PDGFRα+ AND HIC1+ MESENCHYMAL PROGENITORS IN MURINE SKELETAL
TISSUE: ROLES IN HOMEOSTASIS AND REGENERATION

by

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Abstract

Despite its rigid structure, bone is a highly dynamic tissue that is constantly remodeled throughout life to maintain its structure and function. Remodeling is mediated by bone resorbing osteoclasts and bone depositing osteoblasts. While the hematopoietic origins of osteoclasts are well understood, the identity of the skeletal mesenchymal progenitors that give rise to osteoblasts remains elusive. Understanding the identity and biology of these skeletal mesenchymal progenitors is critical to the development of therapeutics for the treatment of bone-related disorders from trauma and surgical-induced heterotopic ossification to osteoporosis-related fractures.

Previous studies have attempted to identify skeletal stem and progenitor cell populations, however, though the periosteum is known to be a source of progenitors crucial to bone regeneration, none of the identified populations were periosteal. While PDGFRα expression has been identified in many putative skeletal progenitors, we show that it is expressed ubiquitously throughout skeletal tissues and therefore does not constitute a unique marker for skeletal progenitors. We identified HIC1 as a marker of a novel injury-inducible periosteal skeletal progenitor that contributes to bone homeostasis and regeneration, an important advancement in the understanding of skeletal biology.

Contrary to skeletal tissues, in skeletal muscle, expression of PDGFRα is restricted to fibro-adipogenic progenitors (FAPs) which have been suggested as the cellular source of heterotopic ossification. Using a new inducible PDGFRα<sup>CreERT2</sup> transgenic model, we confirmed that tissue resident PDGFRα<sup>+</sup> FAPs give rise to HO in skeletal muscle and that they have inherent osteogenic
potential that is induced by an altered inflammatory environment following muscle damage. This presents novel cellular and therapeutic targets for the prevention of acquired HO.

The TGFβ and BMP pathways are well known for their effects on FAPs, inducing ectopic bone formation and fibrosis. Using a PDGFRα^{CreERT2}/Smad4^{Flox} transgenic model, we examined the role of mesenchymal TGFβ and BMP signaling in adult tissues. We found that impairment of SMAD-mediated TGFβ/BMP signaling in PDGFRα^{+} cells induced increased proliferation and uncoupling of bone formation and resorption resulting in high turnover bone loss. This suggests constitutive SMAD-mediated TGFβ/BMP signaling is required for adult murine skeletal homeostasis and deepens our scientific understanding of adult skeletal biology.
Lay Summary

Bone is a dynamic tissue that is constantly remodeled by bone resorbing osteoclasts and bone depositing osteoblasts. The origin of osteoblasts is known to be a mesenchymal progenitor; however, the identity of these mesenchymal progenitors remains unclear. Understanding the identity and biology of these progenitors is crucial for the development of therapeutics to treat various skeletal disorders. The work described here identifies a marker for skeletal progenitor cells that participate in skeletal homeostasis and regeneration. We also identified the cellular source of heterotopic bone and a trigger for their differentiation into bone, presenting new therapeutic targets. Lastly, we identified a molecular signaling pathway required for maintaining adult skeletal homeostasis. The identification of a novel skeletal progenitor population and signaling pathways required for skeletal homeostasis present exciting new advancements in our understanding of skeletal biology and new targets that could be used for the treatment of skeletal disorders.
Preface

Parts of chapter 2 are modified from a manuscript which is being prepared for publication in collaboration with T.M.Underhill. P. Nguyen collected the HIC1 bone marrow cells for single cell analysis. My contributions include development of the concept, performing the experiments, analyzing data, and writing of the chapter and relevant manuscript sections to be included in publication.


Chapter 4 is modified from a manuscript in preparation: Eisner C, Cummings M, Tung LW, Theret M, Rossi FMV. Constitutive Smad4 signaling in PDGFRα+ cells is required for maintenance of adult skeletal homeostasis. Experimental data for Figure 4.4 was produced by M. Theret. L.W. Tung assisted with RNA sequencing analysis. M. Cummings assisted with data collection. My
contributions include experimental design, performing experiments, data analysis, and manuscript preparation.

All research involving animals was conducted following the protocols approved by the UBC Animal Care Committee (protocols A12-0287, A17-0039, A18-0314).
**Table of Contents**

Abstract .................................................................................................................................................. iii
Lay Summary ........................................................................................................................................ v
Preface .................................................................................................................................................. vi
Table of Contents ................................................................................................................................... viii
List of Tables .......................................................................................................................................... xiv
List of Figures ......................................................................................................................................... xv
List of Abbreviations ............................................................................................................................ xix
Acknowledgements .............................................................................................................................. xxi
Dedication .............................................................................................................................................. xxiii

**Chapter 1: Introduction** ..................................................................................................................... 1

1.1 Bone and Regeneration .................................................................................................................. 1

1.1.1 Bone structure and function ........................................................................................................ 1

1.1.2 The cells of bone .......................................................................................................................... 2

1.1.2.1 Osteoclasts ............................................................................................................................. 2

1.1.2.2 Osteoblasts ............................................................................................................................ 3

1.1.2.3 Osteocytes ............................................................................................................................... 4

1.1.3 Bone remodeling .......................................................................................................................... 5

1.1.3.1 Coupling of bone remodeling ................................................................................................. 7

1.1.4 Bone regeneration ....................................................................................................................... 8

1.1.4.1 Inflammatory response and hematoma .................................................................................... 9

1.1.4.2 Soft callus formation .............................................................................................................. 9
1.1.4.3 Hard callus formation .......................................................... 10
1.1.4.4 Callus remodeling .............................................................. 11
1.1.5 Bone disorders and heterotopic bone ........................................ 12
1.2 MSCs and SSCs in Bone .......................................................... 13
1.2.1 What is an MSC? .................................................................. 13
1.2.2 Skeletal stem cells and progenitors ......................................... 15
1.2.2.1 Nestin ........................................................................... 15
1.2.2.2 LepR ............................................................................ 16
1.2.2.3 Grem1 .......................................................................... 17
1.2.2.4 mSSC/BSCP ................................................................. 18
1.2.2.5 Gli1 ............................................................................. 19
1.2.2.6 PTHrP .......................................................................... 20
1.2.2.7 The missing periosteal piece ............................................. 20
1.2.3 Potential SSC or skeletal MSC markers ..................................... 21
1.2.3.1 PDGFRα ....................................................................... 22
1.2.3.2 HIC1 ........................................................................... 23
1.3 TGFβ/BMP Signaling and Bone .................................................. 25
1.3.1 TGFβ superfamily .................................................................. 25
1.3.2 TGFβ and bone ..................................................................... 26
1.3.3 BMP and bone ...................................................................... 27
1.3.4 SMAD4 and TGFβ/BMP signaling in bone ............................ 29
1.3.5 Role in adult skeletal tissues .................................................. 30
Chapter 2: PDGFRα+ and HIC1+ Mesenchymal Progenitors in Bone Homeostasis and Regeneration

2.1 Synopsis ........................................................................................................................................... 34

2.2 Introduction ......................................................................................................................................... 35

2.3 Methods ............................................................................................................................................... 37

2.3.1 Mice and tamoxifen mediated reporter expression ................................................................. 37

2.3.2 Damage model ............................................................................................................................ 38

2.3.3 Histology and immunofluorescence ......................................................................................... 39

2.3.4 Single cell RNA sequencing ...................................................................................................... 39

2.3.5 Statistical analysis ....................................................................................................................... 40

2.4 Results ................................................................................................................................................ 40

2.4.1 PDGFRα and HIC1 lineage tracing models ................................................................................ 40

2.4.2 PDGFRα and HIC1 label separate and distinct populations of cells in skeletal tissues .......... 41

2.4.3 HIC1 labels MSCs that give rise to PDGFRα+ osteoprogenitors and osteocytes ..................... 43

2.4.4 HIC1+ MSCs contribute to various stages of bone regeneration ............................................ 44

2.4.5 HIC1+ Bone Marrow MSCs resemble previously reported murine MSCs ............................... 47

2.5 Discussion .......................................................................................................................................... 49

Chapter 3: Murine tissue-resident PDGFRα+ fibro-adipogenic progenitors spontaneously acquire an osteogenic phenotype in an altered inflammatory environment ........................................ 72

3.1 Synopsis .......................................................................................................................................... 72
3.2 Introduction............................................................................................................. 72
3.3 Methods.................................................................................................................. 74
  3.3.1 Animals and tamoxifen mediated reporter expression................................. 74
  3.3.2 FACS Analysis ................................................................................................. 75
  3.3.3 RNA Sequencing .............................................................................................. 76
  3.3.4 Parabiosis surgery ............................................................................................ 77
  3.3.5 BMP2 induced heterotopic ossification ......................................................... 77
  3.3.6 Notexin muscle damage ................................................................................... 77
  3.3.7 Tissue collection and histology ....................................................................... 77
  3.3.8 Statistical Analysis ........................................................................................... 78
3.4 Results.................................................................................................................... 79
  3.4.1 PDGFRαCT2/td.Tomato labels tissue resident FAPs in muscle and osteogenic cells in murine bone ................................................................. 79
  3.4.2 PDGFRα+ cells are the source of osteogenic cells in BMP2-induced HO .......... 80
  3.4.3 PDGFRα+ cells contributing to heterotopic ossicles are tissue-resident, not bone-marrow derived.............................................................. 81
  3.4.4 Alterations of the inflammatory environment after damage induces PDGFRα+ cells to undergo osteogenic differentiation without the addition of exogenous BMP2 .......... 82
3.5 Discussion ............................................................................................................. 84

Chapter 4: Constitutive SMAD4-mediated TGFβ/BMP signaling in PDGFRα mesenchymal cells is required for murine skeletal homeostasis ......................................................... 100
4.1 Synopsis ............................................................................................................. 100
4.2 Introduction .................................................................................................................. 101
4.3 Methods .................................................................................................................. 104
  4.3.1 Mice .................................................................................................................. 104
  4.3.2 EdU labeling .................................................................................................... 105
  4.3.3 Calcein labeling and analysis ........................................................................ 105
  4.3.4 DigiGait analysis ............................................................................................ 106
  4.3.5 FGF23 antibody treatment ............................................................................. 106
  4.3.6 Whole mount Alizarin Red S/Alcian Blue staining ...................................... 106
  4.3.7 Histology and immunofluorescence ............................................................ 107
  4.3.8 μCT analysis .................................................................................................. 108
  4.3.9 qPCR ................................................................................................................ 108
  4.3.10 FACS analysis .............................................................................................. 109
  4.3.11 Copy number assay ...................................................................................... 109
  4.3.12 RNA sequencing ......................................................................................... 110
  4.3.13 Statistical analysis ....................................................................................... 111
4.4 Results ................................................................................................................... 112
  4.4.1 Deletion of Smad4 in PDGFRα+ mesenchymal progenitors leads to a degenerative skeletal phenotype ............................................................. 112
  4.4.2 Deletion of Smad4 in PDGFRα+ osteogenic cells results in uncoupling of bone formation and resorption ................................................................. 116
  4.4.3 Inhibition of FGF23 does not rescue the smad4^{Fl/Fl} phenotype ............... 120
  4.4.4 Loss of pro-regenerative response in late stage smad4^{Fl/Fl} mice may exaggerate bone loss .................................................................................. 121
4.5 Discussion

Chapter 5: Discussion

5.1 Contributions to the Field and Future Directions

5.1.1 HIC1 labels a previously unidentified periosteal skeletal MSC population

5.1.2 PDGFRα⁺ FAPs are the cellular source of HO and undergo spontaneous osteogenic differentiation in an altered inflammatory environment.

5.1.3 Constitutive SMAD4 signaling is required to maintain adult murine skeletal homeostasis

5.2 Final Conclusions

References
List of Tables

Table 1-1 Summary of selected SSC and skeletal MSC populations reported in the literature ... 33
Table 4-1 Scoring parameters for smad4^{Flox} mice .............................................................. 158
Table 4-2 Complete DigiGait Results for smad4^{Flox} mice ......................................................... 159
List of Figures

Figure 1.1 - Location of SSCs/skeletal MSC populations identified in the literature. .................. 32
Figure 2.1 - PDGFRαEGFP labels all osteogenic lineage cells. ................................................ 52
Figure 2.2 - Structure of the PDGFRαCreERT2 knock-in/knock-out model developed by the Hogan Lab................................................................. 53
Figure 2.3 - Structure of the HIC1CreERT2 knock-in model developed by the Underhill Lab... 53
Figure 2.4 - Expression of PDGFRα and HIC1 in cortical bone .................................................. 54
Figure 2.5 - Expression of PDGFRα and HIC1 in articular cartilage ........................................ 55
Figure 2.6 - Expression of PDGFRα/td.Tomato and HIC1/td.Tomato in the growth plate ....... 56
Figure 2.7 - Expression of PDGFRα and HIC1 in the enthesis ................................................... 57
Figure 2.8 - Expression of PDGFRα and HIC1 in bone marrow ............................................... 58
Figure 2.9 - Expression of PDGFRα and HIC1 in the periosteum and cortical bone. ............ 59
Figure 2.10 - HIC1/td.Tomato+ cells give rise to PDGFRαEGFP+ cortical osteocytes. ........... 60
Figure 2.11 - HIC1CT2/td.Tomato+ cells contribute to all stages of bone regeneration ......... 61
Figure 2.12 - Quantification of HIC1CT2/td.Tomato+ and EdU+ cells during skeletal regeneration............................................................................................................................................ 62
Figure 2.13 - HIC1CT2/td.Tomato+ bone marrow cells resemble previously identified MSC populations........................................................................................................................................... 64
Figure 2.14 - Expression of CAR cell markers in HIC1CT2/td.Tomato+ bone marrow single cell RNASEq clusters ........................................................................................................................................... 65
Figure 2.15 - Expression of pericyte markers in HIC1$^{CT2/td.Tomato^+}$ bone marrow single cell RNASeq clusters

Figure 2.16 - Expression of immune cell markers in HIC1$^{CT2/td.Tomato^+}$ bone marrow single cell RNASeq clusters

Figure 2.17 - Expression of endothelial cell markers in HIC1$^{CT2/td.Tomato^+}$ bone marrow single cell RNASeq clusters

Figure 2.18 - Expression of FAP markers in HIC1$^{CT2/td.Tomato^+}$ bone marrow single cell RNASeq clusters

Figure 2.19 - Expression of osteogenic lineage markers in HIC1$^{CT2/td.Tomato^+}$ bone marrow single cell RNASeq clusters

Figure 2.20 - Proposed hierarchy of HIC1 and PDGFRα during murine skeletal homeostasis and regeneration

Figure 3.1 - PDGFRα$^{CT2/td.Tomato}$ lineage tracing labels osteogenic lineage cells

Figure 3.2 - PDGFRα$^{CT2/td.Tomato}$ lineage tracing labels FAPs in skeletal muscle

Figure 3.3 - PDGFRα$^{CT2/td.Tomato^+}$ cells are the primary source of osteogenic cells in BMP2-induced heterotopic lesions

Figure 3.4 - Heterotopic ossicles originate from tissue-resident PDGFRα$^+$ progenitors, not circulating bone marrow-derived progenitors

Figure 3.5 - GFP$^+$ cells are found within the marrow of heterotopic bone from PDGFRα$^{CT2/td.Tomato}$ parabionts

Figure 3.6 - Cells contributing to heterotopic bone formation in Col1a1*3.6GFP x C57Bl/6J parabionts are tissue resident, not bone marrow derived
Figure 3.7 - An altered inflammatory milieu activates an osteogenic programme in FAPs.

Figure 3.8 - FAPs from CCR2KO mice express high levels of osteogenic genes at D10 after damage.

Figure 3.9 - WT FAPs from undamaged muscle have varied expression of genes previously used to identify putative HO progenitors.

Figure 4.1 - Conditional deletion of Smad4 in PDGFRα+ cells leads to a degenerative motor phenotype.

Figure 4.2 - Digital gait analysis highlights postural gait abnormalities in smad4FliFl mice.

Figure 4.3 - Complete DigiGait Analysis for smad4FliFl mice.

Figure 4.4 - Deletion of Smad4 from PDGFRα+ cells does not affect skeletal muscle at homeostasis.

Figure 4.5 - Deletion of Smad4 from PDGFRα+ cells induces macroscopic skeletal changes.

Figure 4.6 - Deletion of Smad4 results in morphological changes in the bone of smad4Flox mice.

Figure 4.7 - Bone remodeling in smad4Flox mice occurs mainly in the cortical bone compartment.

Figure 4.8 - Increased expression of PDGFRα/td.Tomato in PDGFRαCT2/td.Tomato/smad4FliFl mice is a result of increased FAP proliferation.

Figure 4.9 - Bone mineralization parameters in smad4Flox mice are not significantly altered, but expression of genes associated with mineralization are.

Figure 4.10 - Quantification of bone mineralization parameters in trabecular and cortical bone of smad4Flox mice.

Figure 4.11 - TRAP osteoclast activity is increased in smad4FliFl bone.
Figure 4.12 - TRAP osteoclast analysis from smad4^{Flox} mice. .................................................. 149

Figure 4.13 - αFGF23 treatment does not rescue the smad4^{Fl/Fl} phenotype. .............................. 151

Figure 4.14 - RNASeq GO Bio pathway analysis of differentially expressed genes in PDGFRα^+Scal^+ FAPs from bone of smad4^{Flox} mice. ................................................................. 153

Figure 4.15 - RNASeq GO Bio pathway analysis of differentially expressed genes in whole bone tissue of smad4^{Flox} mice. .................................................................................. 156

Figure 4.16 - Expression of Hif1a and TNF/IL-6 pathways in whole bone tissue of smad4^{Flox} mice.................................................................................................................. 157
List of Abbreviations

BSA – Bovine serum albumin
BMP – Bone morphogenic protein
CAR – CXCL12 abundant reticular cell
CFU-F – Colony forming unit – fibroblast
ECM – Extracellular matrix
EDTA – Ethylenediaminetetraacetic acid
EdU – 5-ethyl-2'-deoxyuridine
FAP – Fibro-adipogenic progenitor
FBS – Fetal bovine serum
FGF – Fibroblast growth factor
GFP – Green fluorescent protein
Grem1 – Gremlin 1
HIC1 – Hypermethylated in Cancer 1
HO – Heterotopic ossification
LepR – Leptin receptor
MSC – Mesenchymal stromal cell
NGS – Normal goat serum
OCT – Optimal cutting temperature
OPG – Osteoprotegerin (Tnfrsf11b)
PBS – Phosphate buffered saline
PDGFRα – Platelet-derived growth factor receptor alpha
PFA – Paraformaldehyde

RANK – Receptor activator of nuclear factor kappa-B (Tnfrsf11a)

RANKL – Receptor activator of nuclear factor kappa-B ligand (Tnfsf11)

Sca1 – Stem cell antigen 1

SSC – Skeletal stem cell

TGFβ – Transforming growth factor beta
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Dedication

For my family and friends

“I was taught that the way of progress was neither swift nor easy”

-Marie Curie
Chapter 1: Introduction

1.1 Bone and Regeneration

Bone is constantly remodeled throughout life by bone resorbing osteoclasts and bone depositing osteoblasts. It has been well established that osteoblasts are derived from mesenchymal progenitors, however the identification of these progenitors remains unclear. As our population ages, osteoporosis and related fractures increase placing a burden on the healthcare system. Thus, it has become more important than ever to identify and understand the biology of skeletal mesenchymal progenitors to provide cellular and molecular targets for therapeutics.

This thesis concerns the role of specific mesenchymal cell populations in bone homeostasis and regeneration. To provide context for these studies, this section provides an overview of basic bone biology, the specialized cells of bone, bone remodeling, fracture healing, and bone disorders, particularly ectopic bone.

1.1.1 Bone structure and function

The skeletal system, composed of bones and cartilage, performs many critical roles including providing structure and support for movement, protection of vital organs, and is the site of hematopoiesis. More recent evidence has also shown that bone performs many endocrine functions, acting as a reservoir for minerals, growth factors, and cytokines. This multitude of functions is mediated by the structure and composition of bone, a mixture of cells and mineralized extracellular matrix. The organic component of the skeletal system makes up about 25% of the
bone volume, with approximately 90% of that volume composed of type 1 Collagen and the remaining 10% as non-collagenous proteins \(^{1-3}\). Another 65% of the bone volume is made up of inorganic components, primarily hydroxyapatite, which gives bone its rigid tensile strength \(^{4}\). Organized into compact cortical bone and woven trabecular or cancellous bone, bone arises during development through either intramembranous ossification – in which bones such as the scapulae and craniofacial bones form directly from mesenchymal cell condensations – or endochondral ossification, in which bones such as the long bones the femur and humerus are formed by the formation of a cartilage template that is subsequently replaced by bone \(^{5}\).

Bones, though highly mineralized, are not static and undergo constant bone remodeling to maintain their strength and participate in endocrine functions \(^{5}\). Bone remodeling which will be discussed in detail further on, is a tightly coupled process by which old bone is resorbed and new bone deposited. These processes are mediated by the specialized cells of bone: osteoclasts, osteoblasts, and osteocytes.

1.1.2 The cells of bone

1.1.2.1 Osteoclasts

Osteoclasts are tissue-specific multinuclear macrophages that are responsible for the resorption of bone \(^{6}\). Haematopoietically-derived from myeloid precursors, differentiation of osteoclasts requires M-CSF and RANKL (Tnfsf11) stimulation which promote the expression of NFATc1 and
DC-Stamp which in turn induce the expression of characteristic osteoclast genes such as Cathepsin K (Ctsk), calcitonin receptor, β3-integrin, and tartrate-resistant acid phosphatase (TRAP, Acp5) (7-10). Mature osteoclasts are activated in part by binding of osteoblast-secreted RANKL to the osteoclast RANK receptor (Tnfrsf11a), which induces polarization of the osteoclast and formation of a tight junction between the bone surface and basal membrane (the ruffled border), forming a sealed compartment below the osteoclast (6). Acidification of the compartment below the ruffled border and secretion of various enzymes such as cathepsin K, and matrix metalloproteinases (MMPs) dissolve hydroxyapatite crystals and degrade bone (11-13). Increases in osteoclast differentiation and activation can result in bone diseases such as osteoporosis and therefore must be closely regulated. Preventing aberrant differentiation and activation of osteoclasts, osteoprotegerin (OPG, Tnfrsf11b) is secreted by osteoblasts and binds to RANKL preventing its binding to the osteoclast RANK receptor making the RANK/RANKL/OPG axis an important mediator of osteoclastogenesis (14).

1.1.2.2 Osteoblasts

Osteoblasts are responsible for bone formation. As their main function is to synthesize bone matrix proteins such as Collagen1α1, osteocalcin, and alkaline phosphatase, they have morphological characteristics of protein-secreting cells including a polarized cuboidal shape, extensive endoplasmic reticulum, prominent Golgi apparatus, and secretory vesicles (15,16). Osteoblasts are mesenchymally-derived though the identity of these mesenchymal progenitors remains in question and will be addressed further on. Mesenchymal differentiation of osteoblasts occurs through a series of steps beginning with the expression of Sox9 which is expressed in committed
osteochondroprogenitors. This is followed by the expression of *Runx2* (CBFA1) and Osterix (OSX, *Sp7*). *Runx2* is widely considered the master regulator of osteoblast differentiation and in the absence of *Runx2* or *Sp7*, osteoblast differentiation fails, and bone is not formed \(^{(17-21)}\). Activating transcription factor 4 (*Atf4*), also has important roles in the maturation of osteoblasts regulating the expression of osteocalcin and RANKL (which regulates osteoclast differentiation and function) as well as promoting efficient amino acid transport to facilitate protein synthesis \(^{(17,22)}\). Following differentiation, mature osteoblasts synthesize bone matrix and produce various extracellular matrix proteins including collagens (primarily collagen type I), non-collagenous proteins (bone sialoprotein II, osteocalcin (OCN/Bglap), osteonectin (ON, *Sparc*), osteopontin (OPN, *Spp1*)), and proteoglycans (decorins and biglycan) \(^{(2,16)}\). Though the precise mechanisms of bone mineralization are still poorly understood and are thought to involve matrix extracellular vesicles that act as nucleation points for mineralization, osteocalcin, osteopontin, osteonectin, and alkaline phosphatase \(^{(2,23,24)}\). At the end of the bone formation phase, osteoblasts either undergo apoptosis, become bone lining cells (quiescent cells on the bone surface), or become osteocytes embedded in the newly deposited matrix.

**1.1.2.3 Osteocytes**

Entombed within individual lacunae of mineralized bone, osteocytes are long lived cells that account for 95% of the cells in mature bone \(^{(25)}\). Osteocytes are connected to each other and the bone surface through canalicular processes and have several important functions in bone including mechanotransduction, regulation of bone remodeling, and regulation of phosphate and calcium homeostasis \(^{(26)}\). The transition from osteoblast to osteocyte is still poorly understood; however, it
is believed to be an active, rather than passive process that occurs through several transitional stages mainly defined by morphology (27-29). Osteocytes can be identified by their expression of FGF23 (Fgf23), and sclerostin (SOST, Sost) as well as several small-integrin binding ligand N-linked glycoprotein (SIBLING) members including DMP1 (Dmp1), MEPE (Mepe), and PHEX (Phex) (30-34).

FGF23 is of particular importance as it is a potent regulator of phosphate homeostasis in the bone/kidney axis and one of the most important osteocyte-secreted endocrine factors (35). DMP1, PHEX, and MEPE act as regulators of FGF23 expression and activity and inactivating mutations have well-characterized effects on the skeletal system (reviewed in (36)). Sclerostin, a Wnt/β-catenin antagonist, has inhibitory effects on bone formation and its expression by osteocytes is regulated by parathyroid hormone (PTH) and mechanical stimulation. In areas of high mechanical strain, sclerostin is downregulated thereby stimulating osteoblast differentiation and matrix deposition (37). Interestingly, osteocyte apoptosis has also been implicated in recruitment of osteoclasts and initiation of the bone remodeling cycle (38). Thus, osteocytes not only act as endocrine cells, but are also involved in regulating the bone remodeling cycle.

1.1.3 Bone remodeling

Together, osteoclasts, osteoblasts, and osteocytes form the basic multicellular unit that remodels bone. Bone remodeling involves a series of five sequential steps occurring simultaneously but asynchronously at multiple skeletal sites: activation, resorption, reversal, formation, and return to quiescence (16,39). While similar to bone remodeling, bone modelling (the uncoupled deposition or
resorption of bone that allows for growth and shaping of bones) is less common in adults than children. Normal bone remodeling is necessary for bone’s endocrine functions, normal growth and strengthening, as well as for fracture healing, however an imbalance of bone resorption and deposition can result in pathologies \(^{(16)}\). As such, bone remodeling is tightly regulated by local and systemic factors that induce targeted or non-targeted remodeling.

