipient mice, and a genetic approach of myeloid-specific $Acvr1^{cR206H}$ expression via generation of $LysM$-Cre;$Acvr1^{cR206H/+}$ mice. For both experiments, I challenged skeletal muscle with cardiotoxin injury and quantified HO development. These experiments would improve our understanding of the cell-autonomous effects of the ACVR1 R206H mutation in immune cells toward directing chondro-/osteogenesis.

The observation that global immunosuppresive medicines can suppress HO formation suggests the immune system as an appealing therapeutic target [Kaplan et al. 2007]. HO development in a ligand-independent, constitutively-active $Acvr1^{Q207D}$ mouse model of HO required an inflammatory millieu, and dexamethasone treatment impaired HO development [Yu et al. 2008], providing a proof-of-principle experiment that inflammatory inhibition may be an effective treatment approach in FOP. Despite the promise of global immunosuppressive therapies, these compounds must be used with caution and are generally not recommended for systemic use in pediatric FOP patients due

102

to adverse events, such as increased propensity for infection, myopathies, and other complications [Poetker et al. 2010].

Targeted immunosuppression of specific immune cells and inflammatory mediators may impair HO formation while also maintaining substantial immune function. I designed two immunosuppressive experiments based on results detailed in Chapter 3, which demonstrated robust upregulation of TNFα and IL-6 in $Acvr1^{cR206H/+}$ lesions and mast cells (Figures 3.9 and 3.15A) and significantly increased macrophage and mast cell density in $Acvr1^{cR206H/+}$ lesions (Figure 3.8B, C). I hypothesized that systemic neutralization of TNFα and IL-6 would inhibit HO formation in $Acvr1^{cR206H/+}$ mice. I also hypothesized that depletion of mast cells and macrophages in $Acvr1^{cR206H/+}$ mice would inhibit HO formation. These *in vivo* experiments provide key insight into the direct immune cellular and molecular contributions to HO formation.

4.3 Results

4.3.1 Reconstitution of an *Acvr1*$^{cR206H/+}$ hematopoietic system in *Acvr1*$^{+/+}$ recipient mice is insufficient to induce HO formation

An unresolved research question is whether expression of the ACVR1 R206H mutation restricted to hematopoietic lineages is sufficient to induce HO formation in an *Acvr1*$^{+/+}$ genetic background. I first investigated this question by conducting a BMT of donor *Acvr1*$^{cR206H/+}$ whole bone marrow into *Acvr1*$^{+/+}$ recipient mice. For this experiment, I used tamoxifen-inducible *ERT2*-Cre;*Acvr1*$^{cR206H/+}$ mice as donors. Because of unknown effects of *Acvr1*$^{cR206H/+}$ increased BMP signaling on hematopoietic survival, engraftment potential, and reconstitution, I used two groups of donor *ERT2*-Cre;*Acvr1*$^{cR206H/+}$ mice: a cohort in which I induced *Acvr1*^{cR206H} allele expression by tamoxifen administration in donor cells prior to transplant, and a cohort in which I induced *Acvr1*^{cR206H} in recipient mice post-transplant after engraftment and reconstitution. I also included two control *Acvr1*$^{+/+}$ donor cell groups; cells that received tamoxifen injections prior to BMT, and cells that received tamoxifen injections post-transplant in recipients. Tamoxifen is not expected to affect *Acvr1*$^{+/+}$ allele expression, nor are these control mice expected to develop HO (Table 4.1). To detect donor marrow engraftment and reconstitution in recipients, I took advantage of murine CD45 receptor biology in which two CD45 isoforms – CD45.1 and CD45.2 – can be used to lineage trace the sources of bone marrow cells [Spangrude et al. 1988]. All donor *ERT2*-Cre;*Acvr1*$^{cR206H/+}$ mice express the CD45.2 isoform, and all recipient B6.SJL mice express CD45.1 [Waterstrat et al. 2010], allowing for flow cytometry detection of donor cell engraftment and reconstitution efficiency post-BMT.

Expression of the *Acvr1cR206H* allele in donor cells prior to BMT was confirmed by PCR (Figure 4.1).

A catalog of all recipient mice, and source/genotype of donor bone marrow, can be found in Table 4.1. Six week old B6.SJL recipient mice were irradiated with 1000 rads and then injected with 5×10^6 whole bone marrow cells from donor mice via tail vein injection (tamoxifen-treated or non-tamoxifen-treated; see Table 4.1). Engraftment and reconstitution occurred over 8 weeks. Following this 8 week period, appropriate cohorts were adminsistered tamoxifen by intraperitoneal injection for four consecutive days. All recipient mice were injured by cardiotoxin injection (100 µL of 10 µM) into right hamstring muscles; contralateral hamstring muscles were injected with PBS. All mice were sacrificed at 21 days post-injury, after which mice were assessed for HO formation by X-ray and µCT. At sacrifice, bone marrow was collected to confirm donor CD45.1 cell reconstitution, as well as to confirm *Acvr1cR206H* allele recombination.

Data analysis suggested that the BMT experiment produced inconclusive results. Mouse #9 received *Acvr1cR206H/+* donor bone marrow, but did not receive tamoxifen injections as a control against possible leaky *Acvr1cR206H* expression. Detection of the recombined *Acvr1cR206H* allele occurred only in three of seven recipients that received *Acvr1cR206H/+* donor bone marrow and tamoxifen injections: mice #11, #12, and #305. Detection of the recombined *Acvr1cR206H* allele was also expected in mice numbers 6, 7, 8, and 10, however these amplicons were not detected (Figure 4.2). Of all *Acvr1+/+* recipient mice that received *Acvr1cR206H/+* donor bone marrow, only one developed any HO, and this amount was very small (Figure 4.3). Engraftment and reconstitution of donor CD45.1$^+$

bone marrow was variable across individual recipients, suggesting that irradiation may have been incomplete in some recipients; however, mature immune cells were detected in all mice analyzed (Table 4.2 and Figure 4.4). Importantly, as a positive control, I included an $Acvr1^{cR206H/+}$ recipient mouse that received $Acvr1^{cR206H/+}$ donor bone marrow (mouse #305). Although this mouse expressed $Acvr1^{cR206H/+}$ globally, and mature mutant immune cells were detected (Table 4.2 and Figure 4.4), no HO was observed (Figure 4.3). These results suggest that $Acvr1^{cR206H/+}$ hematopoietic cells are unable to induce HO formation in an $Acvr1^{+/+}$ background, however possible experimental design limitations and technical issues may prevent conclusive data interpretation (see Chapter 4.4 Discussion).

4.3.2 Myeloid-restricted $Acvr1^{cR206H}$ expression is insufficient to induce HO formation

Based on potential experimental design limitations and technical issues with our BMT experiment, I next used a genetic approach to address the same question: whether expression of the ACVR1 R206H mutation restricted to hematopoietic lineages is sufficient to induce HO formation in an $Acvr1^{+/+}$ genetic background.

Lysozyme M (encoded by the $Lyz2$ gene, also referred to as $LysM$) is a glycoside hydrolase enzyme that is expressed in almost all myelomonocytic lineages, including mature neutrophil granulocytes and macrophages [Cross et al. 1988, Faust et al. 2000]. I generated $LysM$-Cre;$Acvr1^{cR206H/+}$ mice (Figure 2.5) in which recombination and expression of the $Acvr1^{[R206H]FlEx}$ allele occurs exclusively within myelomonocytic lineages. I injected cardiotoxin into the right hamstring muscles into $LysM$-Cre;$Acvr1^{cR206H/+}$ mice and grouped mice into two cohorts: 2 weeks post-injury and 4

weeks post-injury. Two global knock-in *Acvr1$^{cR206H/+}$* mice were injected using same protocol and served as positive controls.

No HO was detected in any *LysM*-Cre;*Acvr1$^{cR206H/+}$* mice, either at 2 weeks or 4 weeks post-injury. HO was detected in both globally-expressing *Acvr1$^{cR206H/+}$* positive-control mice at 2 weeks post-injury (Figure 4.5 A, B).

4.3.3 Systemic neutralization of TNFα and IL-6 reduces inflammation but is insufficient to prevent HO formation in *Acvr1$^{cR206H/+}$* mice

Immunosuppressive corticosteroid therapy has proven effective at halting disease progression in a single FOP patient and in a constitutively-active *Acvr1^{Q207D}* FOP mouse model [Kaplan et al. 2007, Yu et al. 2008]. However, the former data represents only a single adult human patient and has not been extensively evaluated in other FOP patients. Global immunosuppresive regimens are risky for use in pediatric FOP patients due to infectious disease vulnerability, and long-term use of these therapeutics may increase the prevalence of adverse events, such as increased propensity for infection, myopathies, and other complications [Poetker et al. 2010].

Immunomodulary biologics are a class of extremely effective therapeutics used in the clinic for treatment of inflammatory disorders [Sathish et al. 2013]. Biologics are most commonly recombinant DNA-based monoclonal antibodies or decoy fusion receptors that bind and sequester soluble cytokines and chemokines. Due to their target specificity, biologics are remarkably effective and have limited off-target effects.

Based on results from Chapter Three showing that *Acvr1^{cR206H/+}* mice express significantly higher and persistent levels TNFα and IL-6 following skeletal muscle injury (Figure 3.9A, B), I considered the use of biologics as an approach to reduce cytokine levels in *Acvr1^{cR206H/+}* mice. I hypothesized that systemic neutralization of TNFα and IL-6 would inhibit HO formation.

Acvr1^{cR206H/+} mice were injected with cardiotoxin (50 µL of 20 µM concentration) into the gastrocnemius muscle of both hind limbs, with mouse sacrifice and sample collection at 2 weeks post-injury. I neutralized TNF-α and IL-6 using specific neutralizing agents. I neutralized TNF-α via etanercept, a soluble fusion protein of the TNF-α receptor and the IgG1 constant region that binds TNF-α and confers long-term neutralization of TNF-α [Peppel et al. 1991, Kolls et al. 1994]. I neutralized IL-6 using a custom produced mouse-anti-IL-6-antibody. Our experimental design included four *Acvr1^{cR206H/+}* cohorts: PBS vehicle control, etanercept (4.0 mg/kg, 3X week), mouse-anti-IL-6 (150 mg per mouse, 3X week), or etanercept plus mouse-anti-IL-6-antibody (previous concentrations). Neutralizing agents were delivered via retro-orbital delivery into *Acvr1^{cR206H/+}* mice. To confirm efficacy of cytokine neutralization, I quantified serum levels of C-reactive protein (CRP), a pan-inflammatory marker [Pepys et al. 2003], from serum collected throughout the experiment.

Our results show that both etanercept and mouse-anti-IL-6 were effective at reducing systemic inflammation in *Acvr1^{cR206H/+}* mice post injury, with the greatest down-regulation of serum CRP at the intermediate fibroproliferative cell stage (Figure 4.6A). In contrast, vehicle-treated *Acvr1^{cR206H/+}* mice exhibited a 40% increase in serum CRP levels

over baseline measurements. By day 14, CRP levels were equivalent among all groups. Intramuscular mineral content and HO volume were significantly reduced in the etanercept cohort, compared to the anti-IL-6 cohort (Figure 4.6B, C). HO volume was also significantly reduced in the combined etanercept plus anti-IL-6 cohort, compared to the anti-IL-6 cohort (Figure 4.6C). Despite the reduction in ectopic bone parameters in the etanercept cohorts, there was considerable variability in the vehicle positive control mice that prevented conclusive interpretation of results; however, I concluded that mouse-anti-IL-6 is ineffective at preventing HO formation.

Due to the promising etanercept results, I conducted a second experiment of etanercept experimental groups. The second experiment utilized the following dosing regimens: etanercept (4 mg/kg, 2X week; 2 mg/kg, 2X week; 4 mg/kg, 3X week). I quantified HO volume at 2 weeks post-injury and in contrast to the first experiment, HO volume was not reduced in any etanercept-treated $Acvr1^{cR206H/+}$ cohort (Figure 4.6D). Based on the results of both neutralizing agent experiments, I concluded that systemic neutralization of TNF-α and IL-6, while efficiently reducing systemic cytokine levels, is insufficient to inhibit HO formation in $Acvr1^{cR206H/+}$ mice.

4.3.4 Depletion of mast cells and macrophages significantly impairs injury-induced heterotopic ossification development in $Acvr1^{cR206H/+}$ mice

Two immune cell populations previously implicated in HO development are mast cells and macrophages [Gannon et al. 2001, Kan et al. 2009, Kan et al. 2011, Salisbury et al. 2011]. Given the increased cell density and prolonged presence of mast cells and

macrophages in FOP lesions (Figure 3.8B, C), as well as their relevance in multiple HO disorders [Convente et al. 2015], I investigated the contribution of mast cells and macrophages to injury-induced HO by generating immunodeficient $Acvr1^{cR206H/+}$ mice.

The homozygous mutant White Sash $c\text{-}Kit^{W\text{-}sh/W\text{-}sh}$ mouse fully lacks mast cells due to a 3 mb inversion in the upstream regulatory region of the $c\text{-}Kit$ transcriptional start site [Nagle et al. 1995, Grimbaldeston et al. 2005]. The heterozygous mutant $c\text{-}Kit^{W\text{-}sh/+}$ mouse exhibits partial mast cell deficiency [Grimbaldeston et al. 2005]. Importantly, the $c\text{-}Kit^{W\text{-}sh/W\text{-}sh}$ mouse exhibits no other immune cell deficiencies [Grimbaldeston et al. 2005]. To deplete mast cells in the context of global expression of the R206H mutation, I generated $Acvr1^{cR206H/+};c\text{-}Kit^{W\text{-}sh/W\text{-}sh}$ and $Acvr1^{cR206H/+};c\text{-}Kit^{W\text{-}sh/+}$ mice. To deplete macrophages, $Acvr1^{cR206H/+}$ mice were injected with clodronate liposomes (Clo), which selectively induce apoptosis in mature macrophages [van Rooijen et al. 1984, Van Rooijen 1989] (Figure 4.7). This pharmacological approach allowed generation of combined mast cell- and macrophage-deficient mice by injecting $Acvr1^{cR206H/+};c\text{-}Kit^{W\text{-}sh/W\text{-}sh}$ mice with clodronate liposomes. Macrophage depletion was confirmed via flow cytometry (Figure 4.8) and mast cell depletion was confirmed via histological detection (Figure 4.9).

Compared to $Acvr1^{cR206H/+}$ mice, heterozygous $Acvr1^{cR206H/+};c\text{-}Kit^{W\text{-}sh/+}$ mice consistently had a moderate, but statistically insignificant, decrease in HO volume (Figure 4.10A, B). However, fully mast cell-deficient $Acvr1^{cR206H/+};c\text{-}Kit^{W\text{-}sh/W\text{-}sh}$ mice exhibited a ~50% decrease in HO volume relative to $Acvr1^{cR206H/+}$ mice. Macrophage-deficient $Acvr1^{cR206H/+};$Clo-treated mice had a similar ~50% decrease in HO. Combined deficiency of mast cells and macrophages ($Acvr1^{cR206H/+};c\text{-}Kit^{W\text{-}sh/W\text{-}sh};$Clo) resulted in a further

reduction in HO, with a ~75% decrease relative to $Acvr1^{cR206H/+}$ mice (Figure 4.10A, B). These results show that both mast cells and macrophages contribute to HO initiation and development.

4.4 Discussion

4.4.1 $Acvr1^{R206H}$ cells of hematopoietic origin are insufficient to induce heterotopic ossification

The origin of the progenitor cells that differentiate to form heterotopic endochondral ossification in FOP is a major ongoing question of investigation. *In vitro* studies using fibroblastic mesenchymal cells from both human sources and mouse models have demonstrated chondro-/osteogenic potential that is further accelerated with expression of $Acvr1^{R206H}$, providing initial clues about the properties of cells directly contributing to HO [Billings et al. 2008, Culbert et al. 2014]. Additional *in vivo* studies have further expanded information about the identity of cells that directly participate in HO formation, including cells expressing Tie-2, PDGFRα, Mx1, and Scx [Lounev et al. 2009, Wosczyna et al. 2012, Agarwal et al. 2016, Dey et al. 2016]. Although previous work has shown that hematopoietic cells do not participate directly to form ectopic bone [Kaplan et al. 2007], an unresolved question was whether $Acvr1^{R206H}$ hematopoietic cells could induce HO formation in an otherwise $Acvr1^{+/+}$ background via recruitment of chondro-/osteo-progenitor cells to the site of tissue injury.

I showed in Chapter Three that $Acvr1^{cR206H/+}$ mast cells exhibit an upregulated inflammatory response (Figure 3.15A, C), and mast cells are known to recruit fibroblasts to sites of injury and biomaterial implants [Tang et al. 1998, Zhao et al. 2008, Levick et al. 2009, Thevenot et al. 2011]. Additionally, $Acvr1^{+/+}$ chondro-/osteo-progenitor cells participate in ectopic skeletogenesis in the presence of $Acvr1^{R206H}$ cells [Chakkalakal et al. 2012]. Our results from Chapter 3 and supporting literature suggested a hypothesis that

112

enhanced inflammatory signaling present in *Acvr1*$^{cR206H/+}$ immune cells may be sufficient to recruit *Acvr1*$^{+/+}$ fibroblastic progenitor cells and induce HO formation.

The results from our *Acvr1*$^{cR206H/+}$ BMT and *LysM*-Cre;*Acvr1*$^{cR206H/+}$ experiments strongly oppose our hypothesis. HO formed in only one *Acvr1*$^{+/+}$ recipient mouse (#12) that received *Acvr1*$^{cR206H/+}$ donor bone marrow (in which *Acvr1*^{cR206H} expression was confirmed; Figure 4.2). Experimental design and technical issues limit conclusive interpretation of the BMT experiment. I did not fractionate donor bone marrow into a hematopoietic-specific pool; whole bone marrow is a heterogeneous mixture that also contains mesenchymal stromal cells with chondro-/osteogenic potential [Reagan et al. 2016]. Given that *Acvr1*$^{cR206H/+}$ donor engraftment and reconstitution was reasonably high in mouse #12 (Figure 4.4), it is possible that some bystander *Acvr1*$^{cR206H/+}$ stromal cells were also transplanted and contributed to the development of low HO volume in that mouse. Additionally, our experience with *Acvr1*$^{cR206H/+}$ mice suggests that HO is unable to form if the ACVR1 R206H mutation is induced in mice older than ~7-8 weeks of age. Given the 8 week duration for engraftment and reconstitution of donor bone marrow, recipient mice were ~14 weeks of age at time of injury. This length of time (compared to the 4 week old mice used in our standard experimental protocal of HO induction) may explain why our positive-control *Acvr1*$^{cR206H/+}$ mouse (#305) did not develop HO (Figure 4.3), even though it received *Acvr1*$^{cR206H/+}$ syngeneic CD45.2 donor bone marrow (Figures 4.2 and 4.4).

To address the experimental design concerns of the BMT, I utilized an alternative, genetic approach to selectively express *Acvr1*^{cR206H} in cells of hematopoietic lineage. I

113

induced *Acvr1^{cR206H}* expression in myeloid lineage cells due to their significant presence and contribution to FOP and other HO disorders [Convente et al. 2015]. This genetic approach improves the BMT experimental design in two ways: *Acvr1^{cR206H}* expression is restricted to myeloid lineages without bystander stromal cell expression, and this model removes potential mouse age restrictions that impede HO formation. However, I did not detect HO in any *LysM*-Cre;*Acvr1^{cR206H/+}* mice, even at 4 weeks post-injury (Figure 4.5), which is double the normal time needed for HO development.