The primary pathway regulating bone remodeling is the RANK/RANKL/OPG pathway which was identified in the 1990s and which can itself also be regulated by a number of systemic factors\(^{(40)}\). In this pathway, osteoblasts and osteocytes secrete RANKL which binds to the RANK receptor on osteoclast precursors driving their differentiation and activity while OPG, also secreted by osteoblasts and osteocytes, acts a decoy receptor for RANKL inhibiting osteoclastogenesis and bone resorption. RANKL production is induced by IL1, TNF\(\alpha\), 1,25-dihydroxyvitamin D\(_3\), PTH, and PTHrP, whereas OPG production is induced by estrogen and BMP-2 \(^{(39)}\) and the ratio of RANKL:OPG determines the rate of osteoclastogenesis. In targeted bone remodeling, osteocytes produce RANKL and initiate the bone remodeling cycle in response to mechanical loading, thereby directing bone remodeling to areas of microdamage \(^{(41)}\). Non-targeted bone remodeling, also termed stochastic remodeling, is regulated systemically by hormones such as calcitonin, 1,25-dihydroxyvitamin D\(_3\) (calcitriol), estrogens, glucocorticoids, PTH, and PTH related protein (PTHrP) which can have varied and dose-dependent effects on bone remodeling \(^{(39)}\)\(^{(16,24)}\). PTH for example, when administered continuously induces bone loss by increasing osteoblast and osteocyte RANKL production and inhibiting OPG, while intermittent PTH reduces expression of sclerostin, increasing Wnt/\(\beta\)-catenin mediated osteoblast differentiation \(^{(42)}\). Thus, regulation of bone remodeling is the result of multiple signaling pathways.
1.1.3.1 Coupling of bone remodeling

Osteoblasts and osteocytes initiate osteoclast differentiation and action; however, this must be coupled with osteoclast mediated control of osteoblast activity to appropriately regulate bone structure and functions. As previously mentioned, bone acts as a reservoir for many growth factors and as osteoclasts resorb bone, these factors such as b-FGF, TGFβ, BMPs, Insulin-like growth factors (IGFs), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) are released that can then act to recruit osteoblasts and osteoblast precursors, and modulate their activity (reviewed by (43) and (44)). The precise regulation necessary for coupling of bone remodeling due to matrix-derived factors alone seemed improbable, and recent evidence suggests a role for osteoclast membrane bound and secreted factors in the coupling of bone remodeling (44,45). In vivo and in vitro studies have demonstrated that osteoclast expressed EphrinB2 can bind to the EphB4 receptor on osteoblasts and promote osteoblast differentiation while reverse signaling can inhibit osteoclastogenesis, suggesting a mechanism for coupling of bone remodeling, however, contact between osteoblasts and osteoclasts in the BMU is rare, suggesting that direct cell contact may not be a major contributor to coupling (44,46). Osteoclast secreted factors that have been implicated in coupling bone remodeling through genetic studies in vivo and in vitro include sphingosine-1-phosphate (S1P), cardiophrin-1 (CT-1), BMP6, Wnt10b, collagen triple helix repeat containing (CTHRC1) (43). Semaphorins, which include both secreted and membrane-associate molecules, have also been implicated in coupling of bone remodeling with osteoclast transmembrane SEMA4D acting as an inhibitor of bone formation, and SEM3A both inhibiting osteoclastogenesis and enhancing osteoblast differentiation (47,48). More recently, SLIT3 has also
been identified as an osteoclast secreted factor regulating osteoblast migration and differentiation, implicating it in the coupling of bone remodeling (49). Altogether, this evidence suggests that coupling of bone remodeling is a complex process that is unlikely to occur via any single mechanism but through the concerted actions of multiple mechanisms.

1.1.4 Bone regeneration

Bone fractures occur regularly as a result of falls, accidents, and osteoporosis creating a heavy healthcare burden. In 2008, osteoporosis related fractures alone cost the Canadian healthcare system an estimated 2.3 billion dollars (50). However, as a highly dynamic tissue undergoing constant remodeling, bone has an enormous capacity for regeneration after injury. Beyond repair, bone is capable of true regeneration; that is restoration of the original tissues without the formation of a fibrotic scar (51). Following bone injury, the regenerative process can be divided into two histologically distinct fracture healing processes, direct and indirect fracture healing, that recapitulate embryonic developmental processes of intramembranous and endochondral bone formation respectively (52). Though most bone regeneration occurs through some combination of the two processes, stability of the fracture determines which process is predominant (52). Direct healing, in which the bone cortex attempts to directly heal and re-establish continuity through the use of discrete remodeling units known as ‘cutting cones’, recapitulates the embryonic process of intramembranous ossification and occurs primarily when there is rigid internal fixation of the fracture (51-53). Indirect healing, which occurs when the fracture is less stable and there is more interfragmentary motion, relies on endochondral ossification and follows a four-stage regenerative process (52).
1.1.4.1 Inflammatory response and hematoma

Immediately following bone fracture, a hematoma forms at the fracture site containing blood and bone marrow stromal cells as well as inflammatory cells such as macrophages and leukocytes which initiate an inflammatory response peaking 24 hours post injury \(^{(52)}\). Though inflammation, especially chronic inflammation, is usually implicated with negative skeletal effects, an acute inflammatory response is required for proper bone regeneration \(^{(54)}\). TNF\(\alpha\), IL-1, and IL-6 mediate the acute inflammatory response including recruitment of additional inflammatory cells, promoting angiogenesis, and recruitment of cells necessary for bone regeneration \(^{(55)}\). In the absence of TNF\(\alpha\) or IL-6, mesenchymal stromal cell (MSC) differentiation and fracture regeneration are delayed \(^{(56,57)}\). PDGF and TGF\(\beta\) released by thrombin activated platelets in the hematoma also initiate fracture regeneration by recruiting mesenchymal cells and initiating angiogenesis \(^{(58)}\). BMP-2 is also expressed early in the inflammatory response and induces MSC migration and differentiation \(^{(59)}\).

1.1.4.2 Soft callus formation

Following the acute inflammatory response, a fibrin-rich granulation tissue forms followed by the formation of a fibrocartilage callus that stabilizes the fracture and is characteristic of indirect endochondral bone regeneration. In murine models, soft callus formation peaks 7-10 days post injury \(^{(60)}\). The process of soft callus formation requires the recruitment and differentiation of MSCs though the identity and origins of these MSCs remain a topic of scientific interest.
Additionally, there is a subperiosteal intramembranous ossification response that occurs adjacent to the ends of the fracture and provides a semi-rigid structure to the callus (60). TGFβ and BMPs play important roles in fracture healing, and expression of various TGFβ and BMP family members is temporally regulated (reviewed in (53)). In particular TGFβ2 and TGFβ3 are upregulated during the chondrogenic stage of fracture repair and stimulate fibroblast proliferation and chondrocyte proliferation and differentiation (59). BMP-5/6 are also highly expressed at this time suggesting they have roles in intramembranous and endochondral bone formation (59). In the final stages of soft callus formation, the chondrocytes within the cartilaginous callus undergo hypertrophy and the extracellular matrix becomes calcified. VEGF secreted by hypertrophic chondrocytes and osteoblasts promotes angiogenesis and vascularization of the fracture callus in preparation for hard callus formation (61).

1.1.4.3 Hard callus formation

While soft callus formation is predominantly anabolic, the hard callus formation stage is catabolic and after a peak at approximately day 14 post injury, is characterized by a reduction in callus volume (51). During this stage, hypertrophic chondrocytes undergo apoptosis and calcified cartilage is resorbed and replaced by woven bone deposited by osteoblasts. This is possibly regulated by the expression of BMP-3/4/7/8 which are restricted to this time of active osteogenesis (59). Further, this process is heavily reliant on adequate vascularization mediated by VEGF signaling (62). Once the woven bone has replaced the cartilage and bridges the fracture providing rigid mechanical support, bone remodeling begins.
1.1.4.4 Callus remodeling

Remodeling of the callus is necessary to restore its biomechanical properties and while the process begins around 21 days post injury, it can continue for up to several years (54). This period of bone remodeling restores the lamellar structure of the bone and medullary cavity. The process of bone remodeling occurs through the action of osteoclasts and osteoblasts as described earlier and is possibly regulated by TNFα and IL-1 which are highly expressed at this stage (55).

Thus, bone regeneration is a complex biological cascade that includes an inflammatory response, recruitment and differentiation of mesenchymal cells, and re-vascularization. Despite bone’s ability to regenerate, delayed or non-union incidence rates of 2-30% have been reported (63). This has spurred the development of therapies to enhance bone regeneration. These therapies include biophysical enhancements using ultrasound or electromagnetic field stimulation, and local or systemic biological enhancements (51). Local biological enhancements including the use of autologous bone grafts, PDGFs, BMPs, and FGFs to enhance skeletal repair require surgical intervention, thereby limiting their use (51,64). While systemic biological enhancements using PTH and αSOST antibodies have also been used successfully to enhance bone regeneration, MSCs have emerged as both a potential new therapy and cellular target to enhance bone regeneration (51,64,65), however, their use remains limited as there are still significant gaps in the scientific literature about skeletal progenitors and MSCs.
Skeletal dysplasias are a heterogenous group of heritable skeletal disorders affecting bone and cartilage and as of 2019, the Nosology Committee of the International Skeletal Dysplasia Society has identified 461 disorders classified into 42 different groups. For 425 of the listed disorders, 437 genetic alterations have been identified and many affected genes are associated with pathways that have roles in skeletal development and homeostasis, including the TGFβ, BMP, and WNT signaling pathways (66). Fibrodysplasia Ossificans Progressiva (FOP) (OMIM #135100) is an example of such skeletal dysplasias that is characterized by heterotopic ossification (HO), the formation of extraskeletal bone in muscle and soft tissues. FOP results from mutations in the gene Acvr1, which encodes the BMP type 1 receptor ALK2 (ACVR1), resulting in increased BMP signaling and ossification of soft tissues which eventually leads to premature death (67).

Interestingly, non-genetic or acquired HO is vastly more common and the cellular and molecular mechanisms behind it poorly understood, limiting treatment options. Acquired HO commonly arises after injury including orthopedic surgery (68-70), central nervous system trauma (71-73), burns (74,75), and high energy traumas (76,77). Results from animal models suggest that local mesenchymal cells are the source of acquired HO, and recent research has highlighted the existence of resident cells in adipose tissue and the kidney capsule that despite the absence of local skeletal progenitors, can be induced to osteogenesis (78), however the identity of these cells remains unclear. Markers such as Scx (79,80), GLAST (81), Tie2 (82,83), Gli1 (84), and Mx1 (79) have been used to study acquired HO, however their usefulness has been limited by their expression on multiple cell types or incomplete labelling of the induced HO lesions (reviewed in (85)). Intriguingly, amongst all these markers, a subpopulation of PDGFRα+ cells were identified and Tie2+PDGFRα+ cells were
identified as the main cellular source of HO in a murine FOP model \(^{(86)}\). PDGFR\(\alpha\), has become well established as a marker of Fibro-Adipogenic Progenitors (FAPs), a mesenchymal progenitor cells, in various tissues including skeletal muscle with osteogenic potential \textit{in vitro} \(^{(87,88)}\). Finally, there is evidence that FAP-like cells exist in humans \(^{(89)}\). Thus, PDGFR\(\alpha^+\) FAPs (which will be reviewed in the following section), represent a potential cellular source of HO and therapeutic target for HO worthy of further investigation.

1.2 MSCs and SSCs in Bone

1.2.1 What is an MSC?

In the 1970s, Friedenstein et al first identified a subpopulation of non-hematopoietic bone marrow cells which appeared fibroblastic and were able to adhere to plastic. They further demonstrated that these cells could form colonies initiated by a single cell (colony-forming unit fibroblastic, CFU-Fs) and that transplantation of these cells \textit{in vivo} resulted in the differentiation of multiple skeletal tissues including bone, cartilage, fibrous tissue, and adipocytes \(^{(90)}\). Owing to their multilineage potential, Owen and Friedenstein referred to these cells as osteogenic stem cells or bone marrow stromal stem cells \(^{(91)}\). In 1991, Arnold Caplan coined the term ‘Mesenchymal Stem Cell’ to describe these cells with multilineage potential including bone, cartilage, ligament, tendon, stroma, adipocytes, dermis, and connective tissues, drawing parallels with embryonic stem cells \(^{(92)}\). The existence of a stem cell with multilineage potential held great therapeutic potential, drawing enormous scientific interest and putative mesenchymal stem cells were identified in a variety of adult tissues \(^{(93)}\).
The name ‘mesenchymal stem cell’ has since been criticized in great part because there are strict criteria for the definition of stem cells including demonstration of self-renewal and multilineage potential. It was only recently that the ability to self-renew was demonstrated in bone marrow-derived mesenchymal stem cells (94,95). These bone marrow-derived mesenchymal cells were able to generate bone, cartilage, adipocytes, and stroma, recapitulating a bone marrow microenvironment that was capable of supporting hematopoiesis (95). Putative mesenchymal stem cells in other tissues have demonstrated clonogenicity and multilineage potential, however these results were limited to in vitro studies, and their relationship to bone marrow derived cells remain unclear. Thus, some groups have proposed that bone marrow derived cells with confirmed self-renewal and skeletal lineage potential be called skeletal stem cells (SSCs), while cells from other tissues without confirmed in vivo clonogenicity or self-renewal capacity be termed mesenchymal stromal cells or mesenchymal progenitors (96). For the purposes of this thesis, the abbreviation MSC will refer to mesenchymal stromal cells as just described.

Within the bone marrow, in addition to their skeletal lineage potential, MSCs were demonstrated to be important niche cells, supporting the function of hematopoietic stem cells (reviewed in (97)). This suggested that MSCs in other tissues may similarly play supporting or trophic roles for tissue-specific stem cells during regeneration. Indeed, quiescent MSCs have been identified in skeletal (87,88,98) and cardiac muscle (99,100), skin (101,102), lung (103,104), kidney (105,106), and liver (107) that upon damage, proliferate and provide trophic support for regeneration, and in contexts of failed regeneration contribute to the development of fibrosis. Thus, a role for MSCs in extra-skeletal tissues is beginning to emerge. Though these MSC populations were once thought to be similar across tissues, it is becoming increasingly clear, especially in studies of fibrosis, that there are
tissue-specific differences in MSC populations and their lineage potential *in vivo* \(^{(108)}\). Thus, this suggests that it is important to study these MSC populations in tissue-specific and lineage-specific contexts.

### 1.2.2 Skeletal stem cells and progenitors

As the world faces an ageing population susceptible to osteoporosis and bone fractures, the quest to identify skeletal stem cells and understand their biology so they can be used or targeted therapeutically has become more pressing. Like extra-skeletal MSCs, many markers for SSCs and skeletal MSCs have been proposed, all with varying contributions to skeletal development, homeostasis, and regeneration. Here, we provide a review of several proposed murine SSC and MSC populations. The highlighted markers and their characteristics are summarized in Table 1-1.

#### 1.2.2.1 Nestin

In one of the seminal studies of murine MSCs/SSCs, Mendez-Ferrer et al \(^{(95)}\) used Nestin, a marker originally identified in neural stem cells, to identify bone marrow MSCs. Using a Nestin-GFP reporter, they identified a population of perivascular cells with the ability to self-renew and differentiate into osteochondral lineages *in vivo*. Nestin\(^+\) cells were also identified as an important part of the hematopoietic stem cell niche and in a subsequent study by the same group \(^{(109)}\), it was determined that Nestin\(^+\) cells could be divided into Nestin\(^{\text{dim}}\) and Nestin\(^{\text{bright}}\) cells. They demonstrated that Nestin\(^{\text{bright}}\) cells were positive for the pericyte NG2 marker and while still containing high CFU-F potential, were quiescent and more closely associated with hematopoietic
stem cells (HSCs). Nestin<sup>dim</sup> cells conversely, overlapped with LepR<sup>+</sup> cells. These studies were pioneering in demonstrating the self-renewal of SSCs <i>in vivo</i>, however they did not demonstrate whether Nestin<sup>+</sup> cells contribute to bone regeneration. Further, the reliability of Nestin as a marker of SSCs or MSCs has come into question as Nestin lineage tracing using various cre models has revealed differing expression patterns and a contribution to multiple cell populations, including endothelial cells, depending on which model was used<sup>110,111</sup>.

1.2.2.2 LepR

Leptin, a fat-derived hormone, has been implicated in osteogenesis<sup>112,113</sup> and recently, Leptin receptor (LepR) has been identified as a marker of skeletal MSCs. Ding et al<sup>111</sup> originally demonstrated that stromal perivascular LepR<sup>+</sup> cells expressed high levels of stem cell factor (SCF, kit ligand) and CXCL12, essential HSC niche factors, and were required for maintenance of the HSC niche suggesting they highly overlapped with CXCL12-abundant reticular (CAR) cells. It was then further demonstrated by Zhou et al<sup>114</sup> that perivascular LepR<sup>+</sup> cells arise postnatally and are the main source of osteogenic and bone marrow adipogenic cells that arise in adult mice, though little contribution to chondrogenic cells was found at homeostasis. Zhou et al also showed that LepR<sup>+</sup> cells are quiescent but injury responsive. After injury, LepR<sup>+</sup> cells give rise to both osteogenic and chondrogenic cells in the fracture callus. Further, LepR<sup>+</sup> cells expressed PDGFR<sub>α</sub>, PDGFR<sub>β</sub>, CD51, CD105, which have been identified as MSC markers and suggested that LepR<sup>+</sup> cells were enriched for MSCs, though <i>in vitro</i> analysis of LepR<sup>+</sup> CFU-Fs demonstrated variable skeletal lineage potential. More recent evidence using single cell RNA-sequencing has indicated that LepR<sup>+</sup> cells contain at least 4 subpopulations with varied osteogenic and adipogenic markers.
Together, this suggests that LepR$^+$ cells may represent a heterogeneous population of skeletal MSCs.

1.2.2.3 Grem1

BMPs play an important role in skeletal development and recently, Gremlin1 (Grem1), a BMP antagonist, has been used to identify another population of skeletal MSCs. Worthley et al (116) used Grem1 to label what they termed an osteochondroreticular (OCR) cell. Shortly following labelling in the adult, Grem1 labelled a rare population of cells immediately adjacent to the growth plate that contributed to osteogenic, chondrogenic, and reticular stromal cell populations, but not adipogenic cells. Unlike Nestin$^+$ and LepR$^+$ cells, Grem1$^+$ cells contribute to skeletal tissues, both developmentally and in the adult. Interestingly, while trabecular, metaphyseal, and epiphyseal bone were Grem1-derived, very little diaphyseal bone was labelled by Grem1, perhaps reflecting the metaphyseal location of Grem1$^+$ cells at the time of labelling. Worthley et al found that Grem1$^+$ cells were required for postnatal skeletogenesis and in a tibial fracture model, Grem1$^+$ cells also contributed to osteogenic and chondrogenic cells during bone regeneration. While Grem1$^+$ cells were enriched for CD105, they were not further enriched for the murine MSC markers PDGFR$\alpha$ or Sca1. Due to the incomplete contribution of Grem1$^+$ cells to skeletal tissues and lack of overlap with LepR$^+$ cells in vivo, the authors suggest that Grem1$^+$ cells are one of several skeletal MSC populations that make temporal and lineage specific contributions to skeletal development, homeostasis, and regeneration.
1.2.2.4 mSSC/BSCP

Hypothesizing that skeletal system differentiation followed a similar program to hematopoietic cells, Chan et al \(^{(78)}\) sought to identify a multipotent murine skeletal stem cell. Using a Rainbow mouse, Chan et al observed a high level of clonogenicity in the femoral growth plate. Isolating cells from the growth plate, they found a high number of $\text{CD}45^-/\text{Ter}119^-/\text{Tie}2^-/\text{AlphaV}^+$ ($\text{AlphaV}^+$) cells which they then fractionated into 8 distinct subpopulations based on the expression of CD105, Thy, 6C3, and CD200. Different developmental fates (chondrogenic, osteogenic, and stromal) were observed for the different subpopulations suggesting there are distinct progenitors for each lineage. Through a series of elegant experiments, they determined that $\text{AlphaV}^+/\text{Thy}^-/6C3^-/\text{CD}105^-$/$\text{CD}200^+$ (referred to as CD200$^+$ or mSSC) gave rise to the remaining 7 populations \textit{in vitro} and \textit{in vivo} including $\text{AlphaV}^+/\text{Thy}^-/6C3^-/\text{CD}105^+$ cell which they had previously identified as the bone, cartilage, and stromal progenitor (BCSP) \(^{(117)}\). Chan et al found that mSSCs self-renewed \textit{in vivo} and were increased in the fracture callus compared to uninjured bone where they expressed higher levels of osteogenic genes, suggesting they have a role in bone regeneration. While Chan et al also demonstrated that lineage commitment of mSSCs and their subsets could be altered by VEGF and BMP, \textit{no in vivo} lineage tracing or positional information was provided in adult skeletal homeostasis or regeneration, nor was there any indication of whether mSSCs contribute to skeletal development, possibly owing to the complex cell surface phenotype of mSSCs. Nonetheless, Chan et al provided a mSSC lineage map that can be used to identify mSSCs and their progeny.
Indian hedgehog (Ihh) signaling has important roles in embryonic endochondral bone formation, via the Gli transcription factors including Glioma-associated oncogene 1 (Gli1). Using a Gli1\textsuperscript{CreERT2} lineage tracing system, perivascular Gli1\textsuperscript{+} cells were identified as MSCs co-expressing CD29, Sca1, CD44, and CD105 in various tissues including kidney, liver, lung, heart, bone marrow, and muscle where they contributed to injury-induced fibrosis \textsuperscript{(106)}. Shi et al\textsuperscript{(118)} later identified Gli1\textsuperscript{+} cells in the metaphyseal region just below the growth plate that were enriched for several genes used to identify MSCs including CD146, CD44, CD106, PDGFR\textalpha, PDGFR\textbeta, and LepR, and termed these cells “metaphyseal mesenchymal progenitors” (MMPs). Shi et al demonstrated a postnatal developmental contribution of Gli1\textsuperscript{+} cells to osteogenic, chondrogenic, stromal, and adipogenic lineages. They also demonstrated that postnatal Gli\textsuperscript{+} cells contributed to osteogenic and chondrogenic lineages in homeostasis and bone regeneration and were essential for trabecular bone formation. Further, they demonstrated that Gli1\textsuperscript{+} cells gave rise to LepR\textsuperscript{+} stromal cells. Interestingly, Shi et al found that while MMPs were abundant in juvenile mice, they decreased with age and labeling of 4 and 12-month-old mice revealed very few or no Gli1\textsuperscript{+} cells in the metaphyseal region. No work was performed to examine whether Gli1\textsuperscript{+} cells exhibited self-renewal, so it is unclear whether MMPs though multipotent, are truly an SSC population. Overall, this work indicates that MMPs are an important skeletal MSC population during development and bone regeneration and may give rise to other MSC populations such as LepR\textsuperscript{+} cells and supports the idea of several temporal and lineage specific MSC populations contributing to skeletal development, homeostasis and regeneration.
1.2.2.6 PTHrP

Within the growth plate, the resting zone maintains the growth plate by expression of PTHrP which interacts with Ihh released from hypertrophic chondrocytes\(^{(119)}\). Based on this, Mizuhashi et al\(^{(120)}\) used PTHrP to identify a population of skeletal MSCs in the resting zone of the growth plate. They found that PTHrP\(^+\) cells in the resting zone self-renewed \textit{in vivo} and gave rise to columnar chondrocytes and as well as bone and stromal cells below the growth plate, but not adipogenic cells, during postnatal bone growth albeit at a low frequency. The authors also demonstrated that PTHrP\(^+\) cells were necessary for maintaining the growth plate. Unfortunately, the authors did not examine the expression or contribution of PTHrP\(^+\) cells to adult murine bone or in a regenerative context, leaving questions about its role in the adult skeletal system. Interestingly, in addition to \textit{in vivo} self-renewal, Shi et al demonstrated that a portion of PTHrP\(^+\) cells expressed markers associated with mSSCs and BCSPs described by Chan et al\(^{(78)}\) including CD105 and CD200, suggesting that PTHrP\(^+\) cells represent a heterogenous population of MSCs including SSCs.

1.2.2.7 The missing periosteal piece

In summary, multiple SSC and skeletal MSC populations have been reported in the literature, all with distinct anatomical locations and varied lineage potential during development, adult homeostasis, and regeneration. Other candidate markers for skeletal MSCs have also emerged that were not discussed. For example, Hoxa11 was described as a skeletal MSC that gave rise to all skeletal lineages, however its expression is limited to the zeugopod skeleton and therefore cannot be used to identify MSCs in other skeletal sites\(^{(121)}\). Osx, was also suggested as a marker of stromal
progenitors during development, however in the adult, Osx is also expressed in osteoblast precursors and thus does not represent an MSC marker \(^{(122)}\). Interestingly, though the importance of the periosteum to bone regeneration has been known since the 1800s \(^{(123)}\), none of the reported SSC/MSC populations were identified in the periosteum (Figure 1.1). Recent reports have reaffirmed that there is a putative SSC with regenerative potential within the periosteum, however the authors were unable to identify a unique marker for these cells \(^{(124)}\). Another group has proposed Cathepsin K (Ctsk) as a marker for periosteal MSCs, however it is also a commonly used marker of osteoclasts \(^{(125)}\). Thus, this suggests that there is an as yet unidentified population of periosteal MSCs whose identification could make them a potential source and target for therapeutic treatments.

1.2.3 Potential SSC or skeletal MSC markers

MSCs are not uniquely found in skeletal tissues, and many of the studies of SSCs and skeletal MSCs have used markers identified in MSCs from other tissues to confirm the ‘MSC’ identity of their selected cell. It therefore stands, that these markers can be examined using lineage tracing models in the skeletal system to examine their contributions to osteogenic lineages. Here, we discuss 2 such markers, PDGFR\(\alpha\) and HIC1, that have been identified in various tissue resident MSCs, but which have not as yet, been described in the skeletal system.
1.2.3.1 PDGFRα

Stem cell antigen 1 (Sca1, Ly6a) has been used to identify stem and progenitor cells, however its use as an independent marker of MSCs is problematic due to its widespread expression in multiple cell types including endothelial cells, and as it is not expressed in humans, a lack of translation to human models (reviewed in 126). More recently however, Sca1 has been used in conjunction with PDGFRα to label tissue resident MSCs.

Platelet derived growth factor receptor α (PDGFRα), is one of two PDGF receptor subunits that complex to form homo- or heterodimers and interact with PDGF ligands (PDGF-A, PDGF-B, PDGF-C, PDGF-D) to induce various cellular processes such as differentiation, migration, proliferation, and survival 127,128. PDGFRα is expressed during development in the paraxial mesoderm as well as neural crest derived mesenchymal cells and PDGFRαnull/null embryos die at E10, indicating its importance to development 129,130. In the adult, PDGFRα cells are found in stroma of most tissues including bone marrow 131,132, cardiac 99,100 and skeletal muscle 87,88, the central nervous system 133, adipose tissue 134, lung 104, thymus 135, dermis 101, and colon. Our lab has demonstrated that in skeletal and cardiac muscle, CD31CD45PDGFRα+Sca1+ cells are perivascular interstitial cells with fibrogenic and adipogenic potential (Fibro-Adipogenic Progenitors, FAPs). FAPs are quiescent and respond quickly to injury, providing trophic support to tissue-specific stem cells during regeneration and contributing to tissue fibrosis during tissue repair or degeneration 87,100. Further, additional studies demonstrated that skeletal muscle PDGFRα+ cells could generate bone in vitro and in vivo 83,88 and almost all candidate markers for
ectopic bone mesenchymal progenitors were found to contain PDGFRα+ subpopulations \(^{(85)}\). Within the bone marrow, Morikawa et al \(^{(132)}\) identified non-hematopoietic perivascular PDGFRα+Scal+ (PαS) cells that were enriched for CFU-F and exhibited osteogenic, chondrogenic, stromal, and adipogenic lineage potential. Further, PαS cells were found to express other markers associated with MSCs including CD105, and PDGFRβ \(^{(132)}\). Lastly, many putative SSC and skeletal MSC populations discussed above also reported PDGFRα expression by their cells of interest. The ubiquitous use of PDGFRα as an MSC marker in many other tissues and its presence in the bone marrow and other reported SSC/skeletal MSC populations, including those implicated in heterotopic ossification, suggests that it could potentially be a marker for skeletal MSCs with regenerative potential. Yet, despite the longstanding availability of PDGFRα genetic reporter mice \(^{(136)}\), there have been no reports of expression of PDGFRα in the adult murine skeletal system.

### 1.2.3.2 HIC1

Hypermethylated in cancer 1 (HIC1), a zinc finger transcription factor, was originally identified as a candidate tumor suppressor gene that is frequently hypermethylated in various cancers \(^{(137)}\) and is suggested to have a role in maintaining cellular quiescence. More recently, HIC1 has emerged as a potential MSC marker. Scott et al \(^{(98)}\) demonstrated that HIC1 labels multiple quiescent mesenchymal progenitor (MP) subsets in skeletal muscle that are activated after injury and support muscle regeneration. They demonstrated that in skeletal muscle, HIC1 had significant overlap with FAPs and deletion of HIC1 resulted in MP expansion and impaired muscle
regeneration. Scott et al also demonstrated that HIC1 identified tenogenic and myotenogenic progenitors that contributed to mature tenocyte and myotenocyte populations after injury as well as adipocytes, myofibroblasts, and pericytes, confirming multi-lineage potential (138). Similarly, HIC1 labels a population of multipotent mesenchymal progenitors in cardiac muscle that contribute to fibrosis and fatty degeneration after damage (100). In accordance with the results observed by Scott et al, Soliman et al also noted that in the heart, PDGFRα+ cells represented the bulk of HIC1+ cells, indicating there was a high degree of overlap between HIC1 and PDGFRα populations. The Underhill lab has also demonstrated that HIC1 labels perivascular MSC-like populations with multilineage potential in other almost all tissues except cartilage (unpublished data). Together, this places HIC1 as a marker of MSCs across multiple tissues. Interestingly, the Hic1 gene is located in a chromosomal region frequently deleted in patients with Miller-Dieker Syndrome (MDS) (OMIM #247200) (139,140), a syndrome characterized by craniofacial dysmorphism and defects of the digits and limbs, in addition to omphalocele, lissencephaly, and mental retardation (141), suggesting that HIC1 may have roles in skeletal development. In a HIC1null/null model, mice died prenatally with no HIC1null/null mice surviving past E18.5 (142). Examination of embryos revealed that among other defects, many had craniofacial defects such as acrania and exencephaly as well as limb and digit dysmorphologies, further suggesting that HIC1 may have a role in craniofacial and limb/digit development (142). Together, the possible role of HIC1 in skeletal development and evidence as an MSC marker in multiple other tissues suggests it may represent a novel SSC or skeletal MSC population.
1.3 TGFβ/BMP Signaling and Bone

Transforming growth factor receptor β (TGFβ) and bone morphogenetic proteins (BMPs) have important physiological roles in both development and homeostasis as well as fate determination of MSCs. This last point is evidenced by the use of BMPs to induce and study ectopic bone formation, and research demonstrating that TGFβ induces fibrosis in cardiac and skeletal muscle \textit{in vivo}. Within the skeletal system, the TGFβ and BMP signaling pathways play fundamental roles in embryonic skeletal development as well as post-natal skeletal homeostasis. Here, we provide a brief overview of TGFβ and BMP and their roles in bone through canonical and non-canonical signaling pathways.

1.3.1 TGFβ superfamily

The TGFβ superfamily is composed of TGFβs, BMPs, activins/inhibins, and related proteins such as nodal and growth differentiation factors (GDFs). TGFβ members bind to heteromeric cell surface receptor complexes composed of type I and type II receptors and intracellular signaling is mediated through canonical (SMAD-dependent) or non-canonical (SMAD-independent/ mitogen activated protein kinase (MAPK)) mechanisms \(^{(143)}\). In the canonical pathway, TGFβ binds to the TGFβ receptor II (TβRII) which then complexes with TβRI (ALK5) initiating phosphorylation of R-SMAD2/3 which then complex with SMAD4. The Smad complex then translocates to the nucleus where it recruits co-factors such as CREB-binding protein (CBP) and p300 to regulate gene expression. Recent studies have indicated that TβRII can complex with ALK1 or ALK2 to
induce phosphorylation of R-SMAD1/5/8 (144). Similarly, BMP signaling through the canonical pathway involves the binding of BMPs to BMPRII or BMPRIIB which then complexes with type I receptors (BMPRIA/ALK3, BMPRIIB/ALK6, ACVRI/ALK2) to initiate phosphorylation of R-SMAD1/5/8 which then complexes with Smad4 and proceeds as in TGFβ signaling (144). It should be noted here that SMAD4, the only co-Smad, is involved in SMAD-mediated responses for both TGFβ and BMP pathways. Interestingly, nuclear import of the R-Smads does not require SMAD4, however SMAD4 stabilizes R-Smad interactions with CBP and p300, thereby acting as a coactivator (145).

In non-canonical TGFβ/BMP signaling, signaling through the heteromeric receptor complexes activates TGFβ kinase 1 (TAK1) and TAK-binding protein 1 (TAB1) to initiate MKK3-p38 MAPK or -ERK signaling cascades (146).

1.3.2 TGFβ and bone

TGFβ is expressed in 3 isoforms in mammals: TGFβ1, TGFβ2, and TGFβ3 all of which are present in the perichondrium, growth plate, and periosteum during skeletal development (147-149). While TGFβ1 and TGFβ3 appear to be dispensable for embryonic skeletal development based on murine models (150,151), TGFβ2 null mice display severe abnormalities in both intramembranous and endochondral bone, suggesting it is essential for skeletal development (152). Postnatally, TGFβ1, which is released from bone during remodeling, is essential for the coupling of bone formation
and resorption to maintain bone mass by recruiting osteoprogenitors and inducing proliferation as well as regulating the RANKL/OPG ratio produced by osteoblasts (153,154).

Through SMAD-dependent mechanisms, TGFβ regulates osteoblast and chondrocyte differentiation. In osteoprogenitors, TGFβ-induced SMAD2/3 inhibits Runx2, the master regulator of osteoblast differentiation, thereby negatively regulating osteoblast differentiation (155). Similarly, SMAD3 mediated TGFβ signaling inhibits osteoblast apoptosis, delays osteoblast to osteocyte differentiation (156) and inhibits chondrocyte maturation and hypertrophy (157,158).

TGFβ also regulates bone formation through SMAD-independent mechanisms, promoting osteoblast proliferation and early differentiation of mesenchymal progenitors through MAPK pathways (159,160). Overall, TGFβ recruits and induces proliferation of mesenchymal progenitors/osteoprogenitors and inhibits differentiation of mature osteoblasts and chondrocyte hypertrophy through both canonical and non-canonical signaling pathways.

1.3.3 BMP and bone

Of the 14 BMPs that have been identified, BMP2, BMP4, BMP5, BMP6, BMP7, and BMP9 exhibit osteogenic activity (143). Impaired BMP signaling in skeletal development has been extensively studied using transgenic models and almost all have some form of skeletal defect in either chondrocyte or osteogenic compartments (reviewed in (143)) highlighting the importance of BMP signaling throughout endochondral bone development. These studies further indicated that while there are redundancies in BMP signaling during development, BMP7 is required for skeletal
patterning \(^{(161)}\), and though dispensable for development, BMP2 is necessary for the initiation of fracture healing postnatally \(^{(162)}\). In adult animals, BMP2 has been thoroughly studied and can induce robust osteogenic differentiation of mSSCs and MSCs \textit{in vitro} and \textit{in vivo} at extra-skeletal sites \(^{(78,82,83,88,116)}\). Indeed, FOP as reviewed earlier, results from activating mutations of Acvr1 that cause injury-induced heterotopic ossifications, highlighting the ability of BMPs to induce endochondral osteogenesis.

Most BMPs mediate their effects through canonical R-SMAD1/5/8 pathways, activating the transcription factor Runx2 then forming a complex with RUNX2 to initiate further osteoblast gene expression \(^{(160)}\). Interestingly, BMP’s roles in endochondral ossification are primarily mediated by SMAD1 and SMAD5 with SMAD8 contributing less to endochondral bone development \(^{(163)}\). Conversely, BMP3 which is produced by osteoblasts and osteocytes, interacts with Activin Receptor IIb (ACVRIIb) to activate R-SMAD2/3 and opposes the osteogenic action of other BMPs to inhibit osteoblast differentiation \(^{(164)}\).

BMPs can also mediate their actions through non-canonical signaling through TAK1 and p38 MAPK. Deletion of TAK1 results in growth plate defects associated with reduced chondrocyte gene expression \(^{(165)}\) and deletion of MAPK pathway members results in reduced bone mass as a result of impaired osteoblast differentiation \(^{(166)}\). Additionally, there is evidence that MAPKs promote MSC differentiation by positively regulating Runx2 \(^{(160)}\), suggesting a convergence of canonical and non-canonical signaling pathways at Runx2.
1.3.4 SMAD4 and TGFβ/BMP signaling in bone

Of particular interest is SMAD4, the only co-Smad and found at the intersection between TGFβ and BMP signaling pathways. Smad4^{null/null} mice die embryonically at E7.5 due to gastrulation defects\(^{(167)}\), and in humans, mutations in SMAD4 result in various proliferative disorders and Myhre Syndrome (MYHRS) (OMIM #139210), which is characterized by mental retardation, dysmorphic facial features, and skeletal anomalies. Skeletal defects have also been identified in various murine models of conditional Smad4 deletion in various osteogenic cells. Deletion of Smad4 in osteoblasts using Ocn-cre resulted in reduced bone mass, decreased proliferation and differentiation of osteoblasts, and reduced bone formation rate up to 7 months of age which was then followed by increased trabecular bone volume due to reduced osteoclast activity\(^{(168)}\). Deletion of Smad4 in Collagen1a1 (2.3kb) expressing osteoblasts demonstrated decreased osteoclast activity, and increased osteocyte number and density\(^{(169)}\). In another model, deletion of Smad4 in pre-osteoblasts using Osx-cre mice resulted in an osteogenesis imperfecta like phenotype resulting from impaired collagen processing and hypomineralization\(^{(170)}\). Equally, post-natal ablation of Smad4 using an Osx-CreERT2 by the same group resulted in increased proliferation in vivo and decreased osteoblast differentiation and mineralization in vitro though no skeletal phenotypes were reported\(^{(171)}\). Together, these studies suggest that canonical TGFβ and BMP signaling play multiple roles in the skeletal system and effects of Smad4 deletion are cell specific.
1.3.5 Role in adult skeletal tissues

The vast majority of studies that have examined the effects of impaired TGFβ and BMP signaling have been performed developmentally or postnatally using constitutively active cre drivers. As the post-natal period includes the rapid growth that occurs in juveniles which may be more heavily impacted by impaired TGFβ and BMP signaling, their results may not necessarily reflect the effects of TGFβ and BMP signaling in the adult skeleton. Additionally, many studies have utilized cre drivers labeling more differentiated osteogenic or chondrogenic cells and very few have examined the role of TGFβ and BMP signaling in skeletal MSC populations. Combined, this leaves the field open for the study of TGFβ and BMP signaling in the adult murine skeletal system during homeostasis and regeneration using newly available cre drivers for skeletal MSCs.

1.4 Research Aims

The literature reviewed here highlights the dynamic nature of bone and the current lack of understanding of skeletal stem/progenitor cell identity or biology. Here, we present two candidate markers for skeletal MSCs; PDGFRα and HIC1 for which we have unique inducible lineage tracing tools. PDGFRα has also been implicated in heterotopic bone formation and with new lineage tracing models, we can examine the contribution of these tissue-resident cells to heterotopic bone formation. Lastly, these transgenic tools give us a unique opportunity to study the role of TGFβ and BMP signaling in the adult to help better understand the role of these signaling pathways in maintaining skeletal homeostasis.
The aims of this work are three-fold:

**Aim 1:** Understand the role of PDGFRα+ and HIC1+ cells in skeletal tissues during homeostasis and regeneration by using novel lineage tracing models. *Specifically, we wish to understand which skeletal cell populations are labelled by each marker at homeostasis, and in the context of regeneration, what these cells contribute to the regenerative process.*

**Aim 2:** Understand the contribution of PDGFRα+ FAPs to heterotopic bone. Based on previous evidence, *we hypothesize that skeletal muscle resident PDGFRα+ FAPs are the main source of heterotopic bone.*

**Aim 3:** To study the role of SMAD-mediated TGFβ and BMP signaling in adult skeletal tissues *in vivo* using an inducible PDGFRαCreERT2. *We hypothesize that SMAD-mediated TGFβ/BMP signaling is constitutively required to maintain adult murine skeletal homeostasis.*
Figure 1.1 - Location of SSCs/skeletal MSC populations identified in the literature.

Note that though depicted in the diaphysis, Gremlin1 (OCR) cells are found in the stroma and endosteal compartments just below the growth plate.
Table 1-1 Summary of selected SSC and skeletal MSC populations reported in the literature

<table>
<thead>
<tr>
<th>Marker</th>
<th>Refs</th>
<th>Marker overlap</th>
<th>Location</th>
<th>Developmental contribution</th>
<th>Adult Homeostasis</th>
<th>Skeletal Regeneration</th>
<th>Self-renewal/ clonogenicity?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin</td>
<td>[95]</td>
<td>CXCL12</td>
<td>Marrow; perivascular</td>
<td>Y</td>
<td>Y (after 8 months)</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>LepR</td>
<td>[109]</td>
<td>LepR (Nes<em>dim)</em> NG2 (Nes*mut)</td>
<td>Marrow; perivascular around both arterioles and sinusoids</td>
<td>N N N N N</td>
<td>Y N Y Y Y</td>
<td>Y Y Y Y Y (irradiation model)</td>
<td>Clonogenicity; in vivo transplant study</td>
</tr>
<tr>
<td>Greml</td>
<td>[116]</td>
<td>CD105, CD105, PDGFRα, PDGFRβ, CD51, CD105, Sca1</td>
<td>Stromal/endosteal, particularly adjacent to the growth plate</td>
<td>Y Y Y N</td>
<td>Y Y Y Y Y</td>
<td>? ? ? ? ?</td>
<td>Yes; in vivo osteochondrogenic</td>
</tr>
<tr>
<td>CD200 (mSSC)</td>
<td>[78]</td>
<td>CD44, CD106, CD106, CD146, PDGFRα, PDGFRβ, LepR</td>
<td>Growth plate</td>
<td>Y Y Y Y Y</td>
<td>Y Y Y Y Y</td>
<td>Y Y ? ? N/A</td>
<td>Yes; in vivo</td>
</tr>
</tbody>
</table>

Summary of selected SSC and skeletal MSC populations as reviewed in Chapter 2.
Y= Yes, direct evidence, N=No, no evidence, ? = N/A, not addressed in reference.
Chapter 2: PDGFRα⁺ and HIC1⁺ Mesenchymal Progenitors in Bone Homeostasis and Regeneration

2.1 Synopsis

An ageing population and the economic burden of skeletal fractures has incited a rush of research to identify and characterize skeletal stem and progenitor cells that could potentially be used or targeted in the treatment and prevention of fractures. Using PDGFRα, a well characterized marker of mesenchymal stromal cells in various other tissues, we demonstrate that within the murine skeletal compartment, it does not represent a mesenchymal progenitor marker but rather labels all skeletal lineages including bone, cartilage, and cells of the enthesis. Our collaborators have recently identified HIC1 as a potential marker of mesenchymal progenitors with skeletal potential. Utilizing a HIC1 lineage tracing model, we demonstrate that expression of HIC1 in murine skeletal lineages is limited to a small population of periosteal cells that contribute at low levels to bone homeostasis and have extensive regenerative potential after fracture, contributing to both cartilaginous and osseous elements of the callus and regenerated bone. This work helps to further establish a functional hierarchy of skeletal progenitors wherein HIC1 labels periosteal progenitor cells with maintenance and regenerative capabilities, and PDGFRα labels more lineage committed cells within the skeletal compartment. By identifying roles for these two populations of cells in the skeleton, we hope to provide new cellular targets for therapeutics in the treatment of fractures and prevention of bone loss.
2.2 Introduction

Skeletal fractures and other bone disorders represent a substantial burden on the healthcare system and while estimates of the burden for many congenital skeletal disorders are not readily available \(^{(172)}\), the most recent 2008 figures indicate that osteoporosis related fractures alone cost the Canadian healthcare system 2.3 billion dollars, or 1.3% of the federal healthcare budget \(^{(50)}\). In a rapidly aging population, the societal burden of skeletal disorders has spurred a rush of research to identify skeletal stem cells and progenitors for the purpose of better understanding many bone disorders and identifying potential therapeutic targets.

The majority of the skeletal system arises from mesenchymal tissues, suggesting that identification of mesenchymal stromal cells or progenitors may also lead to identification of skeletal stem and progenitor cells. Many markers including Grem1, Gli1, LepR, CD105 (BCSP), PTHrP, and Hoxa11, have been proposed to label skeletal progenitors and mesenchymal cells with varied contributions to the skeletal compartment during development, homeostasis, and regeneration \(^{(114,116-118,120,121)}\). Though there is strong evidence to suggest that a periosteal population of cells plays an important role in bone regeneration, with the exception of Hoxa11\(^+\) and Grem1\(^+\) cells, the remaining proposed mesenchymal populations are found predominantly within the growth plate or bone marrow compartments \(^{(123,173)}\). As Hoxa11 is only expressed in the zeugopod skeleton, making it unsuitable for labelling of all skeletogenic progenitors, and Grem1\(^+\) cells are only found on the endosteal surfaces of bone, this suggests there is an as yet unidentified population of periosteal cells that may have extensive contribution to bone homeostasis and regeneration.
Our lab and others have previously identified PDGFRα as a marker of interstitial, perivascular cells in muscle and adipose tissues, with multi-lineage potential (87,88). Interestingly, many mesenchymal and skeletal progenitor populations, including Gremlin1+, Gli1+, LepR+, and Hoxa11+ progenitors, have all been shown to express PDGFRα. Work by Chan et al identified an AlphaV+, CD200+ murine skeletal stem cell (mSSC) that gives rise to progressively more lineage-restricted cells in a manner reminiscent of hematopoietic stem cells (78). While it is not clear whether PDGFRα is expressed by mSSCs identified by Chan et al or their downstream progeny such as bone-cartilage-stromal-progenitors (BCSPs), due to the ability of PDGFRα+ cells to make bone in vitro and in vivo, we wished to examine whether they also represented a source of endogenous skeletal progenitors (88,174). Interestingly, though various murine reporter and lineage tracing models of PDGFRα have been around since 2002, the expression of PDGFRα in adult murine bone has not previously been reported (136).

Similarly, the Underhill laboratory has more recently identified Hypermethylated in Cancer 1 (HIC1) as a novel marker for mesenchymal progenitors (MPCs) with multi-lineage potential in vitro and in vivo (98). Previous studies of HIC1 have demonstrated its expression in embryonic limb buds and homozygous deletion of the gene results in embryonic or perinatal lethality with mutant embryos exhibiting severe craniofacial and limb abnormalities (142,175). This evidence strongly suggests a role for HIC1 in bone development and as the Underhill laboratory identified HIC1 as a novel marker for MPCs, we wished to determine whether it represented a source of osteogenic cells during adult murine skeletal homeostasis and regeneration.
Here, we focus on the MSC markers PDGFRα and HIC1 identified by the Rossi and Underhill laboratories respectively as potential MSC markers and examine their expression and contributions to the homeostasis and regeneration of murine skeletal tissues. Using novel murine transgenic lineage tracing models, we demonstrate that HIC1 labels a small population of cells with extensive regenerative capacity that resemble previously identified murine skeletal stem cells. We further demonstrate that PDGFRα, which labels all osteolineage cells, cannot be used to distinguish mesenchymal progenitors or murine stem cells in skeletal tissues from their more differentiated osteogenic progeny.

2.3 Methods

2.3.1 Mice and tamoxifen mediated reporter expression

HIC1CreERT2 (HIC1^{CT2}) mice were a kind gift from TM Underhill (University of British Columbia). PDGFRαCreERT2 (PDGFRα^{CT2}) mice were a kind gift from Brigid Hogan (Duke University). B6.129s4- PDGFRα^{tm11(EGFP)Sor}/J (PDGFRαEGFP, Jax Stock #007669), B6.FVB-Tg(Cdh5-cre)7Mlia/J (Cdh5 mice, Jax stock #006137), and B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-tdTOMATO)Hze}/J (Rosa26.tdTomato reporter mice, Jax stock #007909) were purchased from Jackson Laboratories and crossed in-house. All mice were bred and maintained at the Biomedical Research Centre at the University of British Columbia. Mice were fed a 50/50 diet of irradiated PicoLab Mouse Diet 20 and PicoLab Rodent Diet 20 provided ad libitum. To induce reporter expression in CreERT2 mice, tamoxifen dissolved in sunflower oil was administered intraperitoneally (3mg/day for 5 consecutive days) to 8-10-week-old mice. Where required for cell proliferation assays, mice
were given 1mg EdU dissolved in PBS administered intraperitoneally 24 hours before collection. All animal procedures were approved by the University of British Columbia Animal Care Committee (Protocol #A17-0039).

2.3.2 Damage model

To induce bone damage, a stabilized open osteotomy model was used. Mice were anesthetized using isoflurane and given buprenorphine analgesic. An incision was made along the lateral thigh, parallel to the femur and the quadriceps and hamstring muscles gently retracted from the femur. A dental drill equipped with an RA crosscut 701 bur (Sinclair dental) set to 10000rpm was used to cut the femur mid-diaphysis. The osteotomy was cooled and rinsed of bone fragments using sterile saline. A sterile hypodermic needle was then inserted into the proximal medullary cavity of the femur, exiting through the greater trochanter and overlying muscles and skin. The hub of the needle was then trimmed using wire cutters and the end inserted into the distal medullary cavity, aligning the two halves of the bone. The intramedullary pin was then secured by further insertion into the distal femur and the exposed tip trimmed using wire cutters. The muscles were then gently repositioned over the femur and a local dose of bupivacaine administered before closing the skin using dissolvable sutures. Analgesic (buprenorphine) was provided post-operatively every 6-8 hours for 2-3 days.
2.3.3 Histology and immunofluorescence

Bones were collected and fixed in 4% PFA (Sigma) overnight at 4°C. Bones were then rinsed in PBS and decalcified using 0.5M EDTA (Sigma) for 7-10 days at 4°C before being cryoprotected using 20-40% sucrose gradients. Bones were then embedded in OCT (Sakura), sectioned on a Leica cryostat, and collected onto Superfrost Plus slides (Fisher). For immunofluorescent imaging, following blocking with 5% NGS, 1% BSA in PBS, slides were stained with primary antibodies for 1 hour at room temperature or 4°C overnight. Primary antibodies used include CD31 (R&D). Primary antibodies were detected using secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 568, or Alexa Fluor 647 (Invitrogen). For EdU detection, Click-iT Plus EdU detection imaging kits (Invitrogen) were used according to the manufacturer’s directions. Fluorescent microscopy was performed using a Nikon Eclipse Ni microscope equipped with a Lumencor light source. Confocal microscopy was performed using a Nikon C2 laser scanning confocal microscope, Leica SP8 confocal microscope, or a Zeiss LSM 800. Images were assembled using ImageJ (Fiji) and Illustrator CC (Adobe).

2.3.4 Single cell RNA sequencing

Bone marrow from HIC1CT2/td.Tomato mice was collected by perfusing long bones with PBS. Bone marrow plugs were then digested with Collagenase D/Dispase II (Roche) at room temperature for 10 minutes with constant agitation. The cell suspension was then filtered and stained with antibodies against CD45, Ter119, B220, Ly6G, CD3e and CD11b followed by Hoechst and PI. Live, lineage- (CD45-/Ter119-, B220-, Ly6G-, CD3e-, CD11b-) cells were sorted and collected in 20µl of
DMEM+ 5% FBS. RNA and library preparation were performed using the 10X genomics platform. Single cell data was normalized, clustered, and analyzed using Seurat v3 and gProfiler in R.

2.3.5 Statistical analysis

All statistical analysis was performed in Prism (GraphPad). Results shown represent the mean ±SEM unless otherwise indicated. Multigroup analyses were performed using one-way ANOVA with Tukey correction for multiple comparisons. P<0.05 was considered significant.

2.4 Results

2.4.1 PDGFRα and HIC1 lineage tracing models

Though the two proposed MSC markers, PDGFRα and HIC1, have been characterized in various tissues including cardiac and skeletal muscle, they have not previously been examined in the context of skeletal tissues. Using various murine transgenic reporters, we were able to establish roles for the two markers in murine skeletal homeostasis.

The PDGFRαEGFP murine model has been extensively characterized by various groups and expresses a long-lasting nuclear H2B::GFP under the control of the endogenous Pdgfra locus. This model however, in addition to labeling the expected bone marrow stromal cells, perivascular cells, and bone-associated endosteal cells, also labels mature osteocytes within the compact bone (Figure 2.1, arrow). Unsure whether expression of GFP in mature osteocytes was due to the constitutive expression of the reporter throughout development or the long half-life of the H2B::GFP, we turned
to a new conditional PDGFRαCreERT2/td.Tomato murine model developed by Christina Barkaukas of the Hogan Laboratory at Duke University. In this model, a CreERT2pA2/NEO cassette was knocked into the start codon of exon 2 in the Pdgfra locus (Figure 2.2). This allows for cell and temporal-specific tamoxifen mediated reporter expression, however, also results in a non-functional Pdgfra allele. As at least one functional copy of the Pdgfra allele is required for development, mice were maintained as heterozygotes. Similarly, as there were no commercially available HIC1 reporter mice, we used a novel HIC1CreERT2/td.Tomato reporter mouse developed by the Underhill Laboratory at the University of British Columbia. In the HIC1CT2 model, an IRES-CreERT2 cassette inserted into the 3’UTR of the endogenous Hic1 locus allows for cell and temporal-specific tamoxifen-mediated reporter expression (Figure 2.3). This model does not affect the transcription or function of the Hic1 gene, so mice were bred to homozygosity to ensure optimal CreERT2-mediated recombination.

2.4.2 PDGFRα and HIC1 label separate and distinct populations of cells in skeletal tissues

Previous work by the Rossi and Underhill laboratories (98,100) has shown that HIC1 and PDGFRα label similar populations of cells within fat, cardiac muscle, and skeletal muscle, however, when we examined their expression in skeletal tissues, we found that they label discrete populations of cells.