Based on the results of the BMT and *LysM*-Cre;*Acvr1^{cR206H/+}* experiments, I conclude that *Acvr1^{R206H}* cells of hematopoietic origin are insufficient to induce heterotopic ossification. These data are corroborated by a recent publication that reported no HO development in *Vav1*-Cre;*Acvr1^{Q207D}* mice that express constitutively-active *Acvr1^{Q207D}* in all hematopoietic lineage cells [de Boer et al. 2003, Dey et al. 2016].

4.4.2 Neutralization of TNFα and IL-6 is insufficient to inhibit heterotopic ossification

Targeting a subset of the immune system to treat HO is an appealing approach due to potentially reduced adverse events and off-target effects that often results with global immunosuppressive regimens such as corticosteroids. I specifically targeted TNFα and IL-6 for neutralization due to their robust upregulation in *Acvr1^{cR206H/+}* lesions and mast cells (Figures 3.9A, B and 3.15A), as well as their role in chronic inflammatory myopathies [Karalaki et al. 2009, Philippou et al. 2012], a microenvironment mimicked in *Acvr1^{cR206H/+}* lesions (Figure 3.9).

Systemic neutralization of TNFα and IL-6 was effective at reducing inflammation in *Acvr1^{cR206H/+}* mice post-injury (Figure 4.6A), but over the course of two independent experiments, was ineffective to reduce HO. Results from the first experiment were promising; however, HO volume was far lower than normally seen in positive-control *Acvr1^{cR206H/+}* (Figure 4.6C). On our second attempt, HO volume returned to consistent amounts and all regimens of etanercept showed no efficacy at reducing HO (Figure 4.6D).

It is possible that neutralization of TNFα and/or IL-6 is insufficient to reduce HO formation due to compensating effects from other cytokines and enzymes, such as IL-1β, IFN-γ, MCP-1, MMP-9, and chymase, among numerous other candidates. Pro-inflammatory cytokines induce substantial concentrations of chemokines and growth factors that support tissue remodeling and restoration [Karalaki et al. 2009, Philippou et al. 2012], suggesting a more comprehensive anti-inflammatory therapeutic regimen may be necessary to inhibit HO formation.

4.4.3 Depletion of immune cells dramatically impairs heterotopic ossification development

Immune cells are sources of numerous inflammatory mediators that direct a potent inflammatory response to tissue injury. Myeloid-lineage cells including neutrophils, macrophages, and mast cells are known to express and secrete dozens of cytokines, chemokines, enzymes, and other inflammatory small molecules that amplify and sustain an inflammatory response and participate in tissue regeneration [Mosser et al. 2008, Murray et al. 2011, Kolaczkowska et al. 2013, Wynn et al. 2013, Moon et al. 2014, de

Oliveira et al. 2016, Vukman et al. 2017]. Given that immune cells are the primary source of inflammatory factors, depleting immune cells is a proof-of-principle approach that may reduce overall inflammation and impair HO development.

Several previous studies have demonstrated that immuno-ablating select immune cells impairs HO development [Kan et al. 2009, Kan et al. 2011]. Our study is the first to investigate the requirement of mast cells and macrophages for HO formation in the $Acvr1^{cR206H/+}$ mouse model. Our *in vivo* results suggest that mast cells and macrophages are key cells for HO progression in FOP (Figure 4.10). I found that ablation of only mast cells or only macrophages reduced HO formation significantly but not completely, while ablating both resulted in enhanced inhibition, indicating that a single target may be insufficient to completely prevent HO. These data, in combination with our neutralization data, suggest that the inflammatory HO initiation mechanism by mast cells and macrophages may be due to factors not investigated in this project, such as the aforementioned chemokines and growth factors.

Importantly in the context of developing therapeutics to inhibit HO formation in FOP, multiple methods that block mast cell inflammatory signaling, including targeting the Substance P neuroinflammatory pathway, all inhibited HO formation in a BMP2-implant model of HO [Kan et al. 2011, Salisbury et al. 2011]. Macrophages have been shown to produce osteoinductive signals [Champagne et al. 2002], promote osteogenic differentiation of mesenchymal stem cells [Tu et al. 2015], inhibit osteoclastogenesis [Jacquin et al. 2009], and promote fracture repair [Alexander et al. 2011], highlighting their potential relevance to HO development. Our data and these supporting literature suggest

116

that depleting mast cells and macrophages may severely limit their comprehensive inflammatory, pro-fibrotic, and pro-osteogenic functions, ultimately restricting the cascade of events that result in heterotopic endochondral ossification.

Donor Marrow Source	Tamoxifen in donor or recipient	Recipient Mice Genotype	Recipient Mouse Number	HO Response
$Acvr1^{+/+}$	Donor	$Acvr1^{+/+}$	1	-
$Acvr1^{+/+}$	Donor	$Acvr1^{+/+}$	2	-
$Acvr1^{+/+}$	Recipient	$Acvr1^{+/+}$	3	-
$Acvr1^{+/+}$	Donor	$Acvr1^{+/+}$	4	-
$Acvr1^{+/+}$	Donor	$Acvr1^{+/+}$	5	-
$Acvr1^{cR206H/+}$	Recipient	$Acvr1^{+/+}$	6	-
$Acvr1^{cR206H/+}$	Recipient	$Acvr1^{+/+}$	7	-
$Acvr1^{cR206H/+}$	Donor	$Acvr1^{+/+}$	8	-
$Acvr1^{cR206H/+}$	None	$Acvr1^{+/+}$	9	-
$Acvr1^{cR206H/+}$	Donor	$Acvr1^{+/+}$	10	-
$Acvr1^{cR206H/+}$	Donor	$Acvr1^{+/+}$	11	-
$Acvr1^{cR206H/+}$	Donor	$Acvr1^{+/+}$	12	+
$Acvr1^{cR206H/+}$	Recipient	$Acvr1^{cR206H/+}$	305	-

Table 4.1: Bone marrow donors and recipients table
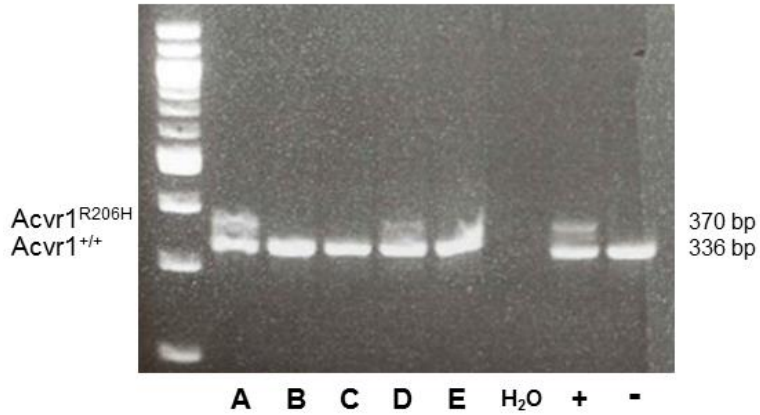
Figure 4.1: Detection of recombined and expressed *Acvr1^cR206H^* allele in donor bone marrow pre-BMT
DNA isolated from donor bone marrow or tail snip was amplified with primers specific to recombination of *Acvr1^[R206H]FlEx^* amplicon (and *Acvr1^+/+^* control amplicon), indicating active *Acvr1^cR206H^* allele expression; Lane A was bone marrow sample from *ERT2*-Cre; *Acvr1^[R206H]FlEx^* + Tam.; Lane B was bone marrow sample from *ERT2*-Cre;*Acvr1^+/+^* mouse + Tam; Lane C was bone marrow sample from *ERT2*-Cre;*Acvr1^[R206H]FlEx^* mouse without Tam; Lane D was tail snip from same mouse as in A; Lane E was tail snip from same mouse as in B. H₂O indicates "water, no DNA control" lane. Abbreviation: Tam. = Tamoxifen-treated.
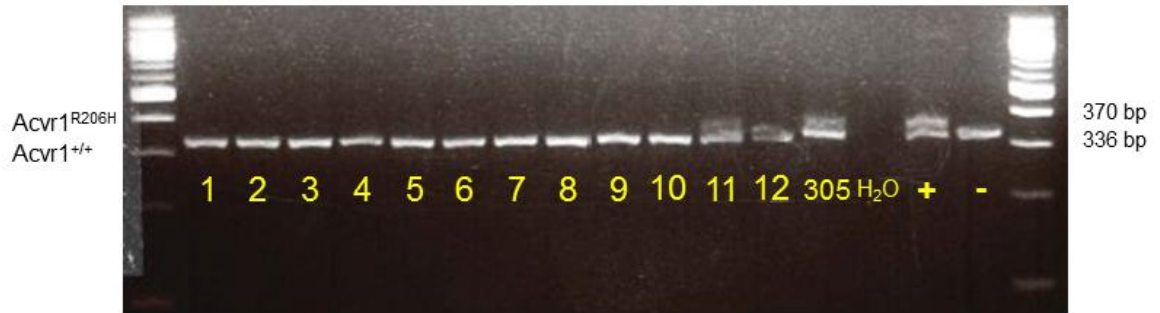
Figure 4.2: Detection of recombined and expressed *Acvr1^cR206H^* reconstituted bone marrow in *Acvr1^+/+^* post-BMT recipients

DNA isolated from reconstituted recipient bone marrow was amplified with primers specific to recombination of *Acvr1^[R206H]FlEx^* amplicon (and *Acvr1^+/+^* control amplicon), indicating active *Acvr1^cR206H^* allele expression. Lane numbers correspond to mice indicated in Table 4.1. H_2O indicates "water, no DNA control" lane.
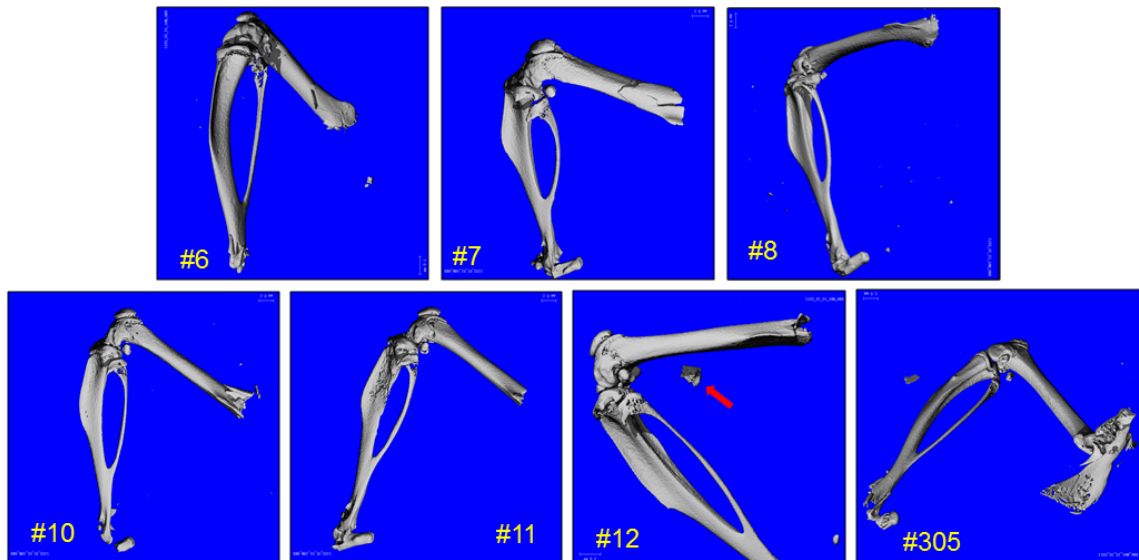
Figure 4.3: *Acvr1^{cR206H/+}* **whole bone marrow is insufficient to generate HO in *Acvr1^{+/+}* recipients**

Six week old *Acvr1^{+/+}* recipient mice (and a positive-control *Acvr1^{cR206H/+}* mouse #305) were irradiated with 1000 rads and injected with 5 X 10^6 *Acvr1^{cR206H/+}* whole bone marrow cells by tail vein injection. Donor marrow engraftment and reconstitution time was 8 weeks. Recipient mouse hamstring muscles were injected with cardiotoxin (100 µL of 10 µM) and sacrificed at 3 weeks post-injury. Hind limbs were analyzed by µCT for visualization of HO. Only recipient #12 developed a small volume of HO (red arrow). The global knock-in *Acvr1^{cR206H/+}* recipient mouse #305 (positive control) did not develop HO.

Mouse Number	B cells		CD4 T cells		CD8 T cells	
	CD45.1 %	CD45.2 %	CD45.1 %	CD45.2 %	CD45.1 %	CD45.2 %
1	0.4	95	7.5	92	8.2	92
2	0.99	96	7.3	92	6.9	93
3	2.0	94	13	85	10.1	90
4	91	0.54	95	0.051	99	0.019
5	5.8	91	37	60	36	64
6	31	63	71	27	45	54
7	91	0.35	97	0.016	99.2	0
8	90	0.39	89	1.2	96	0.23
9	85	0.71	87	1.1	94	0.43
10	66	28	64	33	70	30
11	2.2	95	10	89	13	87
12	65	4.2	35	7	46	51
305	0	92	0.35	99	0.23	99.7

Mouse Number	NK cells		Neutrophils	
	CD45.1 %	CD45.2 %	CD45.1 %	CD45.2 %
1	0.16	99.7	4.7	92
2	0.69	99	0	95
3	0.73	99.1	0	98
4	100	0	96	0.22
5	5.1	95	0.22	93
6	90	9.7	46	48
7	99	0	95	0.24
8	89	2.0	94	0.35
9	92	1.6	90	1.7
10	56	41	57	34
11	5.3	95	2.6	91
12	52.4	43.3	96	0.48
305	0.94	99	0	94

Table 4.2: Donor CD45.2 bone marrow engraftment and reconstitution table
Mice numbers 1-5 received $Acvr1^{+/+}$ donor bone marrow. Mice numbers 6-12 and 305 received $Acvr1^{cR206H/+}$ donor bone marrow.
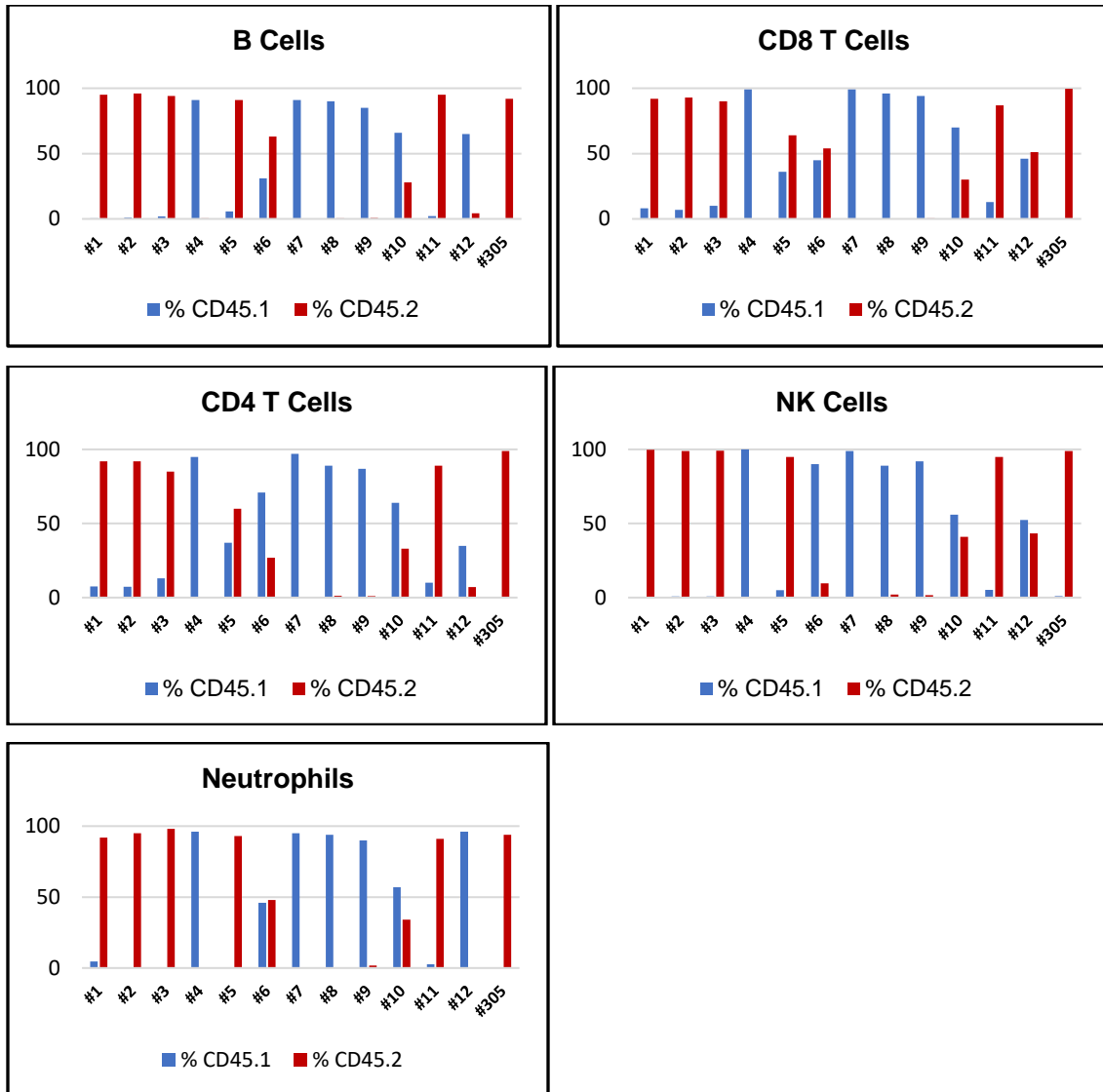
Figure 4.4: Variable efficiency of CD45.2 donor bone marrow engraftment and reconstitution in recipients

Bone marrow was collected from recipient mice at sacrifice for quantification of donor bone marrow engraftment and reconstitution. Bone marrow was incubated with primary antibodies against CD45.1, CD45.2, and a panel of fluoro-tagged antibodies to detect specific immune cell populations (see Chapter 2.8.3). Percentages of donor reconstituted immune cells (CD45.2) and host residual immune cells (CD45.1) are listed for each recipient mouse. Mice numbers 1-5 received $Acvr1^{+/+}$ donor bone marrow. Mice numbers 6-12 and 305 received $Acvr1^{cR206H/+}$ donor bone marrow.