Similar to the PDGFRαEGFP model, the PDGFRαCT2/td.Tomato model continued to label stromal cells and perivascular cells within the bone marrow, as well as bone lining cells of the periosteum and endosteum (Figure 2.4 A). Further, though examined within two weeks of tamoxifen
administration which should only label cells expressing or recently expressing PDGFRα, osteocytes in the PDGFRαCT2/td.Tomato model were also td.Tomato+ suggesting that osteocytes continue to express PDGFRα after their differentiation from osteoblasts. Conversely, using the HIC1CT2/td.Tomato model, though stromal and perivascular cells within the bone marrow were labelled, no endosteal cells and only a small subset of periosteal cells were labelled (Figure 2.4 B). Importantly, HIC1CT2/td.Tomato does not label the vast majority of terminally differentiated osteocytes. The labelling of a subset of periosteal cells and very few to no osteocytes suggested to us that while PDGFRα labeled all osteo-lineage cells, HIC1 seemed to be more restricted, possibly to more ‘upstream’ progenitor cells.

Due to the differential expression patterns of PDGFRα and HIC1 in bone, we examined expression of the two markers in other osteogenic tissues. Long bones such as the femur and tibia that develop through endochondral ossification contain cartilaginous elements that persist into adulthood, particularly the articular surfaces of the bones (articular cartilage), and within the growth plate, which in mice, remains open throughout life.

In adult murine bones, PDGFRαCT2/td.Tomato labels articular chondrocytes and the underlying trabecular bone (Figure 2.5 A). HIC1CT2/td.Tomato however, does not label any articular cartilage cells or the underlying trabecular bone (Figure 2.5 B). Similarly, PDGFRα labels columns of chondrocytes, including proliferative (asterisk) and hypertrophic (arrow) chondrocytes, within the femoral growth plate (Figure 2.6 A). Conversely, in the adult murine skeleton, HIC1CT2/td.Tomato does not label any cells within the growth plate or surrounding trabecular bone (Figure 2.6 B).
Lastly, we examined the expression of PDGFRα and HIC1 within the enthesis, the specialized insertion site of tendon or ligament to bone (176). The enthesis is composed of 3 major zones, bone, fibrocartilage, and tendon/ligament, that contain a range of osseous, cartilaginous, and tendinous or ligamentous cells that together allow attachment of tendons and ligaments to bone. We observed that PDGFRα\textsuperscript{CT2/td.Tomato} labels cells within all zones of the enthesis including the bone, fibrocartilage, and tendon (Figure 2.7 A). HIC1\textsuperscript{CT2/td.Tomato} however, does not label cells within any of the zones of the enthesis at homeostasis, and only labels cells within the epiligament or tendon sheath, the outer, heavily vascularized layer of the ligament or tendon (Figure 2.7 B).

Taken together, this evidence suggests that PDGFRα labels more committed progenitors and terminally differentiated cells in the chondro-osteogenic lineage as well as tenocytes, while HIC1 labels perivascular cells and a subset of periosteal cells with osteogenic potential at homeostasis. We next wished to determine if over time HIC1\textsuperscript{+} cells could give rise to PDGFRα\textsuperscript{+} osteolineage cells through homeostatic skeletal remodeling processes.

2.4.3 HIC1 labels MSCs that give rise to PDGFRα\textsuperscript{+} osteoprogenitors and osteocytes

While our studies of skeletal tissues at homeostasis indicated that HIC1 and PDGFRα label different populations of cells within the bone and bone-associated tissues such as cartilage and the enthesis, both lineage tracing models label cells within the periosteum of bone and within the bone marrow. In order to establish whether there was any overlap in the two cell populations in the periosteum and the bone marrow, we crossed HIC1\textsuperscript{CT2/td.Tomato} mice with PDGFRα\textsubscript{EGFP} reporter mice to label both HIC1\textsuperscript{+} and PDGFRα\textsuperscript{+} cells. Within the bone marrow of
HIC1\textsuperscript{CT2}/td.Tomato/PDGFR\(\alpha\)EGFP mice, many more cells were labelled with GFP than td.Tomato, and in many cases, these cell populations were distinct with little overlap (Figure 2.8). Though less common, GFP\(^+\)/td.Tomato\(^+\) cells were found within the marrow usually adjacent to blood vessels (Figure 2.8 white arrows), suggesting that there may be some overlap in the two populations of cells, or that HIC1\(^+\) cells may give rise to some PDGFR\(\alpha\)^+ cell populations.

Within the periosteum of HIC1\textsuperscript{CT2}/td.Tomato/ PDGFR\(\alpha\)EGFP mice, PDGFR\(\alpha\) and HIC1 label distinct populations of cells but again, do show some overlap (Figure 2.9, white arrow). Further, GFP\(^+\)/tdTomato\(^+\) osteocytes can be seen near the periosteal surface (Figure 2.10) and within the cortical bone, usually near blood vessels. Together, the presence of HIC1\textsuperscript{CT2}/td.Tomato\(^-\) cells and HIC1\textsuperscript{CT2}/td.Tomato\(^+\) PDGFR\(\alpha\)EGFP\(^-\) double positive osteocytes close to the periosteal surface suggest that HIC1 may label a population of progenitor-like MSCs and progenitors that can differentiate into more committed progenitors and even terminally differentiated osteocytes which express PDGFR\(\alpha\).

2.4.4 HIC1\(^+\) MSCs contribute to various stages of bone regeneration

As PDGFR\(\alpha\) labels the majority of osteolineage cells and is not specific to MSCs in the bone, and HIC1 appears to label a population of cells that can give rise to PDGFR\(\alpha\)^+ osteogenic cells at low rates during homeostasis, we wished to see if HIC1\(^+\) cells, which in other tissues are known to be responsive to injury, would have a role in the regeneration of bone. To this end, we damaged the bones of 8-10-week-old HIC1\textsuperscript{CT2}/td.Tomato mice 10 days after tamoxifen administration using an open osteotomy stabilized with an intramedullary pin. Mice were collected at various timepoints
after surgery to determine the contribution of HIC1 cells to the callus and regenerated bone (Figure 2.11 A).

At D3 after damage, during the acute inflammatory phase, 24.05% (±1.569) of cells in the fracture area were td.Tomato+ (Figure 2.12 A) and appeared either fibroblastic or perivascular in nature (Figure 2.11 B, i). During this time, cell proliferation within the callus peaked at 19.32% (±0.2959), with td.Tomato+/EdU+ cells making up 38.43% (±4.494) of EdU+ cells (Figure 2.12 C, D).

At D7 after surgery, coinciding with the peak of soft callus formation (54), callus size had increased significantly (Figure 2.12 B) and the establishment of a cartilaginous callus could be seen. Chondrocytes and fibroblast-like cells could be seen in the callus area (Figure 2.11 C, ii) with td.Tomato+ cells making up 54.49% (±5.80) of cells (chondrocytes and fibroblast-like cells) within the callus (Figure 2.12 A). Cell proliferation decreased to 10.68% (±0.2959), however td.Tomato+/Edu+ cells represented 48.50% (±6.419) of proliferating cells (Figure 2.12 C, D). Interestingly, we noted that the td.Tomato+ cells appeared to be streaming into the fracture site from the periosteal compartment rather than from the bone marrow compartment, suggesting that HIC1/td.Tomato+ cells from the periosteum are responsive to damage, whereas the HIC1/td.Tomato+ cells from the bone marrow may not have regenerative or osteogenic potential, but rather play a role as stromal or niche cells.

By D14 after surgery, hard callus formation peaks in rodent models (54) and we observed that the callus area reached its peak size (Figure 2.12 B) with td.Tomato+ cells making up 47.66% (±2.523) of the callus (Figure 2.12 A) though proliferation continued to decrease to 10.68% (±1.146) with td.Tomtato+ cells making up 59.73% (±6.728) of proliferating cells. td.Tomato+ cells contributed to
both chondrocytes and woven or fibrous bone (Figure 2.11 D, iii) close to the fracture site. Interestingly, we continued to see what appeared to be a migration of periosteal HIC1/td.Tomato$^+$ cells into the fracture site, and little contribution or expansion of the bone marrow HIC1/td.Tomato$^+$ cells. Unlike the rapid endochondral response, remodeling of the hard callus begins 3-4 weeks after damage and continues for up to a year. We examined this process at 3 different timepoints; D21, D28, and D42.

At D21, the hard callus had significantly shrunk in size (Figure 2.12 B) though HIC1$^{CT2}$/td.Tomato$^+$ cells continued to make up 53.0% ($\pm$6.845) of the cells within the callus area, which consisted primarily of woven bone with little to no cartilage visible.

At D28 post fracture there was no significant change in callus area (Figure 2.12 B) or contribution of HIC1$^{CT2}$/td.Tomato$^+$ cells to the callus (Figure 2.12 A). We did note however, the initiation of remodeling of the woven bone in the outer callus into lamellar bone.

By D42 post fracture, the callus had been almost fully remodeled to lamellar cortical bone (Figure 2.11 G, vi) and callus area had decreased significantly (Figure 2.12 B). HIC1$^{CT2}$/td.Tomato$^+$ cells continued to represent 46.53% ($\pm$6.688) of the cells within the regenerated bone, indicating that HIC1$^+$ cells contribute not just to the callus, but to the remodeled, regenerated bone.

Between D21 and D42, cell proliferation continued to decrease from 4.188% ($\pm$1.046) to 0.4733% ($\pm$0.1016) (Figure 2.12 C) as the bone was remodeled. Intriguingly, though cell proliferation decreased significantly over time, the percentage of Edu$^+$ cells that were also td.Tomato$^+$ remained
between 30.51% (±1.412) and 63.51% (±7.694) (Figure 2.12 C, D). As HIC1<sup>CT2</sup>/td.Tomato<sup>+</sup> cells proliferated at roughly the same rate as non-lineage traced cells, this indicated that there may be issues with Cre efficiency or the contribution of another progenitor cell population to the callus during regeneration. These experiments indicated that HIC1<sup>+</sup> periosteal cells not only contributed to bone at homeostasis, but act as a reserve of skeletal progenitors that expand rapidly after damage and contribute greatly to all stage of endochondral bone regeneration. Next, we wanted to know how skeletal HIC1<sup>+</sup> cells compared to previously described populations of skeletal MSCs at the transcriptional level.

2.4.5 HIC1<sup>+</sup> Bone Marrow MSCs resemble previously reported murine MSCs

In order to determine whether HIC1<sup>+</sup> cells resemble previously described populations of MSCs, we performed single cell RNA sequencing (scRNASeq). As periosteal HIC1 labelled cells are found relatively infrequently in the immediate weeks following tamoxifen administration, we were unable to collect enough periosteal HIC1<sup>+</sup> cells for scRNASeq and instead collected HIC1<sup>CT2</sup>/td.Tomato<sup>+</sup> cells from the bone marrow. Clustering of the resulting 757 cells in Seurat v3 identified 7 distinct clusters (Figure 2.13 A) that could be identified by differential gene expression (Figure 2.13 B) and GO biological pathway analysis. We examined the expression of the various candidate genes that have recently been proposed to identify murine skeletal MSCs including LepR, Grem1, Gli1, CD200 (Cd200), Alpha V (Itgav), Endoglin (CD105/Eng), and PDGFRα (Pdgfra) and found that with the exception of Gli1, all were expressed by either some or all clusters of HIC1 labelled cells (Figure 2.13 C), suggesting that bone marrow HIC1<sup>+</sup> cells may encompass a variety of previously described MSCs with different roles. The vast majority of cells clustered into two populations which
we identified as being similar to CXCL12-abundant reticular (CAR) cells (CAR1 47.03%, CAR2 32.89%) due to their expression of CXCL12 (Cxcl12), Kit ligand (Kitl), and IL-7 which are characteristic of CAR cells (Figure 2.14). CAR cells, which have often been conflated with MSCs of various identities, are established as hematopoietic niche cells and other work performed by the Underhill laboratory suggest that HIC1⁺ cells in the bone marrow may also have an important role in maintaining the hematopoietic niche (unpublished data), thus it was unsurprising to find so many cells in these clusters. Other clusters identified included pericytes (4.89%), immune cells (4.09%), and endothelial cells (3.83%) that may have been included due to contamination during sorting (immune and endothelial cells) (Figure 2.15, Figure 2.16, Figure 2.17). A proportion of HIC1⁺ cells in other tissues, such as muscle, have been identified as pericytes by the Rossi and Underhill laboratories (unpublished data), so the inclusion of some labelled HIC1⁺ pericytes in a highly vascularized tissue such as bone marrow was unsurprising.

Of greatest interest to us, we identified a cluster of cells (5.81%) expressing many genes such as Sca-1 (Ly6a), CD90 (Thy1), and CD34; markers which have been used to identify Fibro-adipogenic progenitors (FAPs), MSC populations with multi-lineage potential (Figure 2.13, Figure 2.18) (87,88,177). We also noted that PDGFRα, which is also used as a marker of FAPs, was also expressed by the putative FAP cluster. This suggests that within the bone marrow, there is a small population of HIC1⁺ derived cells that closely resemble FAPs. Interestingly, we also identified a very small cluster of cells (1.45%) that expressed osteogenic genes such as Osteocalcin (Bglap), Collagen1a1 (Col1a1), Osterix (Sp7), and Alkaline Phosphatase (Alpl) (Figure 2.19), suggesting this may be a cluster of more differentiated bone, cartilage and stromal progenitors (BCSPs). Based on the previously identified multilineage potential of FAPs and the expression of osteogenic genes, we
believe the FAP and BCSP clusters may be more similar to the periosteal HIC1$^+$ cells that contribute to adult murine bone maintenance and regeneration and that the high proportion of HIC1$^+$ CAR cells and relatively low proportion of HIC1$^+$ FAP and BCSP cells within the bone marrow could explain why we observed little contribution of bone marrow HIC1$^+$ cells to bone regeneration in our lineage tracing studies.

2.5 Discussion

In effort to identify a skeletal MSC that could translate to humans and present a potential therapeutic target for any number of debilitating bone disorders, many markers have been proposed, all with various contributions to skeletal homeostasis and regeneration. Interestingly, none of the markers has identified a periosteal population of MSCs though the importance of the periosteum to bone regeneration has been known since the 1800s (123). Here we examined the contribution of two candidate MSC markers that are present expressed in the adult murine periosteum.

PDGFR$\alpha$ has frequently been used to identify mesenchymal stromal cells with multilineage potential, including osteogenic potential, in various tissues including cardiac and skeletal muscle, fat, and bone marrow, however its expression in adult skeletal tissues has not previously been reported. Here, using a novel PDGFR$\alpha^{\text{CreERT2}}$ model, we show for the first time that PDGFR$\alpha$ is expressed at homeostasis throughout skeletal tissues and is not restricted to mesenchymal stromal cells or skeletal progenitors. Within the bone, PDGFR$\alpha$ labels terminally differentiated osteocytes as well as bone lining cells of the endosteum and periosteum. Further, PDGFR$\alpha$ labels chondrocytes in the growth plate and articular cartilage as well as fibrocartilage and tenocytes in the enthesis.
This suggests that PDGFRα can be used to label osteogenic and chondrogenic lineage cells, however, it cannot be used to exclusively label the progenitors that give rise to these lineages in the adult and necessitates the use of another marker to label skeletal progenitors.

Other markers such as Nestin, LepR, Grem1, and Gli1 have been proposed to identify MSCs with skeletal stem or progenitor properties however each marker has demonstrated certain limitations to its use. Here, we propose another marker for skeletal MSCs, HIC1. Previous work by the Underhill laboratory has shown that HIC1 is expressed by multipotent progenitors in various tissues including skeletal muscle, fat, and bone marrow. We show that HIC1 expression in skeletal tissues is restricted to a small population of periosteal bone-lining cells that can give rise to PDGFRα⁺ periosteal cells and even PDGFRα⁺ osteocytes. HIC1 cells in other tissues are responsive to damage and we found similar responsiveness of HIC1 cells to bone damage where they contributed to all stages of bone regeneration including cartilaginous and osseous elements. Interestingly, we noted that the response of HIC1 cells seemed to come from the periosteal compartment rather than the bone marrow, where little expansion of HIC1 cells was observed. The labeling of only half the proliferating cells and half of the cells within the callus area at any given time suggests that there may be either inefficiency of labelling the HIC1⁺ cells or the contribution of another population of cells to bone regeneration. As Grem1⁺ endosteal cells have previously been shown to incompletely contribute to adult murine bone regeneration and there is strong evidence for a periosteal contribution of cells, it would appear more likely that HIC1 represents the periosteal population of murine MSCs that contributes to bone regeneration and supports the notion that the regenerative process involves multiple MSC populations. Further, emerging evidence from our lab indicates that bone marrow HIC1⁺ cells act as niche cells for hematopoietic stem cells (unpublished data, paper in progress). This was supported
by our scRNASeq data of bone marrow-derived HIC1+ cells which showed two large clusters of CAR-like cells and a smaller population of FAP or MSC-like cells and more osteo-lineage committed BCSPs. Together, this suggests that HIC1 labels multiple mesenchymal cell populations in skeletal tissues (periosteum and bone marrow) with diverse roles that are dependent on their regionalization.

We therefore propose a functional hierarchy during both homeostasis and regeneration in the adult murine skeleton. HIC1 labels a previously unidentified population of periosteal mesenchymal progenitors that progressively contribute to PDGFRα+ osteoprogenitors and osteocytes through normal processes of bone turnover such as remodeling, but not cartilage or fibrocartilage, during postnatal homeostasis. Comparatively, PDGFRα labels downstream progenitors and committed cells of the osteogenic lineage as well as chondrogenic cells in the adult at homeostasis (Figure 2.20). However, in the context of regeneration after injury, periosteal HIC1 cells are also capable of contributing to chondrogenic cells within the callus (Figure 2.20), though another population of unlabeled cells also may also contribute to the regenerative process. Lastly, by identifying HIC1+ cells as periosteal skeletal MSCs, we identify a previously unidentified population of cells that, in conjunction with endosteal skeletal MSC populations, could be targeted for therapeutic purposes.
Figure 2.1 - PDGFRαEGFP labels all osteogenic lineage cells.

Merged image of cortical bone and bone marrow from PDGFRαEGFP/Cdh5/td.Tomato mice. PDGFRαEGFP+ stromal cells are found throughout the bone marrow (Ma) and adjacent to Cdh5/td.Tomato+ blood vessels (white arrowhead). Within the cortical bone (B), PDGFRαEGFP labels embedded osteocytes (white arrow), and periosteal bone lining cells (asterisk).
Figure 2.2 - Structure of the PDGFRαCreERT2 knock-in/knock-out model developed by the Hogan Lab.

A CreERT2.pA/Neo cassette was inserted into the start site of exon 2 in the endogenous *Pdgfra* locus using a targeting vector with an 8kb homology arm and a 2kb homology arm. Insertion of the CreERT2.pA/Neo cassette into exon 2 results in a non-functional allele and thus mice can only be maintained as heterozygotes; homozygotes exhibit an embryonic lethal phenotype. This work was performed by Christina Barkaukas of the Hogan laboratory at Duke University and gifted to the Rossi Laboratory.

Figure 2.3 - Structure of the HIC1CreERT2 knock-in model developed by the Underhill Lab.

The CreERT2 cassette was inserted into the 3’UTR following exon 3 of the endogenous *Hic1* locus. This knock-in does not affect transcription of the endogenous gene and mice can be bred to homozygosity. This work was performed by the Underhill laboratory of The University of British Columbia and kindly gifted to the Rossi Laboratory.
**Figure 2.4 - Expression of PDGFRα and HIC1 in cortical bone.**

(A) Cortical bone from PDGFRα<sup>CT2</sup>/td.Tomato mouse. Endosteal cells, osteocytes and periosteal bone lining cells are td.Tomato<sup>+</sup>. (B) Cortical bone from HIC1<sup>CT2</sup>/td.Tomato mouse. Only periosteal bone lining cells and occasional osteocytes are td.Tomato<sup>+</sup>. HIC1<sup>CT2</sup>/td.Tomato also labels perivascular cells within the cortical bone. Ma = Marrow, B = Bone, Es = Endosteum, Ps = Periosteum.
Figure 2.5 - Expression of PDGFRα and HIC1 in articular cartilage

(A) Articular cartilage of the distal femur from a PDGFRαCT2/td.Tomato mouse. PDGFRαCT2/td.Tomato labels articular chondrocytes and the underlying trabecular bone osteocytes. (B) Articular cartilage of the distal femur in HIC1CT2/td.Tomato mice. HIC1CT2/td.Tomato does not label articular cartilage or the underlying trabecular bone.
Figure 2.6 - Expression of PDGFRα/td.Tomato and HIC1/td.Tomato in the growth plate

(A) Growth plate in the femur of a PDGFRα<sup>CT2</sup>/td.Tomato mouse in which columns of proliferating and hypertrophic chondrocytes within the growth plate are labelled by td.Tomato. (B) Growth plate from a HIC1<sup>CT2</sup>/td.Tomato mouse. HIC1<sup>CT2</sup>/td.Tomato does not label any chondrocytes in the growth plate. Dashed lines delineate the cartilaginous growth plate (G.Pl).
Figure 2.7 - Expression of PDGFRα and HIC1 in the enthesis.

(A) PDGFRα^{CT2}/td.Tomato labels cells within the various zones of the enthesis (B) HIC1^{CT2}/td.Tomato does not label cells of the enthesis but does label cells of the epiligament or tendon sheath. FC=Fibrocartilage, T=Tendon
Figure 2.8 - Expression of PDGFRα and HIC1 in bone marrow

Bone marrow from a HIC1<sup>CT2</sup>/td.Tomato/ PDGFRα EGFP mouse. PDGFRα EGFP+ cells are found more frequently than HIC1<sup>CT2</sup>/td.Tomato+ cells within the bone marrow. GFP+/td.Tomato+ cells (white arrows) are found within the marrow often adjacent to blood vessels.
Figure 2.9 - Expression of PDGFRα and HIC1 in the periosteum and cortical bone.

Cortical bone and periosteum from HIC1\textsuperscript{CT2}/td.Tomato/ PDGFRαEGFP mice. GFP\textsuperscript{+}/td.Tomato\textsuperscript{+} cells (white arrow) can be found within the periosteum, adjacent to GFP\textsuperscript{+} or td.Tomato\textsuperscript{+} cells. Ma = Marrow, B = Bone, Ps = Periosteum.
Figure 2.10 - HIC1/td.Tomato<sup>+</sup> cells give rise to PDGFRαEGFP<sup>+</sup> cortical osteocytes.

Example of a PDGFRαEGFP<sup>+</sup>/HIC1/td.Tomato<sup>+</sup> osteocyte near the periosteal surface with typical osteocyte morphology including cell processes oriented towards the mineralizing matrix. Dashed line delineates the periosteal surface. Ps = periosteum.
**Figure 2.11 - HIC1^{CT2/td.Tomato} cells contribute to all stages of bone regeneration**

(A) Experimental design for the osteotomy model. (B-G) Immunofluorescent images of the osteotomy at the indicated timepoints after damage. (i-vi) Insets from B-G. Images are representative of N=5 biological replicates per timepoint.
Figure 2.12 - Quantification of HIC1^{CT2}/td.Tomato^+ and EdU^+ cells during skeletal regeneration.

(A) Percentage of Tomato^+ cells within the callus area at the indicated timepoints over the course of bone regeneration. (B) Area of the fracture callus at the indicated timepoint, Mean ± SD. (C) Percentage of proliferating cells within the callus over the course of regeneration. (D) Percentage of td.Tomato^+/EdU^+ cells within the callus over the course of regeneration as a percentage of EdU^+ cells. Td.Tomato^+ cells represented approximately half of the proliferating cells at any time point during regeneration. Mean ± SEM. N=3 biological replicates * p<0.05 ** p<0.005 ***p<0.0005
Figure 2.13 - HIC1^{CT2/td.T} Tomato^+ bone marrow cells resemble previously identified MSC populations

(A) UMAP cluster plot of scRNASeq from murine bone marrow HIC1^{CT2/td.T} cells. 7 unique populations were clustered and identified using differential gene expression and GO biological pathway analysis. (B) Heatmap of the top 10 differentially expressed genes in each cluster (C) UMAP FeaturePlots for various markers used to identify murine skeletal MSCs, demonstrating their expression in bone marrow HIC1^{CT2/td.T} clusters. All markers were identified in one or more clusters, except Gli1.
Figure 2.14 - Expression of CAR cell markers in HIC1^{CT2/td.Tomato} bone marrow single cell RNASeq clusters
Figure 2.15 - Expression of pericyte markers in HIC1\textsuperscript{CT2/td.Tomato}\textsuperscript{+} bone marrow single cell RNASeq clusters
Figure 2.16 - Expression of immune cell markers in HIC1^{CT2/td.Tomato^+} bone marrow single cell RNASeq clusters
Figure 2.17 - Expression of endothelial cell markers in HIC1CT2/td.Tomato+ bone marrow single cell RNASeq clusters
Figure 2.18 - Expression of FAP markers in HIC1^{CT2/td.Tomato} bone marrow single cell RNASeq clusters
Figure 2.19 - Expression of osteogenic lineage markers in HIC1^{CT2/td.Tomato}$^+$ bone marrow single cell RNASeq clusters
Figure 2.20 - Proposed hierarchy of HIC1 and PDGFRα during murine skeletal homeostasis and regeneration

HIC1 labels a population of MSCs that contribute to adult skeletal homeostasis at low levels by differentiation into PDGFRα+ osteo-lineage cells. PDGFRα labels more committed progenitors and terminally differentiated cells within the bone lineage as well as other skeletal-associated tissues such as tendon and cartilage. During regeneration, HIC1 MSCs can also differentiate into chondrocytes that contribute to endochondral ossification.
Chapter 3: Murine tissue-resident PDGFRα+ fibro-adipogenic progenitors spontaneously acquire an osteogenic phenotype in an altered inflammatory environment

3.1 Synopsis

Acquired heterotopic ossifications (HO) arising as a result of various traumas including injury or surgical interventions, often result in pain and loss of motion. Though triggers for HO have been identified, the cellular source of these heterotopic lesions as well as the underlying mechanisms that drive the formation of acquired HO remain poorly understood and treatment options, including preventative treatments, remain limited. Here, we explore the cellular source of HO and a possible underlying mechanism for their spontaneous osteogenic differentiation. We demonstrate that HO lesions arise from tissue resident PDGFRα+ fibro/adipogenic progenitors (FAPs) in skeletal muscle and not from circulating bone marrow derived progenitors. Further, we show that accumulation of these cells in the tissue after damage due to alterations in the inflammatory environment can result in activation of their inherent osteogenic potential. This work suggests a mechanism by which an altered inflammatory cell and FAP interactions can lead to the formation of HO after injury and presents potential targets for therapeutics in acquired HO.

3.2 Introduction

Heterotopic ossification (HO), both inherited and acquired, result in significant health consequences including pain and reduced range of motion (178-180). Compared to the rare forms of
inherited HO, acquired HO is far more common and may arise after orthopedic surgery \(^{(68,69)}\), traumatic or repeated muscle or orthopedic injuries \(^{(69,76,181)}\), burns \(^{(74,75)}\), or central nervous system trauma \(^{(71-73)}\). However, the initial triggers and cellular origin of acquired HO remain unclear and treatment options remain limited.

Recent research has shown the presence of resident cells in adipose tissue and the kidney capsule that, despite the local absence of committed skeletal progenitors, can adopt an osteogenic fate \(^{(78)}\). This has suggested that one or more cell types can be induced to osteogenesis, however the precise identity of the cells giving rise to these osteogenic precursors remains unclear \(^{(78,85)}\). PDGFR\(\alpha^+\) fibro/adipogenic progenitors (FAPs) found throughout most adult tissues, have been shown to have osteogenic potential \textit{in vitro} \(^{(88)}\) and, in situations in which a mutation associated with human FOP is expressed, \textit{in vivo} \(^{(86)}\). Thus these FAPs have been suggested to contribute to heterotopic lesions across tissues after injury \(^{(85)}\). Previous work on FAPs and HO, however, has been concentrated on the use of non-specific lineage-tracing markers such as Tie2 that have significant overlap with other cell populations, or utilize constitutively active cre systems potentially resulting in the early and extensive developmental labeling of mesenchymal tissues \(^{(82,83,86)}\). Further, though it represents a useful tool to study HO in the absence of genetic models of FOP, the use of supra-physiological levels of BMPs has been suggested to induce ‘reprogramming’ of cells to an osteogenic fate \(^{(78,182)}\). While increased levels of BMPs have been implicated in various forms of acquired HO, it is often preceded by an early inflammatory phase \(^{(68,180,183)}\). Prophylactic use of nonsteroidal anti-inflammatory drugs has been reported helpful in attenuating HO post total hip arthroplasty, suggesting a role for inflammation in the pathogenesis of HO \(^{(184)}\). Though a link to inflammation has been established, there has been little research on the cellular mechanisms linking it to HO.
We have previously found that altering the inflammatory environment in muscle after damage causes accumulation of PDGFRα+ FAPs in the tissue, potentially pushing PDGFRα+ cells to spontaneously undergo osteogenic differentiation and cause HO.

Here, we show that PDGFRα+ tissue resident FAPs are the source of osteogenic progenitors in a BMP-induced murine model of acquired HO and that alterations of the inflammatory milieu known to lead to their accumulation in injured tissues, leads to the activation of an osteogenic transcriptional program and HO.

3.3 Methods

3.3.1 Animals and tamoxifen mediated reporter expression

C57Bl/6J (JAX stock #000664), B6.129s4-PDGFRαtm11(EGFP)Sor/J (PDGFRαEGFP, JAX Stock #007669) and B6.129S4-Ccr2tm1Ifc/J (CCR2KO, JAX stock #004999) mice were purchased from The Jackson Laboratories and crossed in-house. Collagen1a1*3.6GFP were a kind gift from D. Rowe (University of Connecticut). PDGFRαCT2 mice were a kind gift from Brigid Hogan (Duke University) and were crossed with B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/J (Rosa26.td.Tomato reporter mice, Jax stock #007909). C57Bl/6-CMV-β actin-EGFP (GFP mice) were a kind gift from I. Weissman (Stanford University). All mice were bred and maintained at the Biomedical Research Centre at the University of British Columbia. To induce reporter expression in PDGFRαCT2/td.Tomato mice, tamoxifen dissolved in sunflower oil was administered intraperitoneally (3mg/day for 5 consecutive days) to 8-10-week-old mice. For all experiments
except parabiosis, both male and female mice were used. All animal procedures were approved by
the University of British Columbia Animal Care Committee.

3.3.2 FACS Analysis

Murine skeletal muscle was collected, digested, and stained as previously described (185,186). To
analyze compact bone by flow cytometry, bones were harvested and mechanically and
enzymatically digested. Briefly, femur and tibia bones were collected from 6-10-week-old mice
and excess muscle removed. Bones were gently crushed in a mortar and pestle with ice cold PBS
to liberate bone marrow and break the bone into small pieces. PBS containing bone marrow was
aspirated and replaced with fresh PBS until bone fragments appeared white and free of marrow
contamination. Bone chips were then transferred to a dish and digested with collagenase II (Sigma)
for 30 minutes at room temperature. Bones were then cut into smaller pieces using a clean razor
blade and further digested with Collagenase D/Dispase II (Roche Biochemicals) for 60 minutes at
37°C with gentle rotation. After digestion, cells were strained through a 70µm cell strainer to
remove remaining bone chips. The resulting cell suspension was then stained for FACS with the
indicated antibodies. Antibodies used for flow cytometry included CD45 (Ablab, clone I3/2),
CD31 (Ablab clone 390, and eBioscience clone MEC 13.3), PDGFRα (CD140a) (eBioscience,
clon APA5), Sca1(eBioscience clone D7). Stained cells were washed and analyzed on a BD
LSRII or CytoFLEX. Further FACS analysis was performed using FlowJo software (FlowJo).
3.3.3 RNA Sequencing

For RNA sequencing, CD31−/CD45−/α7+ /PDGFRα+/Sca1+/FAPs from CCR2KO or WT mice were isolated from skeletal muscle as described above and sorted on a BD Influx cell sorter into DMEM (+5%FBS). Samples were spun down at 800rcf for 5 minutes and resuspended in DEPC treated nuclease-free PBS. RNAzol RT (Sigma) was added and RNA precipitated with LPA(Sigma) and isopropanol at -20°C overnight. Precipitated RNA was washed with ethanol and resuspended in nuclease-free water. Sample quality control was performed using the Agilent 2100 Bioanalyzer. Qualifying samples (N = 2-6) were then prepped following the standard protocol for the TruSeq stranded mRNA library kit (Illumina) on the Illumina Neoprep automated nanofluidic library prep instrument or NEBnext Ultra ii Stranded mRNA (New England Biolabs). Sequencing was performed on the Illumina NextSeq 500 with Paired End 42bp × 42bp reads. De-multiplexed read sequences were then aligned to the Mus Musculus (PAR-masked)/ mm10 reference sequence using TopHat splice junction mapper with Bowtie 2 (http://ccb.jhu.edu/software/tophat/index.shtml) or STAR (https://www.ncbi.nlm.nih.gov/pubmed/23104886) aligners.

DESeq2 and pathway analysis were performed in R using the following packages: org.Mm.eg.db v3.8.2, dplyr v0.8.3, clusterProfiler v3.12.0, magrittr v1.5, AnnotationDbi v1.46.1, pheatmap v1.0.12, ggplot2 v3.2.1, DESeq2 v1.24.0, SummarizedExperiment v1.14.1, DelayedArray v0.10.0, BiocParallel v1.18.1, matrixStats v0.55.0, Biobase v2.44.0, GenomicRanges v1.36.1, GenomeInfoDb v1.20.0, IRanges v2.18.3, S4Vectors v0.22.1, BiocGenerics v0.30.0, RColorBrewer v1.1-2
3.3.4 Parabiosis surgery

Age and weight matched 10-12 week old females mice were used to surgically generate parabiotic mice as previously described (187). Blood chimerism was verified by FACS analysis between 3 and 8 weeks after surgery, before parabionts underwent BMP2-induced heterotopic ossification.

3.3.5 BMP2 induced heterotopic ossification

Recombinant human BMP2 (Peprotech), was reconstituted and mixed with growth factor reduced Matrigel (Corning) at a concentration of 2.5ug/50ul and injected into the mid-belly of the tibialis anterior muscle in 8-12 week old mice as previously described (82). Tissues were collected 21 to 28 days after injection.

3.3.6 Notexin muscle damage

Muscle damage was induced using a single injection of 0.15ug of Notexin (Latoxan) as previously described (188).

3.3.7 Tissue collection and histology

Muscles were dissected into ice-cold PBS and fixed in 4% paraformaldehyde in PBS at 4°C degrees overnight. Following fixation, tissues were rinsed with PBS and decalcified by immersion in Immunocal (StatLab) for 20 minutes at room temperature. Tissues were prepared for
cryoembedding and sectioned using a Leica cryostat. Samples were collected on Superfrost Plus slides (Fisher) and stained for immunofluorescent imaging. Following blocking, sections were stained with primary antibodies overnight at 4°C or 1 hour at room temperature. Primary antibodies used include goat anti-mouse CD31 (PECAM-1) (R&D, cat# AF3628), anti-GFP (Abcam, cat# ab13970), and rabbit anti-mouse laminin (Abcam, cat# ab11575). Primary antibodies were detected using secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 568, or Alexa Fluor 647 (Invitrogen). Fluorescence microscopy was performed using a Nikon Eclipse Ni microscope equipped with a Lumencor light source. Confocal microscopy was performed using a Nikon C2 laser-scanning confocal microscope, Leica SP8 confocal microscope, or a Zeiss LSM 800. Figures were assembled using Fiji (Fiji), Photoshop CC (Adobe), and Illustrator CC (Adobe). Histochemical staining was performed according to standard protocols.

3.3.8 Statistical Analysis

All statistical analysis was performed in Prism6 (GraphPad) or R. Results shown represent the mean ±SD unless otherwise indicated. Two group analyses were performed using an unpaired Student’s T-test, multigroup analyses were performed using one-way ANOVA with Tukey correction for multiple comparisons. P<0.05 was considered significant.
3.4 Results

3.4.1 PDGFRα\textsuperscript{CT2/td.Tomato} labels tissue resident FAPs in muscle and osteogenic cells in murine bone

We previously identified PDGFRα\textsuperscript{+} Fibro/adipogenic progenitors (FAPs) as mesenchymal cells with multilineage potential located in the muscle interstitium \textsuperscript{(87)}. Traditional Cre-loxP systems result in lineage tracing cells from embryonic development onwards, and in the case of PDGFRα, which is expressed in multiple mesodermal precursors early in embryonic development \textsuperscript{(130,189)}, traditional systems result in labeling of multiple cell types and tissues in the adult. To label cells more specifically in a spatial and temporal manner, we utilized a new PDGFRα lineage-tracing mouse developed by the Hogan laboratory in which a CreERT2 construct was inserted into the start site of exon 2 of the endogenous \textit{Pdgfrα} locus to produce a knock-in/knock-out \textsuperscript{(190)}. To validate the expression of td.Tomato in the bone and skeletal muscle of this lineage tracing model, we administered tamoxifen to 8-week-old adult PDGFRα\textsuperscript{CT2/td.Tomato} mice under homeostatic conditions. FACS analysis of adult murine bone from PDGFRα\textsuperscript{CT2/td.Tomato} using lineage mice revealed that similar proportions of CD31⁻/CD45⁻ (Lineage⁻) cells were labelled by td.Tomato (5.613\% ±2.049) and PDGFRα antibody (7.290\% ±3.529) (Figure 3.1 A, B). Gates were selected based on Fluorescent Minus One (FMO controls), as indicated in Figure 3.1 A.

Further, the majority of td.Tomato labelled cells were also stained with PDGFRα antibody and the majority of PDGFRα antibody labelled cells were also positive for td.Tomato (Figure 3.1 A, B), suggesting that the murine model reliably labels PDGFRα\textsuperscript{+} cells within the bone. Upon
histological examination, PDGFRα<sup>CT2</sup> labelled all osteogenic cell populations including cells within the periosteum, endosteum, as well as embedded mature osteocytes within the bone (Figure 3.1 D, i). Due to the KI/KO nature of the lineage tracing model it was impossible to cross PDGFRα<sup>CT2</sup> mice with the well-characterized PDGFRαEGFP knock-in model developed by Philip Soriano, however the expression of td.Tomato<sup>+</sup> cells in murine bone of PDGFRα<sup>CT2</sup> mice closely resembled the that of EGFP in the PDGFRαEGFP model, further supporting its reliability (Figure 3.1 F). Similarly, in adult murine muscle, PDGFRα<sup>CT2</sup>/td.Tomato labels interstitial perivascular cells (Figure 3.2 D), also consistent with what was observed in PDGFRαEGFP reporter mice (Figure 3.2 E). Further though FACS analysis of CD31<sup>-</sup>/CD45<sup>-</sup>/Sca1<sup>+</sup> cells from skeletal muscle using both the td.Tomato lineage tracer and PDGFRα antibody showed a slightly higher percentage of cells labelled with td.Tomato (91.51% ±4.346) than PDGFRα antibody (65.43% ±10.53), the majority of td.Tomato labelled cells were also PDGFRα antibody positive and vice versa (Figure 3.2 A, B). Thus, the PDGFRα<sup>CT2</sup>/td.Tomato mouse provides a useful tool to label tissue resident FAPs in muscle as well as osteogenic lineage cells and bone tissues.

3.4.2 PDGFRα<sup>+</sup> cells are the source of osteogenic cells in BMP2-induced HO

Previous work tracing the cellular source of HO has relied on non-specific markers such as Tie2 and Nestin which have significant overlap with other cell populations and result in only partial labeling of the resulting heterotopic ossicle<sup>(85,191)</sup>. Many of these studies have also shown that a subpopulation of labelled cells express PDGFRα, which has been well characterized as an MSC marker in murine and human tissues<sup>(85)</sup>. While recent studies have implicated skeletal muscle resident PDGFRα<sup>+</sup> cells as drivers of HO in FOP models<sup>(79,86)</sup>, to date there have been no lineage
tracing studies demonstrating PDGFRα+ cells are the primary cellular source of HO in BMP induced models or other muscle injury. In order to substantiate the contribution of skeletal muscle PDGFRα+ cells to HO, we utilized the BMP2-Matrigel model of HO (Figure 3.3 A). While not without its pitfalls, the BMP2-Matrigel model of HO has been well characterized and used in many studies to examine the cellular source of acquired HO (82,83). Injections of Matrigel alone into the TA muscle of PDGFRαCT2/td.Tomato mice resulted in minor muscle damage and a corresponding expansion of td.Tomato+ FAPs, but no ectopic bone (Figure 3.3 B, i). In contrast, mice injected with BMP2 exhibited mature heterotopic ossicles, complete with bone marrow, 21 days after injection (Figure 3.3C, ii). Within the heterotopic bone, td.Tomato+ cells represented 80.01% (±8.290) of all osteogenic cells including osteocytes and bone lining cells. Though it has previously been suggested, the high percentage of td.Tomato labelled cells in the ossicle strongly indicates that PDGFRα+ FAPs, and not another cell type, are the main source of cells contributing to HO.

3.4.3 PDGFRα+ cells contributing to heterotopic ossicles are tissue-resident, not bone-marrow derived.

We next wished to establish whether it is the tissue resident FAPs that give rise to the heterotopic ossicle, and not PDGFRα+ bone marrow derived progenitors recruited to the site of injury via the bloodstream. To this end, we used a parabiotic mouse model. Adult female PDGFRαCT2/td.Tomato mice were induced with tamoxifen and parabiosed to mice ubiquitously expressing GFP (C57Bl/6-CMV-β actin-EGFP mice) (GFP+) (Figure 3.4 A). Once blood sharing between partners was established, the TA muscles of both parabiotic partners were injected with BMP2 and harvested 21 days later. In both PDGFRαCT2/td.Tomato and GFP+ partners, mature heterotopic ossicles
complete with cortical bone and bone marrow developed (Figure 3.4 C-D). In PDGFR$\alpha^{CT2}$ partners, 87.08% (±8.709) of osteocytes and bone lining cells in the heterotopic ossicle were td.Tomato$^+$ (Figure 3.4 C, i, E). td.Tomato$^+$ cells within the bone marrow of the heterotopic ossicle were also visible (Figure 3.4 i, arrowhead), possibly representing stroma. Conversely, in the GFP$^+$ parabiont, no td.Tomato$^+$ cells were observed in either the osteogenic or marrow compartments of the ectopic bone (Figure 3.4 D, ii, E). Interestingly, while no GFP$^+$ cells were found to contribute to the osteogenic cell population of the ossicle in PDGFR$\alpha^{CT2}$/td.Tomato$^+$ mice, occasional GFP$^+$ cells were found within the marrow (Figure 3.5). This suggests that while circulating hematopoietic cells are able to migrate to the heterotopic lesion, bone marrow derived stromal progenitors are not recruited or responsible for the formation of heterotopic bone. We further verified that heterotopic bone-forming cells are tissue-resident using an additional parabiotic model with Col1α1*3.6GFP, a transgenic strain expressing GFP under the control of a collagen enhancer active in the osteogenic lineage, and C57Bl/6J mice and again observed that collagen expressing cells contributing to heterotopic lesions originate from tissue-resident progenitors and do not travel through the circulation to establish heterotopic lesions (Figure 3.6).

3.4.4 Alterations of the inflammatory environment after damage induces PDGFR$\alpha^+$ cells to undergo osteogenic differentiation without the addition of exogenous BMP2

Though useful for producing localized and reproducible acquired HO, the use of supra-physiological levels of exogenous BMPs or transgenic models of altered BMP signaling to induce ectopic bone has been suggested to ‘reprogram’ cells in non-skeletal tissues that would otherwise not have inherent osteogenic potential (78,182). On the other hand, the appearance of HO in traumatic
injury or burn victims supports the notion that there are cells outside of the skeletal system that do have inherent osteogenic potential. Due to the ability of PDGFRα+ cells to form ectopic bone lesions in response to exogenous BMP2 and their osteogenic potential in vitro, they present a potential source of cells with inherent osteogenic potential. We have previously demonstrated that in CCR2KO mice, in which tissues lack the CCR2 receptor, preventing the infiltration of monocytes and macrophages into a tissue after injury, acute muscle injury leads to a delayed regenerative response and to a defect in the clearance of FAPs from muscle leading to their accumulation and spontaneous differentiation along fibrogenic and adipogenic lineages. (192). To test whether in these conditions FAPs could also generate bone, TA muscles of CCR2KO mice were damaged using notexin and tissues were collected 28 days later (Figure 3.7 A). Interestingly, in 54.17% of CCR2KO mice (N=24) we observed the establishment of mature heterotopic ossicles 28 days after acute muscle injury, without the addition of exogenous BMPs (Figure 3.7 B). To determine whether the lesions in the CCR2KO mice were also FAP derived, we crossed PDGFRαEGFP reporter mice and CCR2KO mice (PDGFRαEGFP/CCR2KO). In PDGFRαEGFP/CCR2KO mice that developed HO after muscle damage, the cells contributing to the lesion were PDGFRαEGFP+ (Figure 3.7 C, i).

To verify the activation of osteogenic pathways in FAPs from CCR2KO mice after muscle damage, we performed RNASeq analysis. Analysis 10 days after damage, before the appearance of mature heterotopic ossicles, revealed 397 differentially expressed genes in CCR2KO FAPs compared to WT mice (Figure 3.7 D), including 46 bone-related genes (Figure 3.7 E). Further, GO biological pathway analysis of the differentially expressed genes revealed that genes related to ossification, cartilage development, bone mineralization, and osteoblast differentiation, are also
significantly upregulated in CCR2KO PDGFRα⁺ FAPs at D10 post injury (Figure 3.7 F). When examined over time, we found that while genes associated with the GO terms for ossification, cartilage development, bone mineralization, and osteoblast differentiation are upregulated in both WT and CCR2KO FAPs after damage, however once WT FAPs are cleared by macrophages around D3-4 post injury, osteogenic gene expression is downregulated, while in CCR2KO FAPs, genes associated with these pathways remain high (Figure 3.7 G). Examination of normalized counts for the osteogenic genes Sox9, Runx2, and Spp1 over time also demonstrates that CCR2KO FAPs continue to express these genes at day 10 while in WT FAPs, expression of these genes returns to baseline expression levels (Figure 3.8). This suggests that clearance of activated, “osteogenic” FAPs by infiltrating macrophages inhibits the development of HO, while in CCR2KO mice, in which this mechanism is defective, these cells persist, complete osteoblastic differentiation, and lead to HO. Together, the persistence of osteogenic genes and significantly upregulated osteogenic pathways in PDGFRα⁺ FAPs from CCR2KO animals suggests that an altered inflammatory environment in the skeletal muscle after damage can drive the osteogenic differentiation of resident FAPs, even in the absence of exogenous BMPs.

3.5 Discussion

While the cellular mechanisms underlying bone formation in inherited HO are starting to be understood through transcriptomic analysis and new murine models, there is little understanding of the cells and mechanisms behind the pathogenesis of acquired HO. Several markers such as Tie2 (83), Mx1 (79), Glast (81), Gli1 (84), and Scx (79,80), have been proposed to identify mesenchymal cells with osteogenic potential both within and outside of the skeletal system, however their
usefulness has been limited due to their expression on multiple cell types or incomplete labelling of the heterotopic lesions, suggesting the contribution of additional cell populations to the ossicle. In support of our results however, most of these studies identified PDGFRα+ cells within the heterogenous population of labeled cells (85). Though PDGFRα is a well-known mesenchymal cell marker and is broadly expressed throughout embryonic and adult tissues (193), within skeletal muscle it labels a discrete population of cells that aid in the regeneration of muscle after damage but are also known to differentiate and contribute to muscle degeneration when regeneration is impaired (87,192,194,195). Further, though other cell populations such as Tie2+ cells have not been shown to contribute to the osteogenic lineage of normotopic bone, PDGFRα labels osteogenic cells in skeletal tissues at homeostasis, making it a more robust marker for lineage tracing of osteogenic cells in other tissues. Intriguingly, we found PDGFRα+ FAPs in undamaged muscle also highly express Tie2 (Tek), and at lower levels, Glast (Slc1a3) and Gli1; markers that have been proposed to identify the cells that give rise to HO (Figure 3.9). This suggests that in previous lineage tracing studies, the PDGFRα+ subfraction of heterogenous labelled cells may have been responsible for establishing HO (79,80,83,85). Using a novel PDGFRαCT2/td.Tomato murine knock-in model, we have shown that it is indeed the PDGFRα+ FAPs in muscle give rise to the vast majority of osteogenic cells within heterotopic lesions that form in response to BMP signaling. The relatively small population of unlabeled osteogenic cells can likely be attributed to tamoxifen availability and cre efficiency, rather than alternative progenitor cell populations contributing to ectopic bone formation.

Skeletal stem and progenitor cells have claimed to only exist within the skeletal system, particularly within the bone marrow (182). In addition, adipose deposition in skeletal muscle has
been recently proposed to proceed from FAPs recruited from fat depots through the circulation (196). However, our work using the parabiotic model strongly dispels the theory of a circulating bone marrow or adipose tissue-derived progenitor as the source of ectopic bone. While PDGFRα+ mesenchymal cells are found within the bone marrow under homeostatic conditions, we show that they do not enter circulation and give rise to ectopic lesions at distant sites in response to BMP signaling. This is consistent with work performed by Dey et al (79), who demonstrated that bone marrow (Vav1cre) driven transgenic FOP models and transplantation of FOP transgenic bone marrow were insufficient to induce HO, suggesting hematopoietic cells are not the source of HO. Rather, tissue-resident PDGFRα+ FAPs found within the muscle undergo expansion and osteogenic differentiation. This is consistent with recent work by Chan et al (78) who showed that murine skeletal stem cells may not exist in extra-skeletal tissues at homeostasis, however upon induction with BMPs, can differentiate from yet unknown resident progenitor cells that we now identify as FAPs. Further it is also consistent with work performed by Lees-Shepard et al (86), who demonstrated that FOP transgenic Lin'Sca1+PDGFRα+ cells were sufficient to generate intramuscular HO. Thus, our results suggest that the circulating cells reported to produce collagen in areas of ectopic bone formation (197,198) are likely engaged in matrix remodeling but not in actual bone formation.

Though it has previously been suggested that only bone marrow-derived mesenchymal cells have inherent osteogenic potential, we propose that tissue-resident PDGFRα+ FAPs can acquire such potential and that an altered inflammatory milieu could play a key causal role key in switching from efficient muscle regeneration to the development of trauma-associated HO. Previous studies have successfully used prophylactic anti-inflammatory drugs or in pre-clinical models, clodronate
liposome-mediated macrophage depletion, to prevent HO, suggesting that inflammatory cell stimulation is required for the initiation of HO\(^{(184,191,199)}\). In the case of both NSAIDs and clodronate liposomes however, various other inflammatory cell populations may also be affected and result in decreased FAP activation\(^{(200)}\). In the CCR2KO model, only infiltrating macrophages are inhibited, and other inflammatory cell populations such as eosinophils and neutrophils may be increased, facilitating the activation of FAPs\(^{(201,202)}\). Our previous work has shown that clearance of these activated stromal cells is crucial for normal muscle regeneration and that delay or impairment of their clearance can result in muscle fibrosis\(^{(192)}\), or as we show here, HO and is consistent with recent work by Tirone et al who demonstrated that depletion of macrophages leads to increased HO volume\(^{(203)}\). Further, previous studies have suggested that after local traumatic injuries, osteogenic BMPs may be present in the affected tissues\(^{(204,205)}\). Though further work is required, the combined accumulation of FAPs and the presence of local osteogenic signals such as BMPs could cooperate towards the formation of HO. This points to monocyte driven inflammation as a double-edged sword in the context of muscle injury and the development of HO, similar to other sterile injury models in which monocytes have multiple roles\(^{(206)}\) and suggests that a better understanding of the inflammatory environment and its interactions with local progenitor cell populations after trauma is required to develop better treatments and prophylactics for acquired HO.
Figure 3.1 - PDGFRα<sup>CT2</sup>/td.Tomato lineage tracing labels osteogenic lineage cells.

(A) Representative FACS analysis of CD31<sup>-</sup>/CD45<sup>-</sup> (Lineage<sup>-</sup>) cells isolated from compact and trabecular murine bone of PDGFRα<sup>CT2</sup>/td.Tomato mice 2 weeks after tamoxifen administration stained with PDGFRα antibody. FMO controls are shown in the histograms in black (B) Quantification of PDGFRα<sup>+</sup> skeletal cell populations as analyzed by flow cytometry. (C) Fluorescent Minus One (FMO) controls for PDGFRα antibody and PDGFRα/td.Tomato on FAPs isolated from bone. Samples gated for Lineage<sup>-</sup> and PDGFRα/td.Tomato (left) or PDGFRα Ab (right) used to establish gating. (D) Representative immunofluorescent image of cortical bone from PDGFRα<sup>CT2</sup>/td.Tomato mice with periosteum indicated (Ps). (i) Inset from D showing td.Tomato<sup>+</sup> osteocytes (Ot) with processes oriented towards the endosteum (Es). (E) Comparison of
PDGFRαCT2/Td.Tomato expression and PDGFRαEGFP expression in adult murine trabecular bone. Osteocytes (Ot), bone lining cells (BLC). N=3 biological replicates.
Figure 3.2 - PDGFRα<sup>CT2/td.</sup>Tomato lineage tracing labels FAPs in skeletal muscle.