A

LysM-Cre; *Acvr1*$^{cR206H/+}$
2 weeks post-injury

LysM-Cre; *Acvr1*$^{cR206H/+}$
4 weeks post-injury

Acvr1$^{cR206H/+}$
2 weeks post-injury

B

○ *LysM*-Cre;*Acvr1*$^{cR206H/+}$ - 2 weeks
■ *LysM*-Cre;*Acvr1*$^{cR206H/+}$ - 4 weeks
▲ *Acvr1*$^{cR206H/+}$ - 2 weeks
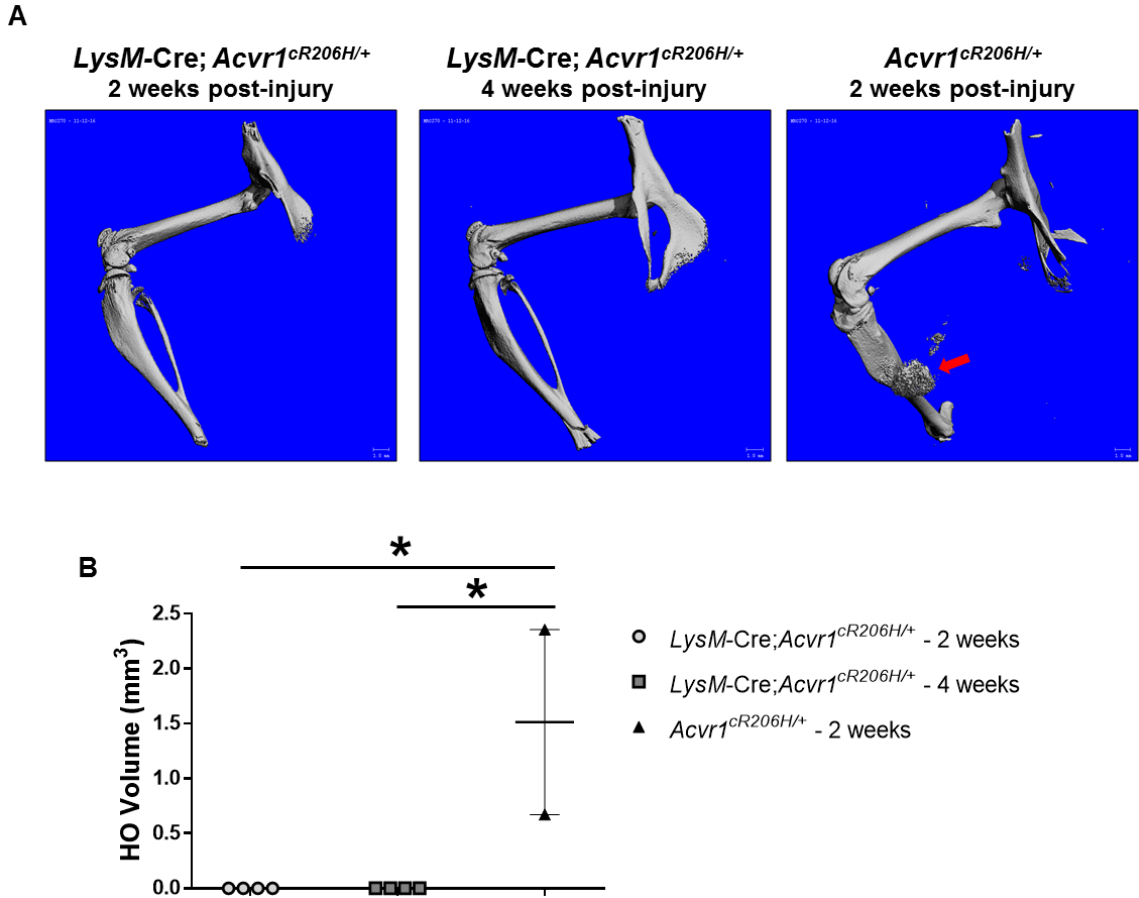
Figure 4.5: Myeloid-restricted *Acvr1*^{cR206H} expression is insufficient to induce HO formation following skeletal muscle injury

Four week old *LysM*-Cre;*Acvr1*$^{cR206H/+}$ mice (and *Acvr1*$^{cR206H/+}$ positive controls) were injected with cardiotoxin (60 µL; 8 µg total peptide) into right hamstring muscles. (A) Hind limbs were analyzed by µCT for visualization of HO. No HO was detected in any *LysM*-Cre;*Acvr1*$^{cR206H/+}$ mouse; HO was detected in both *Acvr1*$^{cR206H/+}$ mice (red arrow). Representative µCT 3D volume renderings per cohort are shown. (B) Quantification of HO for each cohort. n = 4 for each *LysM*-Cre;*Acvr1*$^{cR206H/+}$ cohort, n = 2 for *Acvr1*$^{cR206H/+}$ cohort. Data shown are mean values ± SEM; one-way ANOVA with Tukey's multiple comparisons test compared *Acvr1*$^{cR206H/+}$ vs. other cohorts; * $p < 0.05$

1st Experiment
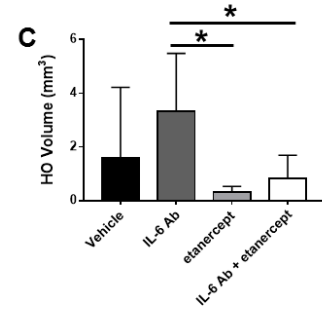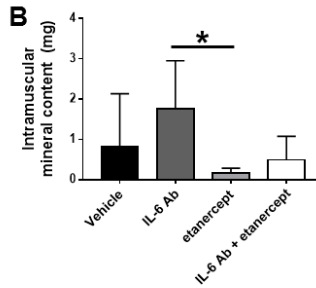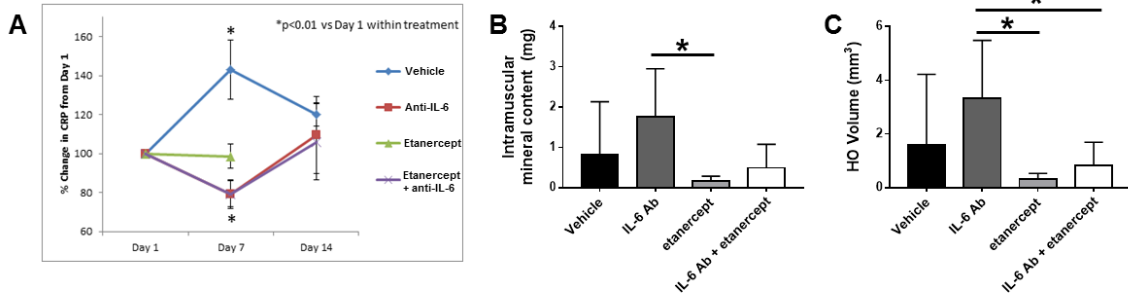
2nd Experiment
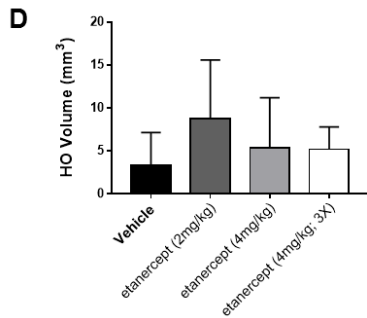
Figure 4.6: TNFα and IL-6 neutralization is insufficient to inhibit HO formation

Four week old *Acvr1^cR206H/+* mice were injected with cardiotoxin (50 μL of 20 μM) into the gastrocnemius muscle of both hind limbs. Four experimental groups: PBS vehicle, mouse-anti-IL-6, etanercept, mouse-anti-IL-6 + etanercept. Mice were sacrificed at 2 weeks post-injury. Hind limbs were scanned by μCT for HO quantification. (A) CRP levels were quantified at the beginning, middle, and end of 2-week experiment. n = 8 for all cohorts except etanercept (n = 6). Data shown are mean values ± SEM; two-way repeated measures ANOVA with Holm-Sidak's multiple comparisons test compared values to baseline measurements; * p < 0.01 versus Day 1 within treatment. (B) Intramuscular mineral content was quantified. (C) HO volume was quantified. n = 8 for all cohorts except etanercept (n = 6). Data shown are mean values ± SEM; one-way ANOVA with Tukey's multiple comparisons test was performed. (D) HO volume was quantified. n = 10 for all groups, except etanercept, 4mg/kg; 3X (n = 2). Data shown are mean values ± SEM; one-way ANOVA with Tukey's multiple comparisons test was performed. Abbreviation: CRP = C-reactive protein
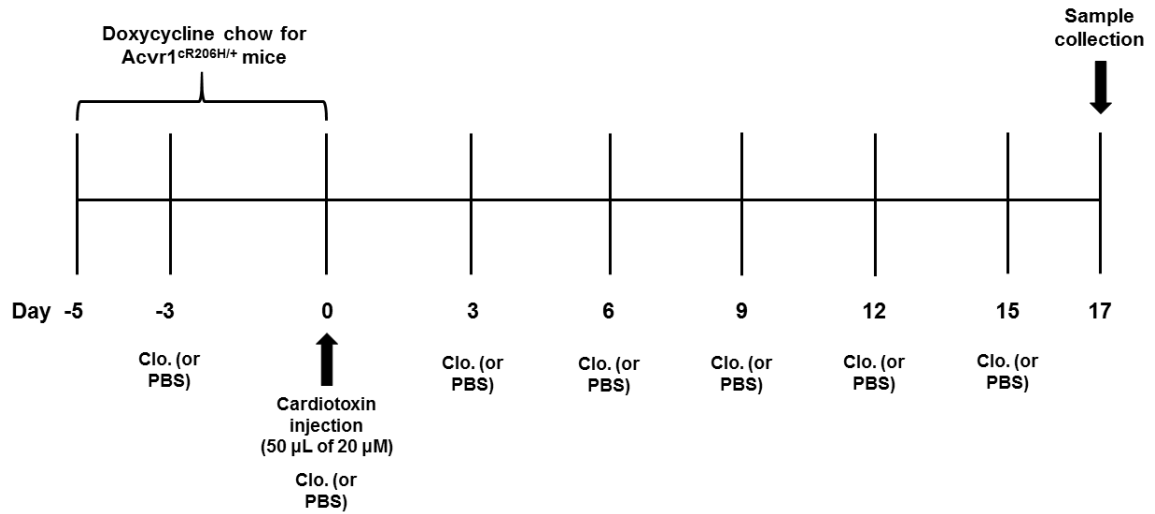
Figure 4.7 Clodronate-liposome-mediated macrophage depletion experimental design *Acvr1cR206H/+* mice were placed on doxycycline chow (625 mg / kg) for five days prior to injury to induce expression of the conditional *Acvr1cR206H* allele. Clodronate-liposomes (100 μL / 10 g bodyweight) were injected intraperitoneally into appropriate mouse cohorts at days indicated; an injection was performed 3 days prior to cardiotoxin injection in order to deplete macrophages at the time of injury. Clodronate-liposomes were injected every 3 days following this first injection to maintain macrophage depletion throughout study. Hamstring muscles were injected with cardiotoxin (50 μL of 20 μM) and samples were collected at Day 17 post-injury. Abbreviation: Clo. = clodronate-liposomes

Figure panel from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1R206H* Mouse Model of Fibrodysplasia Ossificans Progressiva"
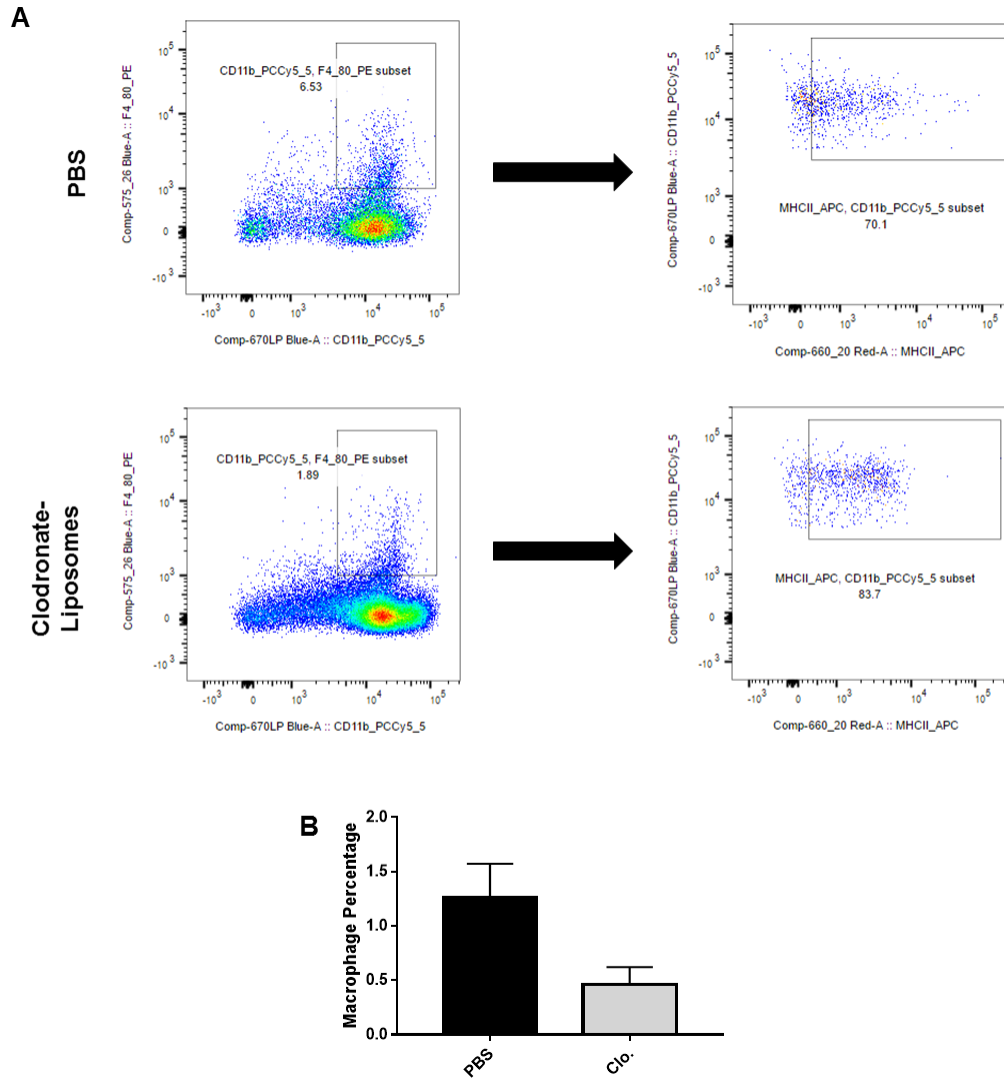
Figure 4.8: Confirmation of macrophage depletion in clodronate-liposome mice
(A) Whole bone marrow was collected from *Acvr1^cR206H/+* mice injected with Clo. or PBS and stained with fluoro-tagged primary antibodies against F4/80, CD11b, and MHC Class II, cell surface receptors that in combination identify macrophages. Cells were first gated for F4/80 and CD11b expression, and then double-positive cells were gated for MHC Class II expression. Numbers listed are cell percentages per gating. (B) Bone marrow macrophage percentages in PBS versus Clodronate-liposome-injected mice. n = 18 for PBS, n = 16 for Clo.; data shown are mean values ± SEM. Abbreviation: Clo. = clodronate-liposomes

Figure panel from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^R206H* Mouse Model of Fibrodysplasia Ossificans Progressiva"
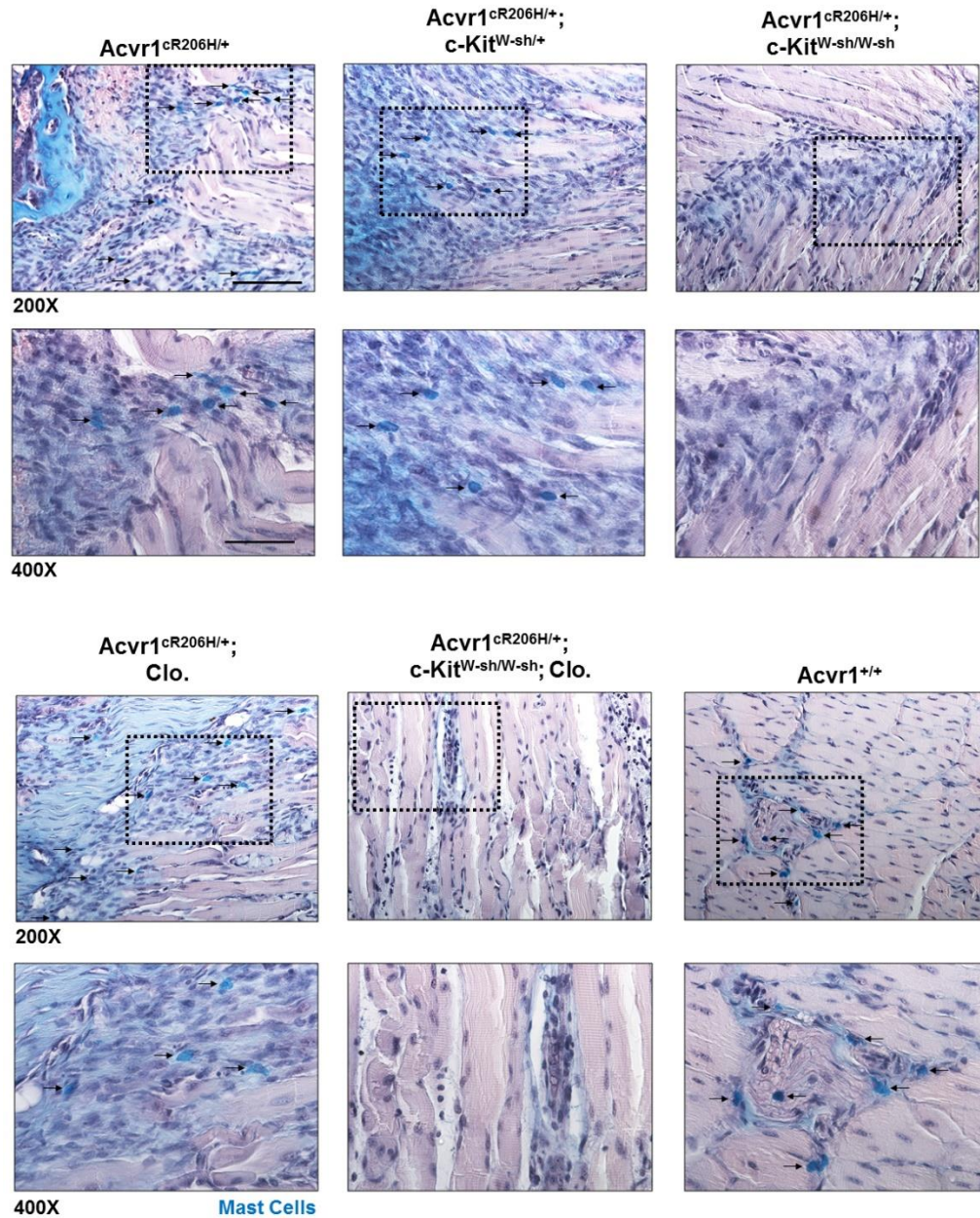
Figure 4.9: Confirmation of mast cell deficiency in *c-Kit^{W-Sh/W-sh}* mice

Mast cells were quantified by C.E.M. stain kit in sections obtained at sacrifice from all cohorts analyzed in Figure 4.10. Representative images at 200X magnification of each cohort are shown, with dotted rectangles indicating further magnification at 400X. Mast cells are indicated by black arrows. Note absence of mast cells in *Acvr1^{cR206H/+};c-Kit^{W-Sh/W-sh}* and *Acvr1^{cR206H/+};c-Kit^{W-Sh/W-sh}*;Clo. mouse cohorts. Scale bar = 100 μm for 200X, 50 μm for 400X. Abbreviation: Clo. = clodronate-liposomes

Figure panel from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^{R206H}* Mouse Model of Fibrodysplasia Ossificans Progressiva"

128
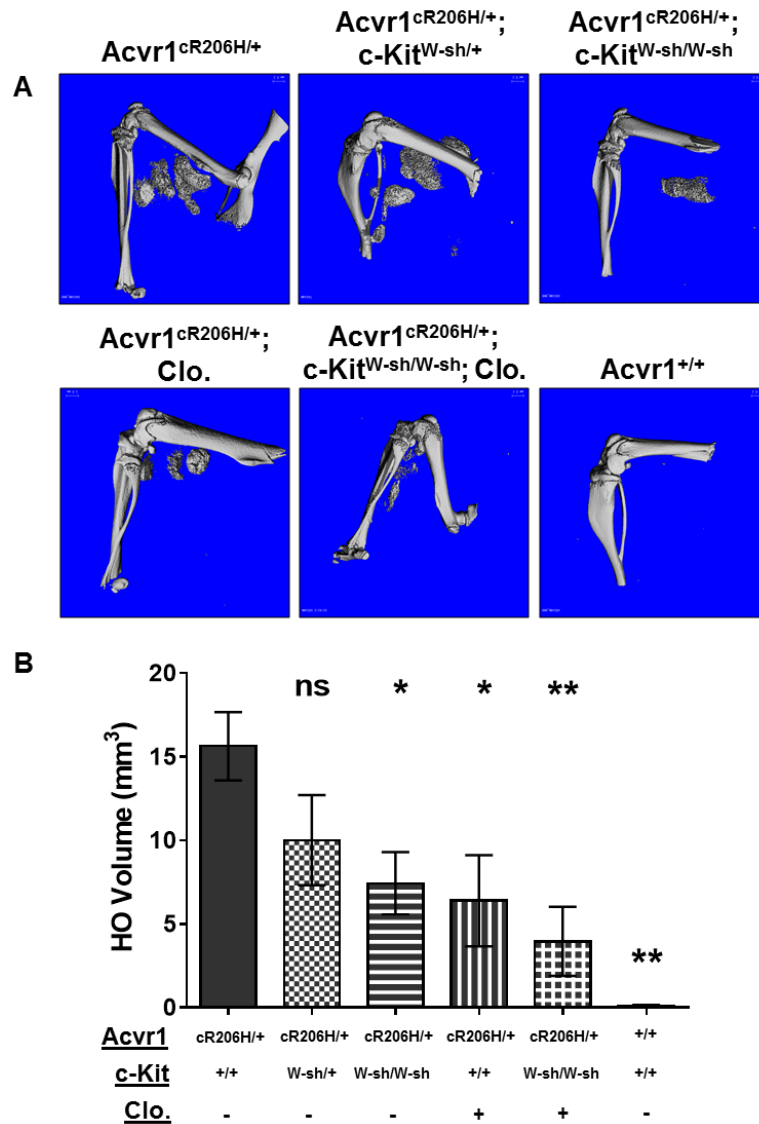
Figure 4.10: Mast cell and macrophage depletion impairs formation of heterotopic ossification in *Acvr1^cR206H/+* mice.

Cardiotoxin-injured skeletal muscles of *Acvr1^cR206H/+* mice with intact or depleted mast cells and/or macrophages were examined by μCT to detect heterotopic ossification after 17 days. Mast cells were genetically ablated in *Acvr1^cR206H/+*;*c-Kit^W-sh/W-sh* mice or partially ablated in *Acvr1^cR206H/+*;*c-Kit^W-sh/+* mice. Macrophages were chemically depleted Clo. Data are compared to *Acvr1^cR206H/+* (positive HO control) and *Acvr1^+/+* (negative control). (A) Representative μCT 3D volume renderings showing mean HO volume per hind limb are shown. (B) Quantification of HO for each cohort (n = 5-20); HO per hind limb was added for total HO per mouse. Data shown are mean values ± SEM; one-way ANOVA with Tukey's multiple comparisons test compared *Acvr1^cR206H/+* vs. other cohorts; * p < 0.05, ** p < 0.01, ns = not significant. Abbreviation: Clo = Clodronate-liposomes.

Figure panel from Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, Kaplan FS, Shore EM. (*submitted*). "Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an *Acvr1^R206H* Mouse Model of Fibrodysplasia Ossificans Progressiva"

129

Chapter Five

Discussion

5.1 Summary

Inflammation is the earliest recognized event of HO formation. Long before the publication reporting the identification of the FOP gene in spring 2006 [Shore et al. 2006], the presence of an inflammatory component was thought to be an essential feature of disease etiology. Identification of the mutation that causes FOP rapidly expanded the knowledge and research tools that enabled investigation of how HO forms in FOP. Studies using the *in vitro* and *in vivo* FOP model systems that followed discovered that the ACVR1 R206H mutation confers increased BMP pathway signaling and accelerates chondrogenesis of fibroblastic progenitor cells. However, similar substantial advances in knowledge regarding the inflammatory role in HO formation remained limited.

In this thesis research, I have conducted the first in-depth investigation on the effects of $Acvr1^{R206H}$ on the inflammatory response in FOP. I focused specifically on the impact of $Acvr1^{R206H}$ on the cellular and molecular inflammatory response to skeletal muscle injury, including the tissue regeneration program. I also investigated whether $Acvr1^{R206H}$ hematopoietic cells are sufficient to induce HO formation in genetically normal backgrounds. Lastly, I examined whether immune cells play a significant role in inducing HO formation via mast cell- and macrophage-depleted $Acvr1^{cR206H/+}$ mouse experiments. Given the shared immunological features of ectopic bone formation in FOP and non-genetic HO disorders, our studies bring new insight into the immunological contributions to HO disorders. These data also support consideration of novel treatment approaches for both FOP and more common non-hereditary forms of HO, potentially benefiting a wide range of patients.

5.2 Discussion

5.2.1 The effect of $Acvr1^{R206H}$ on the inflammatory response to tissue injury

An inflammatory milieu is the earliest recognized phase of lesion formation in FOP, as discussed in Chapter One (Figure 1.4). The response of immune cell influx and cytokine upregulation is qualitatively mirrored in the normal response to tissue injury, however, as discussed in Chapter One (Figure 1.4) and shown in Chapter Three (Figures 3.2, 3.3, 3.4), $Acvr1^{cR206H}$ mutant tissue does not appropriately repair and regenerate skeletal muscle to its pre-injury state, but instead progresses toward heterotopic endochondral ossification. Previous clinical reports and mouse model studies have demonstrated the importance of active inflammation for HO disease progression [Kaplan et al. 2007, Yu et al. 2008], yet no group conclusively demonstrated that the immune response is amplified in FOP. Given the established participation of BMP signaling in activating an inflammatory state [Mohler et al. 2001, Kwon et al. 2009, Lee et al. 2011, Lee et al. 2012, Martinez et al. 2015, Villavicencio et al. 2016], I hypothesized that the enhanced BMP pathway activation by $Acvr1^{cR206H}$ amplifies the inflammatory response to tissue injury beyond the normal cellular and molecular response. To address this hypothesis, I performed a cross-sectional injury experiment (Chapter Three), comparing the inflammatory response and tissue repair programs between $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ mice. I observed that mutant tissue and normal tissue are indistinguishable up to 2-3 days post-injury; however, $Acvr1^{cR206H/+}$ lesions progressively diverged toward terminal heterotopic endochondral ossification instead of appropriately regenerating skeletal muscle, as occurred in $Acvr1^{+/+}$ controls. I showed that the cellular and molecular inflammatory response is robustly amplified in

132

Acvr1$^{cR206H/+}$ mice and persists into the late-stage lesion phase, as opposed to returning to pre-injury levels. Furthermore, I showed that *Acvr1^{cR206H}* exhibits cell-autonomous pro-inflammatory effects in mast cells, as *Acvr1$^{cR206H/+}$* mast cells exhibited increased pro-inflammatory mRNA expression and degranulation.

During the normal skeletal muscle repair program, the early pro-inflammatory response dampens and transitions into an anti-inflammatory response, mediated primarily by M2 macrophages, that facilitates wound repair and tissue regeneration via secretion of IL-4, IL-10, and growth factors [Tidball 2005, Karalaki et al. 2009, Murray et al. 2011, Philippou et al. 2012]. Disruption of this pro-inflammatory to anti-inflammatory transition leads to chronic inflammation and deficient tissue regeneration. Our *in vivo* and *in vitro* data reflect a chronic inflammatory scenario in the presence of the ACVR1 R206H mutation.

The overall amplified and prolonged immune response in FOP could be, at least in part, the result of deficient MSC immunomodulatory function. Upon inflammatory stimulation by lymphocytes and secreted factors such as IFN-γ, IL-1β, and TNFα, MSCs subsequently suppress prolonged inflammation through production of indolamine 2,3-dioxygenase (IDO) and T$_{reg}$ induction in humans, and nitric oxide (NO) via expression of inducible nitric oxide synthase (iNOS) in mice [Shi et al. 2010]. MSCs are able to suppress T and B cell proliferation via cell-cell contact through the programmed death-1 (PD-1) signaling pathway [Augello et al. 2005], NK cell proliferation [Sotiropoulou et al. 2006], and dendritic cell maturation [Zhang et al. 2004]. MSCs are also capable of reduction T cell and monocyte production of IFN-γ and TNFα [Ben-Ami et al. 2011]. Notably, IL-6

produced by MSCs protects lymphocytes and neutrophils from apoptosis [Xu et al. 2007, Raffaghello et al. 2008]. IL-6 is robustly expressed in $Acvr1^{cR206H/+}$ lesions (Figure 3.9), which may contribute to significantly elevated neutrophil and lymphocyte density (Figure 3.8A, C). Investigation of MSC immunomodulary function in FOP could be accomplished via co-culture experiments combining MSCs and immune cell populations, with immune cell proliferation and activation as the primary data point. Assessing MSC immunomodulary function in FOP is a novel area for future investigation.

Our findings show for the first time that the ACVR1 R206H mutation amplifies and prolongs the usual inflammatory response present in tissue injury, mimicking a chronic inflammatory state.

5.2.2 The role of immune cells and inflammatory activation in heterotopic ossification formation

The origin of the progenitor cells that directly differentiate to ectopic bone is a major ongoing research question. Much progress has been made, as several chondro-/osteogenic populations have been suggested to directly contribute to HO in vivo. [Lounev et al. 2009, Wosczyna et al. 2012, Agarwal et al. 2016, Dey et al. 2016]. Other work has also conclusively eliminated the hematopoietic cell lineage as contributing cells to fibroproliferative, chondrogenic, and osteogenic stages of HO [Kaplan et al. 2007]. Given this supporting literature, an unresolved question is what is the role of immune cells in the etiology of HO development, particularly within the context of FOP.

Lesion development in FOP occurs through a series of progressive inflammatory, fibroproliferative, and chondro-/osteogenic cellular and tissue events that results in mature HO. One possibility is that $Acvr1^{R206H/+}$ immune cells are sufficient to initiate and support all stages of lesion formation, from immune cell influx to mature HO. This is an appealing mechanism given that immune cells secrete numerous chemokines and growth factors that support recruitment and proliferation of muscle tissue fibroblasts [Karalaki et al. 2009, Philippou et al. 2012], as well as previous work showing that $Acvr1^{+/+}$ cells can participate in heterotopic endochondral ossification in a mouse model that contains both $Acvr1^{+/+}$ and $Acvr1^{R206H}$ cells [Chakkalakal et al. 2012]. One specific cytokine secreted by activated macrophages is PDGF [Karalaki et al. 2009, Philippou et al. 2012], which is notable given that PDGFRα receptor and cells within muscle tissue exhibit robust BMP-induced osteogenic capacity and participated in HO formation following intramuscular transplant, even in the absence of the ACVR1 R206H mutation [Wosczyna et al. 2012]. Increased activation of PDGFRα disrupts connective tissue development and drives systemic fibrosis [Olson et al. 2009], and a recent publication reported that expression of an endogenous kinase-inactivated decoy PDGFRα receptor during tissue repair attenuates muscle fibrosis [Mueller et al. 2016]. Given previous data and supporting literature, I hypothesized that an $Acvr1^{R206H/+}$ hematopoietic system is sufficient to induce HO formation in an $Acvr1^{+/+}$ background.

Our results from Chapter Four oppose the hypothesis that $Acvr1^{R206H}$ hematopoietic cells alone are sufficient to induce HO formation in a normal background. My bone marrow transplant (BMT) experiment was the most direct approach to address this question,

however this experiment was inconclusive due to experimental design limitations (as discussed in Chapter 4). Bone marrow is a hetergeneous mixture comprised of HSCs, various precursor and mature immune cells, stromal cells, and fat cells [Reagan et al. 2016]. The donor bone marrow sample used for the BMT experiment was not fractionated into an HSC-pure CD34-positive population [Challen et al. 2009] prior to injection. This allows for the possibility of bystander stromal and/or fat cells with chondro-/osteogenic potential being transplanted simultaneously as HSCs, which may contribute to HO formation when expressing *Acvr1^{R206H}* [Augello et al. 2005].

Therefore, I conducted an alternate genetic approach to address the sufficiency of hematopoietic cells for *Acvr1$^{R206H/+}$*-mediated HO, generating *LysM*-Cre;*Acvr1$^{cR206H/+}$* mice that express *Acvr1^{cR206H}* exclusively within myeloid lineages. The results are corroborated by an independent group that recently reported no HO formation in mice expressing the constitutively-active *Acvr1^{Q207D}* receptor in all hematopoietic lineages (as well as endothelial cells) driven by the *Vav1*-Cre promoter [Dey et al. 2016].

Our finding that HO formation was dramatically reduced in mast cell-deficient and macrophage-depleted *Acvr1$^{cR206H/+}$* mice (Chapter Four) suggests that immune cells are necessary participants for HO formation, even if they are alone insufficient to induce HO. Previous studies have reported similar results in non-FOP mouse models of HO [Kan et al. 2009, Salisbury et al. 2010, Kan et al. 2011, Salisbury et al. 2011], bolstering the importance of mast cells and macrophages in HO pathology.

In the context of FOP and HO formation, what are the functions of mast cells and macrophages? During the normal skeletal muscle regeneration program, these cells

potentiate an inflammatory microenvironment, produce enzymes that digest damaged tissue, and recruit fibroproliferative cells that act as intermediaries during normal tissue regeneration [Tidball 2005, Karalaki et al. 2009, Galli et al. 2011, Murray et al. 2011, Philippou et al. 2012, Wynn et al. 2013]. During chronic inflammatory myopathies, the resident inflammatory response is exaggerated, leading to disrupted tissue regeneration and macrophage- and mast cell-mediated fibrosis [Gallant-Behm et al. 2008, Zhao et al. 2008, Levick et al. 2009, Wynn et al. 2010, Philippou et al. 2012, Hugle 2014]. In a non-FOP context, the terminal result of chronic inflammatory myopathy is fibrosis [Wynn 2008, Wynn et al. 2012], however in the context of FOP, the enhanced BMP pathway signaling conferred by $Acvr1^{R206H}$ re-directs the tissue response toward ectopic cartilage and bone [Shen et al. 2009, Culbert et al. 2014]. Our findings from Chapter Three suggest that the amplified and prolonged inflammatory response in an $Acvr1^{cR206H/+}$ background mimics chronic inflammatory myopathy and establishes a conducive microenvironment for the pro-chrondrogenic and pro-osteogenic effects of Acvr1^{R206H} to manifest. Depleting mast cells and macrophages from $Acvr1^{cR206H/+}$ mice may simultaneously reduce levels of multiple inflammatory cytokines, chemokines, and fibroblast growth factors that in unison arrest HO formation (discussed further in Future Directions).

The data presented in this thesis and supporting literature allow consideration of the inflammatory role in FOP disease pathology. The $LysM$-Cre;$Acvr1^{cR206H/+}$ data showing no HO response, in combination with the report that no HO was detected in $Vav1$-Cre;$Acvr1^{Q207D}$ mice, and additional studies that investigated lesion development at different stages, can help direct several remaining questions for FOP etiology, including:

what cell types have the potential to contribute to ectopic cartilage and bone, what cell types require the ACVR1 R206H mutation for participation in lesion development, what are the contributions of tissue-resident vs. migratory progenitor cells, and what is the overall immune system role in disease progression.

Several cell populations with the capacity to contribute to ectopic cartilage and bone have been identified. Our group identified Tie-2$^+$ cells within the fibroproliferative, cartilage, and bone stages of lesion progression in vivo [Lounev et al. 2009]. Double-positive Tie-2$^+$/PDGFRα^+ cells were shown to contribute to HO in response to BMP2 stimulation [Wosczyna et al. 2012]. Two additional cell populations that can contribute to HO formation have also been identified: Mx1$^+$ muscle-resident interstitial cells, and Scx$^+$ tendon-derived progenitor cells [Agarwal et al. 2016, Dey et al. 2016]. Both Mx1$^+$ and Scx$^+$ cells that participated in HO expressed an *Acvr1* mutation, R206H or Q207D; however, Tie-2$^+$ and Tie-2$^+$/PDGFRα^+ cell participation in HO was in a BMP2 stimulation model, and these cells did not express an *Acvr1* mutation, but showed enhanced osteogenesis in response to BMPs [Lounev et al. 2009, Wosczyna et al. 2012].

Fibro/adipogenic progenitors (FAPs) are muscle-resident cells that support proper skeletal muscle regeneration following damage [Joe et al. 2010]. FAPs are defined by a Lin$^-$/Sca-1$^+$/α7 integrin$^-$/CD34$^+$ expression profile, with over 85% of cells in undamaged muscle and up to 98% of cells in damaged muscle also expressing PDGFRα [Joe et al. 2010]. In response to muscle damage, FAPs expand and provide important pro-differentiation signals for proliferating myogenic precursors. Although initially recognized for their ability to differentiate toward fibrogenic and adipogenic lineages, in addition to

138

their primary supportive role for myogenic differentiation, FAPs have recently been appreciated for their role in ectopic bone formation. FAPs express alkaline phosphatase, a key enzyme for mineral deposition during osteogenesis, following stimulation with BMP7 [Uezumi et al. 2010]. Additionally, the muscle-resident Tie-2$^+$/PDGFRα$^+$ cells that can contribute to HO formation in response to BMP2 stimulation, described above [Wosczyna et al. 2012], may be classified as FAPs. The R206H mutation has been shown to accelerate chondrogenic differentiation in MEF cells [Culbert et al. 2014], and it is possible that the mutation may also alter the cell fate of FAPs toward a fibrogenic and heterotopic endochondral ossification differentiation program [A. Bonomi, personal communication].

The above data suggest that while certain cell populations are able to contribute to HO when expressing *Acvr1^{R206H},* and that the R206H mutation is required for initiation of HO in FOP [Chakkalakal et al. 2012], the R206H mutation may not be required in all cell types that particpate in lesion development, including immune cells and fibroblast-like cells that form ectopic cartilage and bone. This was also demonstrated in a chimeric *Acvr1$^{R206H/+}$* mouse model where ~50% of cells in endochondral bone lesions expressed *Acvr1$^{+/+}$* [Chakkalakal et al. 2012]. This surprising result suggests that there may be a threshold percentage and/or lineage subset requirement of cells expressing *Acvr1^{R206H}* in order to support HO formation. Mice expressing *Acvr1^{R206H}* in mesodermal lineage cells using a *Prrx1*-Cre driving system developed robust HO in limbs [Chakkalakal et al. 2016], supporting the possibility of a threshold percentage and lineage restriction of cells with the capacity to form ectopic cartilage and bone.

An ongoing question is the location origin of cells that participate in ectopic cartilage and bone formation. Tissue-resident cells, such as FAPs and other fibroblast-like cells that have been shown to have chondrogenic and osteogenic potential, are prime candidates for cells that form HO. However, there also have been migratory cell populations shown to participate in HO formation. Cells from the endoneurium, which line the myelin sheath of nerves, were observed migrating toward an implanted matrigel BMP2 source and forming HO at their new location in a mouse model of HO formation [Olmsted-Davis et al. 2017]. These cells expressed osterix, a transcription factor that functions as a master regulator of osteoblast differentiation. Additionally, osterix$^+$ cells were observed in proximity to ectopic bone in human patients with HO [Olmsted-Davis et al. 2017]. Immune cells, through chemokine ligand and receptor signaling pathways, have significant roles in recruiting cells to participate in wound healing and immunoprotection of multiple tissues, including skin [Ishida et al. 2007], skeletal muscle [Warren et al. 2005], and lung [Mikhak et al. 2013]. Mast cells contribute to activation of the sympathetic nervous system via degranulation [Salisbury et al. 2011], and mast cells and macrophages control early neutrophil recruitment via the chemokines CXCL1 and CXCL2 [De Filippo et al. 2013]. Given the increased and prolonged immune cell density and activation observed in $Acvr1^{cR206H/+}$ samples, it is possible that the amplified immune response may induce fibroblast-like cell migration, or amplify the migration rate over the normal repair program.