(A) Representative FACS analysis of Lineage<sup>-</sup>/Sca1<sup>+</sup> FAPs isolated from skeletal muscle of PDGFRα<sup>CT2/td.</sup>Tomato mice 2 weeks after tamoxifen stained with PDGFRα Antibody. FMO controls are shown in the histograms in black (B) Quantification of PDGFRα<sup>-</sup>/Sca1<sup>+</sup> muscle cell populations as analyzed by flow cytometry. (C) FMO controls for FAPs isolated from skeletal muscle, gated for Lineage<sup>-</sup>Sca1<sup>+</sup> and PDGFRα/td.Tomato (left) or PDGFRα Ab (right). (D) Representative immunofluorescent cross-section of muscle from PDGFRα<sup>CT2/td.</sup>Tomato mice with PDGFRα/td.Tomato<sup>+</sup> interstitial cells. (E) Comparison of PDGFRα<sup>CT2/td.</sup>Tomato expression and PDGFRαEGFP expression in adult murine skeletal muscle. Sample muscle fibers are delineated with dashed lines. N=3 biological replicates, *p<0.05.
Figure 3.3 - PDGFRα<sup>CT2/td.Tomato</sup> cells are the primary source of osteogenic cells in BMP2-induced heterotopic lesions.
(A) Schematic of the experimental model. (B) Immunofluorescent image of control Matrigel-injected muscle from PDGFRαCT2/td.Tomato mice with Masson’s trichrome stained inset. (i) Higher magnification image from B. (C) Immunofluorescent image of heterotopic bone from PDGFRαCT2/td.Tomato muscle injected with rh-BMP2 with Masson’s trichrome stained inset. td.Tomato+ osteocytes form the majority of cells within the heterotopic ossicle (ii) Higher magnification image from D showing td.Tomato+ osteocytes (arrowhead) and bone lining cells of the ossicle. Mu (muscle), BM (bone marrow).
Figure 3.4 - Heterotopic ossicles originate from tissue-resident PDGFRα⁺ progenitors, not circulating bone marrow-derived progenitors.

(A) Schematic of the experimental design for PDGFRα⁺CT2/td.Tomato and GFP parabionts. (B) Representative FACS plot of blood from control C57Bl/6J mice and PDGFRα⁺CT2/td.Tomato parabiont demonstrating 50% blood sharing (GFP⁺ cells). (C) Immunofluorescent image of BMP2 injected muscle from PDGFRα⁺CT2/td.Tomato parabiont (i) Inset from C with td.Tomato⁺ osteocytes (arrowhead) and td.Tomato⁺ marrow stromal cells (arrow). (D) Immunofluorescent image of BMP2 injected muscle from GFP parabiont. (ii) Inset from D. No td.Tomato⁺ cells were visible in the ectopic bone of the GFP⁺ parabiont (E) Quantification of td.Tomato⁺ osteo-lineage cells (osteocytes and bone-lining cells). Mean ±SD, N=5 biological replicates, P<0.0001.
Figure 3.5 - GFP+ cells are found within the marrow of heterotopic bone from PDGFRαCT2/td.Tomato parabionts.

(A) Immunofluorescent image of heterotopic bone and marrow from PDGFRαCT2/td.Tomato parabiont injected with BMP2 with sample bone marrow GFP+ cell indicated (white arrowhead). Dashed lines indicate area of mineralized ectopic bone.
Figure 3.6 - Cells contributing to heterotopic bone formation in Col1a1*3.6GFP x C57Bl/6J parabionts are tissue resident, not bone marrow derived.

(A) Immunofluorescent image of BMP2 injected muscle from Col1a1*3.6GFP parabiont with GFP+ osteocytes (B) Immunofluorescent image of BMP2 injected muscle from C57Bl/6J parabiont with no GFP+ cells. Dashed lines indicate area of mineralized bone.
Figure 3.7 - An altered inflammatory milieu activates an osteogenic programme in FAPs.

(A) Experimental design for damage-induced HO in CCR2KO mice (B-C) Representative images of a mature heterotopic ossicle from damaged muscle of PDGFRαEGFP/CCR2KO mice stained
by (B) Von Kossa, and (C) immunofluorescence. (i) Inset from C with PDGFRαEGFP+ osteocytes (white arrowheads). (D) Heatmap of differentially expressed genes in PDGFRα+ FAPs from WT and CCR2KO animals 10 days after NTX damage. (E) Heatmap of differentially expressed bone and osteogenesis-related genes in FAPs from CCR2KO mice compared to WT at D10 after NTX damage. (F) Top 40 upregulated GO biological pathways at day 10 after damage highlighting osteogenic and chondrogenic pathways. (G) Number of genes in the associated GO pathways upregulated at the indicated timepoints after damage in WT and CCR2KO FAPs. Osteogenic pathway genes remain upregulated in CCR2KO FAPs at D10 after damage while they are downregulated in WT FAPs.
Figure 3.8 - FAPs from CCR2KO mice express high levels of osteogenic genes at D10 after damage

(A) Normalized transcript counts for *Sox9* in CCR2KO or WT FAPs at the indicated timepoints.
(B) Normalized transcript counts for *Runx2* in CCR2KO or WT FAPs at the indicated timepoints.
(C) Normalized transcript counts for *Spp1* in CCR2KO or WT FAPs at the indicated timepoints.
Figure 3.9 - WT FAPs from undamaged muscle have varied expression of genes previously used to identify putative HO progenitors.

(A) Normalized transcript counts for *Gli1*, *Mx1*, Nestin (*Nes*), Scleraxis (*Scx*), Glast (*Slc1a3*), and Tie2 (*Tek*) in WT FAPs isolated from undamaged muscle. (i) Inset from A showing normalized transcript counts for the above genes with Tie2 removed.
Chapter 4: Constitutive SMAD4-mediated TGFβ/BMP signaling in PDGFRα mesenchymal cells is required for murine skeletal homeostasis

4.1 Synopsis

TGFβ and BMP signaling have important roles in skeletal development and homeostasis regulating cell proliferation, differentiation and even coupling of bone formation and deposition. Tight coupling of bone formation and resorption is required to maintain bone mass and many genetic models of impaired or altered TGFβ and BMP signaling result in aberrant skeletal phenotypes. Here, we used a PDGFRαCreERT2 to ablate Smad4, the common mediator of canonical TGFβ and BMP signaling, in a variety of osteolineage cells including progenitors, osteoblasts, and osteocytes. PDGFRα/smad4Fl/Fl mice developed a severe and rapidly degenerative skeletal phenotype characterized by high turnover bone loss. Cell proliferation and osteocyte density were increased in Smad4 mice though osteoblast differentiation pathways did not appear to be downregulated. Bone remodeling pathways were upregulated, and bone mineralization pathways were slightly downregulated, though osteoclast activity and differentiation were increased in smad4Fl/Fl mice. Further, Tnfsf11 (RANKL) and Tnfrsf11b (OPG) expression were altered in marrow-depleted bone from smad4Fl/Fl mice suggesting an uncoupling of bone formation and resorption. In summary, a constitutive signal through Smad4 acts as a negative regulator of cell proliferation in PDGFRα+ cells and is required for coupling of bone formation and resorption.
4.2 Introduction

Bone is a highly dynamic tissue and skeletal homeostasis is maintained by bone remodeling through osteoclastic bone resorption followed by osteoblast mediated bone formation. The development and action of osteoblasts and osteoclasts are highly regulated and involve multiple signaling pathways such as Wnt/βcatenin, Hedgehog (Hh), Notch, transforming growth factor-β (TGFβ), and bone morphogenic protein (BMP) \(^{207}\). Similarly, coupling of bone resorption and bone formation is tightly regulated to ensure resorbed bone is completely replaced and bone mass is maintained. When this coupling fails and bone resorption outpaces bone formation, bone mass is lost.

The transforming growth factor-β (TGFβ) superfamily composed of TGFβ, activins, and bone morphogenic proteins (BMPs) amongst others, play important roles in skeletal development and postnatal skeletal homeostasis \(^{143}\), regulating both osteoblasts, and osteoclasts. Postnatally, TGFβ plays important roles in the recruitment and expansion of osteoprogenitors and osteoblasts \(^{153,208}\). BMP 2/4/5/6/7 all have potent osteogenic effects and promote osteoblast differentiation and bone mineralization \(^{168,209-211}\).

Importantly, TGFβ also plays important roles in coupling bone formation and resorption. Bone acts as a reserve of latent TGFβ that upon bone resorption by osteoclasts, is released and activated by cleavage from latency-associated protein (LAP) \(^{212}\). There is evidence that TGFβ released during bone resorption recruits osteoprogenitors, thereby coupling bone formation and resorption \(^{208,213}\). Further, TGFβ can modulate osteoclast activation by regulating the RANKL (receptor
activator NF-kB ligand, osteoclast activating)/OPG (osteoprotegerin, osteoclast inhibiting) ratio secreted by osteoblasts, thereby also helping to couple bone formation and resorption \(^{(154)}\).

Signaling of TGFβ superfamily members occurs through heteromeric receptor complexes of specific type I and type II receptors, and intracellular effects are mediated by SMAD complexes or mitogen-activated protein kinase (MAPK) cascades \(^{(143,146)}\). In the SMAD-dependent pathway, receptor activated-smads interact with the common smad (Co-SMAD / SMAD4) and translocate to the nucleus where in conjunction with appropriate co-factors, they regulate gene expression \(^{(143,146,153)}\). While SMAD2/3 mediate TGFβ signaling and SMAD1/5/8 mediate BMP signaling, SMAD4 acts as a common mediator for both pathways \(^{(145)}\).

Mutations of TGFβ and BMP have been identified in a variety of skeletal disorders including Fibrodysplasia ossificans progressive (FOP), brachydactyly, sclerosteosis, Camurati-Engelmann Disease (CED) \(^{(146)}\) and Myhre syndrome \(^{(214)}\). To study the role of TGFβ and BMP signaling in the skeletal system, gene inactivation of various TGFβ and BMP receptors, ligands, and signaling molecules have been used resulting in a wide variety of skeletal phenotypes \(^{(143)}\). While Smad4 null mice are not viable and die prenatally at E7.5-E9.5 due to gastrulation defects \(^{(167)}\), the Smad4\(^{\text{Flox}}\) mouse allows for conditional deletion of Smad4 \(^{(215)}\). Previous studies have examined the role of Smad4 in osteoblasts and osteocytes with complex and at times contradictory results. Ablation of Smad4 in osteoblasts using an Osteocalcin (Bglap) Cre resulted in lower bone volume, lower bone mineral density, decreased number of osteoblasts, and decreased bone formation rate though trabecular bone volume in smad4\(^{\text{Flox}}\) mice increased after 7 months due to reduced osteoclast activity \(^{(168)}\). Embryonic deletion of Smad4 in Osx (Sp7) expressing pre-osteoblasts
resulted in stunted growth, and spontaneous fractures reminiscent of osteogenesis imperfecta, cleidocranial dysplasia, and Wnt-deficiency syndromes (170). These effects were attributed to deficiencies in collagen matrix processing enzymes and hypomineralization (170). Similarly, deletion of Smad4 from Collagen1a1 (2.3-kb) expressing osteoblasts and osteocytes resulted in decreased osteoclast activity and increased osteocyte number and density as a result of decreased apoptosis (169). The majority of these studies however do not truly represent alterations in adult skeletal homeostasis as the use of constitutive cre drivers allows for developmental and bone modeling effects to confound the results. Inducible cre-mediated ablation of Smad4 in the osteoblast lineage (Osx-Cre) resulted in increased cell proliferation in vivo, and delayed osteoblast differentiation and mineralization defects in vitro though no gross morphological defects were reported (171). Together, this suggests that Smad4 plays multiple roles in postnatal skeletal homeostasis that are cell and age dependent.

Using a PDGFRαCreERT2 inducible mouse, we ablated Smad4 in PDGFRα+ mesenchymal progenitors as well as a variety of osteolineage cells including chondrocytes, osteoblasts, and osteocytes. This model produced a rapid and severe degenerative skeletal phenotype characterized by altered gait and bone loss. Deletion of Smad4 in PDGFRα+ cells induced cell proliferation, increased osteocyte density and though no defects in osteoblast differentiation were found, there did appear to be some effects on bone mineralization. Further, osteoclast activity was increased in smad4Fl/Fl mice and the expression of Tnfsf11 (RANKL) and Tnfrsf11b (OPG) were altered suggesting an uncoupling of bone formation and resorption and high-turnover bone loss possibly exacerbated by the activation of inflammatory pathways. Our work suggests that Smad4 is required
in PDGFRα+ cells to maintain adult skeletal homeostasis by preventing aberrant progenitor proliferation and uncoupling of bone remodeling.

4.3 Methods

4.3.1 Mice

C57Bl/6J (JAX stock #000664), Smad4tm2.1Cxd/J (Smad4Flox, JAX Stock #017462), and B6.Cg-Gt(Rosa)26So+tm9(CAG-mdTomato)Hze/J (Rosa26.tdTomato reporter mice Jax stock #007909) were purchased from Jackson Laboratories. HIC1CreERT2 (HIC1CT2) mice were a kind gift from TM Underhill (University of British Columbia). PDGFRαCreERT2 (PDGFRαCT2) mice were a kind gift from Brigid Hogan (Duke University). Mice were crossed and maintained at the Biomedical Research Centre at the University of British Columbia. Mice were fed ad libitum a 50/50 diet of irradiated PicoLab Rodent Diet 20 (5053) and PicoLab Mouse Diet 20 (5058) (LabDiet). To induce reporter expression and cre-loxP recombination for gene deletion in CreERT2 mice, Tamoxifen (Sigma) dissolved in sunflower oil was injected intraperitoneally (IP) (3mg/day for 5 consecutive days) to 8-12 week old mice. PDGFRαCT2/Td.Tomato/SmadFlox mice were monitored weekly starting one week after tamoxifen administration. All animal procedures were approved by the University of British Columbia Animal Care Committee (Protocol A17-0039).
4.3.2 EdU labeling

Where required for labeling of proliferating cells, animals were injected IP with 1mg EdU (Invitrogen) dissolved in Dulbecco’s PBS 24 hours before collection.

4.3.3 Calcein labeling and analysis

Where required for labelling mineralizing bone surfaces, mice were injected IP with 10mg/kg calcein (Sigma) dissolved in 2% NaHCO₃ in PBS. Calcein labels were administered 9d and 2d before collection. For histomorphometric analysis, bones were not decalcified. Bones were fixed in 4% PFA and cryoprotected using a 20-40% sucrose (Sigma) gradient, then embedded in OCT (Sakura). Calcified bones were then sectioned onto specially coated CryoJane slides using a tape transfer system and a Leica cryostat equipped with a CryoJane attachment. Images of trabecular (metaphyseal), and cortical bone were acquired using a Nikon Ni Eclipse microscope and were analyzed using ImageJ (Fiji). Bone surface (BS), single-labeled bone surface (sLS), and double-labeled surface (dLS) were measured separately. In accordance with Dempster et al(216), mineralizing surface was calculated as (dLS+sLS/2)/BS. Mineral apposition rate was calculated as the inter-label thickness (Ir.L.Th) divided by the inter label time (Ir.L.t) (MAR=Ir.L.Th/Ir.L.t). Bone formation rate (BFR) was calculated by MAR*(MS/BS).
4.3.4 DigiGait analysis

Digital gait analysis was performed using a DigiGait Imaging System (Mouse Specifics Inc). Belt speed was set to 12cm/s and belt angle to 0 degrees. A minimum of 2 runs per animal were collected and analyzed. All gait analysis was performed within the DigiGait software.

4.3.5 FGF23 antibody treatment

As part of an academic collaboration, anti-FGF23 antibody and IgG2a isotype control antibody were kind gifts from Amgen (Thousand Oaks, CA). Anti-FGF23 or IgG2a isotype (10mg/kg) were administered IP 3x/week to animals starting 3 days after completion of tamoxifen treatment.

4.3.6 Whole mount Alizarin Red S/Alcian Blue staining

For whole mount staining, mice were collected, eviscerated, and all skin removed. Samples were then fixed in 100% EtOH for 2 days followed by 2 days in 100% acetone. Samples were rinsed with water and stained for 7 days at room temperature using a solution composed of 1 volume Alcian Blue solution (0.3% alcian blue 8GX (Sigma) in 70% EtOH), 1 volume Alizarin Red S solution (0.1% Alizarin Red S (Sigma) in 95% EtOH), 1 volume acetic acid, and 17 volumes 70% EtOH. Samples were rinsed with water and cleared at room temperature for 30 days in a solution of 2% KOH/20% glycerol (Sigma). Stained and cleared samples were stored in 100% glycerol.
4.3.7 Histology and immunofluorescence

Bones were collected and fixed in 4% PFA (Sigma) in PBS at 4°C overnight. For immunofluorescent staining, bones were decalcified using 0.5M EDTA (Sigma) and cryoprotected using a 20-40% sucrose gradient before being embedded in OCT. Bones were then sectioned using a Leica cryostat and mounted on Superfrost Plus slides (Fisher). Following blocking, slides were incubated with primary antibodies for 1 hour at room temperature or overnight at 4°C. Primary antibodies used include CD31 (AF3628, R&D Systems), and FGF23 (MAB26291, R&D Systems). To detect primary antibodies, slides were incubated with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen). Tartrate-Resistant Acid Phosphatase (TRAP) staining was performed using an Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma) according to the manufacturer’s instructions. TRAP images were analyzed using TrapHisto software (Histomorph Software, Liverpool University). Bone outlines were drawn manually, and osteoclasts detected using thresholding according the TrapHisto guidelines. EdU was detected using a Click-iT Plus EdU detection Imaging kit (Invitrogen) according to the manufacturer’s directions. Other histochemical stains were performed according to standard protocols. Brightfield and fluorescent images were acquired on a Nikon Eclipse Ni microscope. Confocal images were acquired on a Leica SP8, Nikon C2, Zeiss LSM800, or Zeiss LSM900 confocal microscope. Images were analyzed using ImageJ (Fiji) and assembled using Illustrator CC (Adobe).
4.3.8 μCT analysis

Whole body MicroCT scans were performed at the Centre for High Throughput Phenogenomics on a μCT 100 (SCANCO Medical AG, Brüttisellen, Switzerland) with an isotropic voxel size of 7.5µm. MicroCT scans for tibial trabecular and cortical analysis were performed at the Centre for Hip Health on a μCT 35 (SCANCO Medical AG, Brüttisellen, Switzerland) with an isotropic voxel size of 3.5µm, integration time 300ms, Energy 70 kVp, Intensity 114µA. A block size of 0.82mm (234 slices) was acquired for both trabecular (metaphyseal) and cortical (mid-diaphysis) scans. Blinded analysis of trabecular and midshaft cortical scans were performed by collaborators at the University of Toronto using CTAN software (Bruker).

4.3.9 qPCR

To obtain RNA for qPCR, bones were collected and the marrow removed by flushing with PBS. Bones were then placed in a mortar, flash frozen with liquid Nitrogen, and crushed into a fine powder. The bone powder was then collected into ice cold RNAzol and RNA isolated according to the manufacturer’s protocol. Total RNA was resuspended in nuclease-free water, quantified using a NanoDrop system, and converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer’s instructions. Custom TaqMan gene expression array plates (Applied Biosystems) were ordered and prepared according to the manufacturers protocol then run on a ViiA 7 Real-Time PCR machine (Applied Biosystems).
4.3.10  FACS analysis

To analyze compact bone by flow cytometry, bones were harvested and mechanically and enzymatically digested. Briefly, femur and tibia bones were collected from 6-10-week-old mice and excess muscle removed. Bones were gently crushed in a mortar and pestle with ice cold PBS to liberate bone marrow and break the bone into small pieces. Bone marrow containing PBS was aspirated and replaced with fresh PBS until bone fragments appeared white and free of marrow contamination. Bone chips were then transferred to a dish and digested with collagenase II (250CDU/ml, Sigma) for 30 minutes at room temperature. Bones were then cut into smaller pieces using a clean razor blade and further digested with Collagenase D (1.5U/ml)/Dispase II (2.4U/ml) (Roche Biochemicals) for 60 minutes at 37°C with gentle rotation. All enzymes were activated with 10µl/ml of CaCl$_2$ (250µM). Digestion was quenched using FACS buffer (2% FBS, 2mM EDTA in PBS), and cells were strained through a 70µm cell strainer to remove remaining bone chips. The resulting cell suspension was then stained for FACS with the indicated antibodies. Antibodies used for flow cytometry included CD45 (clone 30-F11, Ablab, University of British Columbia), CD31 (clone MEC13.3, eBioscience, San Diego CA), Sca1 (clone D7, eBioscience, San Diego CA). Stained cells were washed and analyzed on a BD LSRII. Further FACS analysis was performed using FlowJo software (TreeStar, CA).

4.3.11  Copy number assay

For copy number assays (CNA), genomic DNA (gDNA) was isolated from skeletal muscle sorted td.Tomato$^+$ FAPs cells using a PureLink Genomic DNA kit (Invitrogen) according to the
directions. Digital droplet PCR reactions were prepared using a TaqMan VIC-dye labeled reference primer for *Tfrc* (cat# 4458366, Applied Biosystems) and TaqMan FAM-dye labeled test assay for *Smad4* (Mm00492182_cn, Applied Biosystems). Droplets for DDPCR were generated using a BioRad QX100 droplet generator and PCR run using a T100 Thermal Cycler (BioRad). Droplets were read using a BioRad QX100 droplet reader and analyzed using QuantaSoft (BioRad).

### 4.3.12 RNA sequencing

RNA sequencing was performed on both sorted FAPs and whole bone tissue. For sorted cell RNA sequencing, FAPs from compact bone were isolated after enzymatic digestion using FACS. Sorted CD45⁻/CD31⁻/Sca1⁺/PDGFRL⁺ td.Tomato⁺ cells were collected into DMEM with 5% FBS to preserve viability and then spun down and washed with DEPC treated/nuclease-free PBS. Samples were then resuspended in RNAzol RT (Sigma) and after centrifugation, the aqueous layer collected. GeneElute LPA (Sigma) was added and RNA precipitated with isopropanol at -20°C overnight. RNA was then washed with ethanol and resuspended in RNAse-free water with SUPERase In (Invitrogen) and stored at -80°C until processing for sequencing.

For total bone tissue RNA, bones were collected and marrow removed by flushing with a PBS filled syringe. Bones were then flash frozen using liquid nitrogen and kept in pre-chilled Eppendorf tubes on dry ice. Frozen bones were then placed in tubes containing cold RNAzol RT (Sigma) and immediately homogenized using a tissue homogenizer. Tissue lysate in RNAzol RT was then processed for total RNA isolation according to the manufacturer’s instructions. RNA was resuspended in nuclease free water and stored at -80°C until processing for sequencing.
Sample quality control was performed using the Agilent 2100 Bioanalyzer. Qualifying samples were then prepped following the standard protocols for either the NEBnext Ultra II Stranded mRNA (New England Biolabs), or TruSeq stranded mRNA library kit (Illumina) on the Illumina Neoprep automated nanofluidic library prep instrument. Sequencing was performed on the Illumina NextSeq 500 with Paired End 42bp x 42bp reads. Sequencing data was demultiplexed using Illumina’s bcl2fastq2. Demultiplexed read sequences were then aligned to the Mus Musculus (mm10) reference sequence using either STAR aligner (https://www.ncbi.nlm.nih.gov/pubmed/23104886) or TopHat splice junction mapper with Bowtie 2 (http://ccb.jhu.edu/software/tophat/index.shtml). Sequencing results were analyzed in R using the following packages: DEGreport 1.24.0, ggrepel 0.8.2, forcats 0.5.0, stringr 1.4.0, purrr 0.3.4, readr 1.3.1, tidyr 1.0.3, tibble 3.0.1, tidyverse 1.3.0, org.Mm.eg.db 3.11.1, dplyr 0.8.5, magrittr 1.5, AnnotationDbi 1.50.0, DESeq2 1.28.1, SummarizedExperiment 1.18.1, DelayedArray 0.14.0, matrixStats 0.56.0, Biobase 2.48.0, GenomicRanges 1.40.0, GenomeInfoDb 1.24.0, IRanges 2.22.2, S4Vectors 0.26.1, BiocGenerics 0.34.0, RColorBrewer 1.1-2, pheatmap 1.0.12, ggplot2 3.3.0, clusterProfiler 3.16.0, edgeR 3.30.1, limma 3.44.1, sva 3.35.2, BiocParallel 1.22.0, genefilter 1.70.0, mgcv 1.8-31, nlme 3.1-147, PoiClaClu 1.0.2.1, apeglm 1.10.0

### 4.3.13 Statistical analysis

All statistical analysis was performed in Prism (GraphPad) or R. Results shown represent the mean ±SEM unless otherwise indicated. Individual statistical tests indicated in figure legends, however briefly, two group analyses were performed using unpaired Student’s T-Test, multigroup analysis...
was performed using one-way ANOVA with Tukey correction for multiple comparisons, and grouped data was analyzed using two-way ANOVA with Sidak correction for multiple comparisons. P<0.05 was considered significant.

4.4 Results

4.4.1 Deletion of Smad4 in PDGFRα+ mesenchymal progenitors leads to a degenerative skeletal phenotype.

Previous research on the role of TGFβ and BMP signaling in skeletal homeostasis has predominantly been conducted using constitutively active cre drivers (143), making it difficult to define the role of these pathways in maintaining the adult skeleton. Our previous research demonstrated that PDGFRα labels a variety of osteolineage cells and by using the PDGFRαCT2 mouse, we were able to induce deletion of Smad4 in both mesenchymal progenitors and downstream osteogenic progeny to examine the effects of impaired TGFβ/BMP signaling on the adult skeletal system (Figure 4.1 A). Copy number assays of td.Tomato+ cells isolated from skeletal muscle of smad4Fl/+ and smad4Fl/Fl mice indicated a Smad4 deletion efficiency of 42.32% (±1.388) (Figure 4.1B). Downregulation of Smad4 was also confirmed by RNASeq analysis of FACS sorted Lineage(CD31/CD45)-/td.Tomato+/Sca1+ FAPs from skeletal tissues (Fl/Fl vs Fl+/ log fold change -2.3363, p.adj 5.10x10⁻⁷) (Figure 4.1 C). Strikingly, we noted that after tamoxifen administration, smad4Fl/Fl mice developed a distinct phenotype compared to smad4Fl/+ littermate controls. Smad4Fl/Fl mice developed a hip dysplasia-like phenotype, demonstrated by a ‘frog-like’ sitting stance (Figure 4.1 D, F). Smad4Fl/Fl mice also demonstrated a reduced ability to extend the
hindlimbs (hindlimb clasping), and reduced toe splay when held by the base of the tail (Figure 4.1 E, G). The phenotype appeared degenerative and at later stages, smad4$^{Fl/Fl}$ mice developed kyphosis of the spine (Figure 4.1 H) as well as joint displacements. Joint displacements appeared predominantly in the knee; however slight ankle displacements were occasionally observed. While initially included in our studies, male smad4$^{Fl/Fl}$ mice were excluded from further studies as they developed large scrotal hernias necessitating humane euthanasia before the onset of any hindlimb phenotype. The hindlimb phenotype was scored according to Table 4-1 and appeared on average 3-4 weeks after tamoxifen administration (Figure 4.1 I-L). The hindlimb phenotype could be further stratified using the mobility and hindlimb scores into early (scores 1-3), and late stage (scores of 3-4) but did not result in any significant changes in weight or health. Interestingly, though we also crossed the smad4$^{Flox}$ mouse to HIC1$^{CT2}$ in order to delete Smad4 from upstream quiescent skeletal progenitors, we did not observe any overt phenotypes even up to 6 months post tamoxifen administration.