Although $Acvr1^{R206H}$ expression restricted to hematopoietic lineages is insufficient to cause HO, soft connective tissue repair programs may still be disrupted in this setting. Elevated inflammatory factor expression caused by the R206H mutation in the above

setting may still disrupt the normal skeletal muscle regeneration program, but instead of progressing to mature HO, the tissue morphology only advances to a fibrotic stage, mimicking events that occur in chronic inflammatory myopathies [Mann et al. 2011]. Consideration of the data presented in this thesis, in combination with supporting literature, may explain how the response to skeletal muscle injury may differ depending on the inflammatory reaction and whether the R206H mutation is present.

During the normal skeletal muscle regeneration program, pro-inflammatory activation induces a cellular and molecular response that results in immune cell recruitment and macrophage phagocytosis of damaged tissue and transient accumulation of collagen fibers [Mann et al. 2011]. An anti-inflammatory response, mediated primarily by the class-switching from M1 to M2 macrophages, dampens the pro-inflammatory response and supports myogenic differentiation to restore muscle function. In individuals with chronic inflammatory myopathies, excessive pro-inflammtory activation and elevated numbers of M1 macrophages cause an overabundance of collagen accumulation and impede myogenic differentiation, resulting in fibrosis [Wynn et al. 2010, Mann et al. 2011]. However, since these individuals do not possess the R206H mutation, fibrotic tissue does not progress to ectopic cartilage and bone. In patients with FOP, the R206H mutation amplifies and prolongs the pro-inflammatory response to muscle injury, mimicking chronic inflammation. This overactive immune response may stimulate tissue-resident progenitor cell proliferation, and may recruit additional fibroproliferative cells to the site of injury. These fibroproliferative cells, also expressing $Acvr1^{R206H}$, have the capacity to aberrantly differentiate into ectopic cartilage and bone, resulting in mature HO [Culbert et al. 2014].

141

Mast cells expressing *Acvr1*^R206H^ exhibit an increased pro-inflammatory response, and other immune cells may have a similar inflammatory response. Immune cells expressing *Acvr1*^R206H^ can further amplify the immune response to injury, but cell(s) with chondro-/osteogenic potential must also express the R206H mutation in order for chondrogenesis and osteogenesis to form, as *Acvr1*^R206H^ expression restricted to hematopoietic lineages is insufficient for HO formation (Figure 4.5; [Dey et al. 2016]). Additionally, *Acvr1*^R206H^ expression in immune cells is not required for HO to develop, as has been observed in a *Prrx1*-Cre;*Acvr1*^cR206H/+^ mouse model and a human patient who received an BMT of *Acvr1*^+/+^ donor marrow for treatment of aplastic anemia [Kaplan et al. 2007]. The above literature and data from this thesis suggest that while an immune system expressing *Acvr1*^R206H^ may support a more robust HO response in a global R206H background, a wild-type immune system is also sufficient for HO formation in the same genetic background. Based on this paradigm, the importance of the immune response in FOP may reside in secreted inflammatory factors from immune cells, more so than whether immune cells express the R206H mutation. This is further supported given that non-hereditary HO can form in absence of any causative *ACVR1* mutation, but is often associated with a robust inflammatory phase.

5.2.3 The shared inflammatory features of FOP and non-hereditary heterotopic ossification disorders

An inflammatory component is associated with almost all forms of HO [Convente et al. 2015], including non-hereditary HO disorders associated with severe trauma such as

invasive arthroplasties [Pignolo et al. 2005, Cohn et al. 2011, Bedi et al. 2012], combat blast injuries, and other traumatic wounds [Potter et al. 2010, Alfieri et al. 2012]. Although FOP is an extremely rare genetic disorder, the prevalence of HO in the above scenarios is common, approaching 30% following hip replacement surgery and 65% following combat blast injuries [Neal et al. 2002, Potter et al. 2010]. Strikingly, non-hereditary HO disorders and FOP share immunological mediators. Serum levels of IL-6 were significantly elevated in combat blast injury patients who developed HO [Evans et al. 2012, Forsberg et al. 2014], which is notable given the robust increase in IL-6 in $Acvr1^{cR206H/+}$ lesions and $Acvr1^{cR206H/+}$ mast cells (Chapter Three). MCP-1 was identified as a biomarker that predicted HO development in combat blast injury patients [Evans et al. 2012]. Although MCP-1 expression was equivalent between $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ lesions (Chapter Three), its presence in the FOP setting could contribute to the chronic inflammatory response.

An influx of immune cells is also observed in non-hereditary HO disorders. A robust T- and B-cell influx to sites of injury was shown in a burn injury HO mouse model. In this same study, ablation of mature lymphocytes using Rag1 knock-out mice caused a 60% decrease in bone compared to immunocompetent controls, demonstrating a prominent role for the adaptive immune system in burn injury HO formation [Ranganathan et al. 2016]. Additionally mast cells have been observed in cardiac valve bone formation as well as in BMP ligand-induced HO episodes [Mohler et al. 2001, Kan et al. 2011, Salisbury et al. 2011].

While there may be unique inflammatory contributions to FOP, such as a gain-of-function Acvr1^{R206H} sensitivity to Activin A [Hatsell et al. 2015], common inflammatory

mediators across HO disorders establish the immune system as an appealing treatment target that may benefit large numbers of patients (to be discussed in Future Directions).

5.3 Future Directions

5.3.1 Identifying additional immune cells and inflammatory factors involved in heterotopic ossification etiology

The immune system is an incredibly rich and complex network of cells and factors that act in concert to direct inflammatory responses. There exists dozens of hematopoietic subtypes, interleukins, chemokines, and other mediators that function in response to pathogen invasion and tissue damage [Charo et al. 2006, Akdis et al. 2011, Turner et al. 2014, de Graaf et al. 2016, Vukman et al. 2017]. Our data show upregulation of major pro-inflammatory cytokines in $Acvr1^{cR206H/+}$ mice following injury, including TNFα, IL-1β, IL-6, and Activin A. I also show increased neutrophil, macrophage, mast cell, and T cell density in $Acvr1^{cR206H/+}$ lesions (Chapter Three). However, there are additional immune cells yet to be investigated that may contribute to HO formation, such as T cell subsets (T helper 1 cells, T helper 2 cells, T helper 17 cells, and others), B cells, dendritic cells, and NK cells. I showed that mast cell and macrophage depletion reduced HO by ~75% in $Acvr1^{cR206H/+}$ mice (Chapter Four), suggesting that other inflammatory cells and mediators may have a role in HO development. T cells and B cells have been observed in FOP patient biopsies and in cardiac valve HO [Mohler et al. 2001, Kaplan et al. 2005], and our results have demonstrated a significant role for myeloid lineage cells in HO formation (Chapters Three and Four). These data and supporting literature suggest that dendritic cells and NK cells, which function as intermediaries between the innate and adaptive immune system, may be an appealing area of future investigation.

I focused on investigating the well-defined major pro-inflammatory cytokines TNFα, IL-1β, IL-6, given their significant roles in mediating positive-feedback inflammatory responses [Akdis et al. 2011, Turner et al. 2014] and known involvement in tissue injury response [Gallucci et al. 1998, Luo et al. 2003, Karalaki et al. 2009, Philippou et al. 2012]. Appealing future experiments could investigate a broader profile of inflammatory mediators, including interferons, chemokines, and mast cell-released compounds such as histamine and chymase, that may be involved as part of the total inflammatory contribution to HO formation. The chemokine CX3CR1 is involved in skin wound repair via recruitment of fibroblasts [Ishida et al. 2007], and CXCR6 recruits bone marrow-derived fibroblast precursors that increase susceptibility to renal fibrosis [Xia et al. 2014], highlighting the role of chemokines as potent chemotactic factors for fibro-progenitor cells in injury settings. CX3CR1 is produced by macrophages and CXCR6 is produced by CD4$^+$ and CD8$^+$ T cells [Charo et al. 2006], two immune cell lineages present in FOP lesions.

5.3.2 Investigating the role of *Acvr1^{R206H}* on anti-inflammatory responses during tissue injury

The normal inflammatory response to tissue injury involves an early phagocytic pro-inflammatory phase that eventually transitions into a subsequent regenerative anti-inflammatory phase. Our results from Chapter Three demonstrate that the cellular and molecular pro-inflammatory response in FOP is amplified and prolonged, mimicking chronic inflammatory myopathies.

146

A major event that directs the transition of pro-inflammation to anti-inflammation is the class-switching of M1 macrophages to M2 macrophages [Italiani et al. 2014]. M2 macrophages appear subsequent to early-responding M1 macrophages during the normal tissue repair program. M2 macrophages secrete anti-inflammatory factors, including IL-4, IL-10, TGF-β1, and tissue inhibitors of metalloproteinases (TIMPs), as well as growth factors that suppress fibrosis and mediate tissue regeneration [Mosser et al. 2008, Wynn et al. 2012]. In chronic inflammatory myopathies, the transition of M1 to M2 macrophages is disrupted, maintaining the pro-inflammatory microenvironment that promotes fibrosis at the expense of appropriate tissue regeneration [Wynn et al. 2012].

Our macrophage depletion experiment via clodronate-liposomes depleted macrophages prior to injury and throughout the response and repair program (Chapter Four). An appealing future experiment would be to adjust the timing of macrophage depletion so as to specifically target early M1 or late M2 macrophages for depletion, and examine how that impacts HO formation. Previous work in the musculoskeletal field showed that pro-inflammatory M1 macrophages are essential for skeletal muscle regeneration [Arnold et al. 2007]. However, this study also showed that selectively depleting M2 macrophages after the M1 response resulted in diminished muscle fiber diameter, suggesting that both populations are essential for proper skeletal muscle regeneration [Arnold et al. 2007]. A preliminary experiment quantifying specific M1 and M2 macrophage populations in $Acvr1^{cR206H/+}$ and $Acvr1^{+/+}$ lesions using well-defined markers [Jablonski et al. 2015] would provide initial insight about a more detailed macrophage phenotype in FOP.

A key lymphoid cell involved in dampening inflammation is the regulatory T cell (T_{reg}). T_{regs} are derived from $CD4^+$ T cells and depend on the forkhead transcription factor FoxP3 for specification and function [Josefowicz et al. 2012]. T_{regs} are major sources of IL-10 and IL-2R that function to suppress T cell activation and maintain immune homeostasis in part by inducing $CD4^+$ T cell apoptosis [Pandiyan et al. 2007, Josefowicz et al. 2012]. In the context of muscular disorders, T_{regs} suppress muscle inflammation and injury in the *mdx* mouse model of Duchenne Muscular Dystrophy [Villalta et al. 2014], highlighting a potential role for T_{regs} reducing inflammation in other muscular injury settings, such as occurs in HO disorders.

Future work could also quantify levels of anti-inflammatory cytokines, such as IL-4, IL-10, and TGF-β1. This could be done in primary mast cell and macrophage cultures, as well as serum enzyme-linked immunosorbent assay (ELISA) quantification from new in vivo samples. IHC using sections from the cross-sectional injury study. I quantified TGF-β1 mRNA expression in *Acvr1*$^{cR206H/+}$ and *Acvr1*$^{+/+}$ mast cells and macrophages, but found no differences in expression (Figure 3.15A, B). These preliminary data suggest that the anti-inflammatory response may be unaffected by the ACVR1 R206H mutation, but much additional work must be done for conclusive interpretation.

5.3.3 Targeting the immune system as a novel approach to inhibit heterotopic ossification

There is a pressing need for therapeutics that prevent HO formation in FOP and non-hereditary HO disorders, as current treatments are palliative [Pignolo et al. 2016].

Palovarotene, a retinoic acid receptor-gamma (RARγ) agonist that potently inhibits HO formation in $Acvr1^{cR206H/+}$ mice by disrupting chondrogenesis [Chakkalakal et al. 2016], is currently being investigated in a clinical trial for FOP patients. This represents a major milestone in the search for an effective treatment or cure for FOP. Efforts to find additional treatments for HO are ongoing, and targeting the immune system is an appealing approach given that it is the earliest phase of lesion formation.

Blocking mast cell and macrophage signaling may be an effect method to inhibit HO formation, both in FOP and non-hereditary HO disorders. Multiple methods that block mast cell inflammatory signaling, including targeting the Substance P neuroinflammatory pathway, all inhibited HO formation in a BMP2-implant model of HO [Kan et al. 2011, Salisbury et al. 2011]. An additional option targeting mast cells is the use of imatinib, a c-Kit tyrosine kinase inhibitor that induces mast cell apoptosis. Imatinib has been shown to reduce rheumatoid arthritis inflammation [Juurikivi et al. 2005] and decreased HO in an Achilles tendon injury model of HO [Werner et al. 2013]. Our group showed that imatinib reduced hypoxia-induced HO formation, further bolstering the use of imatinib as an effective HO therapeutic, as well as highlighting the role of hypoxia in HO pathology [Wang et al. 2016]. Notably, imatinib robustly decreases PDGFR phosphorylation and PDGF effector signaling [van Steensel et al. 2009], suggesting its inhibitory effect may be through downregulating the PDGF signaling pathway, and also highlighting PDGF as a potential key factor in HO development.

An additional approach to inhibit mast cell activation is via administration of mast cell stabilizers that block degranulation and release of inflammatory factors. Cromolyn,

quercetin, and other small molecules that block degranulation may be effective and inhibiting HO by preventing the release of cytokines, chemokines, and enzymes that participate in fibrosis during chronic inflammatory myopathies [Galli et al. 2008, Weng et al. 2012, Vukman et al. 2017].

Macrophages have been shown to produce osteoinductive signals [Champagne et al. 2002], promote osteogenic differentiation of mesenchymal stem cells [Tu et al. 2015], inhibit osteoclastogenesis [Jacquin et al. 2009], and promote fracture repair [Alexander et al. 2011], highlighting their potential relevance to HO development. I utilized clodronate-liposomes to transiently deplete macrophages in our immunodeficient $Acvr1^{cR206H/+}$ mouse studies. Although this approach is normally used only for proof-of-principle animal experiments, at least one attempt in humans has been reported [Barrera et al. 2000]. Patients scheduled for knee joint replacement received a single intra-articular injection of clodronate-liposomes into the knee synovium. Biopsy analysis post-surgery showed significantly decreased numbers of CD68$^+$ macrophages, demonstrating drug efficacy. However, this study has significant limitations, as it included only 10 patients receiving a single clodronate-liposome injection. Due to hematopoietic replenishment of depleted macrophages, clodronate-liposomes would have to be administered regularly to maintain cell depletion if used as treatment for HO. Therapeutics that target macrophage activation and inflammatory signaling are a much preferred approach.

Overall, targeting the immune system for treatment of HO in an appealing approach due to early intervention in disease, offering the possibility of halting lesion progression long before maturation into chondro-osseous tissue. Additionally, considering the shared

immunological contributions among FOP and many HO disorders, advancements in this area may provide benefits for FOP and more common non-hereditary forms of HO, which could significantly benefit a wide range of patients.

5.4 Concluding Remarks

In this thesis, I elucidated the pro-inflammatory role of Acvr1^{R206H} in response to skeletal muscle injury and demonstrated via *in vivo* and *in vitro* experiments an amplified and prolonged cellular and molecular inflammatory response that mimics chronic inflammation. I also demonstrated that *Acvr1$^{cR206H/+}$* hematopoietic cells, via BMT and myeloid-specific *Acvr1$^{cR206H/+}$* expression experiments, are insufficient to induce HO formation in an *Acvr1$^{+/+}$* background. Finally, I showed that mast cell and macrophage depletion significantly reduces HO development in *Acvr1$^{cR206H/+}$* mice. In total, these data suggest a role for Acvr1^{R206H} in upregulating the inflammatory response in damaged tissue and recruited immune cells, which supports a comprehensive and chronic inflammatory microenvironment conducive to subsequent fibroproliferative, chondrogenic, and osteogenic events.

Although FOP is a rare disease, non-hereditary HO is fairly common [Neal et al. 2002, Potter et al. 2010]. One of the most remarkable findings of this thesis work is the discovery of shared inflammatory mediators among FOP and many other HO disorders. The work detailed in this dissertation will serve as a foundation for future studies investigating the immunological contributions to HO disorders, which will hopefully uncover new advances in knowledge that facilitate drug discovery efforts to treat HO in FOP and related HO disorders.

Bibliography

Agarwal S, Loder SJ, Cholok D, Peterson J, Li J, Breuler C, Cameron Brownley R, Hsin Sung H, Chung M, Kamiya N, Li S, Zhao B, Kaartinen V, Davis TA, Qureshi AT, Schipani E, Mishina Y, Levi B. "Scleraxis-Lineage Cells Contribute to Ectopic Bone Formation in Muscle and Tendon." Stem Cells. 2016.

Akashi K, Traver D, Miyamoto T, Weissman IL. "A clonogenic common myeloid progenitor that gives rise to all myeloid lineages." Nature. 2000;404(6774):193-7.

Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, Klunker S, Meyer N, O'Mahony L, Palomares O, Rhyner C, Ouaked N, Schaffartzik A, Van De Veen W, Zeller S, Zimmermann M, Akdis CA. "Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases." J Allergy Clin Immunol. 2011;127(3):701-21 e1-70.

Akira S, Uematsu S, Takeuchi O. "Pathogen recognition and innate immunity." Cell. 2006;124(4):783-801.

Akiyama I, Yoshino O, Osuga Y, Shi J, Takamura M, Harada M, Koga K, Hirota Y, Hirata T, Fujii T, Saito S, Kozuma S. "The role of bone morphogenetic protein 6 in accumulation and regulation of neutrophils in the human ovary." Reprod Sci. 2014;21(6):772-7.

Aleman-Muench GR, Soldevila G. "When versatility matters: activins/inhibins as key regulators of immunity." Immunol Cell Biol. 2012;90(2):137-48.

Alexander KA, Chang MK, Maylin ER, Kohler T, Muller R, Wu AC, Van Rooijen N, Sweet MJ, Hume DA, Raggatt LJ, Pettit AR. "Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model." J Bone Miner Res. 2011;26(7):1517-32.

Alfieri KA, Forsberg JA, Potter BK. "Blast injuries and heterotopic ossification." Bone Joint Res. 2012;1(8):192-7.

Allen RE, Boxhorn LK. "Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor." J Cell Physiol. 1989;138(2):311-5.

Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK, Chazaud B. "Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis." J Exp Med. 2007;204(5):1057-69.

Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R, Pennesi G. "Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway." Eur J Immunol. 2005;35(5):1482-90.

Azzolina A, Bongiovanni A, Lampiasi N. "Substance P induces TNF-α and IL-6 production through NFκB in peritoneal mast cells." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2003;1643(1-3):75-83.

Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ, Andrews NC, Lin HY. "Bone morphogenetic

protein signaling by hemojuvelin regulates hepcidin expression." <u>Nat Genet</u>. 2006;38(5):531-9.

Barrera P, Blom A, van Lent PL, van Bloois L, Beijnen JH, van Rooijen N, de Waal Malefijt MC, van de Putte LB, Storm G, van den Berg WB. "Synovial macrophage depletion with clodronate-containing liposomes in rheumatoid arthritis." <u>Arthritis Rheum</u>. 2000;43(9):1951-9.

Barron L, Wynn TA. "Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages." <u>Am J Physiol Gastrointest Liver Physiol</u>. 2011;300(5):G723-8.

Bedi A, Zbeda RM, Bueno VF, Downie B, Dolan M, Kelly BT. "The incidence of heterotopic ossification after hip arthroscopy." <u>Am J Sports Med</u>. 2012;40(4):854-63.

Ben-Ami E, Berrih-Aknin S, Miller A. "Mesenchymal stem cells as an immunomodulatory therapeutic strategy for autoimmune diseases." <u>Autoimmun Rev</u>. 2011;10(7):410-5.

Bentzinger CF, Wang YX, Dumont NA, Rudnicki MA. "Cellular dynamics in the muscle satellite cell niche." <u>EMBO Rep</u>. 2013;14(12):1062-72.

Benveniste P, Cantin C, Hyam D, Iscove NN. "Hematopoietic stem cells engraft in mice with absolute efficiency." <u>Nat Immunol</u>. 2003;4(7):708-13.

Bhatia M, Bonnet D, Wu D, Murdoch B, Wrana J, Gallacher L, Dick JE. "Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells." <u>J Exp Med</u>. 1999;189(7):1139-48.

Billings PC, Fiori JL, Bentwood JL, O'Connell MP, Jiao X, Nussbaum B, Caron RJ, Shore EM, Kaplan FS. "Dysregulated BMP signaling and enhanced osteogenic differentiation of connective tissue progenitor cells from patients with fibrodysplasia ossificans progressiva (FOP)." <u>J Bone Miner Res</u>. 2008;23(3):305-13.

Bischoff SC. "Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data." <u>Nat Rev Immunol</u>. 2007;7(2):93-104.

Boehm T. "Design principles of adaptive immune systems." <u>Nat Rev Immunol</u>. 2011;11(5):307-17.

Borthwick LA, Wynn TA, Fisher AJ. "Cytokine mediated tissue fibrosis." <u>Biochim Biophys Acta</u>. 2013;1832(7):1049-60.

Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A. "Bone morphogenetic proteins: a critical review." <u>Cell Signal</u>. 2011;23(4):609-20.

Brazil DP, Church RH, Surae S, Godson C, Martin F. "BMP signalling: agony and antagony in the family." <u>Trends Cell Biol</u>. 2015;25(5):249-64.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. "Neutrophil extracellular traps kill bacteria." <u>Science</u>. 2004;303(5663):1532-5.

Broere F, Apasov SG, Sitkovsky MV, Van Eden W. "T cell subsets and T cell-mediated immunity." In: Nijkamp FP, Parnham MJ, editors. <u>Principles of Immunopharmacology: 3rd revised and extended edition</u>. 3. Basel: Springer Basel; 2011. p. 15-27.

Buchmann K. "Evolution of Innate Immunity: Clues from Invertebrates via Fish to Mammals." Front Immunol. 2014;5:459.

Chaikuad A, Alfano I, Kerr G, Sanvitale CE, Boergermann JH, Triffitt JT, von Delft F, Knapp S, Knaus P, Bullock AN. "Structure of the bone morphogenetic protein receptor ALK2 and implications for fibrodysplasia ossificans progressiva." J Biol Chem. 2012;287(44):36990-8.

Chakkalakal SA, Zhang D, Culbert AL, Convente MR, Caron RJ, Wright AC, Maidment AD, Kaplan FS, Shore EM. "An Acvr1 R206H knock-in mouse has fibrodysplasia ossificans progressiva." J Bone Miner Res. 2012;27(8):1746-56.

Chakkalakal SA, Uchibe K, Convente MR, Zhang D, Economides AN, Kaplan FS, Pacifici M, Iwamoto M, Shore EM. "Palovarotene Inhibits Heterotopic Ossification and Maintains Limb Mobility and Growth in Mice with the Human ACVR1 Fibrodysplasia Ossificans Progressiva (FOP) Mutation." J Bone Miner Res. 2016;31(9):1666-75.

Challen GA, Boles N, Lin KK, Goodell MA. "Mouse hematopoietic stem cell identification and analysis." Cytometry A. 2009;75(1):14-24.

Champagne CM, Takebe J, Offenbacher S, Cooper LF. "Macrophage cell lines produce osteoinductive signals that include bone morphogenetic protein-2." Bone. 2002;30(1):26-31.

Chargé SBP, Rudnicki MA. "Cellular and Molecular Regulation of Muscle Regeneration." Physiol Rev. 2004;84:209-38.

Charo IF, Ranshohoff RM. "The Many Roles of Chemokines and Chemokine Receptors in Inflammation." N Engl J Med. 2006;354(6):610-21.

Chen L, Flies DB. "Molecular mechanisms of T cell co-stimulation and co-inhibition." Nat Rev Immunol. 2013;13(4):227-42.

Chen W, Ten Dijke P. "Immunoregulation by members of the TGFbeta superfamily." Nat Rev Immunol. 2016;16(12):723-40.

Choi KS, Lee C, Maatouk DM, Harfe BD. "Bmp2, Bmp4 and Bmp7 are co-required in the mouse AER for normal digit patterning but not limb outgrowth." PLoS One. 2012;7(5):e37826.

Cohen RB, Hahn GV, Tabas JA, Peeper J, Levitz CL, Sando A, Sando N, Zasloff M, Kaplan FS. "The natural history of heterotopic ossification in patients who have fibrodysplasia ossificans progressiva. A study of forty-four patients." J Bone Joint Surg Am. 1993;75(2):215-9.

Cohn RM, Schwarzkopf R, Jaffe F. "Heterotopic ossification after total hip arthroplasty." Am J Orthop (Belle Mead NJ). 2011;40(11):E232-5.

Convente M, Towler O, Stanley A, Brewer N, Allen R, Kaplan F, Shore E. "Chapter 30: Fibrodysplasia (Myositis) Ossificans Progressiva." In: Thakker R, Whyte M, Eisman J, Igarashi T, editors. Genetics of Bone Biology and Skeletal Disease: 2nd Edition (in press). 2: Elsevier; 2017.

Convente MR, Wang H, Pignolo RJ, Kaplan FS, Shore EM. "The immunological contribution to heterotopic ossification disorders." Curr Osteoporos Rep. 2015;13(2):116-24.

155

Cooper MD, Alder MN. "The evolution of adaptive immune systems." <u>Cell</u>. 2006;124(4):815-22.

Cooper RN, Tajbakhsh S, Mouly V, Cossu G, Buckingham M, Butler-Browne GS. "In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle." <u>J Cell Sci</u>. 1999;112 (Pt 17):2895-901.

Couteaux R, Mira JC, d'Albis A. "Regeneration of muscles after cardiotoxin injury. I. Cytological aspects." <u>Biol Cell</u>. 1988;62(2):171-82.

Crisan M, Kartalaei PS, Vink C, Yamada-Inagawa T, Bollerot K, van IW, van der Linden R, de Sousa Lopes SM, Monteiro R, Mummery C, Dzierzak E. "BMP signalling differentially regulates distinct haematopoietic stem cell types." <u>Nat Commun</u>. 2015;6:8040.

Crivellato E, Ribatti D. "The mast cell: an evolutionary perspective." <u>Biol Rev Camb Philos Soc</u>. 2010;85(2):347-60.

Cross M, Mangelsdorf I, Wedel A, Renkawitz R. "Mouse lysozyme M gene: isolation, characterization, and expression studies." <u>Proc Natl Acad Sci U S A</u>. 1988;85(17):6232-6.

Culbert AL, Chakkalakal SA, Theosmy EG, Brennan TA, Kaplan FS, Shore EM. "Alk2 regulates early chondrogenic fate in fibrodysplasia ossificans progressiva heterotopic endochondral ossification." <u>Stem Cells</u>. 2014;32(5):1289-300.

Cunningham NS, Paralkar V, Reddi AH. "Osteogenin and recombinant bone morphogenetic protein 2B are chemotactic for human monocytes and stimulate transforming growth factor beta 1 mRNA expression." <u>Proc Natl Acad Sci U S A</u>. 1992;89(24):11740-4.

Dahlin JS, Hallgren J. "Mast cell progenitors: Origin, development and migration to tissues." <u>Mol Immunol</u>. 2014.

Davis EL, Sonnet C, Lazard ZW, Henslee G, Gugala Z, Salisbury EA, Strecker EV, Davis TA, Forsberg JA, Davis AR, Olmsted-Davis EA. "Location-dependent heterotopic ossification in the rat model: The role of activated matrix metalloproteinase 9." <u>J Orthop Res</u>. 2016;34(11):1894-904.

de Boer J, Williams A, Skavdis G, Harker N, Coles M, Tolaini M, Norton T, Williams K, Roderick K, Potocnik AJ, Kioussis D. "Transgenic mice with hematopoietic and lymphoid specific expression of Cre." <u>Eur J Immunol</u>. 2003;33(2):314-25.

De Filippo K, Dudeck A, Hasenberg M, Nye E, van Rooijen N, Hartmann K, Gunzer M, Roers A, Hogg N. "Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation." <u>Blood</u>. 2013;121(24):4930-7.

de Graaf CA, Choi J, Baldwin TM, Bolden JE, Fairfax KA, Robinson AJ, Biben C, Morgan C, Ramsay K, Ng AP, Kauppi M, Kruse EA, Sargeant TJ, Seidenman N, D'Amico A, D'Ombrain MC, Lucas EC, Koernig S, Baz Morelli A, Wilson MJ, Dower SK, Williams B, Heazlewood SY, Hu Y, Nilsson SK, Wu L, Smyth GK, Alexander WS, Hilton DJ. "Haemopedia: An Expression Atlas of Murine Hematopoietic Cells." <u>Stem Cell Reports</u>. 2016.

de Oliveira S, Rosowski EE, Huttenlocher A. "Neutrophil migration in infection and wound repair: going forward in reverse." <u>Nat Rev Immunol</u>. 2016;16(6):378-91.

Deirmengian GK, Hebela NM, O'Connell M, Glaser DL, Shore EM, Kaplan FS. "Proximal tibial osteochondromas in patients with fibrodysplasia ossificans progressiva." J Bone Joint Surg Am. 2008;90(2):366-74.

Derynck R, Zhang YE. "Smad-dependent and Smad-independent pathways in TGF-beta family signalling." Nature. 2003;425(6958):577-84.

Deshmane SL, Kremlev S, Amini S, Sawaya BE. "Monocyte chemoattractant protein-1 (MCP-1): an overview." J Interferon Cytokine Res. 2009;29(6):313-26.

Dey D, Bagarova J, Hatsell SJ, Armstrong KA, Huang L, Ermann J, Vonner AJ, Shen Y, Mohedas AH, Lee A, Eekhoff EM, van Schie A, Demay MB, Keller C, Wagers AJ, Economides AN, Yu PB. "Two tissue-resident progenitor lineages drive distinct phenotypes of heterotopic ossification." Sci Transl Med. 2016;8(366):366ra163.

Douaiher J, Succar J, Lancerotto L, Gurish MF, Orgill DP, Hamilton MJ, Krilis SA, Stevens RL. "Development of mast cells and importance of their tryptase and chymase serine proteases in inflammation and wound healing." Adv Immunol. 2014;122:211-52.

Duchesne E, Bouchard P, Roussel MP, Cote CH. "Mast cells can regulate skeletal muscle cell proliferation by multiple mechanisms." Muscle Nerve. 2013;48(3):403-14.

Dzik JM. "The ancestry and cumulative evolution of immune reactions." Acta Biochim Pol. 2010;57(4):443-66.

Elliott RL, Blobe GC. "Role of transforming growth factor Beta in human cancer." J Clin Oncol. 2005;23(9):2078-93.

Epelman S, Lavine KJ, Randolph GJ. "Origin and functions of tissue macrophages." Immunity. 2014;41(1):21-35.

Evans KN, Forsberg JA, Potter BK, Hawksworth JS, Brown TS, Andersen R, Dunne JR, Tadaki D, Elster EA. "Inflammatory cytokine and chemokine expression is associated with heterotopic ossification in high-energy penetrating war injuries." J Orthop Trauma. 2012;26(11):e204-13.

Fasshauer M, Bluher M. "Adipokines in health and disease." Trends Pharmacol Sci. 2015;36(7):461-70.

Faust N, Varas F, Kelly LM, Heck S, Graf T. "Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages." Blood. 2000;96(2):719-26.

Fiori JL, Billings PC, de la Pena LS, Kaplan FS, Shore EM. "Dysregulation of the BMP-p38 MAPK signaling pathway in cells from patients with fibrodysplasia ossificans progressiva (FOP)." J Bone Miner Res. 2006;21(6):902-9.

Flajnik MF, Kasahara M. "Origin and evolution of the adaptive immune system: genetic events and selective pressures." Nat Rev Genet. 2010;11(1):47-59.

Fong EL, Chan CK, Goodman SB. "Stem cell homing in musculoskeletal injury." Biomaterials. 2011;32(2):395-409.

Forsberg JA, Potter BK, Polfer EM, Safford SD, Elster EA. "Do inflammatory markers portend heterotopic ossification and wound failure in combat wounds?" Clin Orthop Relat Res. 2014;472(9):2845-54.

Frieri M, Patel R, Celestin J. "Mast cell activation syndrome: a review." <u>Curr Allergy Asthma Rep</u>. 2013;13(1):27-32.

Fukuda T, Kohda M, Kanomata K, Nojima J, Nakamura A, Kamizono J, Noguchi Y, Iwakiri K, Kondo T, Kurose J, Endo K, Awakura T, Fukushi J, Nakashima Y, Chiyonobu T, Kawara A, Nishida Y, Wada I, Akita M, Komori T, Nakayama K, Nanba A, Maruki Y, Yoda T, Tomoda H, Yu PB, Shore EM, Kaplan FS, Miyazono K, Matsuoka M, Ikebuchi K, Ohtake A, Oda H, Jimi E, Owan I, Okazaki Y, Katagiri T. "Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva." <u>J Biol Chem</u>. 2009;284(11):7149-56.

Funaba M. "Role of activin A in murine mast cells: modulation of cell growth, differentiation, and migration." <u>J Leukoc Biol</u>. 2003;73(6):793-801.

Gallant-Behm CL, Hildebrand KA, Hart DA. "The mast cell stabilizer ketotifen prevents development of excessive skin wound contraction and fibrosis in red Duroc pigs." <u>Wound Repair Regen</u>. 2008;16(2):226-33.

Galli SJ, Grimbaldeston M, Tsai M. "Immunomodulatory mast cells: negative, as well as positive, regulators of immunity." <u>Nat Rev Immunol</u>. 2008;8(6):478-86.

Galli SJ, Nakae S, Tsai M. "Mast cells in the development of adaptive immune responses." <u>Nat Immunol</u>. 2005;6(2):135-42.

Galli SJ, Borregaard N, Wynn TA. "Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils." <u>Nat Immunol</u>. 2011;12(11):1035-44.

Gallucci S, Provenzano C, Mazzarelli P, Scuderi F, Bartoccioni E. "Myoblasts produce IL-6 in response to inflammatory stimuli." <u>Int Immunol</u>. 1998;10(3):267-73.

Gannon FH, Glaser D, Caron R, Thompson LD, Shore EM, Kaplan FS. "Mast cell involvement in fibrodysplasia ossificans progressiva." <u>Hum Pathol</u>. 2001;32(8):842-8.

Gannon FH, Valentine BA, Shore EM, Zasloff MA, Kaplan FS. "Acute lymphocytic infiltration in an extremely early lesion of fibrodysplasia ossificans progressiva." <u>Clin Orthop Relat Res</u>. 1998(346):19-25.

Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Tsay A, Fitch J, Barnes GL, Graves DT, Einhorn TA. "Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption." <u>J Bone Miner Res</u>. 2003;18(9):1584-92.

Ginhoux F, Jung S. "Monocytes and macrophages: developmental pathways and tissue homeostasis." <u>Nat Rev Immunol</u>. 2014;14(6):392-404.

Glaser DL, Rocke DM, Kaplan FS. "Catastrophic falls in patients who have fibrodysplasia ossificans progressiva." <u>Clin Orthop Relat Res</u>. 1998(346):110-6.

Glaser DL, Economides AN, Wang L, Liu X, Kimble RD, Fandl JP, Wilson JM, Stahl N, Kaplan FS, Shore EM. "In vivo somatic cell gene transfer of an engineered Noggin mutein prevents BMP4-induced heterotopic ossification." <u>J Bone Joint Surg Am</u>. 2003;85-a(12):2332-42.

Gordon S. "Alternative activation of macrophages." <u>Nat Rev Immunol</u>. 2003;3(1):23-35.

Gri G, Frossi B, D'Inca F, Danelli L, Betto E, Mion F, Sibilano R, Pucillo C. "Mast cell: an emerging partner in immune interaction." Front Immunol. 2012;3:120.

Grimbaldeston MA, Chen CC, Piliponsky AM, Tsai M, Tam SY, Galli SJ. "Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo." Am J Pathol. 2005;167(3):835-48.

Groppe JC, Wu J, Shore EM, Kaplan FS. "In vitro analyses of the dysregulated R206H ALK2 kinase-FKBP12 interaction associated with heterotopic ossification in FOP." Cells Tissues Organs. 2011;194(2-4):291-5.

Groppe JC, Shore EM, Kaplan FS. "Functional modeling of the ACVR1 (R206H) mutation in FOP." Clin Orthop Relat Res. 2007;462:87-92.

Hager-Theodorides AL, Ross SE, Sahni H, Mishina Y, Furmanski AL, Crompton T. "Direct BMP2/4 signaling through BMP receptor IA regulates fetal thymocyte progenitor homeostasis and differentiation to CD4+CD8+ double-positive cell." Cell Cycle. 2014;13(2):324-33.

Halova I, Draberova L, Draber P. "Mast cell chemotaxis - chemoattractants and signaling pathways." Front Immunol. 2012;3:119.

Hammond P, Suttie M, Hennekam RC, Allanson J, Shore EM, Kaplan FS. "The face signature of fibrodysplasia ossificans progressiva." Am J Med Genet A. 2012;158A(6):1368-80.

Hansson GK, Edfeldt K. "Toll to be paid at the gateway to the vessel wall." Arterioscler Thromb Vasc Biol. 2005;25(6):1085-7.

Hata A, Lagna G, Massague J, Hemmati-Brivanlou A. "Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor." Genes Dev. 1998;12(2):186-97.

Hatsell SJ, Idone V, Wolken DM, Huang L, Kim HJ, Wang L, Wen X, Nannuru KC, Jimenez J, Xie L, Das N, Makhoul G, Chernomorsky R, D'Ambrosio D, Corpina RA, Schoenherr CJ, Feeley K, Yu PB, Yancopoulos GD, Murphy AJ, Economides AN. "ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A." Sci Transl Med. 2015;7(303):303ra137.

Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Jr., Wrana JL, Falb D. "The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling." Cell. 1997;89(7):1165-73.

Hedger MP, Winnall WR, Phillips DJ, de Kretser DM. "The regulation and functions of activin and follistatin in inflammation and immunity." Vitam Horm. 2011;85:255-97.

Hegyi L, Gannon FH, Glaser DL, Shore EM, Kaplan FS, Shanahan CM. "Stromal cells of fibrodysplasia ossificans progressiva lesions express smooth muscle lineage markers and the osteogenic transcription factor Runx2/Cbfa-1: clues to a vascular origin of heterotopic ossification?" J Pathol. 2003;201(1):141-8.