To better quantify the mobility changes observed in smad4$^{Fl/Fl}$ mice, we performed digital gait analysis. Digital treadmill gait analysis provides insight into the various phases of murine stride (Figure 4.2 A) as well as various other gait parameters such as paw area, stride length and width, step angle, and paw angle (Figure 4.2 B). The ventral view of the mice obtained from the DigiGait system highlighted the hip dysplasia-like phenotype observed in smad4$^{Fl/Fl}$ mice (Figure 4.2 C-D), as well as a reduced stance width and clasping or cupping of the paws (Figure 4.2E-H). While the phenotype was initially most evident in the hindlimbs of smad4$^{Fl/Fl}$ mice, gait analysis indicated there were changes in both hind and forelimb parameters. Though stride length and frequency were not significantly altered in smad4$^{Fl/Fl}$ mice (Figure 4.2 I-J), forelimb % swing stride, the percent of stride during which the paw is in the air, was decreased in smad4$^{Fl/Fl}$ mice (Figure 4.2 K) indicating
that the paw remained in contact with the treadmill for an extended period of the stride. Stance/swing ratio, the ratio of stance phase time to swing phase time, was correspondingly increased in the forelimbs of \( \text{smad4}^{\text{Fl/FI}} \) mice, though it was decreased in the hindlimbs (Figure 4.2 L). Most affected in \( \text{smad4}^{\text{Fl/FI}} \) mice, however, were gait parameters indicating alterations in paw placement. Stance width was decreased in both the fore and hindlimbs of \( \text{smad4}^{\text{Fl/FI}} \) mice (Figure 4.2 M) possibly suggesting postural adjustments for stability. Absolute paw angle of both the fore and hindlimbs of \( \text{smad4}^{\text{Fl/FI}} \) animals was significantly reduced (Figure 4.2 N), and hindlimb paw angle variability was increased (Figure 4.2 O) suggesting inconsistent paw placement, possibly an index of motor control. Overlap distance, the extent of overlap between ipsilateral fore and hindlimbs, was reduced in \( \text{smad4}^{\text{Fl/FI}} \) mice (Figure 4.2 P), and paw placement positioning, a balance metric, was also significantly reduced (Figure 4.2 Q). Additional gait parameters were analyzed (Figure 4.3) and the results summarized in Table 4-2. We noted that though other positional or postural indices did not have significantly different means, the variance between groups (F test) was significantly different (Table 4-2), suggesting that the \( \text{smad4}^{\text{Fl/FI}} \) phenotype is highly variable.

Gait analysis provided valuable insight into how gait was affected, however as PDGFR\(\alpha\) is also expressed in muscle FAPs that aid in muscle regeneration, it did not indicate whether the observed changes were the result of muscular or skeletal abnormalities. Analysis of skeletal muscle from \( \text{smad4}^{\text{Flox}} \) mice at homeostasis (Figure 4.4) did not reveal any significant differences in cross-sectional area, myonuclei per fiber, or collagen deposition, suggesting there were no significant muscular changes in \( \text{smad4}^{\text{Fl/FI}} \) mice at homeostasis and that the phenotype may be due to skeletal changes.
To better visualize any skeletal abnormalities, we performed micro computed tomography (µCT) analysis of the whole skeleton as well as whole mount skeletal staining to observe whether there were changes in the cartilaginous and mineralized bone components of the skeleton. Whole mount staining using Alcian Blue and Alizarin Red S, did not highlight any significant changes in the contribution of cartilage or mineralized bone to the skeletons of smad4\textsuperscript{Fl/Fl} mice (Figure 4.5 A-B), however µCT revealed several changes to the skeleton of smad4\textsuperscript{Fl/Fl} mice. We noted kyphosis of the spine (Figure 4.5 C-D, arrow), as well as lumbar lordosis (Figure 4.5 C-D, arrowhead), and some abnormal curvature in the ribs (Figure 4.5 I-L). Most notably however, we observed evidence of tibial and patellar dislocations (Figure 4.5 E-H, arrow), narrowing of the distal femur with associated pitting of the bone surface (Figure 4.5 G, arrowhead), and almost complete ablation of the pelvic girdle, particularly at the ischiopubic ramus (Figure 4.5 G, asterisk). Some of these phenotypes were present in all smad4\textsuperscript{Fl/Fl} specimens examined, while others such as lumbar lordosis and altered rib morphology, were only present in some smad4\textsuperscript{Fl/Fl} mice (Figure 4.5 M). Interestingly, though we noted changes in the gross morphology of the femurs (Figure 4.6 A), femur weight and length did not differ significantly between smad4\textsuperscript{Fl/Fl} mice and controls (Figure 4.6 B-C). Tibia length, however, was decreased in smad4\textsuperscript{Fl/Fl} mice predominantly at early stage phenotype (Fl/+ 17.98mm ±0.4512, Early Fl/Fl 17.26mm ±0.4021, Late Fl/Fl 17.79mm ±0.1518) (Figure 4.6 D). Histological analysis also indicated notable differences in the bone architecture of smad4\textsuperscript{Fl/Fl} mice such as reduced cortical thickness and erosion of the bone underlying articular surfaces (Figure 4.6 E-F), suggesting quantifiable differences in bone microstructure.
Together, the absence of a muscle phenotype and presence of overt skeletal abnormalities suggested that the smad4 phenotype was due to skeletal defects, and not to muscular defects. These overt skeletal abnormalities suggested that deletion of Smad4 in PDGFRα⁺ cells resulted in rapid changes to skeletal homeostasis that resulted in loss of bone.

4.4.2 Deletion of Smad4 in PDGFRα⁺ osteogenic cells results in uncoupling of bone formation and resorption.

Gross skeletal changes such as those observed using whole body μCT suggested there would also be changes in bone microarchitecture. Using μCT, we scanned a region of trabecular bone in the metaphyseal region of the tibia just below the growth plate and a region of cortical bone in the mid-diaphysis (Figure 4.7 A). While BV/TV and Tb.N appeared to be slightly elevated in smad4Fl/FI mice, none of the trabecular parameters were significantly altered (Figure 4.7 B-E). Confirming our observations of pitting on cortical surfaces, cortical thickness (Ct.Th) was significantly decreased (Fl/+ 0.1349mm ±0.029 vs Fl/Fl 0.1084mm ±0.0242) and cortical porosity (Po(cl)) was significantly increased in smad4Fl/Fl animals (Fl/+ 1.309% ±0.928 vs Fl/Fl 3.105% ±2.462) (Figure 4.7 F-G). As a result of decreased Ct.Th and increased Po(cl), the cortical area fraction (Ct.Ar/Tt.Ar) was decreased in smad4Fl/Fl mice (Fl/+ 94.93% ±1.740 vs Fl/Fl 92.28% ±2.978) (Figure 4.7 J). This suggested that bone remodelling was increased in smad4Fl/Fl mice. RNASeq analysis of whole bone tissue from smad4Flox mice confirmed a significant increase in many genes associated with bone remodelling (GO:0046849), particularly those involved in osteoclast activation or function including Tnfsf11 (RANK), Spp1 (Osteopontin), Snx10, Oscar, and Ctsk (Figure 4.7 K). Similarly bone remodelling genes associated with protective effects such
as *Grem1* and *Tnfrsf11b* (Osteoprotegerin) were significantly downregulated in smad4\(^{Fl/Fl}\) mice. Bone remodelling by osteoclasts and osteoblasts is normally tightly regulated and increased bone remodelling could indicate an increase in bone deposition or resorption.

We therefore examined whether in response to the deletion of *Smad4* in PDGFR\(\alpha^+\) cells, which represent the majority of osteolineage cells, there was a change in the number of PDGFR\(\alpha^+\) cells and found there was a progressive increase in td.Tomato\(^+\) cells late stage smad4\(^{Fl/Fl}\) mice (78.75\% ±13.11) compared to controls (51.39\% ±6.405) (Figure 4.8 A-E). This also corresponded to an increase in osteocyte density in smad4\(^{Fl/Fl}\) mice (Fl/+ 52.97 ±3.892 vs Late Fl/Fl 86.36 ±8.780) (Figure 4.8 F). We also noted that the periosteal surface of the bone at both fibrous entheses and fibrocartilaginous entheses appeared to be eroded and had increased td.Tomato\(^+\) cells (Figure 4.8 A-D). Of note, at late stage, fibrocartilaginous entheses appeared to lose some of their organized structure and become more fibroblastic (Figure 4.8 C-D). These changes to the entheses could potentially affect tendon and ligament attachment to bone and help account for some of the dislocations we observed.

Previous studies have found that deletion of *Smad4* in osteoblasts leads to increased proliferation\(^{(171)}\). To determine if the increase in PDGFR\(\alpha^+/td.Tomato^+\) cells in our model was due to increased proliferation, we treated mice with EdU and found increased proliferation in smad4\(^{Fl/Fl}\) mice (0.7078\% ±0.2105) compared to controls (0.4536\% ±0.1522) (Figure 4.8 G). Genes associated with proliferation such as *Plk1*, *Bub1*, and *Top2a* were upregulated in FACS sorted Lineage(CD31/CD45)/td.Tomato\(^+\)/Sca1\(^+\) cells isolated from bones of smad4\(^{Fl/Fl}\) mice compared to controls (Figure 4.8 H) further supporting that deletion of *Smad4* induced cell proliferation in
PDGFRα+ cells. As PDGFRα+ cells represent the majority of osteolineage cells including bone depositing osteoblasts, and mineralization defects have been reported in other skeletal smad4 models (168,169,171), we next examined whether the increased cell proliferation we observed in smad4Fl/fl mice had an effect on bone mineralization and could account for the changes in bone structure we observed.

Using calcein double labeling, we analyzed the mineralizing surfaces of the bone as well as the mineral apposition rate (MAR) (Figure 4.9 A). Mineralizing surface (MS), MAR, and bone formation rate (BFR) showed similar trends in both cortical and trabecular bone (Figure 4.10) and no significant differences in mineralization parameters were noted between smad4Fl/fl animals and controls (Figure 4.9 B-H). We also examined the expression of genes associated with the regulation of bone mineralization and found many were differentially expressed in bones from smad4Fl/fl mice. Notably, among these differentially expressed genes, those associated with positive regulation of bone mineralization (GO:0030501) (Fam20c, Actn3, Isg15, Mef2c, Fzd9, and Pkdcc) were downregulated and genes associated with negative regulation of bone mineralization (GO:0030502) were upregulated (Grem1, Hif1a, Fgf23, Ccr1, Srgn) (Figure 4.9 I), suggesting that there may be some decrease in bone mineralization that was not captured by the calcein assay. As the main phenotype in smad4 mice indicated a loss of bone and there was no obvious decrease in bone mineralization, we next examined whether there was an increase in bone resorption that could explain the phenotype.

To measure osteoclast activity, we used a tartrate-resistant acid phosphatase (TRAP) assay and noted increased osteoclast staining on cortical surfaces, particularly at fibrous entheses (Figure
Bone perimeter (B.Pm) was increased in smad4\textsuperscript{Fl/Fl} mice (Figure 4.11 C), particularly in trabecular bone (Figure 4.12 A), which was consistent with the trend towards an increased Tb.N we observed using \textmu CT in smad4\textsuperscript{Fl/Fl} mice. Osteoclast perimeter (Oc.Pm), osteoclast number (Oc.N), and osteoclast surface as a percent of the bone surface (Oc.S/BS) all also appeared to be increased in smad4\textsuperscript{Fl/Fl} mice though the results did not reach the threshold for significance (Figure 4.11 D-H). Osteoclast activity patterns appeared similar in both the trabecular and cortical compartments (Figure 4.12) suggesting a trend towards global increased bone resorption in smad4\textsuperscript{Fl/Fl} mice in both the trabecular and cortical compartments. Using RNA sequencing, we also found that many genes associated with osteoclast differentiation (GO:0030316) including \textit{Tnfsf11}, \textit{Ocstamp}, \textit{Dcstamp}, and \textit{Oscar} were significantly upregulated in bone from smad4\textsuperscript{Fl/Fl} mice (Figure 4.11 I), suggesting that osteoclastogenesis is increased and could contribute to increased bone resorption. As bone turnover is a delicate balance between bone resorption and bone deposition, even small changes in bone resorption if not balanced by bone deposition, can lead to irreversible bone loss in what is known as ‘high-turnover’ bone loss \textsuperscript{(217)} and could contribute to the skeletal changes we observed in smad4\textsuperscript{Fl/Fl} mice. RANKL (\textit{Tnfsf11}) and OPG (\textit{Tnfrsf11b}) ratios have been used to examine coupling of bone deposition and resorption as the two genes activate or inhibit osteoclast activity respectively \textsuperscript{(44)}. We found that while the osteoclast activating RANKL (\textit{Tnfsf11}) was upregulated in bone from smad4\textsuperscript{Fl/Fl} mice, osteoprotegerin (\textit{Tnfrsf11b}) was downregulated (Figure 4.11 J) consistent with an uncoupling of bone resorption and bone deposition leading to increased bone loss. Knowing that there was increased bone remodelling due to osteoclast activation, we next examined a subset of bone-related genes to identify a molecular target for the skeletal changes we observed.
4.4.3 Inhibition of FGF23 does not rescue the smad4\textsuperscript{Fl/Fl} phenotype.

Our RNA sequencing data indicated that genes related to bone remodeling were significantly differentially expressed so we examined a subset of these genes using qPCR to identify a target gene that may be have a role in the development of the smad4\textsuperscript{Fl/Fl} phenotype. Confirming our RNASeq results, there was increased expression of genes associated with bone degradation ($Mmp9$ 4.274±3.087, $Mmp13$ 2.836±1.241), and osteoclast differentiation ($Tnfsf11$ 2.548±1.272, $Tnfsf11a$ 2.491±1.956, Oscar 5.219±4.474). Perhaps most notably however, we found there was a 4.501±1.754-fold increase in $Fgf23$ expression (Figure 4.13 A). Interestingly, our RNASeq data also indicated that $Fgf23$ was one of the most upregulated genes in late stage smad4\textsuperscript{Fl/Fl} mice compared to smad4\textsuperscript{Fl/+} controls though the genes most commonly associated with its regulation ($Mepe$, $Phex$, $Dmp1$) \cite{218} were not significantly differentially expressed (Figure 4.13 B). Intriguingly, staining of FGF23 showed reduced numbers of cells expressing FGF23 in smad4\textsuperscript{Fl/Fl} mice (Figure 4.13 C-E) perhaps suggesting that the message was concentrated in a small number of cells. FGF23, secreted by osteoblasts and osteocytes, has roles in phosphate and vitamin D metabolism, and elevated FGF23 levels have been identified in various forms of genetic and acquired hypophosphatemic rickets/osteomalacia \cite{219}. FGF23 is known to inhibit bone mineralization, however whether it does so through direct or indirect mechanisms is still unclear \cite{219,220}. As we also found genes associated bone mineralization were differentially expressed in smad4\textsuperscript{Fl/Fl} mice (Figure 4.9 I), we hypothesized that if the phenotype was due to increased FGF23, inhibition of FGF23 using a neutralizing antibody may delay or inhibit its progression in smad4\textsuperscript{Fl/Fl} mice. Using an anti-FGF23 antibody developed by Amgen, we attempted to neutralize FGF23 in smad4\textsuperscript{Fl/Fl} mice. Administration of 10mg/kg αFGF23 or IgG2a control antibody began 1 week after
tamoxifen treatment, before any phenotype symptoms are observed in smad4<sup>Flo</sup> mice (Figure 4.13 F). While αFGF23 treatment was able to delay the onset of the mobility and hindlimb phenotype by about one week in smad4<sup>F/F</sup> mice compared to IgG2a treated controls and did not appear to affect the health or weight of treated animals, it was insufficient to completely block the phenotype and did not affect the progression of the phenotype once it began (Figure 4.13 G-J). We further analyzed whether αFGF23 antibody treatment had any effect on bone microstructure using µCT (Figure 4.13 K). While there was a treatment based increase of Tb.Th in smad4<sup>F/+</sup> mice treated with αFGF23 antibody, there were no significant treatment effects on other trabecular bone parameters (Figure 4.13 L-O). Similarly, there were no significant effects on cortical parameters, however there was reduced variance in Po(cl) of αFGF23 treated smad4<sup>F/F</sup> mice compared to IgG2a treated controls suggesting that perhaps the αFGF23 antibody treatment may have some minor effects on cortical porosity in smad4<sup>F/F</sup> mice (Figure 4.13 P-T). Together, these results suggested that though Fgf23 is increased in smad4<sup>F/F</sup> mice, it is unlikely that it drives the phenotype and is more likely to be a secondary effect. This indicated that other pathways may be responsible for the smad4<sup>F/F</sup> phenotype, so we turned to gene ontology analysis.

4.4.4 Loss of pro-regenerative response in late stage smad4<sup>F/F</sup> mice may exaggerate bone loss.

To elucidate a possible cause for the smad4 phenotype, we performed RNASeq on both td.Tomato<sup>+</sup>Sca1<sup>+</sup> FAPs from bone and whole bone tissue from smad4<sup>Flo</sup> mice and examined what biological pathways were altered in early and late stages. Amongst the 566 significantly differentially expressed genes (DEGs) in early smad4<sup>F/F</sup> vs smad4<sup>F/+</sup>, and 1087 DEGs in late
smad4^{Fl/Fl} vs smad4^{Fl/+} (Figure 4.14 A-B), pathways associated with extracellular matrix organization, ossification, biomineral tissue development, and wound healing were among the most significantly upregulated pathways in td.Tomato^"Sca1^+ FAPs (Figure 4.14 C-D). Interestingly, we also noted that despite the ablation of Smad4 in FAPs, there was upregulation of pathways associated with response to TGFβ in early and, to a lesser extent, in late smad4^{Fl/Fl} mice, perhaps suggesting a role for SMAD-independent TGFβ signaling in the phenotype (Early, Figure 4.14 C.).

While pro-regenerative pathways were upregulated in PDGFRα^+ FAPs at both early and late smad4^{Fl/Fl} timepoints, this was not reflected at the tissue level. In whole bone tissue at early timepoints, amongst the 319 DEGs (early vs Fl/+, Figure 4.15 A) there was an upregulation of regenerative and osteogenic pathways that was largely absent at later phenotypic stages (Figure 4.15 C). In contrast at late timepoints, amongst the 2124 DEGs (Figure 4.15 B) there was a pronounced upregulation of pathways associated with leukocyte and myeloid cell differentiation, as well as inflammatory pathways (Figure 4.15 C). As osteoclasts are specialized macrophages\(^{(6)}\), upregulation of myeloid cell differentiation pathways could indicate differentiation of osteoclast precursors. Interestingly, pathways such as osteoclast differentiation, bone resorption, bone remodeling, and negative regulation of bone mineralization were also significantly upregulated (Figure 4.15 D) in smad4^{Fl/Fl} bone at late timepoints. Further, inflammation has been implicated in bone loss in many chronic inflammatory diseases, suggesting it could worsen bone loss\(^{(221)}\). Together, this suggests that while there appears to be an early pro-regenerative response mediated by the PDGFRα^+ cell population which maintains a pro-regenerative response throughout, this may be overwhelmed by a later inflammatory and degenerative response in which bone is lost and would be consistent with the bone loss we observed by µCT.
4.5 Discussion

TGFβ and BMP signaling are essential for bone development and homeostasis with SMAD4 acting as a signal transducer central to both. Mutations in *Smad4* have been implicated in various cancers and in Myhre syndrome which is characterized by skeletal abnormalities. Preclinical investigations into the role of *Smad4* using various transgenic models have produced a variety of sometimes conflicting skeletal phenotypes ranging in severity, though most have relied on the use of constitutively active cre drivers with mainly developmental effects (168-171). Here, we used a PDGFRαCT2 model to ablate *Smad4* in a variety of osteolineage cells including osteoblast precursors and showed that constitutive *Smad4* signaling in PDGFRα+ cells is necessary to maintain adult skeletal homeostasis.

Ablation of *Smad4* from PDGFRα+ cells in the adult led to the rapid development of a degenerative skeletal phenotype which primarily affected the hindlimbs and was associated with postural changes in gait which have not previously been reported in other *Smad4* deficient models. While PDGFRα+ cells are also present in the muscle interstitium and have important roles in muscle regeneration (87,88,192), we determined that the phenotype was not due to a skeletal muscle defect, but rather was due to drastic skeletal changes, predominantly in the pelvic girdle and hindlimbs.

Salazar et al (170) previously used an OsxCre model to delete *Smad4*. OsxCre has been shown to have effects on cortical bone independent of any floxed alleles (222) and the use of a constitutively active cre induces developmental effects, however despite this, we noted some similarities in the skeletal
phenotypes. Decreased tibial length, decreased cortical thickness and cortical area fraction were all consistent with the Osx\textsuperscript{Cre}/\textit{smad4}\textsuperscript{Flox} model used by Salazar et al\textsuperscript{(170)} however, while Salazar et al noted delayed osteoblast differentiation and abnormal collagen deposition, we did not find any defect in osteoblast differentiation or expression of bone and cartilage ECM genes, all of which were instead upregulated in \textit{smad4}\textsuperscript{Fl/F1} PDGFR\textalpha\textsuperscript{+} FAPs. Increased osteocyte density which we observed in \textit{smad4}\textsuperscript{Fl/F1} mice, has been reported in other \textit{smad4} murine models\textsuperscript{(169,170)}, though here we attribute this effect to an increase in cell proliferation. As osteoblasts are not reported to express Sca1\textsuperscript{(223)}, Lineage\textsuperscript{-}/PDGFR\textalpha/td.Tomato\textsuperscript{+}/Sca1\textsuperscript{+} FAPs sorted for RNA sequencing represent the more progenitor-like population of PDGFR\textalpha/td.Tomato\textsuperscript{-} lineage traced cells from bone. Gene ontology analysis of PDGFR\textalpha\textsuperscript{+} FAPs showed that genes associated with osteoblast differentiation and extracellular matrix deposition were significantly upregulated in FAPs from both early and late phenotype stages confirming that deletion of \textit{Smad4} did not impair osteoblast differentiation.

While osteoblast proliferation and differentiation are normally associated with SMAD-dependent TGF\textbeta and BMP signaling, there is ample evidence that TGF\textbeta and BMP can regulate osteoblast proliferation and differentiation respectively, in a SMAD-independent manner via MAPK and p38 pathways\textsuperscript{(159,160,166,224-229)}. In line with this, pathways associated with response to TGF\textbeta were upregulated in early stage PDGFR\textalpha\textsuperscript{+} FAPs which represent the osteoprogenitor portion of PDGFR\textalpha/td.Tomato labeled cells. This perhaps represents SMAD-independent TGF\textbeta induced cell proliferation of osteoprogenitors though further work examining the phosphorylation states of the p38 MAPK pathway proteins would be required to confirm this.
Importantly, though there was an increase in PDGFRα/Tomato+ cells, a population which includes osteoblasts, in smad4^{Fl/Fl} mice bone mineralization did not increase proportionally. In fact, consistent with previous work where deletion of Smad4 from osteoblasts resulted in mineralization defects\textsuperscript{(168,169,171)}, in bone from PDGFRα/smad4^{Fl/Fl} mice, genes associated with the negative regulation of bone mineralization were upregulated while those associated with positive regulation were downregulated despite calcein labeling being unchanged, suggesting a slight mineralization defect.

In contrast to several other skeletal Smad4 models where osteoclast activity was decreased\textsuperscript{(168-170)}, we noted increased osteoclast activity and upregulation of genes associated with osteoclast differentiation in smad4^{Fl/Fl} mice which helped explain the bone resorption we noted with µCT, particularly on cortical surfaces. Normally bone resorption and bone deposition are closely coupled and an increase in bone resorption with insufficient increase in bone deposition induces ‘high-turnover’ bone loss\textsuperscript{(217)}. Osteoclast differentiation is primarily mediated by osteoblasts via RANKL (Tnfsf11) and regulated by osteoblast secreted OPG (Tnfrsf11b), and current evidence suggests that the expression ratio of RANKL/OPG determines the degree of osteoclast differentiation and action and therefore represents a measure of ‘coupling’\textsuperscript{(230,231)}. This ratio was skewed in PDGFRα/smad4^{Fl/Fl} mice and we noted significantly increased expression of Tnfsf11 and decreased expression of Tnfrsf11b, suggesting increased osteoclast activation and an uncoupling of bone deposition and resorption. Interestingly, low levels of TGFβ in stromal cell cultures have been reported to promote osteoclast differentiation by regulating the RANKL/OPG ratio secreted by osteoblasts\textsuperscript{(154)}. Latent TGFβ is sequestered in the bone matrix and released in an active form during bone resorption where it acts to recruit osteoblast precursors and initiate
osteogenic differentiation\(^{(208)}\), however high levels of TGFβ inhibit osteoblast mediated differentiation of osteoclasts by reducing RANKL expression and balancing the RANKL/OPG ratio \(^{(154)}\). Thus, our model suggests that TGFβ-mediated regulation of RANKL/OPG occurs via a Smad4 dependent pathway, and in the absence of Smad4, while osteogenic differentiation is not impaired, regulation of RANKL/OPG coupling bone remodeling is impaired and results in a form of high turnover bone loss.

Impairment of mineralization pathways in smad4\(^{Fl/Fi}\) bone was correlated with elevated Fgf23 expression. Osteocyte and osteoblast secreted FGF23 has important roles in Vitamin D synthesis and phosphate homeostasis and mutations in Fgf23 or regulating genes such as Mepe, Phex, and Dmp-1 that result in elevated FGF23 levels, cause skeletal phenotypes characterized by rickets and osteomalacia \(^{(219,232)}\). Recent work has shown that FGF23 has direct and indirect roles inhibiting bone mineralization \(^{(232-235)}\) and we therefore hypothesized that it may have a role in the development of the smad4\(^{Fl/Fi}\) phenotype. Neutralizing antibodies against FGF23 have been used in preclinical models of hypophosphatemic rickets to successfully improve bone mineralization \(^{(235-237)}\), however, in our model neutralizing FGF23 antibody was unable to prevent the smad4\(^{Fl/Fi}\) phenotype. Treatment did appear to delay the onset by about a week and variation in cortical porosity was decreased, however our results suggested that elevated Fgf23 was a secondary effect of the phenotype and not a causative agent. Interestingly, Fgf23 expression is regulated at multiple levels and via a variety of regulators including MEPE, PHEX, and DMP-1 \(^{(36,238)}\). However, Phex, Dmp1, and Mepe were not significantly differentially expressed in smad4\(^{Fl/Fi}\) mice at early or late timepoints (Figure 4.13 B) suggesting that increased Fgf23 expression was not due to their regulatory actions. Inflammation has also been recently implicated in the regulation of Fgf23 via
increased expression of HIF-1α (239). Indeed, inflammatory pathways as well as Hif1a expression (Figure 4.16 A) were significantly upregulated in bone from both early and late stage smad4^{Fl/Fl} mice suggesting that inflammation may be driving the expression of Fgf23 in smad4^{Fl/Fl} mice, and further suggesting that it is a secondary effect of the phenotype.