Heron A, Dubayle D. "A focus on mast cells and pain." J Neuroimmunol. 2013;264(1-2):1-7.

Hess K, Ushmorov A, Fiedler J, Brenner RE, Wirth T. "TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway." Bone. 2009;45(2):367-76.

Hino K, Ikeya M, Horigome K, Matsumoto Y, Ebise H, Nishio M, Sekiguchi K, Shibata M, Nagata S, Matsuda S, Toguchida J. "Neofunction of ACVR1 in fibrodysplasia ossificans progressiva." Proc Natl Acad Sci U S A. 2015;112(50):15438-43.

Hong JH, Lee GT, Lee JH, Kwon SJ, Park SH, Kim SJ, Kim IY. "Effect of bone morphogenetic protein-6 on macrophages." Immunology. 2009;128(1 Suppl):e442-50.

Horsley V, Jansen KM, Mills ST, Pavlath GK. "IL-4 acts as a myoblast recruitment factor during mammalian muscle growth." Cell. 2003;113(4):483-94.

Horvath P, Barrangou R. "CRISPR/Cas, the immune system of bacteria and archaea." Science. 2010;327(5962):167-70.

Hugle T. "Beyond allergy: the role of mast cells in fibrosis." Swiss Med Wkly. 2014;144:w13999.

Huse K, Bakkebo M, Oksvold MP, Forfang L, Hilden VI, Stokke T, Smeland EB, Myklebust JH. "Bone morphogenetic proteins inhibit CD40L/IL-21-induced Ig production in human B cells: differential effects of BMP-6 and BMP-7." Eur J Immunol. 2011;41(11):3135-45.

Huse M, Chen YG, Massague J, Kuriyan J. "Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12." Cell. 1999;96(3):425-36.

Ioannidis V, Beermann F, Clevers H, Held W. "The beta-catenin--TCF-1 pathway ensures CD4(+)CD8(+) thymocyte survival." Nat Immunol. 2001;2(8):691-7.

Ishida Y, Gao JL, Murphy PM. "Chemokine Receptor CX3CR1 Mediates Skin Wound Healing by Promoting Macrophage and Fibroblast Accumulation and Function." The Journal of Immunology. 2007;180(1):569-79.

Italiani P, Boraschi D. "From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation." Front Immunol. 2014;5:514.

Jablonski KA, Amici SA, Webb LM, Ruiz-Rosado Jde D, Popovich PG, Partida-Sanchez S, Guerau-de-Arellano M. "Novel Markers to Delineate Murine M1 and M2 Macrophages." PLoS One. 2015;10(12):e0145342.

Jacquin C, Koczon-Jaremko B, Aguila HL, Leng L, Bucala R, Kuchel GA, Lee SK. "Macrophage migration inhibitory factor inhibits osteoclastogenesis." Bone. 2009;45(4):640-9.

Jamur MC, Oliver C. "Origin, maturation and recruitment of mast cell precursors." Front Biosci (Schol Ed). 2011;3:1390-406.

Joe AW, Yi L, Natarajan A, Le Grand F, So L, Wang J, Rudnicki MA, Rossi FM. "Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis." Nat Cell Biol. 2010;12(2):153-63.

Josefowicz SZ, Lu LF, Rudensky AY. "Regulatory T cells: mechanisms of differentiation and function." Annu Rev Immunol. 2012;30:531-64.

Juurikivi A, Sandler C, Lindstedt KA, Kovanen PT, Juutilainen T, Leskinen MJ, Maki T, Eklund KK. "Inhibition of c-kit tyrosine kinase by imatinib mesylate induces

apoptosis in mast cells in rheumatoid synovia: a potential approach to the treatment of arthritis." Ann Rheum Dis. 2005;64(8):1126-31.

Kalesnikoff J, Galli SJ. "New developments in mast cell biology." Nat Immunol. 2008;9(11):1215-23.

Kan L, Liu Y, McGuire TL, Berger DM, Awatramani RB, Dymecki SM, Kessler JA. "Dysregulation of local stem/progenitor cells as a common cellular mechanism for heterotopic ossification." Stem Cells. 2009;27(1):150-6.

Kan L, Lounev VY, Pignolo RJ, Duan L, Liu Y, Stock SR, McGuire TL, Lu B, Gerard NP, Shore EM, Kaplan FS, Kessler JA. "Substance P signaling mediates BMP-dependent heterotopic ossification." J Cell Biochem. 2011;112(10):2759-72.

Kaplan F, Glaser D, Shore E, Deirmengian G, Gupta R, Delai P, Morhart R, Smith R, Le Merrer M, Rogers J, Connor J, Kitterman J. "The Phenotype of Fibrodysplasia Ossificans Progressiva." Clin Rev Bone Miner Metab. 2005;3:183–88.

Kaplan FS, Pignolo RJ, Shore EM. "Granting immunity to FOP and catching heterotopic ossification in the Act." Semin Cell Dev Biol. 2016;49:30-6.

Kaplan FS, Glaser DL, Shore EM, Pignolo RJ, Xu M, Zhang Y, Senitzer D, Forman SJ, Emerson SG. "Hematopoietic stem-cell contribution to ectopic skeletogenesis." J Bone Joint Surg Am. 2007;89(2):347-57.

Kaplan FS, Shore EM, Gupta R, Billings PC, Glaser DL, Pignolo RJ, Graf D, Kamoun M. "Immunological Features of Fibrodysplasia Ossificans Progressiva and the Dysregulated BMP4 Pathway." Clin Rev Bone Miner Metab. 2005;3(3-4):189-93.

Kaplan FS, Tabas JA, Gannon FH, Finkel G, Hahn GV, Zasloff MA. "The histopathology of fibrodysplasia ossificans progressiva. An endochondral process." J Bone Joint Surg Am. 1993;75(2):220-30.

Kaplan FS, Xu M, Seemann P, Connor JM, Glaser DL, Carroll L, Delai P, Fastnacht-Urban E, Forman SJ, Gillessen-Kaesbach G, Hoover-Fong J, Koster B, Pauli RM, Reardon W, Zaidi SA, Zasloff M, Morhart R, Mundlos S, Groppe J, Shore EM. "Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1." Hum Mutat. 2009;30(3):379-90.

Kaplan FS, Zasloff MA, Kitterman JA, Shore EM, Hong CC, Rocke DM. "Early mortality and cardiorespiratory failure in patients with fibrodysplasia ossificans progressiva." J Bone Joint Surg Am. 2010;92(3):686-91.

Kaplan FS, Pignolo RJ, Shore EM. "The FOP metamorphogene encodes a novel type I receptor that dysregulates BMP signaling." Cytokine Growth Factor Rev. 2009;20(5-6):399-407.

Kaplan FS, Glaser DL, Hebela N, Shore EM. "Heterotopic ossification." J Am Acad Orthop Surg. 2004;12(2):116-25.

Kaplanski G, Marin V, Montero-Julian F, Mantovani A, Farnarier C. "IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation." Trends Immunol. 2003;24(1):25-9.

Karalaki M, Fili S, Philippou A, Koutsilieris M. "Muscle regeneration: cellular and molecular events." In Vivo. 2009;23(5):779-96.

Karlsson G, Blank U, Moody JL, Ehinger M, Singbrant S, Deng CX, Karlsson S. "Smad4 is critical for self-renewal of hematopoietic stem cells." J Exp Med. 2007;204(3):467-74.

Katagiri T, Watabe T. "Bone Morphogenetic Proteins." Cold Spring Harb Perspect Biol. 2016;8(6).

Kaviratne M, Hesse M, Leusink M, Cheever AW, Davies SJ, McKerrow JH, Wakefield LM, Letterio JJ, Wynn TA. "IL-13 Activates a Mechanism of Tissue Fibrosis That Is Completely TGF- Independent." The Journal of Immunology. 2004;173(6):4020-29.

Kawai T, Akira S. "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors." Nat Immunol. 2010;11(5):373-84.

Kitterman JA, Strober JB, Kan L, Rocke DM, Cali A, Peeper J, Snow J, Delai PL, Morhart R, Pignolo RJ, Shore EM, Kaplan FS. "Neurological symptoms in individuals with fibrodysplasia ossificans progressiva." J Neurol. 2012;259(12):2636-43.

Kolaczkowska E, Kubes P. "Neutrophil recruitment and function in health and inflammation." Nat Rev Immunol. 2013;13(3):159-75.

Kolls J, Peppel K, Silva M, Beutler B. "Prolonged and effective blockade of tumor necrosis factor activity through adenovirus-mediated gene transfer." Proc Natl Acad Sci U S A. 1994;91(1):215-9.

Kondo M, Weissman IL, Akashi K. "Identification of clonogenic common lymphoid progenitors in mouse bone marrow." Cell. 1997;91(5):661-72.

Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP. "Neuropeptides activate human mast cell degranulation and chemokine production." Immunology. 2008;123(3):398-410.

Kwon SJ, Lee GT, Lee JH, Kim WJ, Kim IY. "Bone morphogenetic protein-6 induces the expression of inducible nitric oxide synthase in macrophages." Immunology. 2009;128(1 Suppl):e758-65.

Lanchoney TF, Cohen RB, Rocke DM, Zasloff MA, Kaplan FS. "Permanent heterotopic ossification at the injection site after diphtheria-tetanus-pertussis immunizations in children who have fibrodysplasia ossificans progressiva." J Pediatr. 1995;126(5 Pt 1):762-4.

Langen RC, Schols AM, Kelders MC, van der Velden JL, Wouters EF, Janssen-Heininger YM. "Muscle wasting and impaired muscle regeneration in a murine model of chronic pulmonary inflammation." Am J Respir Cell Mol Biol. 2006;35(6):689-96.

Laskin DL, Sunil VR, Gardner CR, Laskin JD. "Macrophages and tissue injury: agents of defense or destruction?" Annu Rev Pharmacol Toxicol. 2011;51:267-88.

LeBien TW, Tedder TF. "B lymphocytes: how they develop and function." Blood. 2008;112(5):1570-80.

Lee KB, Taghavi CE, Murray SS, Song KJ, Keorochana G, Wang JC. "BMP induced inflammation: a comparison of rhBMP-7 and rhBMP-2." J Orthop Res. 2012;30(12):1985-94.

Lee KB, Taghavi CE, Song KJ, Sintuu C, Yoo JH, Keorochana G, Tzeng ST, Fei Z, Liao JC, Wang JC. "Inflammatory characteristics of rhBMP-2 in vitro and in an in vivo rodent model." Spine (Phila Pa 1976). 2011;36(3):E149-54.

Levick SP, McLarty JL, Murray DB, Freeman RM, Carver WE, Brower GL. "Cardiac mast cells mediate left ventricular fibrosis in the hypertensive rat heart." Hypertension. 2009;53(6):1041-7.

Lim J, Tu X, Choi K, Akiyama H, Mishina Y, Long F. "BMP-Smad4 signaling is required for precartilaginous mesenchymal condensation independent of Sox9 in the mouse." Dev Biol. 2015;400(1):132-8.

Litman GW, Rast JP, Fugmann SD. "The origins of vertebrate adaptive immunity." Nat Rev Immunol. 2010;10(8):543-53.

Little SC, Mullins MC. "Extracellular modulation of BMP activity in patterning the dorsoventral axis." Birth Defects Res C Embryo Today. 2006;78(3):224-42.

Lounev VY, Ramachandran R, Wosczyna MN, Yamamoto M, Maidment AD, Shore EM, Glaser DL, Goldhamer DJ, Kaplan FS. "Identification of progenitor cells that contribute to heterotopic skeletogenesis." J Bone Joint Surg Am. 2009;91(3):652-63.

Luo G, Hershko DD, Robb BW, Wray CJ, Hasselgren PO. "IL-1beta stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF-kappa B." Am J Physiol Regul Integr Comp Physiol. 2003;284(5):R1249-54.

MacDonald KM, Swanstrom MM, McCarthy JJ, Nemeth BA, Guliani TA, Noonan KJ. "Exaggerated inflammatory response after use of recombinant bone morphogenetic protein in recurrent unicameral bone cysts." J Pediatr Orthop. 2010;30(2):199-205.

Mann CJ, Perdiguero E, Kharraz Y, Aguilar S, Pessina P, Serrano AL, Munoz-Canoves P. "Aberrant repair and fibrosis development in skeletal muscle." Skelet Muscle. 2011;1(1):21.

Martinez VG, Hernandez-Lopez C, Valencia J, Hidalgo L, Entrena A, Zapata AG, Vicente A, Sacedon R, Varas A. "The canonical BMP signaling pathway is involved in human monocyte-derived dendritic cell maturation." Immunol Cell Biol. 2011;89(5):610-8.

Martinez VG, Sacedon R, Hidalgo L, Valencia J, Fernandez-Sevilla LM, Hernandez-Lopez C, Vicente A, Varas A. "The BMP Pathway Participates in Human Naive CD4+ T Cell Activation and Homeostasis." PLoS One. 2015;10(6):e0131453.

Martinon F, Burns K, Tschopp J. "The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta." Mol Cell. 2002;10(2):417-26.

Massagué J, Seoane J, Wotton D. "Smad transcription factors." Genes Dev. 2005;19(23):2783-810.

Mast BA, Schultz GS. "Interactions of cytokines, growth factors, and proteases in acute and chronic wounds." Wound Repair Regen. 1996;4(4):411-20.

Merad M, Sathe P, Helft J, Miller J, Mortha A. "The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting." Annu Rev Immunol. 2013;31:563-604.

Mikhak Z, Strassner JP, Luster AD. "Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4." J Exp Med. 2013;210(9):1855-69.

Mildner A, Jung S. "Development and function of dendritic cell subsets." Immunity. 2014;40(5):642-56.

Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. "Two major Smad pathways in TGF-beta superfamily signalling." Genes Cells. 2002;7(12):1191-204.

Miyazono K, Kamiya Y, Morikawa M. "Bone morphogenetic protein receptors and signal transduction." J Biochem. 2010;147(1):35-51.

Mogensen TH. "Pathogen recognition and inflammatory signaling in innate immune defenses." Clin Microbiol Rev. 2009;22(2):240-73, Table of Contents.

Mohler ER, Gannon F, Reynolds C, Zimmerman R, Keane MG, Kaplan FS. "Bone Formation and Inflammation in Cardiac Valves." Circulation. 2001;103(11):1522-28.

Moon TC, Befus AD, Kulka M. "Mast cell mediators: their differential release and the secretory pathways involved." Front Immunol. 2014;5:569.

Mosser DM, Edwards JP. "Exploring the full spectrum of macrophage activation." Nat Rev Immunol. 2008;8(12):958-69.

Mueller AA, van Velthoven CT, Fukumoto KD, Cheung TH, Rando TA. "Intronic polyadenylation of PDGFRalpha in resident stem cells attenuates muscle fibrosis." Nature. 2016;540(7632):276-79.

Murray PJ, Wynn TA. "Protective and pathogenic functions of macrophage subsets." Nat Rev Immunol. 2011;11(11):723-37.

Nagle DL, Kozak CA, Mano H, Chapman VM, Bucan M. "Physical mapping of the Tec and Gabrb1 loci reveals that the Wsh mutation on mouse chromosome 5 is associated with an inversion." Hum Mol Genet. 1995;4(11):2073-9.

Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, ten Dijke P. "Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling." Nature. 1997;389(6651):631-5.

Neal B, Gray H, MacMahon S, Dunn L. "Incidence of heterotopic bone formation after major hip surgery." ANZ J Surg. 2002;72(11):808-21.

Nguyen HX, Tidball JG. "Interactions between neutrophils and macrophages promote macrophage killing of rat muscle cells in vitro." J Physiol. 2003;547(Pt 1):125-32.

Notarangelo LD, Kim MS, Walter JE, Lee YN. "Human RAG mutations: biochemistry and clinical implications." Nat Rev Immunol. 2016;16(4):234-46.

O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. "The role of substance P in inflammatory disease." J Cell Physiol. 2004;201(2):167-80.

Oettinger MA, Schatz DG, Gorka C, Baltimore D. "RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination." Science. 1990;248(4962):1517-23.

Olmsted-Davis EA, Salisbury EA, Hoang D, Davis EL, Lazard Z, Sonnet C, Davis TA, Forsberg JA, Davis AR. "Progenitors in Peripheral Nerves Launch Heterotopic Ossification." <u>Stem Cells Transl Med</u>. 2017.

Olson LE, Soriano P. "Increased PDGFRalpha activation disrupts connective tissue development and drives systemic fibrosis." <u>Dev Cell</u>. 2009;16(2):303-13.

Ono Y, Calhabeu F, Morgan JE, Katagiri T, Amthor H, Zammit PS. "BMP signalling permits population expansion by preventing premature myogenic differentiation in muscle satellite cells." <u>Cell Death Differ</u>. 2011;18(2):222-34.

Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. "CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells." <u>Nat Immunol</u>. 2007;8(12):1353-62.

Panzer S, Madden M, Matsuki K. "Interaction of IL-1 beta, IL-6 and tumour necrosis factor-alpha (TNF-alpha) in human T cells activated by murine antigens." <u>Clin Exp Immunol</u>. 1993;93(3):471-8.

Peppel K, Crawford D, Beutler B. "A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity." <u>J Exp Med</u>. 1991;174(6):1483-9.

Pepys MB, Hirschfield GM. "C-reactive protein: a critical update." <u>J Clin Invest</u>. 2003;111(12):1805-12.

Philippou A, Maridaki M, Theos A, Koutsilieris M. "Cytokines in muscle damage." <u>Adv Clin Chem</u>. 2012;58:49-87.

Phillipson M, Kubes P. "The neutrophil in vascular inflammation." <u>Nat Med</u>. 2011;17(11):1381-90.

Pieper K, Grimbacher B, Eibel H. "B-cell biology and development." <u>J Allergy Clin Immunol</u>. 2013;131(4):959-71.

Pignolo RJ, Foley KL. "Nonhereditary Heterotopic Ossification: Implications for Injury, Arthroplasty, and Aging." <u>Clin Rev Bone Miner Metab</u>. 2005;3(3-4):261-66.

Pignolo RJ, Bedford-Gay C, Liljesthrom M, Durbin-Johnson BP, Shore EM, Rocke DM, Kaplan FS. "The Natural History of Flare-Ups in Fibrodysplasia Ossificans Progressiva (FOP): A Comprehensive Global Assessment." <u>J Bone Miner Res</u>. 2016;31(3):650-6.

Poetker DM, Reh DD. "A comprehensive review of the adverse effects of systemic corticosteroids." <u>Otolaryngol Clin North Am</u>. 2010;43(4):753-68.

Potter BK, Forsberg JA, Davis TA, Evans KN, Hawksworth JS, Tadaki D, Brown TS, Crane NJ, Burns TC, O'Brien FP, Elster EA. "Heterotopic ossification following combat-related trauma." <u>J Bone Joint Surg Am</u>. 2010;92 Suppl 2:74-89.

Raffaghello L, Bianchi G, Bertolotto M, Montecucco F, Busca A, Dallegri F, Ottonello L, Pistoia V. "Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche." <u>Stem Cells</u>. 2008;26(1):151-62.

Ramel MC, Hill CS. "The ventral to dorsal BMP activity gradient in the early zebrafish embryo is determined by graded expression of BMP ligands." <u>Dev Biol</u>. 2013;378(2):170-82.

Ranganathan K, Agarwal S, Cholok D, Loder S, Li J, Sung Hsieh HH, Wang SC, Buchman SR, Levi B. "The role of the adaptive immune system in burn-induced heterotopic ossification and mesenchymal cell osteogenic differentiation." <u>J Surg Res</u>. 2016;206(1):53-61.