RNA sequencing of marrow-free whole bone tissue indicated that at early timepoints in smad4^{Fl/Fl} mice there is a pro-regenerative phenotype characterized by pathways associated with wound healing, and ossification. While there was some evidence of inflammatory pathway upregulation at the early timepoint, these pathways were more highly expressed in the late phenotype, and pro-regenerative pathways were almost completely absent. Further, in the late phenotype, pathways associated with bone resorption and osteoclast differentiation were upregulated. Together this suggests that there is an initial pro-regenerative phase that gives way to an overwhelming inflammatory phase in which bone is lost. It should be noted that as osteoclasts are essentially specialized macrophages and derived from a myeloid hematopoietic lineage (6), upregulation of myeloid leukocyte migration and differentiation pathways is consistent with increased osteoclast precursor differentiation. Further, inflammation including many chronic inflammatory conditions, can lead to excessive bone resorption (221). Inflammatory cytokines such as TNF and IL-6 can inhibit osteoblast activity through SMAD-independent pathways, and also act to increase osteoclastogenesis (221). Notably, pathways for TNF production and IL-6 production were also upregulated in bone from late stage smad4^{Fl/Fl} mice (Figure 4.16 B). This suggests that while inflammation may not initiate the phenotype, it may contribute to worsening or acceleration of the bone loss phenotype at the late stage.
One potential limitation of our study was the use of PDGFRα to ablate Smad4 from osteolineage cells. The majority of previous work has been performed using constitutive cre drivers to delete various elements of the TGFβ and BMP pathways and draw conclusions about postnatal bone homeostasis though in many cases confounding development effects during bone modeling could not be excluded \(^{(143)}\). Here, our use of a tamoxifen inducible creERT2 to delete Smad4 in the adult murine skeleton allowed us to avoid confounding developmental bone effects and examine the role of Smad4 in adult skeletal homeostasis. However, as PDGFRα labels a variety of osteolineage cells including progenitors, osteoblasts, osteocytes, and even chondrocytes which were not addressed here, it is unclear which cells were the main effectors of the phenotype and further work will be required to draw this conclusion. Additionally, further work will be required to determine the manner in which SMAD-independent TGFβ or BMP pathways in osteolineage cells can uncouple bone resorption and formation.

Taken together, our work shows an essential function for Smad4 mediated signaling in PDGFRα\(^+\) osteolineage cells to maintain adult skeletal homeostasis. The loss of Smad4 in PDGFRα\(^+\) cells induces cell proliferation and uncoupling of bone formation and resorption, possibly through SMAD-independent TGFβ and BMP signaling resulting in high turnover bone loss and inflammation that may exacerbate the bone loss phenotype. This phenotype has not previously been reported and represents a new avenue for research into adult skeletal homeostasis.
Figure 4.1 - Conditional deletion of Smad4 in PDGFRα+ cells leads to a degenerative motor phenotype.

(A) Schematic of PDGFRαCT2 conditional deletion of Smad4. (B) Excision efficiency of Smad4 from FACS isolated PDGFRα/td.Tomato+ muscle cells. (C) RNASeq centered heatmap of Smad4
and the housekeeping genes \textit{Tbp} and \textit{Hprt}. RNA was isolated from FACS sorted Lin^{-}/td.Tomato^{+}/Sca1^{+} cells from bone. (D-H) Representative images of smad4^{Flox} mice demonstrating normal and altered hip posture (D, F), hindlimb extension (E, G), and kyphosis (H). (I-L) Scoring for hindlimb posture, mobility, health, and weight at the indicated timepoints after tamoxifen administration. N=82-84. (A) N = 2-3 biological replicates, unpaired Student’s T-Test, NS.
Figure 4.2 - Digital gait analysis highlights postural gait abnormalities in smad4^{Fl/Fl} mice.

(A) Schematic outlining the various components of the stride including swing and stance phases that are measured by the DigiGait System. The stance phase can be further divided into brake and
propel phases. (B) Additional gait parameters measured by the DigiGait system include stride width, stride length, paw area, paw angle, and step angle. (C-D) Representative ventral views from the treadmill highlighting the abnormal hip phenotype observed in smad4^{Fl/Fl} mice. (E-H) Representative stride measures and paw inking from the DigiGait system. Smad4^{Fl/Fl} mice exhibit paw cupping and a reduced stance width. (I-Q) Selected measures from the DigiGait analysis. N=6-7, Forelimbs and hindlimb parameters for smad4^{Fl/+} and smad4^{Fl/Fl} mice were analyzed separately using unpaired Student’s T-test, * p<0.05, ** p<0.005
Figure 4.3 - Complete DigiGait Analysis for smad4^{F/F} mice.
N=6-7 biological replicates. Results for forelimbs and hindlimbs were analyzed separately using unpaired Student’s T-Test. *p < 0.05, **p<0.005
Figure 4.4 - Deletion of Smad4 from PDGFRα⁺ cells does not affect skeletal muscle at homeostasis.

(A) Cross-sectional area (CSA) of undamaged skeletal muscle from smad4\(^{Flox}\) mice. (B) Nuclei per fiber in undamaged muscle. (C) Collagen deposition in undamaged muscle of smad4\(^{Flox}\) mice measured by picosirius red staining. (D-E) Representative images of Picosirius Red stained tibialis anterior muscle from smad4\(^{Flox}\) mice. N=2-4 biological replicates. Results analyzed using unpaired Student’s T-Test.
Figure 4.5 - Deletion of Smad4 from PDGFRα⁺ cells induces macroscopic skeletal changes. (A-B) Whole mount Alizarin Red S (bone) and Alcian Blue (cartilage) staining of smad4Flox mice. (C-L) Representative µCT reconstructions. (C-D) Lateral view of the whole skeleton highlighting
kyphosis (arrow) and lumbar lordosis (arrowhead) in smad4^{Fl/Fl} mice. (E-H) Cranial view of femurs and pelvic regions of smad4^{Flox} mice. Narrowing of the distal femur (G arrowhead), knee dislocation (H arrow), and pelvic bone ablation (G-H asterisk) are visible in smad4^{Fl/Fl} mice. (I-J) Lateral view of the ribcage with altered rib morphology in smad4^{Fl/Fl} mice indicated (L arrow). (M) Contingency chart of various skeletal phenotypes observed in smad4^{Flox} mice. Some phenotypes were observed in all smad4^{Fl/Fl} cases studied, while others were only present in some instances. Images representative of N=4 biological replicates.
Figure 4.6 - Deletion of Smad4 results in morphological changes in the bone of smad4\(^{Flox}\) mice.

(A) Macroscopic image of femurs from smad4\(^{Fl/Fl}\) and smad4\(^{Fl/+}\) mice. (B) Femur weight as a percentage of body weight. (C) Femur length in smad4\(^{Flox}\) mice. (D) Tibia length in smad4\(^{Flox}\) mice. (E-F) Representative Masson’s trichrome images of the knee joint (distal femur and proximal tibia) from smad4\(^{Flox}\) mice. (B) N=4-6. (C) N=3. (D) N=5-9, * p=0.0364, ** p=0.0040. N are biological replicates. Results analyzed using one-way ANOVA with Tukey correction for multiple comparisons.
Figure 4.7 - Bone remodeling in smad4Flox mice occurs mainly in the cortical bone compartment.
(A) Representative µCT reconstructions of trabecular and cortical bone from smad4"Flox" mice. (B-J) µCT analysis of trabecular and cortical bone from smad4"Flox" mice. (B). Bone Volume Fraction (BV/TV) (C) Trabecular thickness (Tb.Th) (D) Trabecular Number (Tb.N) (E) Trabecular Spacing (Tb.Sp) (F) Cortical Thickness (Ct.Th) (G) Cortical Porosity (Ct.Po) (H) Cortical Bone Area (Ct.Ar) (I) Total Tissue Area (Tt.Ar) (J) Cortical Area Fraction (Ct.Ar/Tt.Ar). (K) RNASeq heatmap of significantly differentially expressed genes associated with the GO term for bone remodeling (GO:0046849). RNA isolated from whole bone tissue of smad4"Flox" mice. Genes associated with bone remodeling are upregulated in smad4"Fl/Fl" bone, particularly in the late phenotype. (A-J) N=3-11 biological replicates. All results analyzed using one-way ANOVA with Tukey correction for multiple comparisons. *p<0.05.
Figure 4.8 - Increased expression of PDGFRα/td.Tomato in PDGFRα<sup>CT2</sup>/td.Tomato/sm<sup>4</sup>F<sup>Flo</sup> mice is a result of increased FAP proliferation.

(A) Representative images of the distal femur from sm<sup>4</sup>F<sup>lo</sup> mice of the indicated genotype and phenotype stage. (B) Representative images of the periosteum and fibrous entheses on the femurs of sm<sup>4</sup>F<sup>lo</sup> mice. (C) Representative images of fibrocartilaginous entheses of the proximal femur.
of smad4<sup>Flox</sup> mice. (D) Representative images of fibrocartilaginous entheses of the distal femur of smad4<sup>Flox</sup> mice. (E) Quantification of td.Tomato<sup>+</sup> cells in the bones of smad4<sup>Flox</sup> mice. Td.Tomato<sup>+</sup> cells increase significantly in smad4<sup>Fl/Fl</sup> mice and correlate with phenotype stage. (F) Osteocyte density in cortical bone of smad4<sup>Fl/Fl</sup> mice is increased (G) Quantification of proliferating (EdU<sup>+</sup>) cells in the bones of smad4<sup>Flox</sup> mice. Increased td.Tomato<sup>+</sup> cells in the bones of smad4<sup>Fl/Fl</sup> mice are the result of increased cell proliferation. (H) RNASeq heatmap of selected genes associated with cell proliferation in FAPs from smad4<sup>Flox</sup> mice. The majority of selected cell proliferation genes are upregulated, however only Plk1, Bub1, and Top2a are significantly differentially expressed in smad4<sup>Fl/Fl</sup> FAPs (Late vs Fl/+). (A-D) Images representative of N=3 biological replicates. (E) N=7-11 biological replicates. Results analyzed using one-way ANOVA with Tukey correction. **p=0.0063, ***p=0.0006. (F) N=5 biological replicates. Results analyzed using one-way ANOVA with Tukey correction. ***p=0.0007, ****p=<0.0001. (G) N=6 biological replicates. Results analyzed using unpaired Student’s T-Test. * p=0.0375.
Figure 4.9 - Bone mineralization parameters in smad4\(^{\text{Flox}}\) mice are not significantly altered, but expression of genes associated with mineralization are.

(A) Schematic of calcein administration. (B-C) Representative immunofluorescent images of calcein labeled bone from smad4\(^{\text{Fl/+}}\) and smad4\(^{\text{Fl/Fl}}\) mice. (D-H) Quantification of calcein label (I)
RNASeq heatmaps of significantly differentially expressed genes associated with the GO terms for positive and negative regulation of bone mineralization (GO:0030501, GO:0030502). RNA isolated from whole bone tissue of smad4^Fl^ox mice. Genes associated with positive regulation of bone mineralization are downregulated in smad4^Fl/Fl^ mice, and genes associated with negative regulation of bone mineralization are upregulated in smad4^Fl/Fl^ mice. (A-H) N = 5-6 biological replicates. Results analyzed using unpaired Student’s T-Test.
Figure 4.10 – Quantification of bone mineralization parameters in trabecular and cortical bone of smad4Flx mice.

(A-E) Analysis of calcein labeled trabecular bone from smad4Flx mice. (F-J) Analysis of calcein labeled cortical bone from smad4Flx mice. Similar trends are observed in both trabecular and cortical compartments for Mineralizing Surface (MS), Mineral Apposition Rate (MAR), and Bone Formation Rate (BFR).
Formation Rate (BFR). sLS = single labeled surface, dLS = double labeled surface. N=5-6 biological replicates. Results analyzed using unpaired Student’s T-Test.
Figure 4.11 - TRAP osteoclast activity is increased in smad4^{Fl/Fl} bone.

(A-B) Representative images of TRAP staining in trabecular (A) and cortical bone (B) of smad4^{Fl/+} and smad4^{Fl/Fl} mice. (C-H) Quantification of TRAP activity in trabecular and cortical bone. (C)
Bone Perimeter (B.Pm) (D) Osteoclast Perimeter (Oc.Pm) (E) Number of Osteoclasts (N.Oc) (F) Osteoclast Surface/Bone Surface (Oc.S/BS) (G) Number of Osteoclasts/Bone Surface (N.Oc/BS) (H) Number of Osteoclasts/Bone Volume (N.Oc/BV) (I) RNASeq heatmap of significantly differentially expressed genes associated with the GO term for Osteoclast Differentiation (GO:0030316). RNA isolated from whole bone tissue of smad4^Fl/Fl^ mice. Many genes associated with osteoclast differentiation are upregulated in smad4^Fl/Fl^ mice compared to smad4^Fl/+^ controls. (J) Heatmap of RANKL (Tnfsf11) and OPG (Tnfrsf11b) which are significantly differentially expressed in smad4^Fl/Fl^ bone. Tnfsf11 is upregulated and Tnfrsf11b is downregulated. (A-H) N=3-6 biological replicates. Analyzed using unpaired Student’s T-Test. *p = 0.0435.
Figure 4.12 - TRAP osteoclast analysis from smad4Flox mice.

Student’s T-Test. *p<0.05. B.Pm = Bone perimeter, Oc.Pm = Osteoclast perimeter, N.Oc = Number of osteoclasts, Oc.S/BS = Osteoclasts surface/bone surface, N.Oc/BS = Number of osteoclasts/bone surface, N.Oc/BV = Number of osteoclasts/bone volume.
Figure 4.13 - αFGF23 treatment does not rescue the smad4^{F/F} phenotype.

(A) qPCR results of select bone related genes. Fold change smad4^{F/F} vs smad4^{F/+}. (B) RNASeq heatmap of Fg/23 (p.adj=0.0166) and related regulatory genes Mepe (p.adj=0.5228), Phex (p.adj=0.7479), and Dmp1 (p.adj=0.6769) from whole bone tissue of smad4^{Flox} mice. (C-D)
Representative FGF23 immunofluorescent staining in smad4Fl/+ bone (C), and smad4Fl/Fi bone (D). (E) Quantification of FGF23 staining. (F) Schematic of the aFGF23 antibody intervention. (G-J) Mobility, hindlimb, health scores, and weight of treated mice. (K) Representative µCT scans of trabecular and cortical bone from IgG2a and aFGF23 treated smad4Fl/Fi mice. (L-O) µCT analysis of trabecular bone parameters (L) Bone Volume Fraction (M) Trabecular thickness (N) Trabecular number (O) Trabecular spacing. (P-T) µCT analysis of cortical bone parameters (P) Cortical thickness (Q) Cortical porosity (R) Total Area (S) Cortical Area (T) Cortical area fraction. (E) N=4-5 biological replicates. One outlier removed using Grubbs (alpha=0.1). Analyzed using one-way ANOVA with Tukey correction for multiple comparisons. ** P= 0.0062. (L-T) N=2-3 biological replicates. Grouped analysis performed using two-way ANOVA with Sidak correction for multiple comparisons.
Figure 4.14 - RNASeq GO Bio pathway analysis of differentially expressed genes in PDGFRα+Scal+ FAPs from bone of smad4Flox mice.

(A) Heatmap of the top 50 most significantly differentially expressed genes in early stage smad4Fl/+ mice compared to smad4Fl/Fl. (B) Heatmap of the top 50 most significantly differentially
expressed genes in late stage smad4\textsuperscript{Fl/Fl} mice compared to smad4\textsuperscript{Fl/+}. (C) Top 20 significantly upregulated GO Bio pathways in FAPs from early phenotype smad4\textsuperscript{Fl/Fl} mice compared to smad4\textsuperscript{Fl/+} controls. Regenerative, extracellular matrix (ECM) organization, and osteogenic pathways are highlighted. (D) Top 20 significantly upregulated GO Bio pathways in FAPs from late phenotype smad4\textsuperscript{Fl/Fl} mice compared to smad4\textsuperscript{Fl/+} controls. Pathways associated with regeneration, ECM organization, and ossification are highlighted.
Figure 4.15 - RNASEq GO Bio pathway analysis of differentially expressed genes in whole bone tissue of smad4^{Flox} mice.

(A) Heatmap of the top 50 most significantly differentially expressed genes in early smad4^{Fl/Fl} mice compared to smad4^{Fl/+}. (B) Heatmap of the top 50 most significantly differentially expressed genes in late stage smad4^{Fl/Fl} mice compared to smad4^{Fl/+} controls. (C) Top 20 significantly upregulated GO Bio pathways in bone of early phenotype smad4^{Fl/Fl} mice compared to smad4^{Fl/+} controls. Osteogenic and regenerative pathways are highlighted. (D) Top 20 significantly upregulated GO Bio pathways in bone of late phenotype smad4^{Fl/Fl} mice compared to smad4^{Fl/+} controls. Pathways related to leukocyte and myeloid cell activation and differentiation are highlighted. (E) Additional osteoclast and bone related GO Bio pathways significantly upregulated in bone of late phenotype smad4^{Fl/Fl} mice compared to controls. Similar pathways were not upregulated in early phenotype RNASEq data.
Figure 4.16 - Expression of Hif1a and TNF/IL-6 pathways in whole bone tissue of smad4^Flox^ mice

(A) Normalized counts of Hif1a in bone from smad4^Flox^ mice. (B) GO pathway analysis of TNF and IL-6 pathways which are significantly upregulated in late smad4^Flox/Flox^ mice compared to controls.
### Table 4-1 Scoring parameters for smad4<sup>Flox</sup> mice

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<th>Hindlimb</th>
<th>Health</th>
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<tbody>
<tr>
<td>1 Normal</td>
<td>1 Normal</td>
<td>1 Normal (BAR)</td>
</tr>
<tr>
<td>2 Minor reduced mobility/reluctance to stand up</td>
<td>2 Reduced extension/ minor joint/ hip dislocation</td>
<td>2 Slight hunching/ piloerection/ squinting</td>
</tr>
<tr>
<td>3 Reduced mobility/cannot fully stand up</td>
<td>3 No extension/ moderate joint/ hip dislocation</td>
<td>3 Hunched/ piloereact/ eyes squinted</td>
</tr>
<tr>
<td>4 Severely reduced mobility/unable to stand up</td>
<td>4 Legs under body at all times, severe joint/hip dislocation</td>
<td>4 Severe hunching/ piloerection/ eyes almost closed. Appears moribound</td>
</tr>
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<td>Forelimb</td>
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<td>-------------------------------</td>
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<td>----------</td>
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<td>Pvalue</td>
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</tr>
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| Paw Drag    | NS       | 0.7187        | NS       | 0.4628        | N/A
Chapter 5: Discussion

5.1 Contributions to the Field and Future Directions

Bone remodeling is required for skeletal function yet imbalances in bone deposition and resorption quickly lead to skeletal disorders. The majority of therapeutics for bone disorders have been focused on inhibiting osteoclast activity to increase bone mass, and very few treatments outside of BMPs have been identified to target bone deposition. This is in part due to poor biological understanding of osteoblast progenitors (skeletal MSCs) and the mechanisms of their regulation in the adult. The concept of mesenchymal stem cells originated in the bone marrow, and while this has expanded to include MSCs in other tissues, there has recently been a resurgence in research to identify endogenous skeletal stem and progenitor cells for therapeutic applications. The work described here describes a role for PDGFRα+ cells in skeletal homeostasis and heterotopic ossification and identifies HIC1 as a novel marker of periosteal skeletal MSCs. The importance of these findings is presented below.

5.1.1 HIC1 labels a previously unidentified periosteal skeletal MSC population

Many markers for skeletal MSCs have been identified all with differing contributions to skeletal development and regeneration, suggesting that multiple progenitor populations may be involved in these processes. Notably, all the candidate skeletal MSC populations were found within the growth plate, endosteum, or bone marrow. Though the importance of the periosteum as a cellular
source of MSCs in regeneration was established in the 1800s (123) and re-confirmed recently (124), the identity of these periosteal cells has remained elusive.

In chapter 2, we investigated the distribution of PDGFRα+ and HIC1+ cells in skeletal tissues. PDGFRα was selected as a marker as it has been well established as an MSC marker in skeletal muscle by our lab and subsequently as an MSC marker in many other tissues since. Similarly, HIC1 has recently emerged as a novel marker of injury responsive quiescent MSCs, suggesting that one of these markers may also identify skeletal MSCs. While PDGFRα reporter mice have been around since 1997 (129), the expression pattern of PDGFRα in skeletal tissues has not been reported.

We demonstrated that while PDGFRα preferentially labels MSCs in skeletal muscle, it labels all skeletal lineages including chondrocytes (articular and in the growth plate), osteoblasts, osteocytes, as well as stromal cells and thus cannot be uniquely used to identify skeletal MSC populations.

We also showed that HIC1 labels stromal cells in the bone marrow and a small population of periosteal cells that is relatively quiescent at homeostasis but can contribute to PDGFRα+ osteocytes during normal bone remodeling. We next demonstrated that HIC1+ cells are injury responsive and contribute to all stages of bone regeneration. While HIC1 did not label all cells within the fracture callus, this is consistent with multiple MSC populations contributing to bone regeneration. We noted that the contribution of HIC1+ cells to the fracture callus was overwhelmingly periosteal and single cell RNA-sequencing analysis of bone marrow HIC1+ cells
suggest that within the bone marrow, the majority of HIC1\(^+\) cells are CAR-like niche cells, indicating that periosteal and bone marrow HIC1\(^+\) cells have diverse roles that depend on their location.

Additional work is being performed by the Underhill laboratory to characterize HIC1\(^+\) cells as MSCs \textit{in vitro} and \textit{in vivo}, however the small number of periosteal cells labeled during quiescence presents a unique challenge for their characterization. Further, work is required to demonstrate whether HIC1\(^+\) cells have the capacity to self-renew and could thus be classified as SSCs. Additionally, lineage identification of unlabeled cells in the fracture callus could help determine which other skeletal MSC populations contribute and coordinate with HIC1\(^+\) cells to regenerate bone.

To our knowledge this is the first report of a marker uniquely identifying periosteal MSCs with regenerative potential, helping to fill the gap in scientific understanding of the periosteal contribution to bone regeneration and presenting a novel therapeutic target.

5.1.2 \textbf{PDGFR\(\alpha^+\) FAPs are the cellular source of HO and undergo spontaneous osteogenic differentiation in an altered inflammatory environment.}

Therapeutics for HO have largely been limited due to a lack of understanding of the cells and mechanisms behind its pathogenesis. Though it is now accepted that HO arises from mesenchymal progenitors, many proposed markers have significant expression in multiple cell types (i.e. Tie2)
The majority of proposed MSC populations were found to contain subpopulations of PDGFRα+ cells, a marker our lab identified as labeling FAPs in skeletal muscle.

In chapter 3, we examined the role of PDGFRα+ cells in HO using a newly developed PDGFRαCT2 lineage tracing model and confirmed that they are the main source of osteogenic cells. The expression of Tie2, Glast, and Gli1 by PDGFRα+ cells suggests that in previous lineage tracing studies (81,83,84), the PDGFRα+ subfraction of heterogenous cells may have been the drivers of HO. Further, we demonstrated that these ossifications arise from local progenitors and are not recruited from the bone marrow or circulating progenitors as has previously been speculated.

Lastly, we showed that PDGFRα+ FAPs have inherent osteogenic potential in vivo and do not require reprogramming by exogenous BMPs. Alterations of the inflammatory environment preventing the clearance of FAPs after damage was sufficient to induce the continued expression of osteogenic genes and the formation of HO.

Together, these studies confirmed tissue resident PDGFRα+ cells are the cellular source of HO and suggest a possible mechanism by which injury and an impaired inflammatory response can induce osteogenic differentiation and HO.

We noted incomplete penetrance of the CCR2KO phenotype after damage, consistent with the spontaneous development of HO after surgery or trauma. Future studies should examine how subtle changes in the inflammatory environment after damage drive osteogenic differentiation and the development of HO.
5.1.3 Constitutive SMAD4 signaling is required to maintain adult murine skeletal homeostasis

TGFβ and BMP signaling are not only required for skeletal development and homeostasis, but also act as drivers of osteogenesis and fibrosis of MSCs in other tissues. Most studies of impaired TGFβ and BMP signaling have utilized constitutive cre drivers which can lead to confounding developmental effects and thus, little is known about the in vivo effects on adult tissues. This prompted us to examine the role of these signaling pathways in the adult skeletal system using PDGFRα<sup>CT2/Smad4<sup>Fl</sup>ox</sup> mice to impair both TGFβ and BMP signaling after postnatal growth was completed.

We found that ablation of Smad4 in PDGFRα<sup>+</sup> cells resulted in the rapid development of a novel skeletal phenotype characterized by gait abnormalities and bone loss. Characterization of this phenotype indicated changes in the gross morphology of bones and changes in the bone microarchitecture that were predominantly cortical.

We demonstrated that consistent with previously reported results, PDGFRα mediated Smad4 ablation lead to increased proliferation and increased osteocyte density though it did not seem to impair the expression of genes associated with osteoblast differentiation or ECM deposition.

While other groups have found that ablation of Smad4 in skeletal populations led to decreased osteoclast activity (168-170), we showed that there was increased expression of genes associated with
osteoclast differentiation and activity in smad4^Fl/Fl^ mice. We also demonstrated altered RANKL/OPG ratios that suggested uncoupling of bone remodeling that could result in high turnover bone loss.

Fgf23 was highly upregulated in smad4^Fl/Fl^ mice and has previously been implicated in the development of hypophosphatemic rickets and osteomalacia, however we demonstrated that it was unlikely to be a causal driver of the observed phenotype.

Lastly, we found that following an early pro-regenerative response, later phenotypic stages were characterized by an upregulation of inflammatory pathways that may exacerbate the bone loss phenotype though the mechanisms by which this may occur requires further study.

There were several limitations to this study. As PDGFRα labels all osteogenic lineages in adult skeletal system, deletion of Smad4 occurred in multiple cell types and thus further study is required to determine which subpopulation of PDGFRα^+^ cells are the main effectors of the Smad4 phenotype. Further, PDGFRα is not exclusively expressed by skeletal lineages, and thus some of the effects we observed such as elevated inflammatory pathways, may be mediated by effects of Smad4 ablation in PDGFRα^+^ cells in non-skeletal tissues.

Additionally, further work is required to determine whether TGFβ or BMP signaling are the drivers of skeletal homeostasis in the adult as there is evidence in the literature that both have roles in regulating bone remodeling.
This study characterized an adult skeletal phenotype that has not previously been reported and study highlights the importance of constitutive Smad4 signaling in maintaining adult skeletal homeostasis. While it may not provide a therapeutic target, it contributes to our scientific understanding of the roles of TGFβ and BMP signaling in the adult skeleton without confounding developmental effects.

5.2 Final Conclusions

In conclusion, this thesis highlights the roles of PDGFRα⁺ and HIC1⁺ cells in various aspects of murine skeletal homeostasis, regeneration, and as a driver of HO. We present HIC1 as a novel marker of periosteal skeletal MSCs and highlight the importance of PDGFRα⁺ cells in the pathogenesis of HO, contributing to our understanding of skeletal MSC biology. We also demonstrate the importance of SMAD-mediated TGFβ/BMP signaling in maintaining the adult skeletal system, providing characterization of a novel phenotype. We hope that this work furthers the understanding of skeletal MSC biology and eventually contributes to therapeutic strategies targeting MSCs to treat skeletal disorders.
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