Rath D, Amlinger L, Rath A, Lundgren M. "The CRISPR-Cas immune system: biology, mechanisms and applications." <u>Biochimie</u>. 2015;117:119-28.

Reagan MR, Rosen CJ. "Navigating the bone marrow niche: translational insights and cancer-driven dysfunction." <u>Nat Rev Rheumatol</u>. 2016;12(3):154-68.

Rhodes CP, Ratcliffe NA, Rowley AF. "Presence of coelomocytes in the primitive chordate amphioxus (Branchiostoma lanceolatum)." <u>Science</u>. 1982;217(4556):263-5.

Ricklin D, Hajishengallis G, Yang K, Lambris JD. "Complement: a key system for immune surveillance and homeostasis." <u>Nat Immunol</u>. 2010;11(9):785-97.

Rodenberg E, Azhdarinia A, Lazard ZW, Hall M, Kwon SK, Wilganowski N, Salisbury EA, Merched-Sauvage M, Olmsted-Davis EA, Sevick-Muraca EM, Davis AR. "Matrix metalloproteinase-9 is a diagnostic marker of heterotopic ossification in a murine model." <u>Tissue Eng Part A</u>. 2011;17(19-20):2487-96.

Rubartelli A, Lotze MT. "Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox." <u>Trends Immunol</u>. 2007;28(10):429-36.

Salazar VS, Gamer LW, Rosen V. "BMP signalling in skeletal development, disease and repair." <u>Nat Rev Endocrinol</u>. 2016;12(4):203-21.

Salisbury E, Sonnet C, Heggeness M, Davis AR, Olmsted-Davis E. "Heterotopic ossification has some nerve." <u>Crit Rev Eukaryot Gene Expr</u>. 2010;20(4):313-24.

Salisbury E, Rodenberg E, Sonnet C, Hipp J, Gannon FH, Vadakkan TJ, Dickinson ME, Olmsted-Davis EA, Davis AR. "Sensory nerve induced inflammation contributes to heterotopic ossification." <u>J Cell Biochem</u>. 2011;112(10):2748-58.

Sambasivan R, Yao R, Kissenpfennig A, Van Wittenberghe L, Paldi A, Gayraud-Morel B, Guenou H, Malissen B, Tajbakhsh S, Galy A. "Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration." <u>Development</u>. 2011;138(17):3647-56.

San Antonio JD, Winston BM, Tuan RS. "Regulation of chondrogenesis by heparan sulfate and structurally related glycosaminoglycans." <u>Dev Biol</u>. 1987;123(1):17-24.

Sartori R, Sandri M. "BMPs and the muscle-bone connection." <u>Bone</u>. 2015;80:37-42.

Sartori R, Schirwis E, Blaauw B, Bortolanza S, Zhao J, Enzo E, Stantzou A, Mouisel E, Toniolo L, Ferry A, Stricker S, Goldberg AL, Dupont S, Piccolo S, Amthor H, Sandri M. "BMP signaling controls muscle mass." <u>Nat Genet</u>. 2013;45(11):1309-18.

Sathish JG, Sethu S, Bielsky MC, de Haan L, French NS, Govindappa K, Green J, Griffiths CE, Holgate S, Jones D, Kimber I, Moggs J, Naisbitt DJ, Pirmohamed M, Reichmann G, Sims J, Subramanyam M, Todd MD, Van Der Laan JW, Weaver RJ, Park BK. "Challenges and approaches for the development of safer immunomodulatory biologics." <u>Nat Rev Drug Discov</u>. 2013;12(4):306-24.

Scarlett RF, Rocke DM, Kantanie S, Patel JB, Shore EM, Kaplan FS. "Influenza-like viral illnesses and flare-ups of fibrodysplasia ossificans progressiva." Clin Orthop Relat Res. 2004(423):275-9.

Schaefer L. "Complexity of danger: the diverse nature of damage-associated molecular patterns." J Biol Chem. 2014;289(51):35237-45.

Schaffer AA, Kaplan FS, Tracy MR, O'Brien ML, Dormans JP, Shore EM, Harland RM, Kusumi K. "Developmental anomalies of the cervical spine in patients with fibrodysplasia ossificans progressiva are distinctly different from those in patients with Klippel-Feil syndrome: clues from the BMP signaling pathway." Spine (Phila Pa 1976). 2005;30(12):1379-85.

Schatz DG, Swanson PC. "V(D)J recombination: mechanisms of initiation." Annu Rev Genet. 2011;45:167-202.

Schultz E, Jaryszak DL, Valliere CR. "Response of satellite cells to focal skeletal muscle injury." Muscle Nerve. 1985;8(3):217-22.

Shaw MH, Reimer T, Kim YG, Nunez G. "NOD-like receptors (NLRs): bona fide intracellular microbial sensors." Curr Opin Immunol. 2008;20(4):377-82.

Shen Q, Little SC, Xu M, Haupt J, Ast C, Katagiri T, Mundlos S, Seemann P, Kaplan FS, Mullins MC, Shore EM. "The fibrodysplasia ossificans progressiva R206H ACVR1 mutation activates BMP-independent chondrogenesis and zebrafish embryo ventralization." J Clin Invest. 2009;119(11):3462-72.

Shi S, Hoogaars WM, de Gorter DJ, van Heiningen SH, Lin HY, Hong CC, Kemaladewi DU, Aartsma-Rus A, ten Dijke P, t Hoen PA. "BMP antagonists enhance myogenic differentiation and ameliorate the dystrophic phenotype in a DMD mouse model." Neurobiol Dis. 2011;41(2):353-60.

Shi Y, Hu G, Su J, Li W, Chen Q, Shou P, Xu C, Chen X, Huang Y, Zhu Z, Huang X, Han X, Xie N, Ren G. "Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair." Cell Res. 2010;20(5):510-8.

Shi Y, Wang YF, Jayaraman L, Yang H, Massague J, Pavletich NP. "Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling." Cell. 1998;94(5):585-94.

Shi Y, Massague J. "Mechanisms of TGF-beta signaling from cell membrane to the nucleus." Cell. 2003;113(6):685-700.

Shore EM, Kaplan FS. "Inherited human diseases of heterotopic bone formation." Nat Rev Rheumatol. 2010;6(9):518-27.

Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, Choi IH, Connor JM, Delai P, Glaser DL, LeMerrer M, Morhart R, Rogers JG, Smith R, Triffitt JT, Urtizberea JA, Zasloff M, Brown MA, Kaplan FS. "A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva." Nat Genet. 2006;38(5):525-7.

Sierra-Filardi E, Puig-Kroger A, Blanco FJ, Nieto C, Bragado R, Palomero MI, Bernabeu C, Vega MA, Corbi AL. "Activin A skews macrophage polarization by promoting a proinflammatory phenotype and inhibiting the acquisition of anti-inflammatory macrophage markers." Blood. 2011;117(19):5092-101.

Simoes Sato AY, Bub GL, Campos AH. "BMP-2 and -4 produced by vascular smooth muscle cells from atherosclerotic lesions induce monocyte chemotaxis through direct BMPRII activation." <u>Atherosclerosis</u>. 2014;235(1):45-55.

Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter H, Hainzl A, Schatz S, Qi Y, Schlecht A, Weiss JM, Wlaschek M, Sunderkotter C, Scharffetter-Kochanek K. "An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice." <u>J Clin Invest</u>. 2011;121(3):985-97.

Smith C, Kruger MJ, Smith RM, Myburgh KH. "The inflammatory response to skeletal muscle injury: illuminating complexities." <u>Sports Med</u>. 2008;38(11):947-69.

Smith R. "Fibrodysplasia (myositis) ossificans progressiva. Clinical lessons from a rare disease." <u>Clin Orthop Relat Res</u>. 1998(346):7-14.

Song GA, Kim HJ, Woo KM, Baek JH, Kim GS, Choi JY, Ryoo HM. "Molecular consequences of the ACVR1(R206H) mutation of fibrodysplasia ossificans progressiva." <u>J Biol Chem</u>. 2010;285(29):22542-53.

Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. "Interactions between human mesenchymal stem cells and natural killer cells." <u>Stem Cells</u>. 2006;24(1):74-85.

Spangrude GJ, Heimfeld S, Weissman IL. "Purification and characterization of mouse hematopoietic stem cells." <u>Science</u>. 1988;241(4861):58-62.

Starr TK, Jameson SC, Hogquist KA. "Positive and negative selection of T cells." <u>Annu Rev Immunol</u>. 2003;21:139-76.

Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, Figueiredo JL, Kohler RH, Chudnovskiy A, Waterman P, Aikawa E, Mempel TR, Libby P, Weissleder R, Pittet MJ. "Identification of splenic reservoir monocytes and their deployment to inflammatory sites." <u>Science</u>. 2009;325(5940):612-6.

Takeuchi O, Akira S. "Pattern recognition receptors and inflammation." <u>Cell</u>. 2010;140(6):805-20.

Tang L, Jennings TA, Eaton JW. "Mast cells mediate acute inflammatory responses to implanted biomaterials." <u>Proc Natl Acad Sci U S A</u>. 1998;95(15):8841-6.

Taylor SS, Yang J, Wu J, Haste NM, Radzio-Andzelm E, Anand G. "PKA: a portrait of protein kinase dynamics." <u>Biochim Biophys Acta</u>. 2004;1697(1-2):259-69.

Thevenot PT, Baker DW, Weng H, Sun MW, Tang L. "The pivotal role of fibrocytes and mast cells in mediating fibrotic reactions to biomaterials." <u>Biomaterials</u>. 2011;32(33):8394-403.

Tidball JG. "Inflammatory processes in muscle injury and repair." <u>Am J Physiol Regul Integr Comp Physiol</u>. 2005;288(2):R345-53.

Tomiya A, Aizawa T, Nagatomi R, Sensui H, Kokubun S. "Myofibers express IL-6 after eccentric exercise." <u>Am J Sports Med</u>. 2004;32(2):503-8.

Tu B, Liu S, Liu G, Yan W, Wang Y, Li Z, Fan C. "Macrophages derived from THP-1 promote the osteogenic differentiation of mesenchymal stem cells through the IL-23/IL-23R/beta-catenin pathway." <u>Exp Cell Res</u>. 2015;339(1):81-9.

168

Tucker JA, Mintzer KA, Mullins MC. "The BMP signaling gradient patterns dorsoventral tissues in a temporally progressive manner along the anteroposterior axis." <u>Dev Cell</u>. 2008;14(1):108-19.

Turner MD, Nedjai B, Hurst T, Pennington DJ. "Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease." <u>Biochim Biophys Acta</u>. 2014;1843(11):2563-82.

Uezumi A, Fukada S, Yamamoto N, Takeda S, Tsuchida K. "Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle." <u>Nat Cell Biol</u>. 2010;12(2):143-52.

Urist MR. "Bone: formation by autoinduction." <u>Science</u>. 1965;150(3698):893-9.

van Dinther M, Visser N, de Gorter DJ, Doorn J, Goumans MJ, de Boer J, ten Dijke P. "ALK2 R206H mutation linked to fibrodysplasia ossificans progressiva confers constitutive activity to the BMP type I receptor and sensitizes mesenchymal cells to BMP-induced osteoblast differentiation and bone formation." <u>J Bone Miner Res</u>. 2010;25(6):1208-15.

Van Rooijen N. "The liposome-mediated macrophage 'suicide' technique." <u>J Immunol Methods</u>. 1989;124(1):1-6.

van Rooijen N, van Nieuwmegen R. "Elimination of phagocytic cells in the spleen after intravenous injection of liposome-encapsulated dichloromethylene diphosphonate. An enzyme-histochemical study." <u>Cell Tissue Res</u>. 1984;238(2):355-8.

van Steensel L, Paridaens D, Schrijver B, Dingjan GM, van Daele PL, van Hagen PM, van den Bosch WA, Drexhage HA, Hooijkaas H, Dik WA. "Imatinib mesylate and AMN107 inhibit PDGF-signaling in orbital fibroblasts: a potential treatment for Graves' ophthalmopathy." <u>Invest Ophthalmol Vis Sci</u>. 2009;50(7):3091-8.

Villalta SA, Rosenthal W, Martinez L, Kaur A, Sparwasser T, Tidball JG, Margeta M, Spencer MJ, Bluestone JA. "Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy." <u>Sci Transl Med</u>. 2014;6(258):258ra142.

Villavicencio AT, Burneikiene S. "RhBMP-2-induced radiculitis in patients undergoing transforaminal lumbar interbody fusion: relationship to dose." <u>Spine J</u>. 2016.

Vincent L, Vang D, Nguyen J, Gupta M, Luk K, Ericson ME, Simone DA, Gupta K. "Mast cell activation contributes to sickle cell pathobiology and pain in mice." <u>Blood</u>. 2013;122(11):1853-62.

Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, Ugolini S. "Innate or adaptive immunity? The example of natural killer cells." <u>Science</u>. 2011;331(6013):44-9.

Vukman KV, Forsonits A, Oszvald A, Toth EA, Buzas EI. "Mast cell secretome: soluble and vesicular components." <u>Semin Cell Dev Biol</u>. 2017.

Wang H, Lindborg C, Lounev V, Kim JH, McCarrick-Walmsley R, Xu M, Mangiavini L, Groppe JC, Shore EM, Schipani E, Kaplan FS, Pignolo RJ. "Cellular Hypoxia Promotes Heterotopic Ossification by Amplifying BMP Signaling." <u>J Bone Miner Res</u>. 2016.

Wang T, Li BY, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, Martin J, Manganaro T, Donahoe PK. "The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors." <u>Cell</u>. 1996;86(3):435-44.

Wang YX, Rudnicki MA. "Satellite cells, the engines of muscle repair." <u>Nat Rev Mol Cell Biol</u>. 2012;13(2):127-33.

Warren GL, Hulderman T, Mishra D, Gao X, Millecchia L, O'Farrell L, Kuziel WA, Simeonova PP. "Chemokine receptor CCR2 involvement in skeletal muscle regeneration." <u>FASEB J</u>. 2005;19(3):413-5.

Watabe T, Miyazono K. "Roles of TGF-beta family signaling in stem cell renewal and differentiation." <u>Cell Res</u>. 2009;19(1):103-15.

Waterstrat A, Liang Y, Swiderski CF, Shelton BJ, Van Zant G. "Congenic interval of CD45/Ly-5 congenic mice contains multiple genes that may influence hematopoietic stem cell engraftment." <u>Blood</u>. 2010;115(2):408-17.

Weller CL, Collington SJ, Brown JK, Miller HR, Al-Kashi A, Clark P, Jose PJ, Hartnell A, Williams TJ. "Leukotriene B4, an activation product of mast cells, is a chemoattractant for their progenitors." <u>J Exp Med</u>. 2005;201(12):1961-71.

Weng Z, Zhang B, Asadi S, Sismanopoulos N, Butcher A, Fu X, Katsarou-Katsari A, Antoniou C, Theoharides TC. "Quercetin is more effective than cromolyn in blocking human mast cell cytokine release and inhibits contact dermatitis and photosensitivity in humans." <u>PLoS One</u>. 2012;7(3):e33805.

Werner CM, Zimmermann SM, Wurgler-Hauri CC, Lane JM, Wanner GA, Simmen HP. "Use of imatinib in the prevention of heterotopic ossification." <u>Hss j</u>. 2013;9(2):166-70.

Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. "Neutrophils: Molecules, Functions and Pathophysiological Aspects." <u>Laboratory Investigation</u>. 2000;80(5):617-53.

Wosczyna MN, Biswas AA, Cogswell CA, Goldhamer DJ. "Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification." <u>J Bone Miner Res</u>. 2012;27(5):1004-17.

Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J. "TGF beta signals through a heteromeric protein kinase receptor complex." <u>Cell</u>. 1992;71(6):1003-14.

Wu M, Chen G, Li YP. "TGF-beta and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease." <u>Bone Res</u>. 2016;4:16009.

Wu MY, Hill CS. "Tgf-beta superfamily signaling in embryonic development and homeostasis." <u>Dev Cell</u>. 2009;16(3):329-43.

Wu X, Yung LM, Cheng WH, Yu PB, Babitt JL, Lin HY, Xia Y. "Hepcidin regulation by BMP signaling in macrophages is lipopolysaccharide dependent." <u>PLoS One</u>. 2012;7(9):e44622.

Wynn TA. "Cellular and molecular mechanisms of fibrosis." <u>J Pathol</u>. 2008;214(2):199-210.

Wynn TA, Ramalingam TR. "Mechanisms of fibrosis: therapeutic translation for fibrotic disease." <u>Nat Med</u>. 2012;18(7):1028-40.

Wynn TA, Barron L. "Macrophages: master regulators of inflammation and fibrosis." <u>Semin Liver Dis</u>. 2010;30(3):245-57.

Wynn TA, Chawla A, Pollard JW. "Macrophage biology in development, homeostasis and disease." Nature. 2013;496(7446):445-55.

Xia Y, Yan J, Jin X, Entman ML, Wang Y. "The chemokine receptor CXCR6 contributes to recruitment of bone marrow-derived fibroblast precursors in renal fibrosis." Kidney Int. 2014;86(2):327-37.

Xu G, Zhang Y, Zhang L, Ren G, Shi Y. "The role of IL-6 in inhibition of lymphocyte apoptosis by mesenchymal stem cells." Biochem Biophys Res Commun. 2007;361(3):745-50.

Yadin D, Knaus P, Mueller TD. "Structural insights into BMP receptors: Specificity, activation and inhibition." Cytokine Growth Factor Rev. 2016;27:13-34.

Yamtich J, Sweasy JB. "DNA polymerase family X: function, structure, and cellular roles." Biochim Biophys Acta. 2010;1804(5):1136-50.

Yang X, Ricciardi BF, Hernandez-Soria A, Shi Y, Pleshko Camacho N, Bostrom MP. "Callus mineralization and maturation are delayed during fracture healing in interleukin-6 knockout mice." Bone. 2007;41(6):928-36.

Yoshioka Y, Ono M, Osaki M, Konishi I, Sakaguchi S. "Differential effects of inhibition of bone morphogenic protein (BMP) signalling on T-cell activation and differentiation." Eur J Immunol. 2012;42(3):749-59.

Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Bouxsein ML, Hong DW, McManus PM, Katagiri T, Sachidanandan C, Kamiya N, Fukuda T, Mishina Y, Peterson RT, Bloch KD. "BMP type I receptor inhibition reduces heterotopic [corrected] ossification." Nat Med. 2008;14(12):1363-9.

Zhang W, Ge W, Li C, You S, Liao L, Han Q, Deng W, Zhao RC. "Effects of mesenchymal stem cells on differentiation, maturation, and function of human monocyte-derived dendritic cells." Stem Cells Dev. 2004;13(3):263-71.

Zhang X, Goncalves R, Mosser DM. "The isolation and characterization of murine macrophages." Curr Protoc Immunol. 2008;Chapter 14:Unit 14 1.

Zhao E, Xu H, Wang L, Kryczek I, Wu K, Hu Y, Wang G, Zou W. "Bone marrow and the control of immunity." Cell Mol Immunol. 2012;9(1):11-9.

Zhao XY, Zhao LY, Zheng QS, Su JL, Guan H, Shang FJ, Niu XL, He YP, Lu XL. "Chymase induces profibrotic response via transforming growth factor-beta 1/Smad activation in rat cardiac fibroblasts." Mol Cell Biochem. 2008;310(1-2):159-66.

Zon LI. "Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal." Nature. 2008;453(7193):306-13.

Zuniga-Pflucker JC. "T-cell development made simple." Nat Rev Immunol. 2004;4(1):67-72.